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FUNGAL DISEASES

Involvement of *ThSNF1* in the development and virulence of biocontrol agent *Trichoderma harzianum*

Luis Galarza[1] · Yasunori Akagi[2] · Kazumi Takao[1] · Esther Peralta[3] · Efrén Santos[3] · Motoichiro Kodama[1]

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Abstract *Trichoderma harzianum*, a biocontrol agent for various plant pathogens, is known to degrade fungal cell walls; this mycoparasitism is believed to require secretion of cell-wall-degrading enzymes against host pathogens. In this study, we identified a homologue of yeast *SNF1* (sucrose nonfermenting 1) encoding protein kinase in *T. harzianum* (*ThSNF1*) by draft genome sequencing of strain T36. Targeted gene disruption of *ThSNF1* was performed using the PEG method with fusion PCR products. Growth of mutant *ΔThSNF1* was markedly less than for the wild-type strain on minimal medium with chitin as a carbon source. The mutant exhibited reduced expression of the genes encoding chitinase and polygalacturonase and markedly reduced spore production. Mycoparasitism against plant pathogens such as *Fusarium oxysporum* f. sp. *cubense* (Panama disease) and *Fusarium graminearum* (Fusarium head blight) was clearly impaired in the mutant. The results suggest that *ThSNF1* is critical for asexual development, utilization of certain carbon sources and virulence on fungi, and is therefore important for the biocontrol ability of *T. harzianum.*

Keywords *Trichoderma harzianum* · *ThSNF1* · Mycoparasitism · Biocontrol · Cell-wall-degrading enzymes

Introduction

Members of the genus *Trichoderma* are known as biocontrol agents because of their mycoparasitism against many plant pathogenic fungi. Some *Trichoderma* spp. can penetrate hyphae and kill the host fungus (Abdullah et al. 2007). *Trichoderma* spp. have been reported to control several pathogens of diverse crops via various mechanisms, such as the production of antifungal metabolites, competition for nutrients and space, mycoparasitism and efficiency in promoting plant defense mechanisms (Hoyos-Carvajal et al. 2008; Woo and Lorito 2007). The mycoparasitism of *Trichoderma* is characterized by hyphae that coil around host hyphae and penetrate host cells (Abdullah et al. 2007). Release of a range of enzymes, such as β-1,3-glucanase, pectinase, xylanase and chitinases, is thought to be important for the biocontrol activity because they enable *Trichoderma* to degrade the host's cell walls (Hjeljord and Tronsmo 1998). Involvement of specific chitinase genes in the biocontrol properties of *Trichoderma reesei* was investigated using genome-wide analysis of chitinase genes (Seidl et al. 2005).

Serine/threonine protein kinase is an important mediator of fungal proliferation and development, signal transduction and infection-related morphogenesis in filamentous fungi (Dickman and Yarden 1999). Carbon catabolite repression is a universal regulatory principle that leads to the

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✉ Motoichiro Kodama
mk@muses.tottori-u.ac.jp

[1] The United Graduate School of Agricultural Sciences, Tottori University, 4-101 Koyama-Minami, Tottori 680-8553, Japan

[2] Faculty of Agriculture, Tottori University, 4-101 Koyama-Minami, Tottori 680-8553, Japan

[3] Biotechnology Research Center of Ecuador, Higher Polytechnic College of the Littoral CIBE-ESPOL. Campus Gustavo Galindo, Km 30, 5 Perimetral Road, Guayaquil, Ecuador

inhibition of expression of genes encoding enzymes needed to utilize complex carbon sources such as glucose and other simple sugars. In yeast, release from catabolite repression requires expression of the Snf1p protein kinase (Celenza and Carlson 1984; Ruijter and Visser 1997). Treitel et al. (1998) described Snf1p (encoded by *SNF1*) as a protein kinase that phosphorylates the DNA-binding transcriptional repressor Mig1p (called *creA* in filamentous fungi) (Ronne 1995).

