Deletion of the Nonribosomal Peptide Synthetase Gene *nps1* in the Fungus *Clonostachys rosea* Attenuates Antagonism and Biocontrol of Plant Pathogenic *Fusarium* and Nematodes

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ABSTRACT

Secondary metabolites produced by biological control agents may influence the outcome of their interactions with plant pathogenic microorganisms and plants. In the present study, we investigated the role of the nonribosomal peptide synthetase gene *nps1* expressed by the biocontrol fungus *Clonostachys rosea*. A gene expression analysis showed that *nps1* was induced during confrontations with the plant pathogenic fungus *Botrytis cinerea*. Gene deletion strains of *nps1* displayed increased growth rates and conidiation. However, the nematocidal activity of culture filtrates from *C. rosea* $\Delta nps1$ strains was significantly weaker than that from wild-type filtrates ($P \leq 0.001$); after 24 h of incubation with culture filtrates from *nps1* deletion strains, only 13 to 33% of a mixed community of nematodes were dead compared with 42% of nematodes incubated with wild-type culture filtrates. The $\Delta nps1$ strains

also showed reduced biocontrol efficacy during pot experiments, thus failing to protect wheat seedlings from foot rot disease caused by the plant pathogenic fungus *Fusarium graminearum*. Furthermore, *C. rosea* $\Delta nps1$ strains were not able to reduce populations of plant-parasitic nematodes in soil or in roots of wheat as efficiently as the wild-type strain. Both *C. rosea* wild-type and $\Delta nps1$ strains increased the dry shoot weight and shoot length of wheat by 20 and 13%, respectively. We showed that NPS1, a putative nonribosomal peptide synthetase encoded by *nps1*, is a biocontrol factor, presumably by producing a hitherto unknown nonribosomal peptide compound with antifungal and nematocidal properties that contributes to the biocontrol properties of *C. rosea*.

Keywords: antagonism, plant growth, wheat

Nonribosomal peptide synthetases (NRPSs) are multifunctional, modular, megasynthases that have a role in the biosynthesis of small bioactive nonribosomal peptides (NRPs) because of their thiotemplate mechanism that is independent of the ribosomal protein synthesis machinery (Finking and Marahiel 2004; Grünwald and Marahiel 2006; Mootz et al. 2002; Reiber et al. 2005; Sieber and Marahiel 2003; Stein et al. 1996). NRPSs synthesize peptides using modules, with each module responsible for incorporating a specific amino acid in the final peptide product. Each module typically contains at least three core domains: an adenylation domain, a thiolation or peptidyl carrier protein domain, and a condensation domain. The adenylation domain recognizes and activates the substrate by using ATP as an energy source (Conti et al. 1997; May et al. 2002; Stachelhaus et al. 1999). The thiolation or peptidyl carrier protein domain accepts the activated substrate, which is bound to a 4'-phosphopantetheine cofactor as a thioester bond (Weber et al. 2000), and it transfers the substrate to a condensation domain, which is responsible for the peptide bond formation between adjacent substrates on the megasynthase complex (Finking

and Marahiel 2004; Keating et al. 2000, 2002; Weber et al. 2000). To date, numerous NRPS genes (*nps*) have been reported, mostly from bacteria and filamentous fungi, although their biological functions are mostly unknown (Bushley and Turgeon 2010; Oide et al. 2006). However, in past decades, a few functional studies have shown that NRPSs have roles in bacteria and fungi that are related to cell surface properties, morphological and reproductive development, nutrient acquisition, virulence, and protection against oxidative stress (Hahn and Dubnau 1991; Kim et al. 2007; Lee et al. 2005; Oide et al. 2006, 2007). Biotechnological applications have been described, with several NRPSs used as antibiotics or anticancer agents (e.g., bacitracin, gramicidin, penicillin, and vancomycin) and immunosuppressing agents (e.g., rapamycin and cyclosporine) (Challis et al. 2000; Keating et al. 2001; Keating and Walsh 1999; Marahiel et al. 1997; Schaeffer 1969).

The ascomycete *Clonostachys rosea* (Link: Fr.; Schroers, Samuels, Seifert & W. Gams, comb. nov.) (Schroers et al. 1999) is a mycoparasitic fungus that can control plant diseases caused by an extensive range of pathogenic fungi, such as *Alternaria* spp. (Jensen et al. 2004), *Bipolaris sorokiniana* (Jensen et al. 2002), *Botrytis cinerea* (Sutton et al. 1997), *Fusarium culmorum* (Jensen et al. 2000), *Fusarium graminearum* (Hue et al. 2009), and *Sclerotinia sclerotiorum* (Rodríguez et al. 2011). However, recent studies have shown that certain *C. rosea* strains also have a negative impact on a range of different plant-parasitic nematodes (Iqbal et al. 2018b; Zou et al. 2010a, b). Several different biocontrol mechanisms have been reported for *C. rosea*, including direct parasitism (Li et al. 2002; Yu and Sutton 1997), antibiosis through the production of secondary metabolites and enzymes (Dong et al. 2005; Dubey et al. 2014; Fatema et al. 2018; Gan et al. 2007; Iqbal et al. 2018b; Pachenari and Dix 1980; Rodríguez et al. 2011; Tzelepis et al. 2015; Zou et al. 2010b), plant growth promotion

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(Ravnskov et al. 2006; Roberti et al. 2008), and the induction of plant defense reactions (Lahlali and Peng 2014; Mouekouba et al. 2014; Roberti et al. 2008).

The genome of *C. rosea* strain IK726 was recently sequenced (Karlsson et al. 2015) and shown to contain high numbers of genes putatively involved in the biosynthesis of natural products, including polyketides and NRPs, but a comparatively low number of chitinases (Tzelepis et al. 2015). Strain IK726 is an efficient biocontrol agent against many plant pathogenic fungi and oomycetes (Jensen et al. 2007) and plant-parasitic nematodes (Iqbal et al. 2018a). The IK726 genome contains 17 *nps* genes, including the *nps1* gene, which was predicted to encode an epipolythiodiketopiperazine (ETP) synthetase (Karlsson et al. 2015), which is possibly involved in the biosynthesis of nematocidal epipolythiodiketopiperazine compounds (Dong et al. 2005) or glioperazine (Usami et al. 2004). ETPs are derivatives of cyclic peptides and constitute a poorly studied class of secondary metabolites (Gardiner and Howlett 2005). They typically contain disulfide bridges that allow ETPs to cross-link with proteins via cysteine residues and produce reactive oxygen species (Patron et al. 2007).

