Enhanced biocontrol activity of *Trichoderma* through inactivation of a mitogen-activated protein kinase

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The production of lytic enzymes in *Trichoderma* is considered determinant in its parasitic response against fungal species. A mitogen-activated protein kinase encoding gene, *tvk1*, from *Trichoderma virens* was cloned, and its role during the mycoparasitism, conidiation, and biocontrol was examined in *tvk1* null mutants. These mutants showed a clear increase in the level of the expression of mycoparasitism-related genes under simulated mycoparasitism and during direct confrontation with the plant pathogen *Rhizoctonia solani*. The null mutants displayed an increased protein secretion phenotype as measured by the production of lytic enzymes in culture supernatant compared to the wild type. Consistently, biocontrol assays demonstrated that the null mutants were considerably more effective in disease control than the wild-type strain or a chemical fungicide. In addition, *tvk1* gene disruptant strains sporulated abundantly in submerged cultures, a condition that is not conducive to sporulation in the wild type. These data suggest that Tvk1 acts as a negative modulator during host sensing and sporulation in *T. virens*.

Many species of *Trichoderma* have been used as potent biocontrol agents for a variety of soil-borne phytopathogenic fungi (1). The response of *Trichoderma* to the presence of a potential host includes production of antibiotic compounds, formation of specialized structures, and degradation of the host's cell wall followed by the assimilation of its cellular content, namely mycoparasitism (2). This last phenomenon has been proposed as the central mechanism accounting for the antagonistic activity of *Trichoderma* species. Hydrolytic enzymes produced by *Trichoderma*, such as chitinases, β-1-3 glucanases, β-1-6 glucanases, and proteases (2), facilitate penetration of the host. Strains of *Trichoderma* transformed to overexpress hydrolytic enzymes have been shown to be better biocontrol agents than their corresponding parental strains (2). Consequently, most of these hydrolytic enzyme-encoding genes have been designated as mycoparasitism-related genes (MRGs). The ability of *Trichoderma* to sense and respond to different environmental conditions, including the presence of a potential host, is essential for successful colonization of soil, organic material, and developing plant roots. Sensing of such environmental conditions may occur through a variety of transduction pathways, which determine the adequate cellular response. Mitogen-activated protein kinase (MAPK) pathways transduce a large variety of signals, including those associated with pathogenesis. Parasitism by *Trichoderma* resembles in many aspects the interaction of phytopathogenic fungi with their host. In this sense, MAPKs have been directly implicated in pathogenicity in *Magnaporthe grisea* (Pmk1, Pms1), *Botrytis cinerea* (Bmp1), *Fusarium oxysporum* (Fmk1), *Cochliobolus heterostrophus* (Cmk1), and *Ustilago maydis* (Ubc3/Kpp2) (3). In several fungal systems, including phytopathogenic fungi, homologues to the MAPK Kss1 from *Saccharomyces cerevisiae* have been implicated in the expression of cell-degrading enzymes. In *F. oxysporum*, the induction of the pectate lyase-encoding gene (*pl1*) was abolished in the *fmk1* null mutant (4). Similarly, Bmp1 from *B. cinerea* positively controls the expression of those enzymes involved in plant penetration (5). In *Trichoderma atroviride*, a species closely related to *Trichoderma virens*, transcription of a proteinase-encoding gene (*prb1*) in response to nitrogen limitation was blocked by the addition of a specific inhibitor of MAP kinase kinases (6).

To test the hypothesis that MAPKs are directly involved in the establishment of the parasitic relationship between *Trichoderma* and its hosts, we have cloned a MAPK from *T. virens* that is similar to Pmk1. Here we report the role of this gene in several aspects of the life cycle of *T. virens*, including growth, conidiation, expression of MRGs, secretion of cell wall-degrading enzymes, and biocontrol activity.

Materials and Methods

Fungal Strains. *T. virens* Gv29-8 (wild type) and arginine auxotrophic Tv10.4 strains were used in this study (7). *Rhizoctonia solani* AG-4 and *Pythium ultimum* were used as hosts. These plant pathogens were isolated from the roots of dying cotton seedlings. The fungal strains were maintained on Potato Dextrose Agar (Difco), unless otherwise indicated.