The *SNF1*-mediated process controls expression of multiple cell-wall-degrading enzyme genes (Tonukari et al. 2000). Therefore, modification of this process through disruption of *SNF1* homologues in fungi could lead to loss of production of multiple cell-wall-degrading enzymes and hence be useful for investigations into the role of these enzymes in regulating the expression of virulence genes in plant pathogens (Tonukari et al. 2000). In *Cochliobolus carbonum, SNF1* homologue *ccSNF1* controls expression of genes for several cell-wall-degrading enzymes and is also important for virulence against the maize host (Tonukari et al. 2000). Disruption of *Fusarium oxysporum SNF1* (*FoSNF1*) reduces virulence on cabbage and *Arabidopsis* (Ospina-Giraldo et al. 2003). In *Gibberella zeae*, *GzSNF1* is reported to be required for normal sexual and asexual development (Lee et al. 2009).

These investigations have demonstrated that *SNF1* is involved in development, production of cell-wall-degrading enzymes and virulence of plant pathogens. However, the role and importance of the gene in mycoparasitism in *Trichoderma* and other biocontrol agents is currently unknown. To address this question, we analyzed the draft genome sequences of the fungus and identified the *T. harzianum SNF1* homologue (*ThSNF1*) and functionally characterized the gene. This is the first report of a functional analysis of an *SNF1* ortholog in the biocontrol fungus *T. harzianum.*

Materials and methods

Fungal strains and culture conditions

In this study, *T. harzianum* strain T36 was used as the wild-type strain. The isolate was obtained from stock collections at the Biotechnology Research Center of Ecuador (CIBE-ESPOL). The wild type and transformants were maintained on potato dextrose agar (PDA, Difco, Detroit, MI, USA) at 25 °C. Mycelial fragments were stored in 20 % glycerol at -80 °C. The strains were cultured in potato dextrose broth (PDB) and yeast peptone glucose (YPG) medium for DNA extraction. *F. oxysporum* f. sp. *cubense* (Fo-01) (Panama disease) was obtained from stock collection at the CIBE-ESPOL, and *Fusarium graminearum* (No. 201) (Fusarium head blight) was kindly provided by Dr. Suga (Gifu University, Japan). The isolate of *F. oxysporum* f. sp. *cubense* was used under the special permission from the Minister of Agriculture, Forestry and Fishery of Japanese Government. Culture media with different carbon sources were prepared with minimal medium (2 g/L KH_2PO_4, 1.4 g/L $(NH_4)_2SO_4$, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 0.3 g/L $CaCl_2{\cdot}2H_2O$, 0.005 g/L $FeSO_4{\cdot}7H_2O$, 0.002 g/L $ZnSO_4{\cdot}7H_2O$ and 0.002 g/L $MnSO_4{\cdot}H_2O$) by adding 1 % glucose or 1 % colloidal chitin (Wako Chemicals, Osaka, Japan).

Isolation and gene targeting of *ThSNF1*

The sequences of the PCR primers used in this study are shown in Table 1. The gene encoding SNF1 protein kinase homologue *ThSNF1* (GenBank accession LC002817) in *T. harzianum* was determined by analyzing the draft sequence data of the T36 strain obtained with Illumina HiSeq 2000 using *SNF1* genes from *Saccharomyces cerevisiae* (Celenza and Carlson 1984), *F. oxysporum* (Ospina-Giraldo et al. 2003) and *C. carbonum* (Tonukari et al. 2000) as queries. The size of the full-length *ThSNF1* gene is 2361 bp, and it encodes a protein containing 710 amino acids.

An outline of the PCR approach (Kuwayama et al. 2002; Nayak et al. 2006; Ninomiya et al. 2004) for constructing

Table 1 Oligonucleotide primers used in this study

Primers	Sequence (5′–3′)
Thsnf1AF	aagtcaaaaacgggacggca
Thsnf1AR	cgctcaagctggtcaagggt
Thsnf1BF	gatcgctgcggttttgaaca
Thsnf1BR	aaaaagattgaccgccgcag
HphF	gacgtctgtcgagaagtttc
HphR	gtattgaccgattccttgcg
Thsnf1inF	gccgagtcgagcgtgagatt
Thsnf1inR	ttgaggaagttgccgtcggt
Thsnf1homoF	tgcctcaaatcaagcccgtt
HphhomoR	caatagctttgggacgatgcaag
HphhomoF	ttacaacgtcgtgactggga
Thsnf1homoR	gcggccatgggtaaaggagt
fushphF	gatgctagcatcgacctgatttacactttatgcttccg
fushphR	acatctggtagcactcgcatcttcgctattacgcca
βTub 1	tccgtcgtgccttcccccaaggtctccgac
βTub 2	ggagcgaatccgaccatgaagaagtggaga
ThTUBF	gtccaaccctgcctacggtg
ThTUBR	cctcaacctccttcatggcg
Chi33H3F	cgacttcaactgggcgacct
Chi33H3R	cgtaacaatctgcacggcca
Thpgx1F	ttgggcttgaaagacacgca
Thpgx1R	cgtttgcagccgagcctact