This study was designed to characterize the biological function of the *C. rosea nps1* gene. We hypothesized that NPS1, a nonribosomal peptide synthetase encoded by *nps1*, is involved in the biosynthesis of compounds with antifungal and/or nematocidal activity that contribute to the biocontrol properties of *C. rosea*. By generating *C. rosea nps1* deletion strains, we demonstrated that NPS1 plays a role in growth, conidiation, in vitro antagonism against nematodes, and the biocontrol of Fusarium foot rot disease and nematode root diseases of wheat.

MATERIALS AND METHODS

Fungal strains and maintenance conditions. *C. rosea* strain IK726 (wild-type [WT]) and mutants derived from the WT, *B. cinerea* strain B05.10, *F. graminearum* strain PH-1, and *Rhizoctonia solani* strain SA1 were maintained on potato dextrose agar (PDA) medium (Oxoid; Basingstoke, Hampshire, England) at 25°C in the dark. Petri plates with PDA were inoculated using stock cultures preserved in 10% (wt/vol) glycerol at –80°C.

Sequence and phylogenetic analyses. The *nps1* gene was retrieved from the *C. rosea* IK726 genome sequence (EMBL accession no. PRJEB4200) (Karlsson et al. 2015). Conserved protein domains were identified using the Conserved Domain Database (Marchler-Bauer et al. 2017) and Simple Modular Architecture Research Tool (SMART), which is database used for protein prediction (Letunic and Bork 2018). SignalP version 4.1 was used to identify signal peptides for targeting proteins to the secretory pathway (Nielsen 2017).

A reference set of fungal NRPS protein sequences was retrieved for the phylogenetic analysis (Bushley and Turgeon 2010). Adenylation and condensation domains were predicted using SMART (Letunic and Bork 2018). Adenylation and condensation domain sequences were extracted and aligned separately using Clustal W, and a phylogenetic tree was constructed using MEGA version 6 (Tamura et al. 2013). The evolutionary history of NRPS families based on adenylation and condensation domains was inferred using a maximum likelihood method based on the Le and Gascuel (LG) model, and the G + F (gamma distribution with amino acid frequencies) amino acid substitution model (Le and Gascuel 2008) was identified as optimal for modeling gamma rates distributed among sites and partial deletions of gaps for adenylation while using all sites to construct a condensation domain-based phylogenetic tree. Statistical support was determined by 1,000-iteration bootstrap resampling.

Experimental setup for gene expression analysis. *C. rosea nps1* gene expression was assessed during fungal–fungal interactions using quantitative reverse-transcription PCR (qRT-PCR).

C. rosea was confronted in a dual-plate assay with *B. cinerea* (Cr–Bc), *F. graminearum* (Cr–Fg), or with itself (Cr–Cr) on 9-cm diameter Petri plates containing PDA. Cultures were incubated at 25°C in darkness. *C. rosea* was inoculated 5 days before inoculating *B. cinerea* or *F. graminearum* to compensate for differences in the mycelial growth rate. The growing front (7 to 10 mm) of the *C. rosea* mycelium was harvested at the time of contact with *B. cinerea* or *F. graminearum* mycelia. Mycelium harvested at the same stage when *C. rosea* confronted itself served as the control treatment. Each treatment was replicated five times.

Nucleic acid isolation and manipulation. Genomic DNA was extracted from *C. rosea* as described previously (Nygren et al. 2008). RNA extraction was performed using a Qiagen RNeasy kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). RNA was treated with RNase-free DNase I (Fermentas, St. Leon-Rot, Germany), and the concentration was determined spectrophotometrically using a NanoDrop-1000 (Thermo Scientific, Wilmington, DE). One microgram of total RNA was reverse-transcribed in a total volume of 20 µl using the Maxima first strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany), followed by 12-fold dilution and storage at –20°C. The following protocol was used for reverse-transcription: 5 min at 25°C and 30 min at 42°C, followed by enzyme inactivation at 85°C for 5 min.

Gene expression analysis using qRT-PCR. Specific PCR primers targeting the *C. rosea nps1* gene (Table 1) were designed using the PrimerSelect software implemented in the DNASTAR Lasergene version 10 software package (DNASTAR Inc., Madison, WI). Primer amplification efficiency was determined based on the amplification of serial dilutions of *C. rosea* genomic DNA. Transcript levels were quantified using an iQ5 qPCR system (Bio-Rad, Hercules, CA) using the SsoFast EvaGreen Supermix (Bio-Rad) as described previously (Kamou et al. 2016). Subsequent to the quantitative PCR reactions, a melt curve analysis was performed to confirm that the signal was the result of a single product amplification. Relative expression levels of the *nps1* gene were calculated in relation to the reference gene β -tubulin (*tub2*) (Mamarabadi et al. 2008) using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) with five biological replicates, each based on two technical replicates.

TABLE 1. List of primers used in this study

Name	Target gene ^a	Sequences (5' → 3')
Tub CF	<i>tub2</i>	ttc aga ccg gtc agt gcg ta ^b
Tub CR		gcc aga aag cag ca caa t ^b
actin_F	<i>actin</i>	ggt ctg gat tcc ggt gat ggt gtc
actin_R		tgc gca gtg gtg gag aag gtg t
NPS1-ups F	<i>nps1</i>	<u>gggg</u> aca act ttg tat aga aaa <u>gtt g</u> att gtt
		gtc cgc cgc ttt gat ^c
NPS1-ups R		<u>gggg</u> ac tgc ttt ttt gta caa act tg ggg caa
		ata gct cgc aca aat ^c
NPS1-ds F	<i>nps1</i>	<u>gggg</u> aca gct ttc ttg tac aaa <u>gtg g</u> ccc tga
		caa tgg aac cta ca ^c
NPS1-ds R		<u>gggg</u> ac aac ttt gta taa taa <u>agt tg</u> ggt gac
		tgc ttt ggt ttt tag ag ^c
NPS-ko F	<i>nps1</i>	tgc ggt tta gta cgc tga gga a
NPS-ko R		gaa agt ccg ggc gaa caa ac
Hyg F	<i>hph</i>	gcg cgc aat taa ccc tca c ^b
Hyg R		gaa ttg cgc gta cag aac tcc ^b
HygF_qPCR	<i>hph</i>	acg ggc gga gat gca ata ggt ^b
HygR_qPCR		gct tgc atg tag gag ggc gtg g ^b
NPS1_F_val	<i>nps1</i>	gct tgc ccc caa ccg taa gag a
NPS1_R_val		agt cgg caa tca cat ggg agg taa g
NPS1_F_exp	<i>nps1</i>	ggc gag ccc ttt tcc aat cc
NPS1_R_exp		aac cca gac caa cgc cca tac taa

^a *tub2* = β -tubulin, *hph* = hygromycin B phosphotransferase, *nps1* = nonribosomal peptide synthetase.

