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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

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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 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′-GCNTAYGGNRTNGTNTG-3′ and MAPK-R: 5′-CATYTCNGCNARDATRCANCC-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 *Agrobacterium tumefaciens* strain containing the disruption construct pTSZ-Δ tmk1, in which the entire *tmk1* coding region is replaced by an *hph* (hygromycin B phosphotransferase-encoding) expression cassette (Zeilinger, 2004a), fungal transformants were selected on PDA containing 200 μg/ml hygromycin B. Transformants were recovered and purified to mitotic stability by repeated transfer to selective medium and by two rounds of single spore isolation.

For complementation assays, an ~3.5-kb fragment bearing the *tmk1* gene and its 5′ and 3′ regulatory regions from the parental strain *T. atroviride* P1 was reintroduced into the Δ*tmk1*-12 mutant by co-transformation with plasmid p3SR2 (Kelly and Hynes, 1985), a vector containing the *Aspergillus nidulans amdS* gene. Transformants were selected on acetamide-containing SM medium without any nitrogen source and 2% (w/v) of Agar-Nobel (Difco, BD, Germany) and purified to mitotic stability by two rounds of single spore isolation. Re-integration of the *tmk1* gene was verified by Southern analysis and a transformant strain named *tmk1*-re carrying one copy of *tmk1* was selected for further experiments.

2.4. DNA and RNA manipulations

Genomic DNA was isolated as previously described (Gruber et al., 1990); Southern hybridizations were carried out as described by Sambrook et al. (Sambrook et al., 1989). RNA was isolated as described by Chomczynski and Sacchi (1987).

2.5. Real-time reverse transcriptase-polymerase chain reaction

Real-time reverse transcriptase PCR of total RNA was carried out as described previously (Zeilinger et al., 2005). For amplification of a ~170-bp fragment of the actin-encoding gene *act1* as reference gene, real-time RT-PCR was carried out with primers actinP1F (5′-GCACGGAATCGCTCGTTGC-3′) and actinP1R (5′-TTCTCCACCACCGCCAAGC-3′) in the same run than the transcripts of interest. A ~90-bp fragment of *nag1* was amplified using primers Fnag1Taq (5′-TGTCCTACAGCCTCTGCTGCAAAAGTTC-3′) and Rnag1Taqkurz (5′-CATCTCCTCACAGACAAGCGGTGAAAG-3′) and the following program: 40 cycles consisting of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s. A fragment of the endochitinase-encoding gene *ech42* was amplified with primers ech42 P1 forward (5′-CGCAACTTCCAGCCTCAGAACC-3′) and ech42 P1 reverse (5′-TCAATACCATCGAAACCCCAGTCC-3′) using 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s. A ~140-bp fragment of the condensation domain-encoding region of the peptide synthetase Tex1 was amplified with primers tex1_Ta_f (5′-GGTACACGTCTCTGCCGCTATGCTATC-3′) and tex1_Ta_r (5′-CATTTCGGTGCCAGCGTACGCGG-3′) using 40 cycles consisting of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s. All determinations were performed three times from two

biological replicates with three different sample dilutions. The efficiency of the real-time RT-PCR was calculated from the given threshold cycles in Bio-Rad iCycler (Bio-Rad) according to the equation: $E = 10^{-1/\text{slope}}$ (Rasmussen et al., 2001). The relative transcription ratio was determined according to the mathematical model published by Pfaffl (Pfaffl, 2001).

2.6. Transmitting light microscopy

Microscopic studies were mainly performed as previously described by Lu et al. (2004). Glass slides, on which 500 μl of PDA was spread onto, were inoculated and incubated on a moistened filter paper (Gel-blotting paper; Roth, Karlsruhe, Germany) at 28 °C in a Petri dish sealed with parafilm. Additionally, sterile nylon 66 fibers (approximate diameter 14 μm; Nilit, Migdal-Haemek, Israel) were placed on the media before inoculation. For mycoparasitic studies of the antagonistic behavior, *T. atroviride* and *R. solani* were inoculated on opposite sides of the glass slides. After 48–72 h, the fungal hyphae were viewed under a Leitz Aristoplan (Wetzlar, Germany) microscope, and pictures were taken using an Olympus DP 10 (Olympus America Inc., Melville, NY) camera.

2.7. Antagonistic and biocontrol assays

Plate confrontation assays with *R. solani* or *B. cinerea* as hosts were performed as previously described (Lorito et al., 1996; Zeilinger et al., 1999). Plant experiments for *in vivo* biocontrol tests were conducted with *Phaseolus vulgaris L. (var. nanus L.)* (Austrosaat, Vienna, Austria) and *R. solani*-infested soil as previously described (Brunner et al., 2003). The height of surviving plants was investigated for a period of 6 days after germination and related to the number of growing beans. The vitality was calculated from the relative plant height with *Trichoderma* compared to a control experiment without *Trichoderma*. Additional control experiments were conducted without *R. solani* and a total of 14 bean seeds per attempt.

2.8. Antibiotic activity assays

As described previously (Graeme-Cook and Faull, 1991), an agar plug with *Trichoderma* was placed on a PDA plate covered with a cellophane or a dialysis tubing cellulose membrane (cut off 12 kDa; Sigma–Aldrich, St. Louis, MO), and cultivated for 3 days. The area covered by the colony was marked and the membrane carrying the fungus was removed. A spore suspension containing 10^7 *B. cinerea* spores was spread over the surface of the plate which was further incubated for 7 days at room temperature (RT). The antibiotic activity secreted by the *Trichoderma* colony was detected as a zone of inhibition of germination of *B. cinerea* spores. The inhibition index (an extent for inhibition) was calculated as (diameter of the zone of inhibition)/(diameter of the colony growth). These assays were set up in triplicate.

2.9. Combined antagonistic and antibiotic activity assays

Plate confrontation assays were carried out on PDA plates covered with cellophane or dialysis tubing cellulose membranes and incubated for 4 days at 28 °C. The areas covered by the fungal colonies as well as the confrontation zone were marked on the plate and the

membrane was removed. A lawn of 10^7 *B. cinerea* spores was spread over the surface of the plate and incubated at RT for 7 days. The inhibition index for this assay was calculated as (area of the zone of inhibition)/(area of fungal growth).

2.10. Enzyme assays

Chitinase activity in the culture filtrates was determined in triplicate. Enzyme assays were performed as previously described (Harman et al., 1993) using the substrates *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide for determination of *N*-acetyl-glucosaminidase and 4-nitrophenyl β-D-*N*,*N*′,*N*″-triacetylchitotriose (all from Sigma) for determination of endochitinase activity as substrates. Enzyme activity was measured as U/ml (one unit is defined as the release of 1 μmol of nitrophenol per minute) and related to biomass by determination of the mycelial dry weight resulting in expression of enzyme activity as U/g mycelial dry weight.