the gene disruption vectors is shown in Fig. 1a. Genomic DNA of *T. harzianum* T36 was used to amplify a 994-bp fragment (left-side arm of the vector) and a 603-bp fragment (right-side of the vector) from *ThSNF1* with PCR primers Thsnf1AF/Thsnf1AR and Thsnf1BF/Thsnf1BR, respectively. The primer sets were designed for the deletion of the *ThSNF1* internal sequence, which encodes a serine/threonine protein kinase (SNF1) homologue. The *hph* marker cassette was amplified by PCR from p71*sfi* plasmid, which contains a hygromycin B phosphotransferase gene, with primers fushphF/fushphR. The final fusion products were amplified using primers Thsnf1AF/Thsnf1BR. The PCR was performed using a Thermal Cycler Dice TP650 (Takara Bio, Ohtsu, Japan) with an initial denaturing step of 5 min at 95 °C; followed by 30 cycles of 15 s at 95 °C, 15 s at 59 °C, and 30 s at 72 °C; and a final extension of 5 min at 72 °C. The final fused products were purified with a QIAquick Kit (Qiagen, Tokyo, Japan) before transformation of the *T. harzianum* T36 strain.

Fungal protoplasts were prepared using the method previously described by Akamatsu et al. (1997) with modifications. Protoplasts at a concentration of 1.25×10^8 protoplasts/mL in a final volume of 80 μL were transformed with the disruption vector as previously described (Akamatsu et al. 1997). To identify *ThSNF1* deletion mutants, we used three sets of primers for hygromycin