Bacterial Strains and Plasmids. *Escherichia coli* strains DH5α (Bethesda Research Laboratories) and JM103 (Invitrogen) were used for all DNA manipulations. The plasmids used were pBluescript (Stratagene) and pCB1004 (Fungal Genetics Stock Center). All PCR products were cloned in pCR2.1 (Invitrogen). Probes used for Northern blot analysis were obtained as follows. A 1.3-kb *Hin*dIII/*Bam*HI fragment of *Tv-prb1* cDNA was cut from pPOE. A 1.4-kb *Hin*dIII/*Xba*I fragment corresponding to *Tv-cht1* was removed from pCOE plasmid (8). From pSZD2, a 0.52-kb *Pst*I/*Xho*I fragment corresponding to *Tv-bgn2* was excised. Two fragments corresponding to *Tv-cht2* (from position 988-1417) and *Tv-nag1* (from position 513-1608) were obtained from *T. virens* genomic DNA by PCR by using oligonucleotides designed based on the sequences reported by Kim *et al.* (9).

DNA and RNA Manipulations. Plasmid DNA was isolated by using a commercial kit (Qiagen). DNA from *Trichoderma* was obtained as described (10). Total RNA was isolated by using phenol:chloroform extraction according to the protocol of Jones *et al.* (11). Southern and Northern blots were performed by using Hybond-N+ membranes (Amersham Biosciences) according to the manufacturer's recommendations.

Western Blot Analysis. Proteins extracts were prepared as described (6), and protein concentration was determined by using the Bradford assay (Bio-Rad) with BSA as a standard. Equivalent amounts of protein (25 μg) from each sample were resuspended in Schägger 2× buffer (12) and boiled after adding

Abbreviations: ERK, extracellular-regulated kinase; MAPK, mitogen-activated protein kinase; MRG, mycoparasitism-related gene.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY162318).

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2-mercaptoethanol (5%). Proteins were fractionated by SDS/PAGE on 10% gels according to Schägger and Von Jagow (12). Gels were transferred to Hybond-C extra membranes (Amersham Biosciences) and probed according to the instructions of the Phospho Plus p42/p44 MAP Kinase (Thr202/Tyr204) Antibody kit (Cell Signaling Technology, Beverly, MA).

Cloning and Sequencing of *tvk1*. Genomic DNA of *T. virens* Gv29-8 was used as a template for PCR reactions with the primers described in ref. 13. The PCR product was cloned and sequenced by the dideoxynucleotide chain termination method of Sanger (14) with a Sequenase kit (Version 2.0, United States Biochemical). A fragment that had high similarity with the *pmk1* gene from *M. grisea* was selected and used as a probe to screen a cosmid DNA library of *T. virens* Gv28-9. Three clones were identified and one was selected for sequencing. Southern analysis allowed the identification of a 3.3-kb *Bam*HI fragment containing the *tvk1* gene, which was subcloned into the *Bam*HI site of plasmid pCB1004 (pDXG35). This fragment was sequenced entirely, followed by a BLAST DNA–protein sequence database search.

Construction of the Gene Replacement Vector *pTVK1*::*arg2*. To construct the *tvk1* gene replacement vector, a 1.48-kb *Eco*RV/*Sal*I fragment containing part of the *tvk1* coding region (from amino acid 88 until the end of the protein) was replaced by a 3.2-kb *Sma*I/*Eco*RV fragment of the *T. virens arg2* gene (7). Briefly, a *Bam*HI/*Eco*RV fragment of pDGX35 was subcloned into *Bam*HI/*Eco*RV sites of pBluescript SK(−) to generate plasmid pAM1. The pAM1 plasmid was digested with *Eco*RV and ligated to an *Eco*RV/*Sma*I fragment of the *arg2* gene (pAM2). To obtain the C-terminal region of Tvk1, we used a 0.4-kb *Sal*I/*Bam*HI fragment from pDGX35 to probe cosmid DNA digested with several enzymes. A 3.2-kb *Sal*I fragment was cloned in pBluescript KS(+) and then subcloned as a *Cla*I/*Apa*I fragment into pAM2. The resulting vector (*pTVK1*::*arg2*) was linearized and used for transformation of the *T. virens* arginine auxotrophic strain Tv10.4 (Fig. 1*A*).