^b *tub2* primers and *hph* primers (from Mamarabadi et al. 2008 and Dubey et al. 2016, respectively).

^c attB and attBr sequences are underlined.

Construction of gene deletion cassette, transformation, and mutant validation. A three-fragment multisite gateway cloning system (Invitrogen, Carlsbad, CA) was used to construct an *nps1* gene deletion cassette. PCR amplification ≈ 1 kb of the 5' flank and the 3' flank regions of the *nps1* gene was performed using *C. rosea* genomic DNA using the gene-specific primer pairs NPS1-ups F/ups R and NPS1-ds F/ds-R (Table 1), respectively, as

described in Figure 1. Gateway entry clones of the purified 5' flank and 3' flank PCR fragments were generated according to the manufacturer's instructions (Invitrogen). We used a gateway entry clone for the hygromycin-resistance cassette (*hygB*) that was constructed during our previous studies (Dubey et al. 2012, 2013a,b). The gateway LR recombination reaction was performed using the entry plasmid of the respective fragments and the

Fig. 1. Schematic representation of deletion cassettes and mutant validation using PCR and reverse-transcription PCR (RT-PCR). **A**, Organization of the *nps1* locus in wild-type and mutant strains of *Clonostachys rosea*. The *nps1* coding region was replaced by a *hygB* cassette by homologous recombination, resulting in the generation of $\Delta nps1$ strains. The short, thin arrows indicate the location of primers used to construct the deletion cassette and the analysis of mutants using PCR. LB = left border, RB = right border. **B and C**, PCR verification of $\Delta nps1$ using primers located in the *hygB* cassette (Hyg F/Hyg R) in combination with primers located upstream and downstream from the deletion cassette (NPS1-ko F/NPS-ko R). PCR products of ≈ 2.7 kb using primers NPS-ko F/HygR_qPCR and ≈ 2.9 kb using NPS-ko R/HygF_qPCR were expected from a correct gene replacement. M, gene ruler DNA ladder mix; 1 to 7, independent $\Delta nps1$ mutants; WT, wild-type strain. **D**, RT-PCR analysis of *nps1* gene expression in WT and deletion strains using *nps1*-specific primers. **E**, RT-PCR analysis of *nps1* gene expression in WT and deletion strains using hygromycin-resistance gene *hph*-specific primers. Primer combinations used for PCR and RT-PCR are provided above each image. 1 to 5, independent $\Delta nps1$ mutants used in all phenotypic analyses; NC = negative control.

destination vector pPm43GW (Karimi et al. 2005) to generate a deletion cassette following the conditions described by the manufacturer (Invitrogen).

C. rosea was transformed using *Agrobacterium tumefaciens*-mediated transformation (ATMT) as described previously (Utermark and Karlovsky 2008). Putative transformants were selected and subcultured on PDA plates containing hygromycin (200 µg/ml). To validate the homologous integration of the deletion cassette into putative transformants, a PCR screening approach was performed with primer combinations specific to the hygB cassette (Hyg F/Hyg R) and sequences flanking the deletion vector (NPS1-ko F and NPS1-ko R) (Table 1), as described previously (Dubey et al. 2014, 2016). An RT-PCR analysis was performed for *C. rosea* WT and *nps1* deletion strains using a Maxima first-strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) and primers specific for *nps1* (NPS1 F_val and NPS1 R_val), the hygromycin-resistance gene *hph* (HygF_qPCR and HygR_qPCR), and actin (*act*; ActinF_qPCR and ActinR_qPCR) (Table 1). PCR-positive transformants were tested for mitotic stability, and then purified by two rounds of single spore isolation, as described previously (Dubey et al. 2014, 2016). The length of the *nps1* gene prevented the construction of a gene complementation cassette; therefore, all phenotypic analyses were performed using five independent positive transformants to ensure that any phenotypic effect was caused by the *nps1* gene deletion, not by ectopic insertions.

Phenotypic analyses of *nps1* deletion mutants. *Growth and conidiation rates.* To obtain growth rate measurements for *C. rosea* $\Delta nps1$ strains and WT, a 5-mm-diameter agar plug excised from the mycelial front was transferred to PDA and Czapek Dox agar (CZ; Sigma-Aldrich, Steinheim, Germany) plates. Cultures were incubated at 25°C under dark conditions, and the colony diameter was measured daily up to 7 days postinoculation (DPI), with three replicates of each isolate. To determine conidiation rates of the $\Delta nps1$ strains and WT, conidia were harvested from 13-day-old cultures maintained on PDA and CZ plates at 25°C under darkness by adding 7 ml of sterile water to the culture and then scraping the surface of the mycelium with a glass rod to release the conidia. Conidia were counted using a hemocytometer (Hausser Scientific, Horsham, PA), with four biological replicates of each isolate.

In vitro antagonism assays. The *in vitro* antagonistic ability of *C. rosea* $\Delta nps1$ strains and WT against *B. cinerea* and *F. graminearum* was determined by performing a dual-plate confrontation assay. A 9-cm PDA plate was inoculated with a 5-mm-diameter mycelial agar plug of a *C. rosea* strain on one side of the plate. After 7 days of incubation at 25°C in darkness, an agar plug of *B. cinerea* or *F. graminearum* was inoculated at an equal distance on the opposite side of the plate, and mycelial growth was measured daily for up to 5 DPI.

Mycelial biomass production, the production of fungal growth-inhibiting compounds, and nematocidal inhibition levels in liquid cultures of *C. rosea* $\Delta nps1$ strains and WT were measured by inoculating 100 ml of potato dextrose broth (PDB) in 500-ml E-flasks with 2×10^6 conidia/flask of *C. rosea* $\Delta nps1$ strains or WT, followed by incubation at 25°C on a rotating shaker (120 rpm) for 2 weeks under dark conditions. The fungal biomass was separated from the broth by filtering it through three layers of filter paper (Ahlstrom-Munksjö, Helsinki, Finland). The culture filtrate was further sterile-filtrated through a 0.45-µm cellulose membrane (Sarstedt, Nümbrecht, Germany), as described previously (Iqbal et al. 2018b). Subsequently, 10 ml of culture filtrate was transferred to a 50-ml E-flask and inoculated with a 5-mm-diameter agar plug from a 3-day-old culture of *B. cinerea*, *F. graminearum*, or *R. solani* and incubated at 25°C on a rotary shaker (120 rpm) in darkness. The dry weight biomass of the plant pathogenic fungi was recorded at 5 DPI.