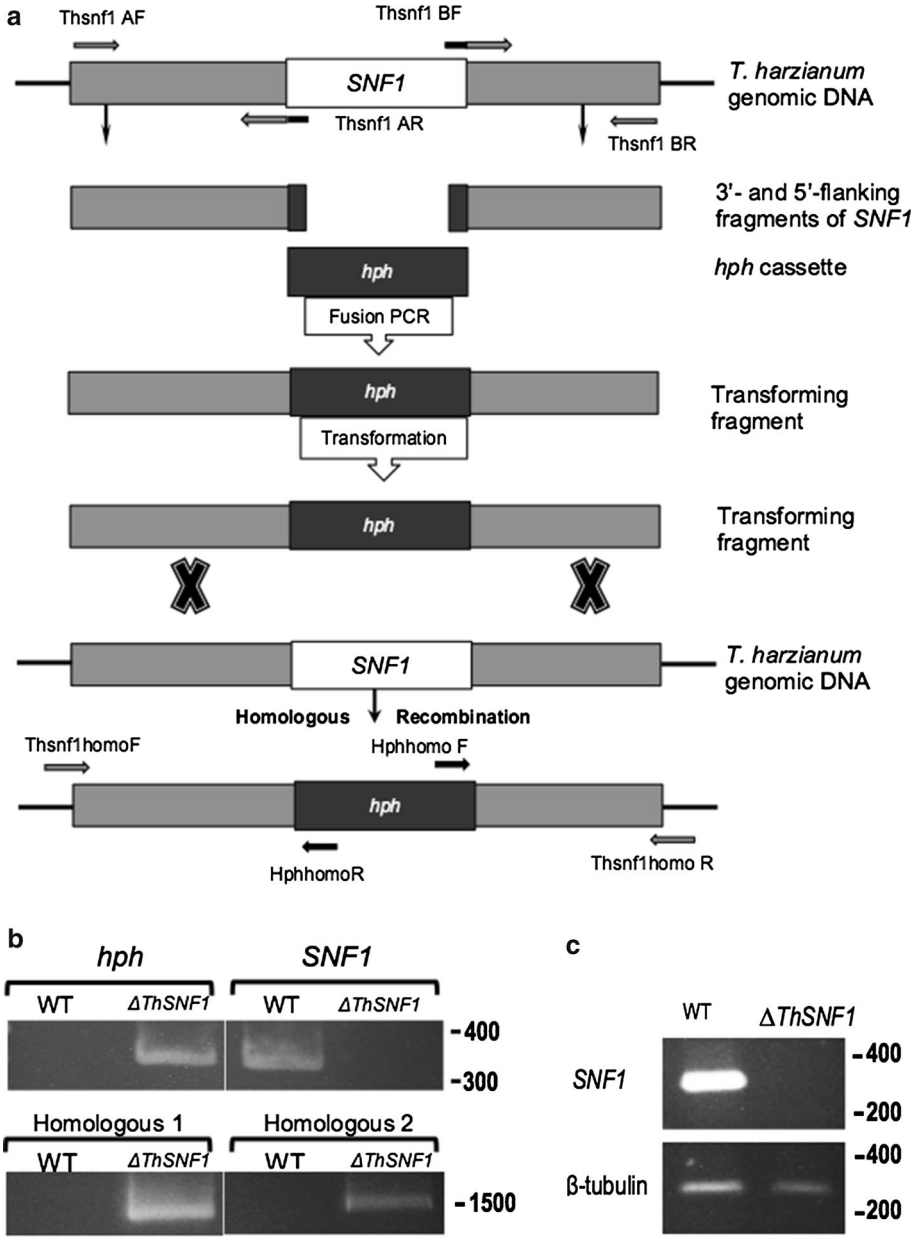

Fig. 1 Deletion strategies for *ThSNF1* in the genome of the *Trichoderma harzianum* T36 strain. **a** A fusion PCR method was used to construct the *ThSNF1* replacement vector. All PCR primers used are listed in Table 1. The 5′ region of *SNF1* was amplified by PCR with primer pair ThSNF1AF/ThSNF1AR; the 3′ region was amplified with primer pair ThSNF1BF/ThSNF1BR. The *hph* gene was amplified with primer pair fushphF/fushphR. The three PCR products were then used as a template for fusion PCR using primer pair ThSNF1AF/ThSNF1BR; the resulting PCR product was used for transformation. *White*, *black* and *gray* indicate sequences of *SNF1*, *hph* and flanking regions of *SNF1*, respectively. **b** PCR analysis of gene replacement events in *ThSNF1* in the wild-type strain (WT) and the *ΔThSNF1* mutant using primer pairs hphF/hphR (**b**)-hph, Thsnf1inF/Thsnf1inR (**b**)-*SNF1*, Thsnf1homoF/HphhomoR (**b**)-Homologous 1, and HphhomoF/Thsnf1homoR (**b**)-Homologous 2. **c** Expression of *ThSNF1* in the wild-type T36 (WT) and *ΔThSNF1* strains of *T. harzianum.* RT-PCR primer sets listed in Table 1 were used to detect *ThSNF1* and the β-tubulin gene of *T. harzianum*

B-resistant colonies (Table 1). Initially, a pair of primers for the *hph* cassette was used to verify the insertion of the vector. Next, primer set Thsnf1inF/Thsnf1inR was used to verify the insert. Primer pairs Thsnf1homoF/HphhomoR and HphhomoF/Thsnf1homoR were used to examine integration of the *hph* cassette by a double-crossover homologous recombination event at the *ThSNF1* locus. Putative mutant strains determined by the expected diagnostic amplification fragments were purified by single spore isolation.

Gene expression analysis

For expression analysis, total RNA was extracted from fungal mycelia grown in minimal medium supplemented with glucose or the autoclaved *F. oxysporum* f. sp. *cubense* mycelium (0.5 % w/v) as the carbon source as previously described (Vieira et al. 2013). After 3 days of culture, the mycelia were harvested through filter paper and washed with distillated water. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated with DNaseI (Takara Bio) to remove traces of contaminating DNA. A total of 1 μg of the RNA sample was converted into cDNA using the PrimeScript RT-PCR Kit (Takara Bio) with random 6-mer primers according to the manufacturer's instructions. The resulting cDNA was used as a template for RT-PCR with primer sets Thsnf1inF/Thsnf1inR, Chi33H3F/Chi33H3R, Thpgx1F/Thpgx1R and ThTUBF/ThTUBR for *ThSNF1*, *Chi18-17* (chitinase), *PGX1* (polygalacturonase) and the β-tubulin gene, respectively. The PCR primer sequences of those genes were designed by using the draft genome data of the *T. harzianum* strain T36.