Fungal Transformation. Preparation and transformation of *T. virens* protoplast were performed according to the method described in ref. 7. Prototrophic transformants were selected on Vogel's minimal medium containing sucrose as a sole carbon source (VMS). Disruption of the *tvk1* gene in selected transformants was confirmed by Southern and Western blot analysis.

Submerged Culture Analysis. Spores (1×10^6 spores per ml) of the wild-type and Δtvk1 mutants (Δtvk24 and Δtvk133) were inoculated into Potato Dextrose Broth (Difco), Vogel's medium (VMS), or minimal medium (MM) (15) and incubated for 72 h at 28°C. Then samples were analyzed by using a light microscope (BX60, Olympus, Melville, NY). Images were captured and modified by using the programs IMAGE-PRO PLUS 4.0 (Media Cybernetics, Silver Spring, MD) and PHOTOSHOP (Adobe Systems, Mountain View, CA), respectively.

Simulated Mycoparasitism Assay. *Trichoderma* spores (1×10^6 spores per ml) were germinated and grown for 48 h in VMS. Mycelia were then harvested and transferred to fresh media. Vogel's minimal medium without carbon or nitrogen source (VM or VM-N, respectively) was used to evaluate the effect of nutrient limitation; VMS plus 0.5% *R. solani* cell walls (VMSR) and Vogel's medium without nitrogen or carbon source plus 0.5% *R. solani* cell walls (VM-NR or VMR, respectively) were used to simulate a mycoparasitic condition. VMS was used as control. Samples were collected after 3, 6, and 24 h of incubation, frozen in liquid nitrogen, and stored at −70°C until used. For analysis of enzymatic activities, the culture filtrate was recovered and frozen at −20°C until used.

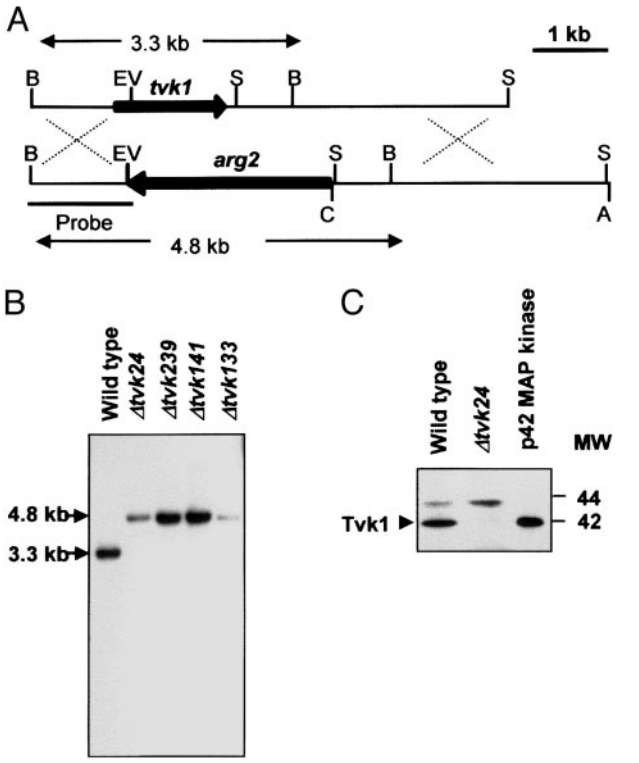

Fig. 1. Strategy for *tvk1* gene disruption and analysis of transformants. (*A*) Schematic representation of Tvk1 replacement. Thick arrows represent the *tvk1* and *arg2* coding regions. Lines represent the 5′ and 3′ regions of the *tvk1* gene. Crossover events are indicated by dotted lines. EV, *Eco*RV; B, *Bam*HI; S, *Sal*I; C, *Cla*I; A, *Apa*I. (*B*) Southern analysis of transformants. Genomic DNA was digested with *Bam*HI. Lanes 2–5 represent four independent transformants; lane 1 is the wild type. The blot was hybridized with the probe indicated in *A*. (*C*) Immunoblot analysis of crude extracts from *T. virens* (wild-type and Δtvk24 strains) with an ERK1/ERK2-specific polyclonal antibody. The arrow indicates the signal corresponding to Tvk1. p42 MAPK is a recombinant MAPK protein used as positive control.