To determine the level of nematocidal activity, culture filtrate (800 µl) or liquid media (control) was mixed with 200 µl of water

containing 100 nematodes (a mixed community extracted from soil) in wells (in a 24-well plastic plate) and incubated at 25°C in darkness for 24 h. The number of viable nematodes was determined by washing the nematodes with cold water and by performing a touch assay under a light microscope (Wild M5A; Heerbrugg, Switzerland) at 50× magnification (Gill et al. 2003).

To examine the involvement of NPS1 during secondary metabolite production, the aforementioned culture filtrates were further analyzed by performing ultra-high-performance liquid chromatography–mass spectrometry (UHPLC-MS). UHPLC-MS analyses were performed using an Agilent 1290 Infinity II UHPLC (Agilent, Palo Alto, CA) attached to a Bruker maXis Impact QTOF MS (Bruker Daltonics GmbH, Bremen, Germany). Samples (1 µl) were analyzed on a reversed-phase UHPLC column (2.1 × 50 mm, 1.5 µm; Accucore Vanquish; Thermo Scientific, Waltham, MA) and eluted with a gradient of acetonitrile (MeCN) in water with 0.2% formic acid (10 to 95% MeCN for 3 min and 95% MeCN for 1.2 min at 0.9 ml/min). The MS was used in the positive mode with a range of *m/z* 50 to 1500. Compass Data Analysis 4.3 software (Bruker Daltonics) was used to calibrate mass spectra against sodium formate clusters to create extracted ion chromatograms corresponding to expected substances and to convert data to the mzXML format. Ion chromatogram peak picking was conducted using the R software environment and the XCMS program (Smith et al. 2006) and centWave method (Tautenhahn et al. 2008). All mycelial biomass production measurements, *in vitro* antagonism assays, and chemical analyses were performed using five biological replicates.

In planta biocontrol assays. The *in planta* biocontrol ability of $\Delta nps1$ strains and WT were evaluated with two different experiments:

- I. A Fusarium foot rot assay on wheat (*Triticum aestivum*, winter wheat cultivar Stava) was performed using five biological replicates. Each replicate consisted of 12 to 15 plants and followed the procedure described previously (Dubey et al. 2014, 2016; Knudsen et al. 1995). Surface-sterilized wheat seeds were air-dried in a laminar flow cabinet and inoculated with *C. rosea* WT or *nps1* deletion strain conidia (1×10^7 conidia/ml) during a 30-min period on a rotary shaker (150 rpm). Water-soaked seeds were used as a control. Three seeds were sown in a 2-cm hole in moistened sand in individual plastic pots (5 × 5 × 5 cm). Pathogen inoculation was performed by placing a 5-mm-diameter PDA plug with *F. graminearum* mycelium in the hole close to the seeds (Roberti et al. 2008). A sterile PDA plug without any *F. graminearum* mycelium was used as a control. After inoculation, holes were covered with moist sand. Pots were maintained in a growth cabinet under controlled conditions (i.e., 12-h/12-h light/dark photoperiod with 150 µmol·m⁻²·s⁻¹ light intensity, temperature of 15°C ± 1°C, and relative humidity of 80 ± 5%). Seedlings were harvested 3 weeks postinoculation, and disease symptoms were scored based on a scale of 0 to 4, as described previously (Dubey et al. 2014, 2016; Knudsen et al. 1995).
- II. An assay of nematode root disease in wheat was performed with eight biological replicates using naturally infested soil, as described previously (Iqbal et al. 2018b). Briefly, soil was collected in December 2016, from a field in Säby (coordinates: lat. 59° 51' 10.98"N, long. 17° 40' 37.344"E), which is close to Uppsala, Sweden. Soil samples were collected from three distinct locations within a single field during fallow conditions after wheat. Samples were pooled, mixed thoroughly, and maintained in plastic bags at 4°C prior to experiments. Immediately before setting-up the experiment, the soil was cleaned to remove clumps and mixed with sand (particle size, 0.50 mm; Rådasand, Lidköping, Sweden) to a final content of 20% vol/vol. To formulate *C. rosea* $\Delta nps1$ and WT strains as peat–wheat bran powder formulations, we

adapted the method described by Jensen et al. (2000) by using a coffee mill. Spore viability and the concentration of *C. rosea* strains in the formulations were determined as described previously (Iqbal et al. 2018b). *C. rosea* formulations were mixed with soil to reach a final concentration of 2.5×10^5 viable CFU/g of soil. Plastic pots ($9 \times 9 \times 10$ cm) were filled with 480 g of *C. rosea*-inoculated or uninoculated soil (control). Eight wheat seeds were planted in each pot and placed in a growth cabinet with a 12-h/12-h light/dark photoperiod at 15°C and relative humidity of $70 \pm 5\%$. All pots were subjected to the same watering regime throughout the 8-week experimental period.

At the end of the experiment, plants were harvested by cutting aerial parts at the root–shoot junction at the surface of the soil, and plant shoot dry weight (g) and shoot length (cm) were recorded. Living nematodes were extracted from 15 g of soil and 1 g of harvested roots using a modified Baermann funnel method (Baermann 1917; Viketoft et al. 2005). Counting of the number of nematodes was conducted under a light microscope (Wild M5A; Heerbrugg, Switzerland). The classification of nematodes to the genus level was based on an analysis of morphological characters. Reliable and nonoverlapping morphological characters, such as the type of body cuticle, labial regions (e.g., amphidial apertures and stylet), pharynx and tail shape, and the position of the excretory and nerve ring were considered. The male and female reproductive system characters were also considered during identification (Decraemer and Hunt 2013; Hunt et al. 2018).