2.11. Quantitative 6-pentyl-α-pyrone analysis

Quantification of 6-pentyl-α-pyrone (6-PP) from PDA plates previously inoculated with Δ*tmk1*-12 strain and *T. atroviride* parental strain, respectively, was performed as described (Reithner et al., 2005).

2.12. Qualitative analysis of peptaibol production

Sterilized synthetic medium composed of (0.5% w/v) glucose, (0.08% w/v) KH_2PO_4, (0.07% w/v) KNO_3, (0.02% w/v) $CaCl_2$, (0.05% w/v) $MgSO_4{\cdot}7H_2O$, (0.001% w/v) $MnSO_4{\cdot}5H_2O$, (0.0005% w/v) $CuSO_4{\cdot}5H_2O$, and (0.0001% w/v) $FeSO_4{\cdot}7H_2O$ was inoculated with conidial suspensions of *T. atroviride* P1 and the Δ*tmk1*-12 mutant. The stationary cultures were incubated at 28 °C for 10 days. Five microliters of a mixture containing 0.5 ml of the culture filtrate and 1 ml of mobile phase A (acetonitrile/water/formic acid; 28:72:0.1) were used for LC-MS/MS analysis (QTrap 4000 LC-MS/MS system; Applied Biosystems, Foster City, CA, USA). The chromatographic separation was carried out on a BDS Hypersil® C-18 column (5 μm, 150 × 2.1 mm; Thermo Electron, Woburn, MA, USA) using a linear gradient elution starting with mobile phase A and ending with mobile phase B (acetronitrile/water/formic acid; 100:0:0.1). The LC flow rate was set to 0.3 ml/min at a column oven temperature of 25 °C. The ESI interface was used in positive ion mode at 450 °C with the following settings: nitrogen curtain gas (CUR) at 69 kPa, nebuliser gas (GS1) nitrogen at 345 kPa, auxiliary gas (GS2) nitrogen at 345 kPa, ion spray voltage (IS) +4000 V, collision-activated dissociation gas (CAD) “high”, interface heater (ihe) on. Enhanced product ion scan (EPI) was used as a hybrid triple quadrupole-linear ion trap (LIT) scan mode, involving selection of the precursor in quadrupole 1, fragmentation by collision with CAD gas molecules in quadrupole 2, trapping and scanning product ions in quadrupole 3 operated as LIT. For screening of atroviridins, three sequential EPI experiments in a single period with precursor ions corresponding to $[M+2H]^{2+}$ species of atroviridins A–C (Oh et al., 2002) were used: atroviridin A: *m/z* 982.2, scan range 150–1965 amu; atroviridin B: *m/z* 989.2, scan range 150–1980 amu; atroviridin C: *m/z* 996.2, scan range 150–1995 amu. For all experiments the following settings were kept constant: the average of three successive mass spectra were recorded (CE 10, 30, and 50 V); scan rate

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4000 amu/s, step size 0.12 amu, Q1 unit resolution, Q3 entry barrier 8 V, declustering potential (DP) +50 V.

3. Results

3.1. Cloning and characterization of tmk1

PCR amplification with degenerate primers designed according to conserved regions of fungal MAPKs resulted in a 747-bp product. The PCR product was used to screen a *T. atroviride* P1 genomic γ BlueStar library, where multiple clones carrying inserts of 10–15 kb were obtained. Sequencing with internal primers Tmk1intF1, F2, R1, and R2 showed that *T. atroviride tmk1* (GenBank Accession No. AF452096) consists of an open reading frame of 1335-bp interrupted by three putative introns at positions conserved in other fungal MAPK homologs, e.g. *M. grisea* Pmk1 (Xu et al., 1998), and *Fusarium oxysporum* Fmk1 (Di Pietro et al., 2001). The deduced protein sequence of the *tmk1* gene comprises 355 amino acids and is 99% identical to *T. virens* TmkA and 97% identical to *M. grisea* Pmk1 with most of the changes located in the N-terminal parts of the proteins. Analysis of Tmk1 by the PROSITE website (http://us.expasy.org/prosite/; (Falquet et al., 2002) revealed a consensus sequence typical for MAP kinases between residues 57 and 159 (TXY+F-X (19)-R-E-X (72, 86) R-D-X-K-X (9)-C) and a nuclear localization sequence at aa 256–272. The catalytic domain is located between aa 23 and 311 with residues 29–53 being responsible for ATP binding. Based on Southern analysis with genomic DNA of *T. atroviride* digested with several restriction enzymes and the 747-bp PCR product as a probe, *tmk1* is present as single copy in the genome of *T. atroviride* (data not shown).

3.2. Generation and characterization of tmk1 mutants

tmk1 gene deletion by *Agrobacterium*-mediated transformation resulted in seven transformants with single-copy integration of the *hph* marker cassette at the homologous *tmk1* gene locus (Zeilinger, 2004a). Because of identical phenotypes of the obtained Δ*tmk1* mutants, two of them (Δ*tmk1*-11 and Δ*tmk1*-12) were chosen for further studies and compared to the parental strain and a respective rescued transformant (*tmk1*-re). *tmk1*-re was derived from mutant Δ*tmk1*-12 by replacing the integrated deletion cassette by a single functional copy of the *tmk1* gene. Δ*tmk1* mutants grew at about 80–85% the rate of the parental strain and *tmk1*-re on PDA plates (Zeilinger, 2004a), but produced ~3-times more conidia under these conditions. They began to sporulate at the center of the colony—similar to the phenotype described for *T. virens* Δ*tmkA* mutants (Mukherjee et al., 2003). Conidia of *T. atroviride* Δ*tmk1* mutants appeared to be pale green and, in contrast to the parental and the *tmk1*-re strain, were produced light-independently. Microscopic analysis of Δ*tmk1* mycelia showed an increased tendency of the mutant's hyphae to coil around themselves compared to the parental and the *tmk1*-re strain (Fig. 1a–c). To further elucidate this abnormal hyper-coiling, the behavior of the Δ*tmk1* mutants in the presence of autoclaved, uncoated nylon fibers was examined. The fibers should mimic the physical appearance of a host hyphae without the presence of a contact stimulant, e.g. lectins. As shown in Fig. 1d, the Δ*tmk1*-12 mutant still grew towards and along the nylon fibers and attached to them. The same effect was observed with Δ*tmk1*-11 (data not shown). As expected, neither directed

growth nor attachment to the plain nylon fibers was noticed with the parental strain *T. atroviride* P1 (Fig. 1e) or the *tmk1*-re strain (Fig. 1f).