Confrontation Assays. *Trichoderma* strains were subjected to confrontation assays without contact by using *R. solani* as a host, as described by Cortés *et al.* (15). Confrontation was carried out on modified VMS agar (mVMS) containing 0.75 g/liter sucrose and 0.45 g/liter NH_4NO_3. *Trichoderma* mycelia were collected from the zone of interaction between the fungi.

Biocontrol Assays. Assays were conducted as described (8). Briefly, cotton seeds (cultivar 112, Stoneville, Memphis, TN) were coated with *Trichoderma* strains and planted into a nonsterile soilless medium (Metro Mix, 366, Scotts, Marysville, OH) infested with *R. solani* or *P. ultimum*. Seeds planted into noninfested medium and seeds treated with the commercial fungicide Apron XL LS (Syngenta, Guelph, ON, Canada; active against *P. ultimum*) were used as positive controls. Healthy, surviving seedlings were counted after a 10-day incubation at 25°C in a growth chamber. Additionally, the extension of the disease symptoms in the root system was evaluated in *R. solani*-infected plants by using an arbitrary scale of 0 (no symptoms) to 5 (entire root system discolored and decayed) with a maximum of 6 for nongerminated/dead seeds. Each treatment was replicated six times, with 10 seeds each, and the entire experiment was repeated twice.

Results

Isolation of *tvk1*, a Gene Encoding a MAPK from *T. virens*. Southern blot analysis indicated that *tvk1* is present as a single copy in *T. virens*. The cloned *tvk1* gene contains four exons interrupted by three introns as reported for other MAPK encoding genes in fungi (GenBank accession no. AY162318). The deduced protein sequence of the gene has 360 amino acids with an estimated molecular mass of 41.6 kDa and a pI of 6.44. Alignment of the sequence by using the MEGALIGN-CLUSTAL program (DNASTAR, Madison, WI) indicated that Tvk1 corresponds to the recently reported TmkA protein from *T. virens* IMI306092 (16), sharing 98% identity with Tmk1 from *T. atroviride*, 95% identity to Cmk1, Pmk1, and Fmk1 from *F. oxysporum*, *M. grisea*, and *Colletotrichum lagenarium*, respectively (4, 13, 17), and 54% identity with Kss1 from *S. cerevisiae*. The region comprised between residues 58 and 160 contains the typical sequence observed in several members of the MAPK family: F-X (10)-R-E-X (72–82)-R-D-X-K-X (9)-C (18). Tvk1 also contains the residues T (184)-E-Y (186) required for its phosphorylation and activation by a MAP kinase kinase homologue.

Generation of *tvk1* Null Mutants. Disruption of *tvk1* in 20 stable transformants was evaluated by Southern blotting with a 1.4-kb *Bam*HI/*Eco*RV fragment from plasmid pDGX35 as a probe. A 3.3-kb hybridizing fragment was expected in the wild type, a 4.8-kb fragment was expected in null mutants, and both bands were expected in case of ectopic integration events (Fig. 1*B*). Seven transformants with the expected hybridization pattern were identified and further analyzed by Southern blot to verify that no additional integration events had occurred. None of the selected transformants showed additional bands other than that corresponding to the replacement event. Transformants designated Δtvk24 and Δtvk133 were arbitrarily chosen for further phenotypic and physiological studies.

To verify that Tvk1 was not produced in the gene disruptants, total protein extracts from *T. virens* wild type and Δtvk24 strains were probed with a polyclonal antibody that recognizes the p42/p44 MAPKs from mammalian cells (Fig. 1*C*). As expected, both 42- and 44-kDa proteins were detected in the wild-type strain (Fig. 1*C*, lane 1). In contrast, only the signal corresponding to the 44-kDa MAPK was found in Δtvk24 (Fig. 1*C*, lane 2). These results demonstrated that the Δtvk24 strain did not produce a p42 MAPK homologue. Similar results were obtained after the analysis of other gene disruptants.