Statistical analyses. Gene expression and phenotypic data were analyzed using an analysis of variance (ANOVA) with a general linear model approach implemented in Minitab Statistical Software version 18.1 (Minitab Inc., State College, PA). Pairwise comparisons were performed using Fisher's least significant difference or the Tukey–Kramer method at the 95% significance level. Statistical analyses of the UHPLC-MS data were performed using MetaboAnalyst 4.0, a web-based tool suite for metabolomic data analysis (Chong et al. 2018). After sample normalization by sum, log transformation, and Pareto scaling (division by the square root of the respective standard deviation) (van den Berg et al. 2006), differences between sample groups (mutants and WT) were analyzed using a principal component analysis and orthogonal partial least squares discriminant analysis, along with ANOVA or *t* tests.

RESULTS

Sequence analysis of *nps1*. The full-length sequence of *nps1* was retrieved from the genome of the *C. rosea* strain IK726 (Karlsson et al. 2015). The open reading frame of *nps1* was 6621 bp (excluding three introns of 56, 55, and 60 bp) and was predicted to encode a polypeptide composed of 2206 amino acid residues (protein ID: BN8691_T00013362_1). The conserved domain and SMART protein analysis of the translated amino acid revealed that the NPS1 protein was composed of three parts, i.e., an adenylation or AMP-binding domain (amino acid positions 1078 to 1461), a thiolation or phosphopantetheine attachment site (amino acid positions 1584 to 1647), and a condensation domain (amino acid positions 1673 to 1955). A SignalP analysis did not identify an N-terminal secretion signal peptide.

Adenylation domain-based phylogenetic analysis resolved nine major NRPS subfamilies (Fig. 2), as described previously (Bushley and Turgeon 2010). Two adenylation domains were present in the predicted NPS1 protein sequence. The first predicted NPS1 adenylation domain clustered in the ChNPS11/ETP module 1 synthetase subfamily of toxins (e.g., gliotoxin and sirodesmin) with 99% bootstrap support, whereas the other adenylation domain clustered in the ChNPS12/ETP module 2 subfamily (Fig. 2). More specifically, NPS1 clustered with Afu6g_09660_A1_2 from *Aspergillus fumigatus* with 96% bootstrap support (Fig. 2). A phylogenetic analysis using the condensation domains resulted in the same clustering (Supplementary Fig. S1).

***Nps1* gene expression analysis.** The primer pair NPS1_F_exp and NPS1_R_exp (Table 1) specifically amplified a 177-bp amplicon of the *nps1* gene with PCR amplification efficiency of 90%. The relative expression of the *nps1* gene was 18-fold higher during confrontations between *C. rosea* and *B. cinerea* ($P \leq 0.05$) than that induced by confrontations with *F. graminearum* or the self-confronting control treatment (Fig. 3).

Generation and validation of *nps1* deletion strains. The *nps1* gene was replaced with the hygB selection cassette by homologous recombination using ATMT. A total of 470 hygromycin-resistant *C. rosea* colonies were obtained over the course of four independent transformations on selection plates containing cefotaxime (200 µg/ml) and hygromycin (200 µg/ml). After subculturing colonies on fresh selection plates, 430 hygromycin-resistant colonies remained. Successful PCR amplification of products of the expected size using primers located upstream and downstream of the deletion construct in combination with primers located in the hygB cassette (Fig. 1A) was observed for seven $\Delta nps1$ strains, whereas no amplification of the WT was observed (Fig. 1B and C). Furthermore, the RT-PCR analysis confirmed loss of *nps1* expression in five randomly selected mutants ($\Delta nps1$ -A, $\Delta nps1$ -B, $\Delta nps1$ -C, $\Delta nps1$ -D, and $\Delta nps1$ -E), whereas expression was detected in the WT (Fig. 1D). *Hph* expression was detected in the five $\Delta nps1$ strains, whereas no amplification was observed in the WT (Fig. 1E). Successful RT-PCR amplification of *act* in all mutants and WT indicated that the cDNA was intact.

Phenotypic analyses. Deletion of *nps1* increased the growth and conidiation rate of *C. rosea*. The growth rate of all *C. rosea* *nps1* deletion strains was, on average, 21% higher than the WT growth rate on PDA and 25% higher on CZ ($P \leq 0.001$) (Fig. 4A and B). However, there was no significant difference ($P = 0.098$) in biomass production when *nps1* deletion strains and WT were cultured in liquid PDB. However, the conidiation of *nps1* deletion strains was significantly greater ($P \leq 0.001$) than that of WT when cultured on PDA (average, 203%) or CZ (average, 87%) (Fig. 4C and D).

Deletion of *nps1* in *C. rosea* reduces in vitro antagonism against nematodes. *B. cinerea* and *F. graminearum* showed reduced growth rates in dual-plate confrontation assays with *C. rosea* WT ($P = 0.001$) compared with their growth rates when grown alone, unchallenged by *C. rosea* WT (Supplementary Fig. S2A). Furthermore, *B. cinerea* showed a reduced growth rate ($P = 0.016$) at 3 DPI during interactions with *nps1* deletion strains compared with that observed during interactions with *C. rosea* WT. However, this effect was not statistically significant at 5 DPI. The growth rate of *F. graminearum* was not statistically different in dual-plate assays with WT or $\Delta nps1$ strains at 3 or 5 DPI ($P \geq 0.12$) (Supplementary Fig. S3A). However, a zone of inhibition was observed during interactions with WT at 3 weeks after inoculation, but not during interactions with $\Delta nps1$ strains. Additionally, there was no significant difference in the amount of mycelial biomass produced by *B. cinerea*, *F. graminearum*, or *R. solani* when grown in culture filtrates of *nps1* deletion strains compared with that of WT.

The nematocidal activity of culture filtrates from *nps1* deletion strains was significantly weaker than that of WT ($P \leq 0.001$); after 24 h of incubation with culture filtrates from *nps1* deletion strains, only 13 to 33% of a mixed community of nematodes were dead compared with 42% of nematodes incubated with WT culture filtrates (Fig. 5). A closer inspection of the nematode mortality data revealed that the mortality level of plant-parasitic nematode genera (i.e., *Pratylenchus*, *Boleodorus*, *Merlinus*, *Rotylenchus*, *Helicotylenchus*, *Tylenchorhynchus*, *Heterodera* [juvenile stage, J2], and *Paratylenchus*) was significantly ($P \leq 0.001$) higher than that of nonparasitic nematode groups (bacterivores, fungivores, and omnivores) when incubated in culture filtrates of *nps1* deletion strains and WT (Supplementary Fig. S4).

