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Signaling via the *Trichoderma atroviride* mitogen-activated protein kinase Tmk1 differentially affects mycoparasitism and plant protection

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Abstract

Trichoderma atroviride is a mycoparasite of a number of plant pathogenic fungi thereby employing morphological changes and secretion of cell wall degrading enzymes and antibiotics. The function of the *tmk1* gene encoding a mitogen-activated protein kinase (MAPK) during fungal growth, mycoparasitic interaction, and biocontrol was examined in *T. atroviride*. Δ*tmk1* mutants exhibited altered radial growth and conidiation, and displayed de-regulated infection structure formation in the absence of a host-derived signal. In confrontation assays, *tmk1* deletion caused reduced mycoparasitic activity although attachment to *Rhizoctonia solani* and *Botrytis cinerea* hyphae was comparable to the parental strain. Under chitinase-inducing conditions, *nag1* and *ech42* transcript levels and extracellular chitinase activities were elevated in a Δ*tmk1* mutant, whereas upon direct confrontation with *R. solani* or *B. cinerea* a host-specific regulation of *ech42* transcription was found and *nag1* gene transcription was no more inducible over an elevated basal level. Δ*tmk1* mutants exhibited higher antifungal activity caused by low molecular weight substances, which was reflected by an over-production of 6-pentyl-α-pyrone and peptaibol antibiotics. In biocontrol assays, a Δ*tmk1* mutant displayed a higher ability to protect bean plants against *R. solani*.

Keywords: *Trichoderma atroviride*; Mycoparasitism; Mitogen-activated protein kinase (MAPK); Chitinase; Antifungal metabolites; Peptaibols

1. Introduction

Members of the genus *Trichoderma* are potent mycoparasites as they attack and parasitize plant pathogens, and therefore they are commercially applied as biocontrol agents (Hjeljord and Tronsmo, 1998). What currently is defined as biocontrol is a combination of different mechanisms like formation of infection structures (e.g. coiling), production of hydrolytic enzymes, secretion of antifungal metabolites, and induction of defense responses in plants, that work synergistically to achieve disease control (Harman et al., 2004; Howell, 2003).

After recognizing the presence of a potential host fungus, *Trichoderma* inhibits or kills the plant pathogen by parasitizing its hyphae, thereby employing hydrolytic enzymes like chitinases and glucanases to degrade the host's cell wall (Chet et al., 1998; Kubicek et al., 2001). Sensing of the host's presence may involve a variety of signal transduction pathways resulting in the expression of mycoparasitism-related genes. The current model is that both enzyme production and infection structure formation are induced responses triggered by molecules released from the host fungus (e.g. degradation products from its cell wall) or located on its surface (e.g. lectins). Recent findings

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suggest that additionally the production of antimicrobial metabolites could be enhanced by the presence of the host, as 6-pentyl-α-pyrone production in *T. harzianum* is elicited by *Rhizoctonia solani* (Serrano-Carreon et al., 2004).

Highly conserved mitogen-activated protein kinase (MAPK) cascades found in animals, plants, and fungi are involved in the transmission of extracellular and intracellular signals, thereby often regulating transcription factors by MAPK-mediated phosphorylation (Schaeffer and Weber, 1999). The study of fungal MAPKs revealed their involvement in several essential developmental processes such as sporulation, mating, hyphal growth, and pathogenicity (Gustin et al., 1998; Xu, 2000; Zeilinger, 2004b). In the model pathogenic fungus *Magnaporthe grisea* three MAPK-encoding genes have been characterized, among which *pmk1* (an ortholog of *FUS3*/*KSS1* of *Saccharomyces cerevisiae*) and *mps1* (an ortholog of *S. cerevisiae SLT2*) were found to be essential for pathogenicity-related processes like appressoria formation, host tissue colonization, and penetration of the host cuticle (Dixon et al., 1999; Xu and Hamer, 1996; Xu et al., 1998). All examined Pmk1 homologs from other phytopathogenic fungi were also shown to be involved in pathogenicity, and some of them to regulate the induction of secreted plant cell wall-degrading enzymes (Di Pietro et al., 2001; Jenczmionka and Schaeffer, 2005; Lev and Horwitz, 2003; Zheng et al., 2000).