Morphology and colony growth

Phenotypes of the mutant were examined on minimal media supplemented with different carbon sources or on PDA. Colony morphology and radial growth of the mutant and the wild-type strains were checked every day. Conidia were counted as previously described by López-Mondéjar et al. (2009).

In vitro mycoparasitism assay

The antagonism test was performed in triplicate on PDA by placing a mycelial disc (5 mm in diameter) of each pathogenic fungus (*F. oxysporum* f. sp. *cubense* or *F. graminearum*) on one side of a Petri dish; the wild type or *ΔThSNF1* mutant of *T. harzianum* was placed on the other side. After 10 days, mycoparasitism by *Trichoderma* spp. was evaluated using the following scale (Ezziyyani et al. 2004) based on the percentage of the colony surface of the pathogen covered by *Trichoderma* and sporulation by *Trichoderma*: 0: 0 % coverage; 1: 25 % coverage; 2: 50 % coverage; 3: 100 % coverage; 4: 100 % coverage plus sporulation.

Results

Cloning and targeted disruption of *ThSNF1* in *T. harzianum*

The gene encoding the serine/threonine protein kinase *SNF1* homologue from *T. harzianum* was identified by analyzing the draft sequence of the T36 strain and was designated *ThSNF1* (GenBank accession number LC002817). The size of the full-length *ThSNF1* gene was 2361 bp, encoding a protein of 710 amino acids. The deduced amino acid sequences of *ThSNF1* showed homology to *S. cerevisiae SNF1* (Celenza and Carlson 1984), *C. carbonum ccSNF1* (Tonukari et al. 2000), *F. oxysporum FoSNF1* (Ospina-Giraldo et al. 2003), *G. zeae GzSNF1* (Lee et al. 2009) and other fungal *SNF1* homologues. Alignment of the amino acid sequences of *ThSNF1* with ascomycetes *SNF1* orthologs *FoSNF1* and *ccSNF1* showed high homology, especially in the serine/threonine protein kinase catalytic domain (Suppl. Figure 1).

To examine the role of *ThSNF1* in morphology, growth, development and mycoparasitism of *T. harzianum*, the gene was deleted from the fungus using transformation-mediated gene disruption. The targeting vector containing the 3′- and 5′-flanking sequences of *ThSNF1* was constructed to disrupt the gene by homologous recombination (Fig. 1a). Transformation of T36 protoplasts with the *ThSNF1*-disruption vector resulted in hygromycin B-resistant colonies; homologous integration of the transformants was further examined by PCR screening. Primer set HphF/HphR produced the expected 0.4-kb band from the *ΔThSNF1* mutant (Fig. 1b). Primer set Thsnf1inF/Thsnf1inR resulted in no amplified fragments from the *ΔThSNF1* mutant (Fig. 1b), suggesting that *ThSNF1* was deleted by homologous integration of the vector. To confirm *ThSNF1* disruption, we used primer combinations Thsnf1homoF/HphhomoR and HphhomoF/Thsnf1homoR to detect the junctions between the recipient *ThSNF1* region and the integrated vectors, respectively (Fig. 1b). With these primer combinations, the PCR failed to amplify any DNA fragments from the wild-type strain. By contrast, primer combinations Thsnf1homoF/HphhomoR and HphhomoF/Thsnf1homoR amplified the expected-sized bands in the mutant (Fig. 1b). The deletion strain was used for further work.

The expression of *ThSNF1* in the wild-type strain and the *ΔThSNF1* mutant was confirmed by RT-PCR analysis.

ThSNF1 expression was not detected in the mutant strain (Fig. 1c).