Known nematicidal dioxopiperazines produced by *C. rosea* (i.e., gliocladin A–E, verticillin A, 11-deoxyverticillin A, Sch52900, and Sch52901) (Dong et al. 2005) were not detected by UHPLC-MS analyses of culture filtrates from *C. rosea* WT and *npsI* mutant

strains. Furthermore, an untargeted comparison of the metabolite profiles of *C. rosea* WT and *npsI* mutant strains did not reveal any consistent or significant differences in metabolite profiles (Supplementary Fig. S5).

Fig. 2. Phylogenetic tree of major NRPS subfamilies based on adenylation domains. Amino acid sequences of NRPSs were retrieved (Bushley and Turgeon 2010). Adenylation domains were predicted using SMART, a protein prediction tool, aligned by Clustal W, and used to construct a phylogenetic tree using a maximum likelihood method in MEGA version 6. Branch support values (bootstrap proportion ≥ 50) are associated with nodes. The nine major subfamilies of NRPS are shown in the tree. The scale bar indicates the branch length measured as the average number of substitutions per site. Ac = *Acremonium chrysogenum*, Af = *Aspergillus fumigatus*, An = *Aspergillus nidulans*, Bd = *Batrachochytrium dendrobatidis*, Ch = *Cochliobolus heterostrophus*, Cr = *Clonostachys rosea*, Fg = *Fusarium graminearum*, Mg = *Magnaporthe oryzae*, Nl = *Nocardia lactamdurans* (bacterium), Pc = *Penicillium chrysogenum*, Pp = *Postia placenta*, Tr = *Trichoderma reesei*, and Um = *Ustilago maydis*.

Deletion of nps1 reduces C. rosea biocontrol efficacy against Fusarium foot rot disease. Coating wheat seeds with WT *C. rosea* conidia provided very good protection ($P < 0.05$) against *Fusarium* foot rot disease caused by *F. graminearum* (Fig. 6). In contrast, all *C. rosea nps1* deletion strains failed ($P > 0.05$) to protect wheat seedlings against the disease (Fig. 6). The disease severity on wheat seedlings grown from seeds coated with $\Delta nps1$ strains was higher than that on seedlings grown from seed coated with WT (Fig. 6), although this increase was not significant for strains $\Delta nps1$ -B and $\Delta nps1$ -D. The shoot weight of $\Delta nps1$ -treated and WT-treated wheat seedlings was not significantly different ($P = 0.204$) (Supplementary Fig. S6).

Deletion of nps1 reduces C. rosea biocontrol efficacy against nematode root disease. The number of nematodes recovered from soil 8 weeks after inoculating the soil with either the *C. rosea* WT or $\Delta nps1$ strain was 31% and 19 to 26% lower, respectively, than that recovered from the control treatment ($P < 0.001$) (Fig. 7A). Inoculation of soil with $\Delta nps1$ strains reduced ($P < 0.001$) the number of recovered nematodes to a significantly lesser degree than the WT treatment (Fig. 7A). The nematode mortality data were then split into two groups comprising plant-parasitic nematode genera (27% of all nematodes) and nonparasitic nematodes (i.e., bacterivores, fungivores, or omnivores; 73% of all nematodes). Reanalysis of the data revealed that the number of plant-parasitic nematode genera (*Pratylenchus*, *Boleodorus*, and *Merlinus*) recovered from soil inoculated with *C. rosea* WT was lower ($P < 0.05$) than that recovered from the other soils (Fig. 7B). The same effect was also seen for nonparasitic nematode groups (Fig. 7C), although the effect was less pronounced than it was for the plant-parasitic genera. In contrast, soil inoculated with *C. rosea nps1* strains reduced the number of plant-parasitic nematode genera that were recovered ($P < 0.05$) (Fig. 7B), but not the numbers of bacterivore, fungivore, and omnivore groups recovered (Fig. 7C).

Furthermore, adding *C. rosea* WT to soil reduced ($P < 0.001$) the number of *Pratylenchus* (migratory endoparasitic) and *Heterodera* (J_2 , endoparasitic) nematodes extracted from roots of wheat by 46% compared with the control treatment (Fig. 8). Although inoculating soil with *nps1* mutant strains reduced ($P < 0.001$) the number of endoparasitic nematodes recovered from wheat roots by 22%

compared with the control treatment, the effect of this treatment was less pronounced compared with that of the WT treatment ($P < 0.001$) (Fig. 8).

Wheat grown in soil inoculated with either *C. rosea* WT or $\Delta nps1$ strains showed increased ($P < 0.001$) shoot length (13%) and dry shoot weight (20%) compared with the control treatment (Fig. 9A and B); however, the strain used to inoculate the soil did not significantly affect shoot length or dry shoot weight. In addition, yellowing of leaves and chlorosis were observed in control plants, but not in plants grown in soil treated with *C. rosea* WT or $\Delta nps1$ (data not shown).

DISCUSSION

Out of the 17 NRPS genes in the *C. rosea* genome, *nps1* is the only gene predicted to encode an ETP synthetase (Karlsson et al. 2015), which is further supported by our phylogenetic analysis, which clustered the predicted NPS1 into the ChNPS11/ETP module 1 and ChNPS12/ETP module 2 subfamilies. The predicted modular structure of NPS1 contains all the necessary domains (adenylation, thiolation or peptidyl carrier protein, and condensation) that are required for a functional NRPS to perform peptide synthesis (Conti et al. 1997; Keating et al. 2000, 2002; May et al. 2002; Stachelhaus et al. 1999; Weber et al. 2000). The induction of the *nps1* gene during interactions with *B. cinerea* suggests that NPS1 has a role during interspecific fungal interactions. The gene was also expressed during interactions with *F. graminearum*, although expression was not significantly higher than that induced by the control treatment, which did not contradict a role in mycoparasitism.

Other NRPS genes in mycoparasitic fungi have been reported to be differentially expressed in interspecific fungal interactions; for example, *nps13* in *C. rosea* strain IK726 was induced during confrontations with *B. cinerea*, but not during confrontations with *F. graminearum* (Nygren et al. 2018). Furthermore, the NRPS-like gene TOPH 03459 in the truffle parasitizing fungus *Tolypocladium ophioglossoides* was highly upregulated during growth in yeast malt broth compared with broths containing material from the *Elaphomyces muricatus* truffle host (Quandt et al. 2016). Peptaibols are a large and complex group of NRPs with antibiotic properties that have an important role in antagonism and mycoparasitism in *Trichoderma atroviride* strain P1 (Lorito et al. 1994, 1996; Schirmböck et al. 1994) and *C. rosea* (Rodríguez et al. 2011). The *nps1* gene was also expressed during *C. rosea* self-interactions, which showed that interspecific fungal interactions are not crucial for the transcriptional activation of *nps1* and may suggest more diverse roles for NPS1.