Tvk1 Mutants Show Altered Vegetative Growth and Conidiation. Colonies of Δtvk1 mutants showed a reduction in the rate of colony growth and development of aerial hyphae on solid media. Conidial suspensions of wild type showed an intense green color compared with the pale green color of suspensions of the mutant conidia. In addition, null mutants produced two times less conidia than the wild type when grown on Potato Dextrose Agar with no significant changes in conidiophore morphology. When growth was analyzed in liquid media, all Δ*tvk1* mutants formed mycelial pellets smaller than those generated by the wild-type strain. Surprisingly, Δtvk1 mutants conidiated massively in late submerged cultures (72 h), whereas no conidia appeared in cultures of the parental strain even after 7 days of culture. Fig. 2*A* shows liquid cultures of *T. virens* wild type (Gv29-8) (leftmost flask), Δtvk1 mutants (Δtvk24 and Δtvk133) (two center flasks), and the parental strain (Tv10.4) (rightmost flask) after 72 h of incubation in VMS. The ability of Δtvk1 mutants to conidiate in liquid culture seems to be independent of the media used, because conidiation was evident either in VMS or Potato Dextrose Broth. Microscopic observations of samples from Δtvk24 and Δtvk133 sporulating liquid cultures showed normal conidiophore development, resembling those produced in aerial hyphae (Fig. 2 *C* and *D*). In contrast, the WT strain did not produce conidia in the same media (Fig. 2*B*).

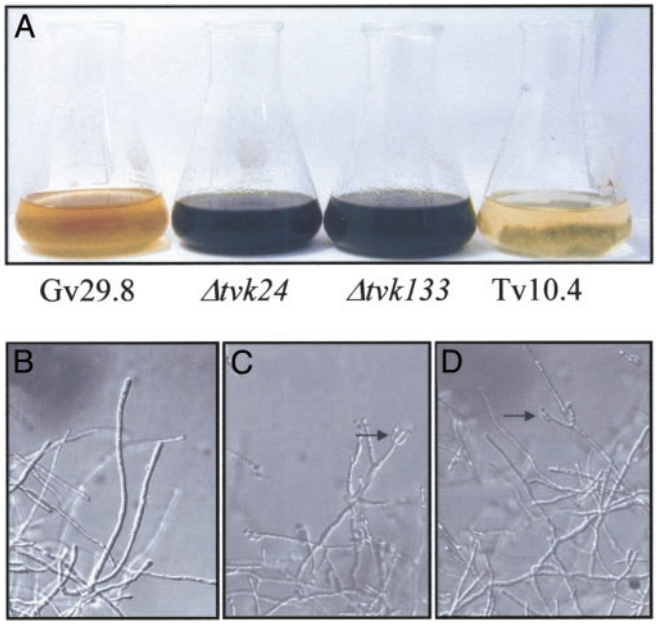

Fig. 2. Conidiation in submerged culture. (*A*) Overview of *T. virens* parental and mutant strains liquid cultures after 72 h in Potato Dextrose Broth. (*B–D*) Microscopic analysis of *T. virens* wild type (Gv29.8) (*B*), Δtvk24 (*C*), and Δ*tvk133* (*D*). Conidiophores are indicated by arrows.

Increased Expression of MRGs in Δtvk24 Under Simulated Mycoparasitism. Northern blot analysis showed that in the wild type the *N*-acetylglucosaminidase-encoding gene *Tv-nag1* was expressed in medium with no carbon source 3 h after transfer but no message was detected after 6 h. Whereas in the mutant the expression was detected after 3 h and increased by 6 h. In medium containing *R. solani* cell walls the gene was expressed only after 6 h for both the wild-type and the mutant strains but reached much higher levels in the mutant (Fig. 3*A*, *Tv-nag1*). The second chitinase-encoding gene analyzed (*Tv-cht1*) was expressed in both strains in the absence of carbon source but reached higher levels in Δtvk24 by 6 h. *Tv-cht1* was clearly induced by cell walls in the wild-type strain with maximum expression by 6 h. In contrast, no obvious induction could be observed in the mutant strain, which reached the same level observed under carbon limitation conditions, except that this occurred earlier (Fig. 3*A*, *Tv-cht1*). The endoglucanase-encoding gene *Tv-bgn2* was induced by cell walls by 6 h, but no difference in expression was observed for the wild type and the mutant, and the gene was not expressed in medium with no carbon source (Fig. 3*A*, *Tv-bgn2*). The pattern of expression of a third chitinase gene (*Tv-cht2*) was similar to that of *Tv-cht1*, except that maximum expression was reached after 24 h under simulated mycoparasitism. Consistent with the previous expression pattern of other MRGs analyzed, Δtvk24 showed a much more pronounced induction of *Tv-cht2* than the wild-type strain. No expression of these genes was detected in minimal media supplemented with primary nitrogen and carbon sources (data not shown).