Phenotypic characterization of the *ThSNF1*-targeted strain

The effects of the *ΔThSNF1* mutation on morphology, conidiation and vegetative growth were examined. Agar blocks from colonies grown on PDA plates were transferred onto minimal media supplemented with different nutritional sources. In the presence of glucose, the growth rate of the *ΔThSNF1* mutant was not markedly reduced compared with that of the wild type (Fig. 2a). In contrast, growth was clearly reduced when chitin was added as the sole carbon source (Fig. 2a), indicating that the mutant had decreased ability to utilize chitin. Additionally, there was a significant difference in the conidial yield between the wild type and the mutant strain on PDA and on minimal medium supplemented with glucose (MMG) (Fig. 2b). However, conidial morphology and the germination rate of the mutant were the same as those of the wild type (data not shown).

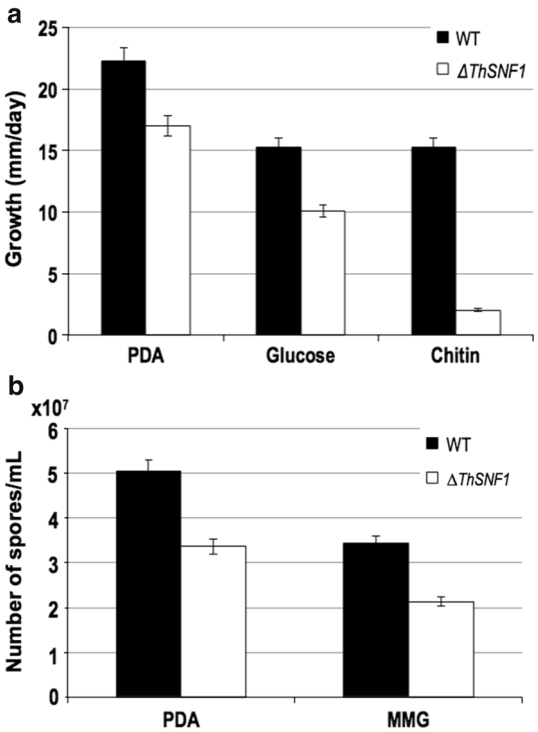

Fig. 2 Colony growth and sporulation of wild-type strain T36 (WT) and mutant *ΔThSNF1* of *Trichoderma harzianum*. **a** Growth rates between the wild-type T36 (WT) and mutant *ΔThSNF1* on minimal media supplemented with different carbon sources. Colony growth was measured daily for 7 days to calculate the mean growth rate (mm/day). **b** Mean number (± SD) of conidia produced by wild-type T36 (WT) and mutant *ΔThSNF1* of *T. harzianum.* Conidia from 7-day-old cultures grown on PDA or minimal media supplemented with glucose (MMG) were harvested and counted. $N = 3$

The expression of genes encoding cell-wall-degrading enzymes in the *ThSNF1*-targeted strain

The wild-type and mutant strains were grown in liquid shake cultures for 3 days. The expression of a chitinase gene (*Chi18-17*) (Seidl et al. 2005) and a polygalacturonase gene (*PGX1*) of *T. harzianum* was examined by RT-PCR. Involvement of these enzyme genes in mycoparasitism has been previously reported (Seidl et al. 2005; Viterbo et al. 2001). Expression of the genes was undetectable in the *ΔThSNF1* mutant under conditions that ordinarily would induce these genes (Fig. 3).

Mycoparasitic ability of the *ThSNF1*-targeted strain

Loss of mycoparasitic ability of the *ΔThSNF1* mutant against two pathogens (*F. oxysporum* f. sp. *cubense* and *F. graminearum)* on dual culture plates was evident 10 days after inoculation. Mycelia of *T. harzianum* came into contact with the pathogen colonies. Following contact, mycelia of the wild-type strain covered the pathogen colonies and sporulated, indicating strong mycoparasitism of the pathogens (Fig. 4). The colonies of the pathogens were obscured compared with the colonies cultured with the *ΔThSNF1* mutant. In contrast, the *ΔThSNF1* mutant did not overgrow the pathogen or sporulate on the plates, and the pathogen colonies continuously expanded after contact (Fig. 4a, b).