Elucidation of signaling pathways of *Trichoderma* affecting mycoparasitism recently started and confirmed the involvement of conserved signaling components. In *T. atroviride* and *T. virens,* α-subunits of heterotrimeric G proteins were demonstrated to play important roles in the antagonism of plant pathogens (Mukherjee et al., 2004; Reithner et al., 2005; Rocha-Ramirez et al., 2002; Zeilinger et al., 2005), and in *T. virens* in addition a MAP kinase was found to affect mycoparasitism-related processes (Mendoza-Mendoza et al., 2003; Mukherjee et al., 2003) as well as plant systemic resistance (Viterbo et al., 2005). Although both species are closely related, examination of G protein signaling revealed significant differences among these biocontrol agents. Whereas in *T. atroviride* the subgroup I Gα protein Tga1 affects chitinase and antifungal metabolite production (Reithner et al., 2005), and was shown to be indispensable for the overgrowth of the host *R. solani* (Rocha-Ramirez et al., 2002), *T. virens* TgaA is involved in antagonism against *Sclerotium rolfsii* but not *R. solani* and Δ*tgaA* loss-of-function mutants sporulate and coil similar to the parental strain (Mukherjee et al., 2004).

In this study, we describe the characterization of the *T. atroviride* MAP kinase-encoding gene *tmk1* according to its influence on mycoparasitism-related processes and plant protection. Δ*tmk1* mutants showed reduced mycoparasitism in direct mycoparasite–host interactions on plates and a host-specific regulation of *ech42* gene transcription. In addition, they exhibited a 10-times elevated production of peptaibols and displayed a higher ability to protect bean plants against *R. solani* infection. To our knowledge, this is the first report on increased secondary metabolite synthesis in a fungal MAPK deletion mutant.

2. Materials and methods

2.1. Strains and culture conditions

Trichoderma atroviride strain P1 (formerly *T. harzianum,* ATCC 74058) was used for this study and grown on potato-dextrose agar (PDA; Merck, Germany) at 28 °C until sporulation. *Botrytis cinerea* and *R. solani* were used as pathogens and were obtained from the collection of the Institute of Plant Pathology, Università degli Studi di Napoli Federico II, Naples, Italy. *Escherichia coli* JM 109 was the host for plasmid amplification.

Trichoderma atroviride was grown in liquid synthetic medium (SM) as described previously (Brunner et al., 2003). For induction experiments, the fungus was pre-cultivated for 36 h in SM containing 1% (w/v) glycerol as carbon source, harvested by filtration, washed with sterile tap water and transferred to fresh SM medium containing 1% (w/v) colloidal chitin or 1% (w/v) *N*-acetyl-β-D-glucosamine.

For the determination of conidia production *T. atroviride* was incubated on PDA at 28 °C for 7 days with daily exposure to sunlight for 30 min. Spores were collected and counted in a counting chamber. The results are representing the total amount of conidia produced on one plate determined in four replicates.

2.2. Cloning of tmk1

A ~750-bp product was obtained by PCR amplification of genomic DNA from *T. atroviride* P1 with degenerate oligonucleotide primers based on conserved regions of MAPK sequences (MAPK-F: 5′-GCNTAYGGNRTNG TNTG-3′ and MAPK-R: 5′-CATYTCNGCNARDATRC ANCC-3′). The product was subcloned into pGEM-T (Promega, Madison, WI) and sequenced. For screening a genomic λ BlueStar library of *T. atroviride* P1, the *tmk1*-containing PCR fragment was radioactively labeled with [α^{32}P]dCTP by random priming and used as a probe. The genomic sequence of the isolated clones carrying the *tmk1* gene and its flanking regions was determined by primer walking using the following internal primers: Tmk1intF1: 5′-GTCCGTTGGCTGTATC-3′ (bp 815–830), Tmk1intF2: 5′-GTATTCTGGTTCACTAC-3′ (bp 1603–1619), Tmk1intR1: 5′-CTTACCAAACAACACCGTAG-3′ (bp 98–117), and Tmk1intR2: 5′-CAAGGCAATA ATTC AGGAG-3′ (bp 688–670).

2.3. Fungal transformation

For obtaining Δ*tmk1* mutants, *Agrobacterium*-mediated transformation of *T. atroviride* was carried out as previously described (Zeilinger, 2004a). Briefly, after co-cultivating a conidial suspension (10^7 spores ml^{-1}) for 24 h with an