Expression of the protease-encoding gene, *Tv-prb1*, was determined under simulated mycoparasitism both in the absence of an alternative carbon source (Fig. 3*A*, *Tv-prb1*) and in VM-NR that contains no ammonia (Fig. 3*B*, *Tv-prb1*). Induction by cell walls was observed for both strains, although higher transcript levels were detected under carbon starvation than under nitro-

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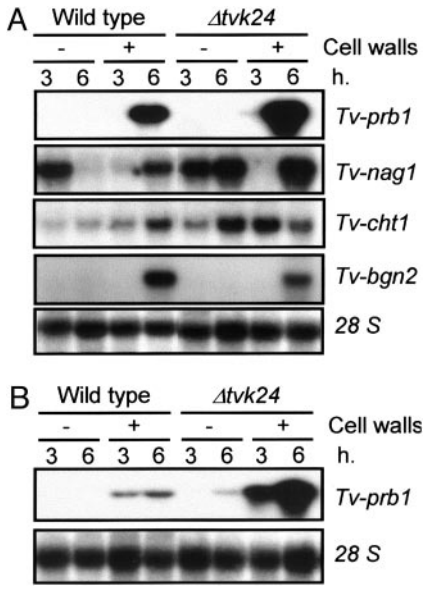

Fig. 3. Expression of MRGs in simulated mycoparasitism. (*A*) Northern analysis of the genes *Tv-prb1*, *Tv-nag1*, *Tv-cht1*, and *Tv-bgn2* under carbon limitation with and without *R. solani* cell walls in the wild type and the Δtvk24 mutant. Numbers indicate time of induction. (*B*) Northern analysis of *Tv-prb1* under nitrogen limitation and simulated mycoparasitism. + and −, presence and absence of *R. solani* cell walls, respectively. Fifteen micrograms of total RNA was loaded in each lane. 28S ribosomal RNA was used as load control.

gen limitation conditions. In both cases, induction was much higher for Δtvk24 than for the wild-type strain. Δtvk24 showed the greatest level of expression after 6 h, 7- to 10-fold higher than the expression in wild type. The mutant showed detectable levels of *Tv-prb1* expression after 6 h when grown in VM-N (Fig. 3*B*, *Tv-prb1*). Accordingly, in-gel protease, endochitinase, *N*-acetylglucosaminidase, and glucanase activity analyses of the culture filtrates showed up to 10-fold higher activity levels in the mutants than in the wild type. Interestingly, the presence of an additional endochitinase activity band in the null mutants under carbon source limitation was observed (Fig. 6, which is published as supporting information on the PNAS web site).

High Levels of MRGs Are Detected in Δtvk24 During Confrontation with *R. solani*. Expression analysis of the same set of genes was carried out in Δtvk24 and wild-type strains during confrontation assays with mVMS plates (Fig. 4*A*). When Δtvk24 overgrew the colony of *R. solani* (TR), a clear induction of *Tv-prb1*, *Tv-nag1*, *Tv-cht1*, and *Tv-bgn2* was detected as compared with the control condition where the mutant strain was growing alone (T) (Fig. 4*B*). Interestingly, there was a detectable level of expression of all genes in the control (T). Transcript accumulation of all MRGs in Δtvk24 was not only increased in the presence of the host but also reached much higher levels than for the wild-type strain. Unexpectedly, at the time point sampled, no difference in the expression of MRGs was detected when the wild-type strain was growing alone (T) or in the presence of *R. solani* (TR) (Fig. 4*B*).