Discussion

Trichoderma spp. are useful in agriculture, and *T. harzianum* is a well-known, effective biological control agent for alternative pathogen control (Chet 1987). Among the various mechanisms used by antagonistic *Trichoderma* spp. (Hoyos-Carvajal et al. 2008; Woo and Lorito 2007), cell wall degradation of host plant pathogenic fungi is an important strategy for mycoparasitism (Benítez et al. 2004).

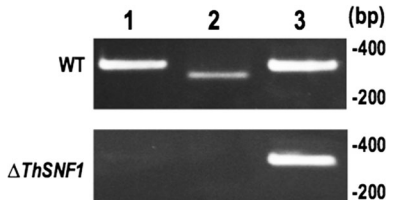

Fig. 3 Expression of genes encoding cell-wall-degrading enzymes in wild-type T36 (WT) and mutant *ΔThSNF1* of *Trichoderma harzianum.* Total RNA was extracted from fungal mycelia grown in minimal medium supplemented with glucose as the only carbon source. RT-PCR primer sets listed in Table 1 were used for detection of chitinase gene *Chi18-17* (*lane 1*), polygalacturonase gene *PGX1* (*lane 2*) and the β-tubulin gene (*lane 3*) of *T. harzianum*

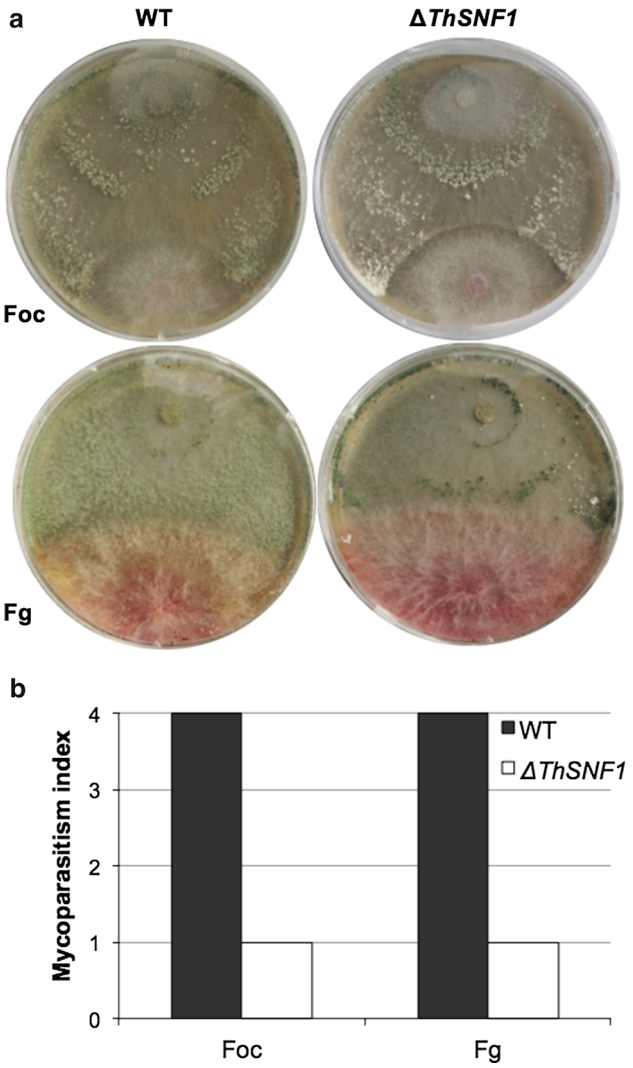

Fig. 4 Dual-culture antagonism test of wild-type (WT) and mutant *ΔThSNF1 Trichoderma harzianum* against either *Fusarium oxysporum* f. sp. *cubense* (Foc) or *Fusarium graminearum* (Fg) A 5-mm-diameter mycelial disk of each fungus was placed on PDA; pathogen is on lower side, *Trichoderma* on upper side. **a** The antagonism test was performed on PDA by placing a mycelium disc (5 mm in diameter) of each pathogenic fungus) on one side of a Petri dish; the opposite side of each dish was inoculated with the *Trichoderma* strains. The plates were incubated at 25 °C for 10 days. **b** Mycoparasitism of the *Trichoderma* strains against the pathogens as determined in triplicate using the scale described in the "Materials and methods". Each value is the mean of three replicates per treatment

Because chitin is the major cell wall component of many plant pathogenic fungi, the role of chitinase and its gene in mycoparasitism and biocontrol activity has been investigated (Seidl et al. 2005; Viterbo et al. 2001). A comprehensive survey of *Trichoderma* chitinase genes using genome-wide analysis revealed multiple chitinase gene homologues in the *Trichoderma* genome (Seidl et al. 2005).