The results presented above show the involvement of the Tmk1 MAP kinase in the production of morphological changes as *tmk1* gene deletion causes permanent coiling around hyphae or attachment to hyphal-like structures in the absence of a host-derived signal.

3.3. Mycoparasitic abilities of Δtmk1 strains

In order to investigate whether this release from host-derived signals for initiating primary steps of mycoparasitism is reflected by an increased host attack, we performed direct plate confrontation assays against *R. solani* and *B. cinerea* as fungal hosts. In contrast to the parental strain *T. atroviride* P1 and the rescued transformant *tmk1*-re, which already overgrew and lysed *R. solani* 7 days after inoculation of the mycoparasite and the host on opposite sides of the plate (data not shown), the Δ*tmk1* mutant just contacted and started to overgrow the host fungus during this period. After 7 more days the hyphae of *Rhizoctonia* were almost completely lysed by *T. atroviride* P1 and the *tmk1*-re strain, while the Δ*tmk1* mutant did not completely overgrow and lyse the host fungus even under prolonged incubation times (reflected by still intact hyphae of the host fungus; Fig. 2a). Although the mycoparasitic activity of the Δ*tmk1* mutant was reduced, typical morphological changes associated with mycoparasitism (e.g. attachment to the host's hyphae and growth alongside these hyphae) could be detected microscopically (Fig. 2b).

When the Δ*tmk1* mutants were inoculated opposite to *B. cinerea* on PDA plates, no formation of a zone with direct contact could be observed with the naked eye, while the control strains (parental strain and rescued transformant) touched and started to overgrow the host (Fig. 2a). Nevertheless, microscopic analysis revealed single Δ*tmk1* hyphae attaching to and growing alongside *B. cinerea* hyphae (data not shown).

3.4. nag1 and ech42 gene transcription in Δtmk1 mutants

Besides attaching to and coiling around the host's hyphae, the mycoparasitic interaction of *Trichoderma* also involves the secretion of cell wall-degrading enzymes preceded by the induction of the respective genes (Mach et al., 1999; Zeilinger et al., 1999). The transcription of two mycoparasitism-related genes coding for the Ech42 chitinase and the Nag1 *N*-acetyl-glucosaminidase was examined in the Δ*tmk1*-11 mutant when grown in liquid culture under chitinase-inducing conditions and in dual plate cultures when confronted with *R. solani* or *B. cinerea* as hosts.

Three hours after transfer to liquid growth medium containing *N*-acetyl-β-D-glucosamine, *nag1* transcript formation in the Δ*tmk1* mutant and the parental strain was at its maximum and decreased again in both strains 5 h after transfer to induction medium (Fig. 3a). Upon transfer to liquid growth medium containing colloidal chitin, *nag1* transcript levels were at their maximum at 48 h in both the Δ*tmk1* mutant and the parental strain. Under both conditions, the Δ*tmk1* mutant attained higher *nag1* transcript levels compared to the parental strain *T. atroviride* P1. When analyzing *ech42* gene transcription, the Δ*tmk1* mutant again

displayed enhanced mRNA levels compared to the parental strain at all time points tested (Fig. 3b).

In direct plate confrontation assays, induction of *nag1* gene transcription reached its maximum in the parental strain *T. atroviride* P1 upon direct contact with the host fungi *R. solani* and *B. cinerea*. Interestingly, in the Δ*tmk1* mutant neither host fungus was able to significantly enhance *nag1* gene transcription over the basal level detected when the fungus was grown alone on PDA plates (Fig. 3c).

When analyzing *ech42* gene transcription at different mycoparasitic stages, we found enhanced mRNA levels in the parental strain already before contact with the host fungi, whereas in the Δ*tmk1* mutant *ech42* gene transcription was only significantly induced upon direct contact with *R. solani* but remained un-induced upon confrontation with *B. cinerea* (Fig. 3d).

Interestingly, the Δ*tmk1* mutant produced elevated basal *nag1* and *ech42* mRNA levels in the control condition (growth on PDA in the absence of a host fungus) when compared to the parental strain, indicating a de-regulated production of these cell wall-degrading enzymes under non-inducing conditions.

To summarize, *tmk1* gene deletion results in a de-regulated transcription of the two mycoparasitism-related genes studied as even under non-inducing conditions elevated mRNA levels were found in the Δ*tmk1* mutant. Nevertheless, *nag1* gene transcription could not be induced over the basal level by the presence of a host fungus, whereas induction of *ech42* gene transcription was triggered in a host-specific manner in the Δ*tmk1* mutant.

3.5. Extracellular chitinase activities in Δtmk1 mutants

In the culture supernatants of the parental strain *T. atroviride* P1 increasing amounts of extracellular chitinases (*N*-acetyl-glucosaminidases (NAGase) as well as endochitinases) were detected during induction with *N*-acetylglucosamine or colloidal chitin in liquid culture (Table 1). Upon induction with *N*-acetyl-glucosamine the Δ*tmk1*-11 mutant produced similar NAGase activities as the parental strain for the first 5 h of induction. After 10 h under these conditions NAGase production in the Δ*tmk1*-11 mutant was 1.6-times higher compared to the parental strain. When induced with colloidal chitin, the Δ*tmk1*-11 mutant showed slightly increased NAGase activities compared to the parental strain after 36 h; at 48 h after induction Δ*tmk1*-11 showed an about 1.8-fold higher extracellular activity than the parental strain. Similar to the NAGase activities, 48 h after induction with colloidal chitin endochitinase activities of the Δ*tmk1*-11 mutant were increased ~1.5-fold. Enzyme activities measured for cultures of the rescued transformant *tmk1*-re were comparable to those of the parental strain (data not shown). These results suggest that Tmk1 negatively influences the production of extracellular chitinases during later points of induction.

3.6. Production of antifungal metabolites

Since the mycoparasitic action of *Trichoderma* is not merely an effect of hydrolytic enzymes and direct contact to the host fungus but also due to the secretion of antimicrobial substances (Howell, 1998), the antifungal activity of one Δ*tmk1* mutant was determined. In the *B.*

cinerea spore germination assay the Δ*tmk1*-12 mutant showed higher biological activity (inhibition index of 0.22 when pre-cultivated on cellophane and 0.06 when pre-cultivated on dialysis tubing cellulose membrane; standard deviation for the experiments are displayed in Fig. 4) compared to the parental strain (inhibition index of 0.06 on cellophane and no detectable inhibition index on dialysis tubing membrane; Fig. 4a). When the inhibition indices were calculated after plate confrontation assays against *B. cinerea*, the Δ*tmk1*-12 mutant and the parental strain secreted similar amounts of inhibitory substances into the media (inhibition index of 0.74 and 0.59, respectively), independent from the membrane type used (Fig. 4b). These data indicate that a fraction of secreted metabolites smaller than 12 kDa is significantly increased in the deletion strains when grown alone, whereas production of antifungal substances remains inducible by the presence of a host fungus to a similar extent in all strains tested. Higher molecular weight antifungal substances were secreted to an even higher extent by the parental strain when confronted with the host (Fig. 4b).