Biocontrol Activity. The biocontrol ability of Δtvk24 and Δtvk133 was tested *in vivo* against two different root pathogens, *R. solani* and *P. ultimum*. Plant mortality was very low in treatments with *R. solani* (10%). Although protection by Δtvk24 and Δtvk133 was higher (mortality 0% and 5%, respectively) than with wild type (mortality 8%), the differences were not statistically significant. However, when the root systems of these plants were evaluated for disease symptoms, significant differences were detected (Fig. 5*A*). The disease index was significantly less for the treatments

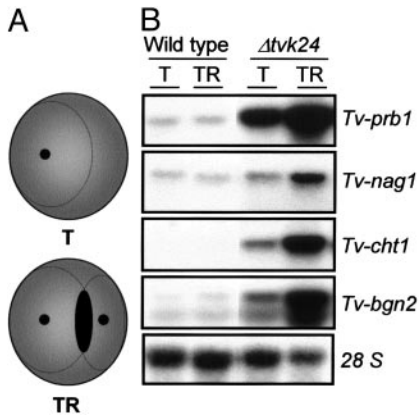

Fig. 4. Expression of MRGs in direct confrontation with *R. solani*. (*A*) Schematic representation of the interaction between *T. virens* strains and *R. solani*. T, *T. virens* strain growing alone in VMS plates; TR, *T. virens* strain growing in presence of *R. solani*. The interaction zone is colored black. (*B*) Expression of MRGs in *T. virens* strains. Samples were collected when *T. virens* strains grew 1.0 cm over the colony of *R. solani*. The probes used for the Northern blot analysis were the same as those indicated in Fig. 3. Fifteen micrograms total RNA were loaded in each lane.

with the Δ*tvk1* mutant strains than for the wild type. When cotton plants were infected with *Pythium*, only 10% of the nontreated seeds survived. However, survival increased to 70% and 80% in treatment with Δtvk24 and Δtvk133, respectively, compared to only 20% in wild-type strain. This level of protection afforded by the two mutant strains was comparable and even significantly higher than the protection obtained by treatment of the seeds with the commercial fungicide Apron (Fig. 5*B*).

Discussion

The morphological and molecular responses observed in *Trichoderma* when confronted by a host depend on the capacity of

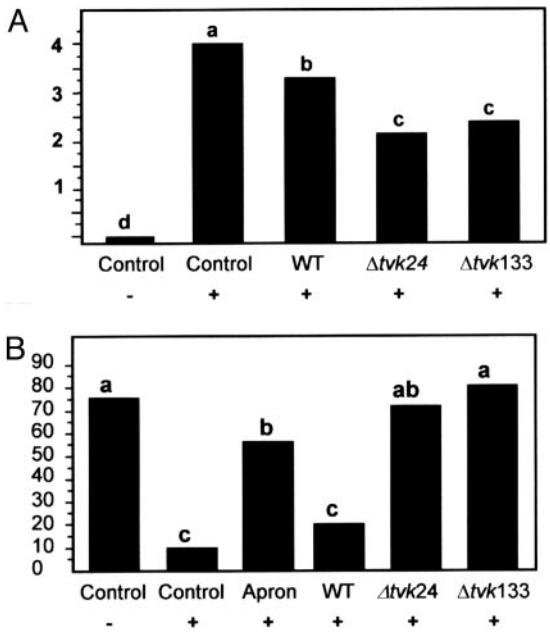

Fig. 5. Biocontrol activity of wild type, Δtvk24, and Δtvk133. (*A*) Disease index of the root systems from cotton plants infected with *R. solani*. (*B*) Percentage of healthy plants after *P. ultimum* treatment. Columns with a letter in common did not differ significantly according to Fisher's protected least significant difference test at a significance level of 5%. + and −, presence and absence of the pathogen, respectively.

Trichoderma to sense and respond to external stimuli and to adjust its intracellular activities accordingly. In this study, we isolated and characterized a MAPK-encoding gene from *T. virens*, *tvk1*. The deduced protein sequence of *tvk1* showed high similarity with other MAPKs reported for phytopathogenic fungi, especially Pmk1 from *M. grisea* (13). The *tvk1* gene contains three intron sequences also reported for other MAPK genes from fungi. Even though *tmkA* and *tvk1* encode the same protein in different strains of *T. virens*, *tmkA* appears to have lost a conserved intron found in diverse genes belonging to Pmk1 homologues in filamentous fungi, including *tvk1* (16), suggesting that *tmk*A from *T. virens* IMI306092 has suffered recent evolutionary changes. Tvk1 belongs to a family of kinases regulated by external signals (extracellular-regulated kinases, or ERKs), which constitute part of the MAPK superfamily. The signature sequence present in the Tvk1 protein indicates that it is related to the YERK1 family (yeast and fungal ERK1) (19).