Functional analysis of genes encoding these cell-wall-degrading enzymes is difficult due to the multiple copies of the genes in the fungal genome, as well as for the genes encoding enzymes that degrade the cell walls of plant pathogens (Tonukari et al. 2000; Walton 1994). The major obstacle in examining the role of such genes and enzymes is redundancy. Plant pathogens have multiple genes for multiple cell-wall-degrading enzymes, including chitinase, glucanase and pectinase (Walton 1994). Therefore, mutation of these genes using molecular techniques results in retention of at least some residual enzyme activity. This technical obstacle has been resolved through loss of function of the *SNF1* homologue in the plant pathogenic fungus *C. carbonum* (Tonukari et al. 2000). Because the yeast *SNF1* ortholog in *C. carbonum* (*ccSNF1*) is required for derepression of catabolite-repressed genes, mutation of the gene in the pathogen caused downregulation of catabolite-repressed cell-wall-degrading enzymes. Therefore, *SNF1*-disrupted mutants were useful for determining whether the cell-wall-degrading enzyme complex is important for fungal pathogenicity in hosts.

An *SNF1* ortholog in *Trichoderma* has been identified and analyzed in the cellulolytic industrial species *T. reesei* (Cziferszky et al. 2003). The Snf1 kinase of the fungus phosphorylates regulation-relevant serine residues in the yeast carbon catabolite repressor Mig1, but not in the filamentous fungal counterpart Cre1 (Cziferszky et al. 2003). However, the role of the *SNF1* ortholog in *Trichoderma* spp. in mycoparasitism during biocontrol has not yet been elucidated.

The *SNF1* ortholog (*ThSNF1*) in biocontrol strain T36 of *T. harzianum* was identified in this study using draft genome data of the strain. Involvement of the gene in mycoparasitism activity was examined using a *ΔThSNF1* mutant. The *ThSNF1* gene is structurally and functionally related to the *SNF1* orthologs in *F. oxysporum* (*FoSNF1*) (Ospina-Giraldo et al. 2003), *C. carbonum* (*ccSNF1*) (Tonukari et al. 2000) and others. These proteins showed high similarity, particularly in the serine/threonine protein kinase catalytic domain. Previous studies indicated that *SNF1* homologues in *C. carbonum* and *F. oxysporum* were involved in the utilization of certain sugars as carbon sources (Ospina-Giraldo et al. 2003; Tonukari et al. 2000). Moreover, these *SNF1* homologues control expression of genes for several cell-wall-degrading enzymes and, hence, contribute to virulence against host plants.

Deletion of *ThSNF1* in *T. harzianum* resulted in a phenotype similar to those pathogens, including impaired ability to utilize certain carbon sources such as chitin, reduced expression of genes encoding cell-wall-degrading enzyme and reduced virulence/mycoparasitism against *Fusarium* pathogens. The production of cell-wall-degrading enzymes such as chitinase is an important factor for mycoparasitism by *Trichoderma* spp. (Seidl et al. 2005). Transgenic plants that expressed the *T. harzianum* chitinase

gene became resistant to several plant pathogenic fungi, indicating the involvement of the genes in the antagonistic and antifungal activities of the biocontrol fungus *T. harzianum* (Lorito et al. 1998). Thus, the impaired production of such enzymes as a result of defects in *ThSNF1* most likely disturbed mycoparasitic invasion of host fungi, the most common mechanism in the biocontrol activity of *Trichoderma* spp.

The results of the *SNF1* mutation do not differentiate the role of each individual cell-wall-degrading enzyme during mycoparasitism because all of the enzymes might be downregulated. However, the *SNF1* modification is a valuable strategy to examine the contribution of genes for the cell-wall-degrading enzyme complex, including the chitinase, polygalacturonase and glucanase genes, in virulence against host plants or fungi by plant pathogenic or mycoparasitic fungi.

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