Since one of the major antimicrobial substances produced by *Trichoderma* is 6-pentyl-α-pyrone (6-PP) (Claydon et al., 1987; Ghisalberti and Sivasithamparam, 1991; Simon et al., 1988), we were interested in its production in the Δ*tmk1* mutants. Quantitative analysis of 6-PP revealed 1.6-fold elevated amounts secreted into PDA plates by the Δ*tmk1*-12 mutant (1.23 ± 0.16 g 6-PP/g mycelial dry weight) compared to the parental strain (0.75 ± 0.11 g 6-PP/g mycelial dry weight).

To obtain insights into the production of peptaibols, another important class of antifungal metabolites (Correa et al., 1995), the transcription ratio of the coding region of the peptaibol-specific α-aminoisobutyric acid (AiB) condensation domain of a non-ribosomal peptide synthetase of *T. atroviride* P1 (GenBank Accession No. DQ973296), with high similarity to *T. virens* Tex1, compared to *act1* as reference gene was determined by real-time RT-PCR. After growth under peptaibol-producing conditions for 10 days, the transcription ratio of the Δ*tmk1*-12 mutant was 2.5-fold (± 0.1) higher induced compared to the parental strain. The culture filtrates, obtained from the same cultivations, were screened for the presence of peptaibols by LC-MS/MS analysis. The chromatograms revealed that the peptaibol production pattern and peak intensities differed between the Δ*tmk1*-12 mutant and the parental strain (Fig. 5a). When the peak intensities of the Δ*tmk1*-12 mutant and *T. atroviride* P1 were related to the mycelial dry weight of the cultures and compared with each other, it was found that the production of peptaibols was approximately 10-times higher in the mutant. However, when the $[M+2H]^{2+}$ species were taken as precursors for MS/MS fragmentation, the spectra showed the presence of AiB indicated by the fragments differing in 85 amu (Fig. 5b). It shall be noted that the tandem mass spectra did not match with those expected for the atroviridins and neoatroviridins. Thus we conclude that these peptaibols were absent in all samples tested. Nevertheless, the presence and position of AiB strongly suggests significant over-production of yet uncharacterized peptaibols of the trichorzianin-family in our Δ*tmk1*-12 mutant.

3.7. In vivo biocontrol abilities of Δtmk1 mutants

To test the *in vivo* biocontrol abilities of our Δ*tmk1* mutants, assays with beans planted in *R. solani*-infected soil were performed. In these experiments the vitality of the beans coated with spores of the Δ*tmk1*-12 mutant was 30% higher than of beans protected by *T. atroviride* P1 when considering plant growth in the control experiment with uncoated beans (Fig. 6). To approve the exclusion of a possible influence of the deletion of *tmk1* on the vitality of beans, control assays without addition of *R. solani* to the soil were conducted. It was found that neither Δ*tmk1*-12 mutant nor the parental strain had a statistically significant influence on the plant vitality (Fig. 6). Thus we conclude that in contrast to a reduced mycoparasitic activity, plant protection is increased in Δ*tmk1* mutants. This enhanced protection is clearly not due to a growth promoting effect on the plants but to a direct (mycoparasite–host interaction) or indirect (increased plant resistance) effect.

4. Discussion

In phytopathogenic fungi, signal transduction via MAPK cascades is, among other processes, involved in the parasitic interaction (Banuett and Herskowitz, 1994; Di Pietro et al., 2001; Horwitz et al., 1999; Lev et al., 1999; Takano et al., 2000; Xu and Hamer, 1996; Xu et al., 1998). So far at least three different MAPKs were found to be functional in most of these fungi. MAPKs homologous to *T. atroviride* Tmk1 like *M. grisea* Pmk1 and *S. cerevisiae* FUS3/KSS1 were previously shown to be involved in the regulation of mating and fusion (Madhani et al., 1997), as well as appressorium formation and invasive growth (Xu and Hamer, 1996).

Tmk1 function was analyzed by generating and characterizing respective *T. atroviride* gene deletion mutants in which *tmk1* was replaced by the *hph* (hygromycin B phosphotransferase-encoding) gene (Zeilinger, 2004a). To clearly relate all observed alterations to *tmk1* gene deletion, a rescued transformant again exhibiting the characteristics of the parental strain was generated. Thus the observed phenotypic aberrations like reduction of the growth rate and light-independent formation of conidia in Δ*tmk1* mutants are truly due to *tmk1* deletion, which is in accordance to the fact that similar phenotypes were also reported for *T. virens* Δ*tmkA* and Δ*tvk1* mutants (Mendoza-Mendoza et al., 2003; Mukherjee et al., 2003). Further investigations on the growth behavior of Δ*tmk1* mutants demonstrated a permanent induction of the coiling response in the absence of host-derived signals as the mutant coiled around its own hyphae and around plain nylon fibers in the absence of a host fungus.

To reveal the role of *T. atroviride* Tmk1 during mycoparasitism, mycoparasitism-related processes like chitinase gene expression, infection structure formation (coiling), and the secretion of antifungal substances were examined in Δ*tmk1* mutants. *T. atroviride* Δ*tmk1*-11 mutant showed enhanced transcription of the mycoparasitism-related genes *ech42* and *nag1* and increased production of cell wall-degrading chitinases when cultivated in liquid culture under inducing conditions. Similar findings were also described for *T. virens* Δ*tvk1* strains (Mendoza-Mendoza et al., 2003).

Results from direct plate confrontation assays against *R. solani* und *B. cinerea* as hosts suggested that Tmk1 affects the host specificity of *T. atroviride* as the Δ*tmk1* mutant still

could parasitize *R. solani* (although less effectively than the parental strain) whereas it was no longer able to overgrow *B. cinerea*, although attachment to and growth alongside of *Botrytis* hyphae could still be observed microscopically (data not shown). This host-dependent behavior of the Δ*tmk1* mutant was also reflected by the fact that *ech42* gene transcription was inducible upon direct contact with *R. solani*, whereas it remained un-induced when the Δ*tmk1* mutant was confronted with *B. cinerea*. These findings are similar to the host-specific loss of the biocontrol potential of *T. virens tmkA* mutants which were as effective against *R. solani* as the parental strain whereas they failed to colonize *S. rolfsii* sclerotia (Mukherjee et al., 2003).