Furthermore, the deletion of *nps1* was associated with an increase in the growth rate and conidiation of *C. rosea*. This suggested that the NPS1 product metabolite(s) may exert an inhibitory effect on *C. rosea* growth. Alternatively, deletion of the *nps1* gene may allow the allocation of resources for growth and conidiation that would otherwise be used for secondary metabolite biosynthesis (Calvo et al. 2002); in other words, there could be a trade-off between investing resources in growth versus secondary metabolite production. Similar results were observed in a study involving the mycoparasitic fungus *Trichoderma virens*, in which the deletion of *tex7*, *tex8*, and *tex13* NRPS genes resulted in mutants with higher growth rates compared with that of the WT (Mukherjee et al. 2012). Our results were also in line with those of a previous study that showed that deletion of the polyketide synthase genes *pks22* and *pks29* in *C. rosea* IK726 resulted in increased conidiation (Fatema et al. 2018), suggesting that secondary metabolites may influence the growth and development in *C. rosea*.

The involvement of NRPSs in fungal growth, morphology, pigmentation, and reproduction has been reported by studies involving the plant pathogen *Cochliobolus heterostrophus*. Oide et al. (2007) indicated that the *nps2* gene is essential for ascospore

Fig. 3. Expression analysis of *nps1* during interactions with fungal prey. Jitter plot showing gene expression of *nps1* determined by qRT-PCR during the interaction of *Clonostachys rosea* with *Botrytis cinerea* (Cr–Bc), *C. rosea* with *Fusarium graminearum* (Cr–Fg), and *C. rosea* with itself (Cr–Cr, control). Relative expression is calculated as the ratio between the *nps1* gene and tubulin using the $2^{-\Delta\Delta CT}$ method. Gene expression during the interaction of Cr–Bc was significantly higher ($P \leq 0.05$) than that during the interaction of Cr–Cr or Cr–Fg, as determined by the Tukey–Kramer test. The statistical analysis was performed for a minimum of three biological replicates. One biological replicate in the Cr–Cr treatment deviated considerably from the other four replicates and was considered an outlier; therefore, it was excluded from the calculations.

development in *C. heterostrophus* given that $\Delta nps2$ strains failed to form asci or ascospores in crosses where the $\Delta nps2$ mutation was homozygous. Furthermore, *C. heterostrophus* $\Delta nps6$ strains showed a reduction in pigmentation and in asexual sporulation compared with the WT (Oide et al. 2006). However, the lack of severe developmental or morphological defects in *C. rosea* $\Delta nps1$ strains did not support a similar direct involvement of NPS1 in reproduction.

The reduced growth rate of *B. cinerea* during dual-plate interactions with $\Delta nps1$ strains compared with the WT at 3 DPI may be associated with the higher growth rate of $\Delta nps1$ strains. However, this is only a minor effect because it was not observed at 5 DPI or during interactions with *F. graminearum*. In contrast, the effects of WT versus $\Delta nps1$ strains against nematodes were considerable. The lower nematode mortality in culture filtrates from $nps1$ deletion strains compared with that from WT suggested that NPS1 biosynthesizes a metabolite with nematocidal properties. Dong et al. (2005) isolated nine verticillin-type epipolysulfanyldioxopiperazine (gliocladin A–E, verticillin A, 11-deoxyverticillin A, Sch52900, and Sch52901) compounds from *C. rosea* strain 1A, and all compounds showed toxicity toward *Caenorhabditis elegans* and *Panagrellus redivivus* nematodes, with >50% mortality after 24 h of incubation. The failure to identify these metabolites in this

study may have been attributable to differences in growth conditions (wheat kernels vs. PDB) or between strains. Mining the UHPLC-MS data did not result in identifying the putative NPS1 product metabolite because the detected metabolites were not present in WT; however, they were absent in $\Delta nps1$ strains. This may have been attributable to a number of reasons, including the presence of the metabolite at a concentration that was too low for detection in the investigated cultures. Furthermore, growth in PDB may not be optimal for producing the NPS1 product metabolite. Culture filtrates from $\Delta nps1$ strains still possessed a certain level of nematocidal activity, which may have been caused by the production of additional compounds or enzymes (e.g., serine proteases) with nematocidal activity. Previously, it was shown that *C. rosea* strain 611 produces the extracellular serine protease PrC during growth in PDB, which accounted for 80% mortality of *P. redivivus* within 48 h of incubation with PrC (Li et al. 2006). Serine protease genes were also induced in *C. rosea* strain IK726 during confrontation with *F. graminearum* (Iqbal et al. 2018b) and with the silver scurf pathogen of potato *Helminthosporium solani* (Lysøe et al. 2017), suggesting that serine proteases may be involved in several different biotic interactions.

Interestingly, plant-parasitic nematodes had higher mortality rates in culture filtrates from *C. rosea* compared with nonparasitic

Fig. 4. Growth and conidiation rate of *Clonostachys rosea* wild-type (WT) and $nps1$ deletion strains on potato dextrose agar (PDA) and Czapek-dox medium (CZ). **A and B**, Growth rate on PDA and CZ media, respectively. **C and D**, Conidiation on PDA and CZ media, respectively. WT and $nps1$ deletion strains were inoculated on solid agar medium and incubated at 25°C. The growth rate was measured daily up to 7 days postinoculation (DPI), whereas conidiation was measured 13 DPI. The growth rate experiment was performed using three biological replicates, whereas conidiation was performed using four biological replicates. Error bars represent the standard deviation. Different letters indicate statistically significant differences ($P \leq 0.05$) between treatments within an experiment based on the Tukey–Kramer test.

nematodes, which suggested that plant-parasitic nematodes are particularly sensitive to certain metabolites or proteins secreted by *C. rosea*. This effect has been reported not only for metabolites and proteins secreted by *C. rosea* (Iqbal et al. 2018b) but also for *Fusarium oxysporum* (Hallmann and Sikora 1996) and *Purpureocillium lilacinum* (previously *Paecilomyces lilacinus*) (Cayrol et al. 1989). Our in vitro and in planta data are consistent with the possibility that the NPS1 biosynthesis pathway is directly involved in producing compounds with specificity toward plant-parasitic nematodes. However, this hypothesis requires the identification of the NPS1 product metabolite(s) for validation.