As the production of secondary metabolites was reported to be associated with the formation of conidiospores (Bu'Lock, 1961) and the inhibition of sporulation was shown to be accompanied by an inhibition of aflatoxin production in *Aspergillus parasiticus* (Reiss, 1982), we hypothesized that the light-independent conidiation of our Δ*tmk1* mutants may correlate with changes in the production of antimicrobial substances. Qualitative and quantitative analysis of the secreted metabolites demonstrated an increased production of 6-pentyl-α-pyrone and peptaibols upon deletion of *tmk1*; additionally also the pattern of the secreted peptaibols differed compared to the parental strain. Nevertheless, the secretion of antifungal substances could still be induced during direct confrontation with *B. cinerea* compared to the constitutive level during sole growth on PDA. Summarizing, while *tmk1* gene deletion resulted in a de-regulation of the production of antifungal metabolites, their formation still was inducible by the presence of a host fungus. Therefore, although the lack of Δ*tmk1* mutants to overgrow *B. cinerea* would suggest a loss of their ability to appropriately react on signals derived from this host, the enhanced secretion of antifungal substances in the presence of this pathogen evidences a still remaining host–pathogen cross-talk. Thus we speculate that deletion of *tmk1* at least not completely impairs sensing of this host fungus.

The finding that mycoparasitism-related processes like infection structure formation (coiling) as well as chitinase and antifungal metabolite production (Chet et al., 1998; Kubicek et al., 2001) were unaltered or even enhanced upon *tmk1* gene deletion somehow contrasts the reduced/lost ability of the Δ*tmk1* mutants to control host fungi in direct confrontation assays. On the other hand, in greenhouse experiments the examined Δ*tmk1*-12 mutant was able to protect bean plants against *R. solani* infection even better than the parental strain indicating a negative regulatory role of Tmk1 on *Trichoderma*-triggered plant resistance. Similar results were reported by Mendoza-Mendoza et al. (Mendoza-Mendoza et al., 2003) for *T. virens* Δ*tvk1* mutants which showed a greater capacity to control and reduce damage by *R. solani* and *Pythium ultimum* on cotton plants, whereas for *T. virens* Δ*tmkA* mutants attenuated mycoparasitism and a failure to induce full systemic resistance against the bacterial leaf pathogen *Pseudomonas syringae* pv. *lacrymans*, but similar effectivity as the wild-type in biocontrol activity against *R. solani* on bean plants was found (Viterbo et al., 2005). These findings strongly suggest the presence of further, still unknown, mycoparasitism-related factors which are missing in our Δ*tmk1* mutants and which are therefore affected by a signaling pathway involving Tmk1.

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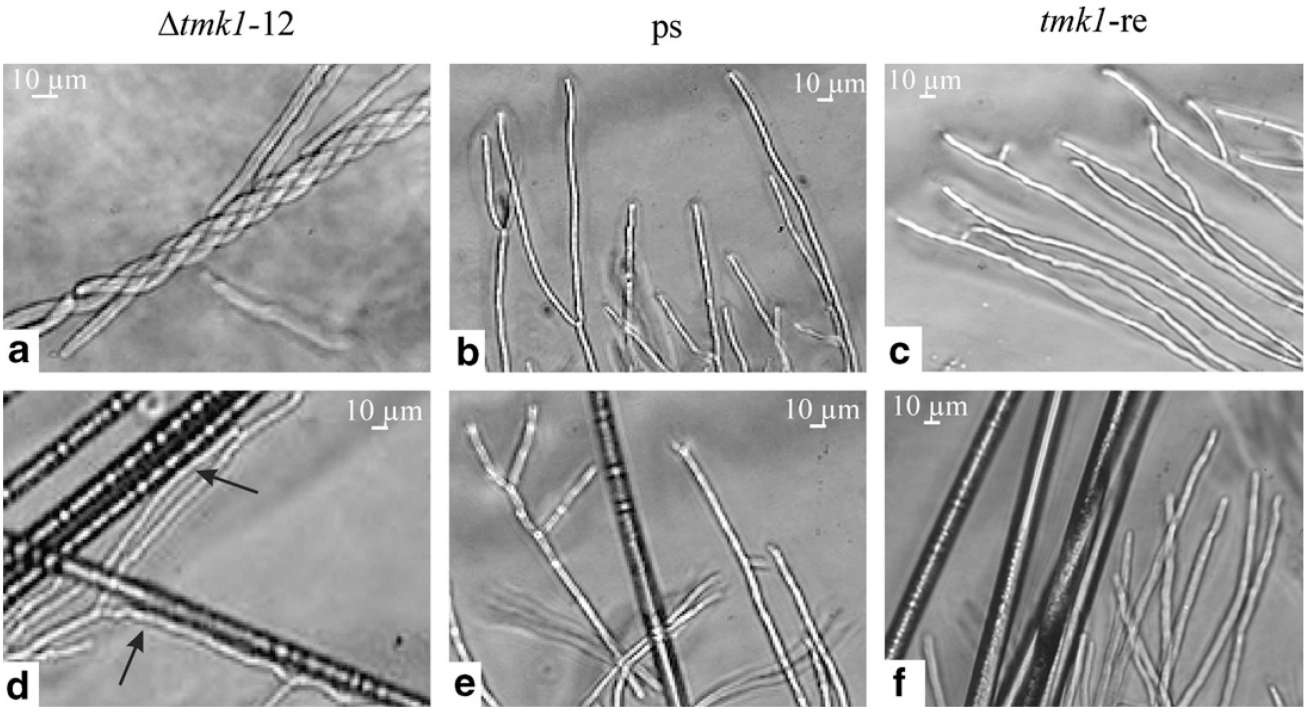

Fig. 1.

Light microscopic examination of the Δ*tmk1*-12 mutant, the *T. atroviride* parental strain P1 (ps), and the rescued transformant *tmk1*-re after growth for 48–72 h on PDA without (a–c) and with (d–f) plain nylon 66 fibers (approximate diameter 14 µm). Arrows mark the attachment to and the growth along the nylon fibers; the thick, dark structures in panels (d–f) represent the nylon fibers.