An in planta bioassay showed that the *nps1* gene is required for efficient biocontrol of *Fusarium* foot rot disease on wheat caused by *F. graminearum*. Previously, the lower growth rate of *C. rosea*

Fig. 5. Nematode mortality in culture filtrates of *Clonostachys rosea* wild type (WT) and *nps1* deletion strains in potato dextrose broth. Nematode mortality was assessed in culture filtrates from *C. rosea* WT, *Δnps1* strains, and in a control medium after 24 h of incubation at 25°C. Error bars represent the standard deviation based on five biological replicates. Different letters indicate a statistically significant difference ($P \leq 0.05$) between treatments as determined by Fisher's least significant difference test.

Fig. 6. In planta bioassay to test the biocontrol efficacy of *Clonostachys rosea* wild-type (WT) and *nps1* deletion strains against *Fusarium graminearum* foot rot disease on wheat. Wheat seeds were coated with *C. rosea* conidia (WT or *Δnps1* strains) and planted in moist sand together with an *F. graminearum* agar plug. Seedlings were harvested 3 weeks postinoculation, and disease symptoms were scored on a scale of 0 to 4. Error bars represent the standard deviation based on five biological replicates. Each replicate comprised the mean of 12 to 15 plants. Different letters indicate statistically significant differences ($P \leq 0.05$) between treatments based on Fisher's least significant difference test.

Fig. 7. Effect of *Clonostachys rosea* wild-type (WT) and *nps1* deletion strains on nematode populations recovered from soil planted with wheat. **A**, Effects of soil applications of *C. rosea* WT and *Δnps1* strains on the overall nematode population recovered from soil, including plant-parasitic nematode genera (*Pratylenchus*, *Rotylenchus*, *Helicotylenchus*, *Tylenchorhynchus*, *Merlinus*, *Boleodorus*, *Heterodera* [J2], and *Paratylenchus*), and other trophic groups (bacterivores, fungivores, and omnivores). **B**, Effect on plant-parasitic nematode genera. **C**, Impact on other trophic groups recovered from the soil. The control treatment corresponds to soil without *C. rosea* application. Error bars represent the standard deviation based on eight biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) between treatments as determined by Fisher's least significant difference for **B** and by the Tukey–Kramer test for **A** and **C**.

Fig. 8. Effect of *Clonostachys rosea* wild-type (WT) and *nps1* deletion strains on nematode populations recovered from wheat roots. The data include both migratory endoparasitic nematodes from the genus *Pratylenchus* and endoparasitic nematodes from the genus *Heterodera* (J₂). The control treatment corresponds to plants grown in soil without *C. rosea* application. Error bars represent the standard deviation based on eight biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) between treatments as determined by the Tukey–Kramer test.

Fig. 9. Effect of soil applications of *Clonostachys rosea* wild-type (WT) and *nps1* deletion strains on shoot length and shoot weight of wheat. **A**, Average shoot length (cm) and **B**, average dry shoot weight (g) of plants grown in soil treated with *C. rosea* WT or *Δnps1* strains or in soil that was not treated with *C. rosea* application (control). Error bars represent the standard deviation based on eight biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) between treatments as determined by the Tukey–Kramer test.

zearalenone lactonohydrolase *zhd101* gene deletion strains partially explained a reduction in biocontrol activity (Kosawang et al. 2014). However, because *Δnps1* strains display a higher, not lower, growth rate on solid media than the WT, it is more likely that the reduced biocontrol efficiency depends on the inability to produce a metabolite with fungal growth inhibitory activity. Similar results have been reported by Fatema et al. (2018), who showed that deletion of the *pks29* polyketide synthase gene in *C. rosea* reduced the biocontrol ability of *C. rosea* toward *F. graminearum*, resulting in increased disease severity in barley. NRPS genes are also involved in the virulence of several different plant pathogenic fungi, including *nps6* in *C. heterostrophus* (Lee et al. 2005), *AMT* in *Alternaria alternata* (Johnson et al. 2000), *HTS1* in *Cochliobolus carbonum* (Panaccione et al. 1992), and *esyn1* in *Fusarium scirpi*, and in other *Fusarium* species (Haese et al. 1993; Herrmann et al. 1996). Certain NRPS genes also encode NRPs that act as siderophores, thereby influencing virulence. Deletion of the *nps1* gene in *F. graminearum* resulted in mutant strains that were incapable of producing the extracellular ferrichrome-type siderophore malonichrome, with negative consequences for sexual development and virulence (Emery 1980; Oide et al. 2015).

Our results concerning the ability of *C. rosea* to control plant-parasitic nematodes are in accordance with previous findings that showed that *C. rosea* decreased the number of plant-parasitic nematodes by 40 to 70% when applied to soil, which resulted in increased growth of wheat and carrot (Iqbal et al. 2018b). Iqbal et al. (2018b) further showed that *C. rosea* did not reduce the populations of bacterivore, fungivore, or omnivore nematodes in soil, which was in contrast with the results of this study. One explanation for this discrepancy may be the notable difference in the proportion of plant-parasitic nematodes present in the soil used by the two studies compared with the proportion of nonparasitic nematodes; in this study, 73% of the nematodes were nonparasitic compared with 36% in the study by Iqbal et al. (2018b).

Deletion of the *nps1* gene reduced the ability of *C. rosea* to control plant-parasitic nematodes in soil and roots of wheat, showing that NPS1 is a biocontrol factor against both fungal and nematode diseases. Previously, the PrC serine protease, the PacC transcription factor for pH-dependent gene expression, and several epipolysulfanyldioxopiperazine compounds produced by *C. rosea* have been reported as virulence factors against nematodes based on in vitro assays (Dong et al. 2005; Li et al. 2006; Zou et al. 2010a,b). However, the reduced biocontrol ability of *Δnps1* strains was not accompanied by a reduction in wheat seedling growth. This may have been attributable to the relatively low proportion of plant-parasitic nematodes in the soil used for the experiment, as already mentioned, or to a plant growth promotion effect of *C. rosea* (Ravnskov et al. 2006; Roberti et al. 2008) that is mechanistically separated from that of NPS1.

In summary, we demonstrated that the *nps1* gene product influences growth and conidiation, in vitro antagonism, and biocontrol of *Fusarium* foot rot disease and nematode root disease in wheat. This study further emphasized the role of secondary metabolites in determining the outcomes of microbial biocontrol interactions.

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