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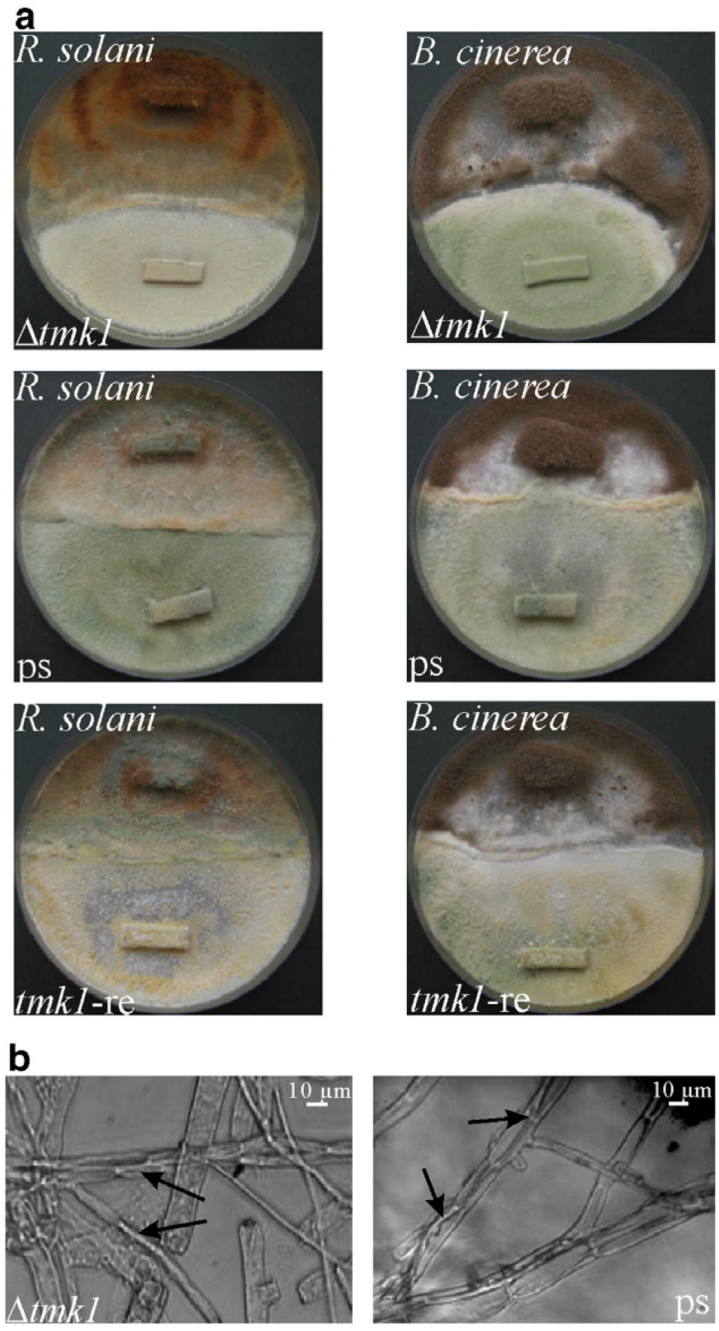

Fig. 2.

(a) Plate confrontation assays of Δ*tmk1* mutant *tmk1*-11 and the rescued transformant *tmk1*-re in comparison to the parental strain *T. atroviride* P1 (ps) against *R. solani* and *B. cinerea* for analyzing the mycoparasitic ability. Pictures were taken 14 days after inoculation of the two fungi on opposite sides of the plate. (b) Microscopic analyses of the confrontation zone between *T. atroviride* (thin hyphae) and *R. solani* (thick hyphae). The interaction of *Trichoderma* hyphae with the host is marked with arrows.

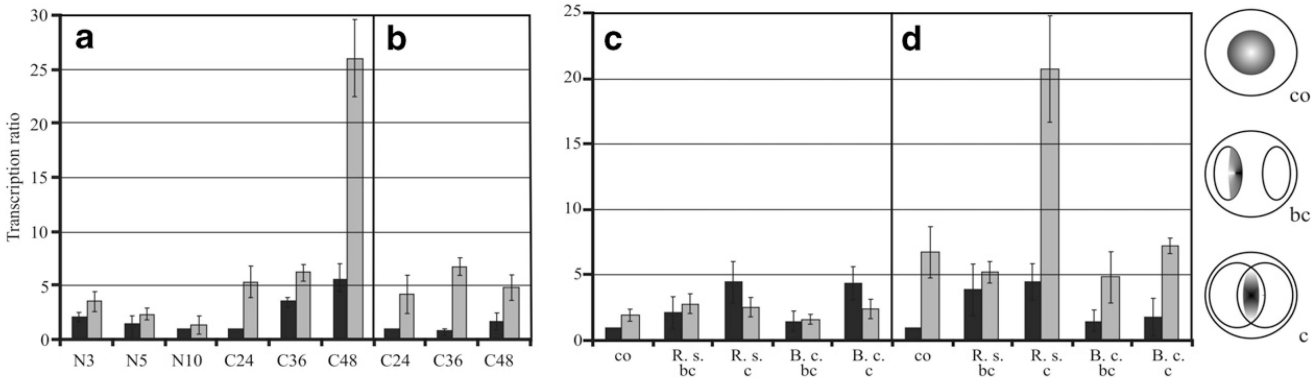

Fig. 3.

Relative transcription ratios of the chitinase-encoding genes *nag1* (a and c) and *ech42* (b and d) in the Δ*tmk1*-11 mutant (grey bars) compared to the parental strain *T. atroviride* P1 (black bars). (a and b) Real-time RT-PCR analyses were performed 3, 5, and 10 h after transfer of mycelia to liquid growth medium containing 1% *N*-acetylglucosamine (N) and 36 and 48 h after transfer to 1% colloidal chitin (C). The samples of the parental strain showing the lowest mRNA levels within one PCR run were arbitrarily assigned the factor 1 (N10, C24). (c and d) Direct confrontation assays with *R. solani* (R.s.) and *B. cinerea* (B.c.) as host fungi. Samples were collected from a control (co) where *T. atroviride* was grown alone, from an early stage before direct contact between the two fungi (bc), and from a later stage of direct contact—if present—with an interaction zone of 1.0 cm (c) and subjected to real-time RT-PCR. The control sample of the parental strain was arbitrarily assigned the factor 1.

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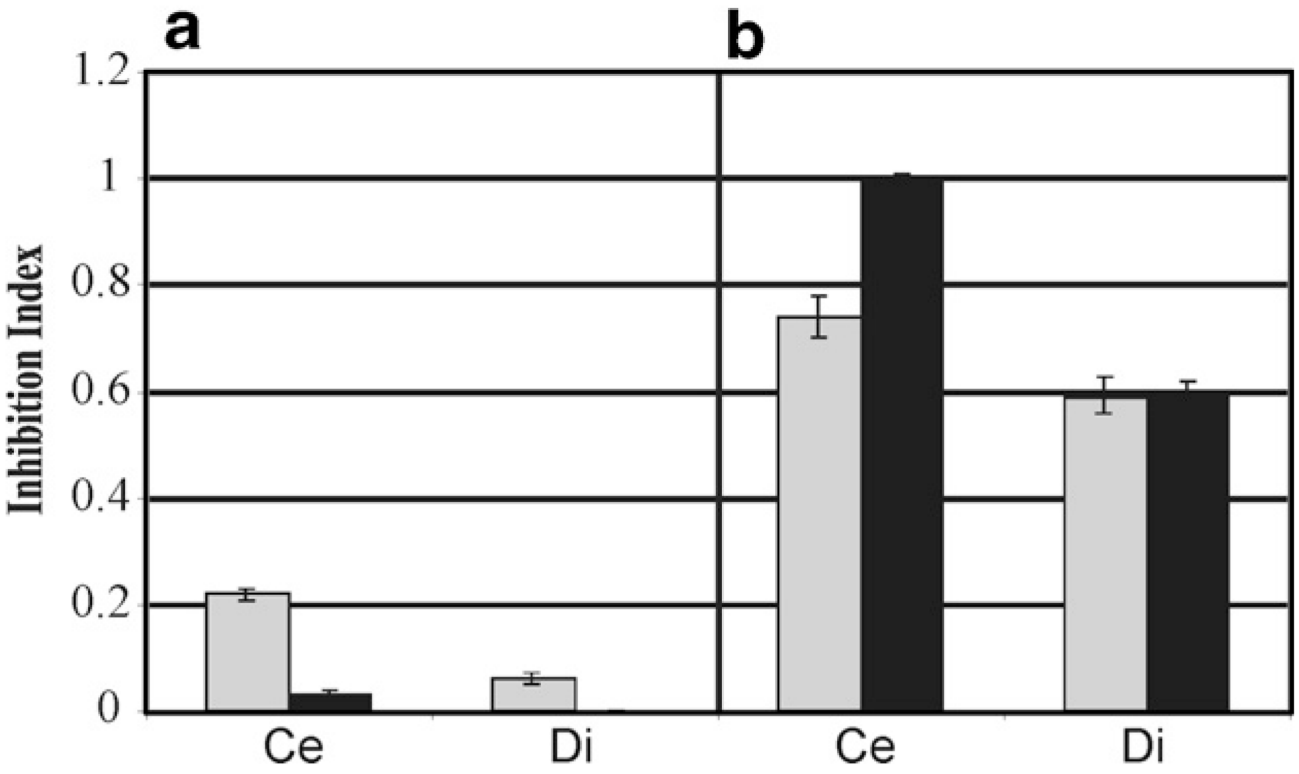

Fig. 4.

Inhibition indices for *T. atroviride* P1 (▪) and the Δ*tmk1*-12 mutant (■) obtained from *B. cinerea* spore germination assays. The strains were grown on PDA for 7 days on plates covered by a cellophane (Ce) or a dialysis tubing cellulose membrane (Di) either alone (a) or were confronted against *B. cinerea* (b). Each bar represents the average of at least three experiments; error bars indicate standard deviation which is less than 15% for all experiments.

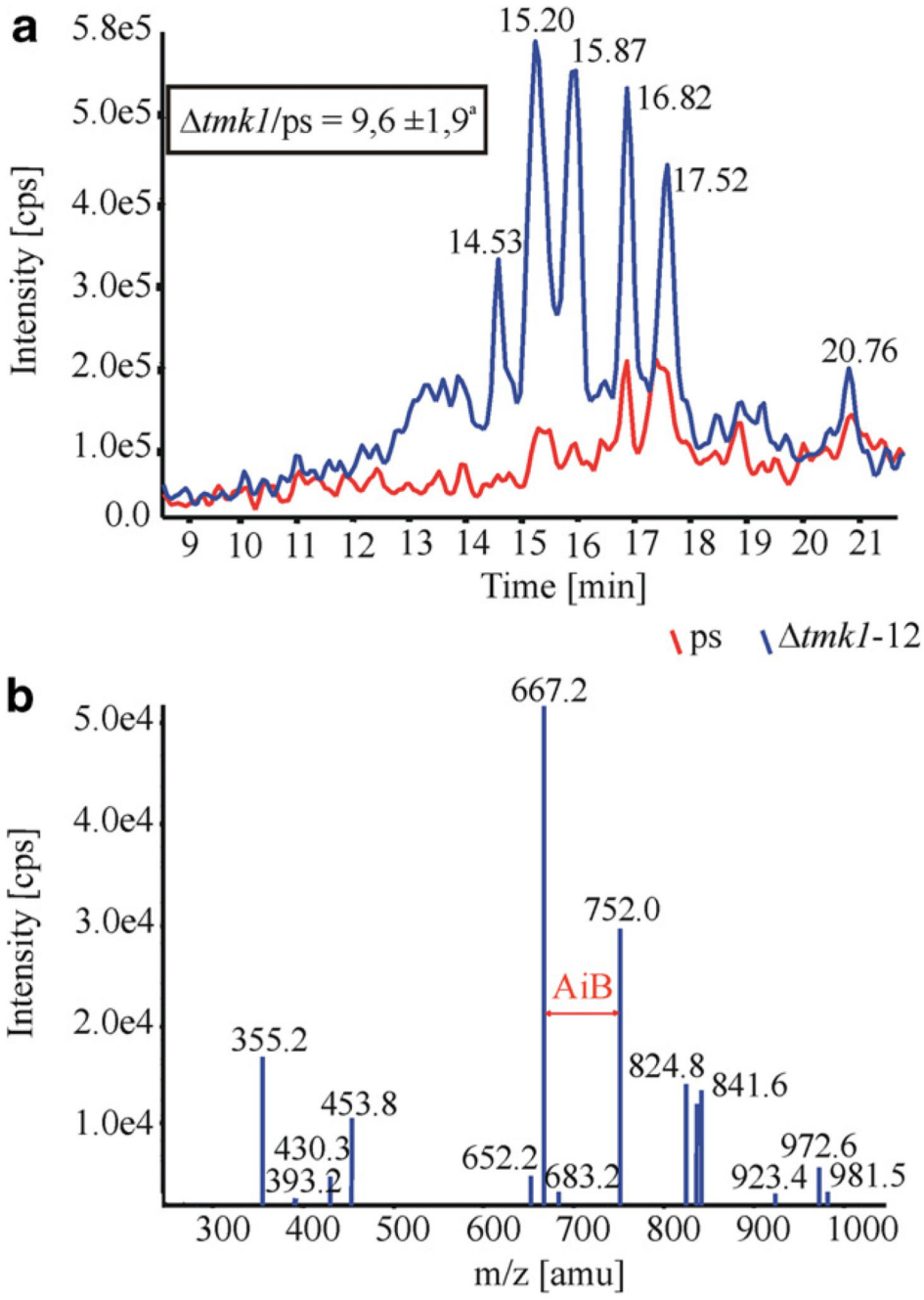

Fig. 5.

LC-MS/MS screening for peptaibols (a) of culture filtrates from the Δ*tmk1*-12 mutant (blue graph) and the parental strain (red graph). The *m/z* ratio 982.2 of the peak with the retention time of 15.87 min suggests the presence of atroviridins; nevertheless fragmentation of this peak related these peptaibols to the trichorzianin family (b). [a]The inset indicates the ratio of the peak-intensities related to the mycelial dry weight of the strains tested.

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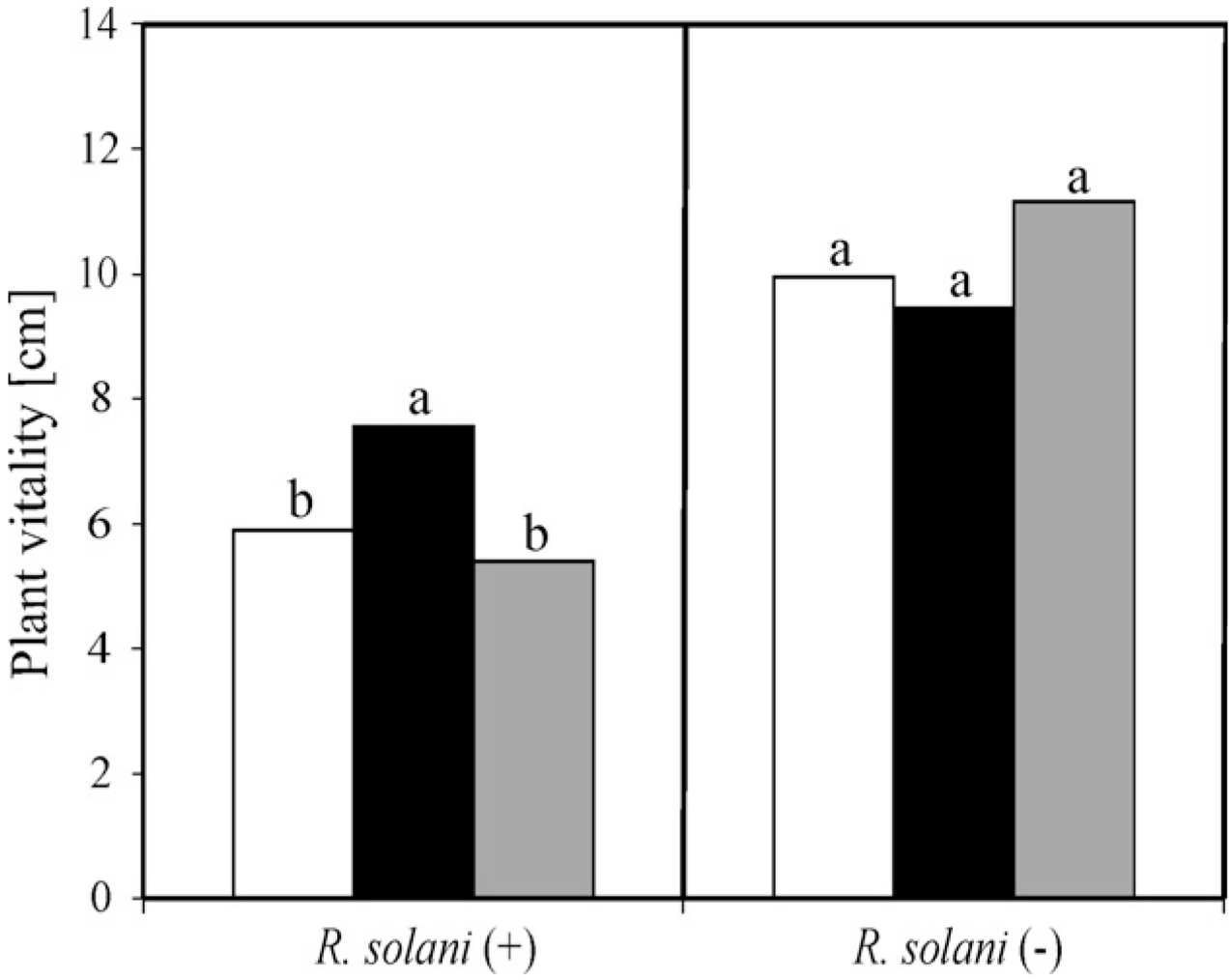

Fig. 6.

Effect of *T. atroviride* parental strain P1 and the Δ*tmk1* mutant on the vitality of beans infected with *R. solani* (+). Bean seeds were either coated with spores of the parental strain (□), the Δ*tmk1*-12 mutant (▪) or were uncoated (without *Trichoderma*) as a control (■). Additional control experiments were carried out without pathogen (−). Vitality of the plants is given in plant height (cm) 6 days after germination. Columns marked with the same letter differed significantly according to the Mann–Whitney test at a significance level of 10%.

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Table 1

Extracellular *N*-acetyl-glucosaminidase (NAGase) and endochitinase activities in culture filtrates of *T. atroviride* parental strain and the Δ*tmk1*-11 mutant

Carbon source	Strain	Induction time [h]	NAGase-activity [U/g][a]	Endochitinase-activity [U/g][a]
N-Acetylglucosamine	Parental strain	3	11.3 ± 3.0	n.d.[b]
		5	25.8 ± 2.0	n.d.
		10	47.2 ± 2.1	n.d.
	Δ*tmk1*-11	3	11.0 ± 2.1	n.d.
		5	23.7 ± 3.0	n.d.
		10	77.8 ± 1.1	n.d.
Colloidal chitin	Parental strain	36	82.1 ± 0.4	17.3 ± 0.1
		48	132.7 ± 5.9	30.4 ± 0.3
	Δ*tmk1*-11	36	92.5 ± 4.5	14.2 ± 1.4
		48	242.5 ± 18.5	46.3 ± 0.9

[a] Values are related to the mycelial dry weight and are means ± standard deviations of three independent experiments.

[b] Not determined.