MAPK pathways have been implicated in controlling cellular growth in a variety of eukaryotic organisms. Tvk1 null mutants showed a reduction in growth rate as reported for *tmkA* mutant strains (16). In addition, Δ*tvk1* mutants produced less conidia than the wild-type strain on solid media (data not shown). Similar morphological alterations have been reported for mutants in homologues of *tvk1* in other fungi. The analysis of MAPK null mutants from *C. lagenarium*, *U. maydis*, and *C. heterostrophus* has established that these *tvk*1 homologues are required for sporulation (17, 20, 21). In contrast, mutants in the corresponding gene in *M. grisea*, *F. oxysporum*, and *B. cinerea* showed no alterations in spore production (4, 5, 13). All Δtvk1 strains analyzed showed reduced conidial pigmentation with loss of the characteristic dark green color observed in the wild type. The pigment associated with the spore surface of *T. viride*, a closely related species to *T. virens*, has been reported to be a nonindolic melanin-like polyphenol (22). In *C. lagenarium*, the expression of genes involved in melanin synthesis is regulated by the Cmk1-MAPK. Mutations in *cmk1* result in the production of albino spores. In *T. virens*, Δtvk1 strains produced spores with decreased pigmentation on solid media, without becoming albinos. In contrast, all Δtvk1 strains produced abundant conidia that developed the characteristic dark green color of the wild type in submerged cultures independently of the media used. These results suggest that Tvk1 may differentially regulate melanin biosynthesis as reported for *C. lagenarium* (17).

In general, filamentous fungi grow by hyphal elongation in submerged cultures, but nutrient depletion may induce conidiation, as in *Neurospora crassa* (23). Growth of Δtvk1 strains under nutrient limitation led to the early production of spores (24 h) when compared with the 72 h required to detect conidia in complete medium. However, no change in vegetative hyphal growth pattern was observed in the wild-type strain when grown under starvation conditions. Additionally, sporulation in Δtvk1 strains is completely dependent on the availability of nutrients, because continuous replacement of fresh medium to the culture blocked conidial formation.

As a mycoparasite, *Trichoderma* depends on the production of hydrolytic enzymes for the colonization of host fungi. Recently, Xu (3) suggested that Pmk1 and its homologues might be involved in the positive regulation of the expression of cell wall-degrading enzymes. In support of this hypothesis, a Δkss1 mutant of *S. cerevisiae* and a Δbmp1 mutant of *B. cinerea* were unable to trigger the expression of the endopolygalacturonases PGU1 and BCPG1, respectively (5, 24), and Δfmk1 mutants of *F. oxysporum* showed considerably reduced transcript levels of *pl1*, a gene encoding pectate lyase (4). To test this hypothesis for a mycoparasite, we analyzed the expression of lytic enzymes associated with the parasitic process in the wild type and the Δ*tvk1* mutants of *T. virens*. Unexpectedly, Δtvk24 highly expressed most of the lytic enzyme-encoding genes selected as compared with the wild-type strain. These results are in clear contrast to the hypothesis suggested by Xu (3). However, *mpk4* null mutants from *Arabidopsis thaliana* showed constitutive overexpression of several chitinase and glucanase genes, suggesting a negative regulation role for this MAPK in the expression of the genes (25). In contrast, Δtvk1 mutants still require nutrient limitation combined with the presence of cell walls to express the hydrolytic enzymes. Among the MRGs analyzed in the Δtvk24 strain, the expression pattern of the *Tv-prb1* gene illustrated several interesting features. In *T. atroviride prb1* is subjected to nitrogen catabolic repression (NCR) as well as being induced by the presence of *R. solani* cell walls, but this response is completely dependent on the absence of a primary nitrogen source (6). Interestingly, the expression of *Tv-prb1* seems to be equally dependent on NCR in both wild-type and Δtvk24 strains, because no transcript was detected when the strains were grown in VMSR. *Tv-prb1* expression associated with the sole lack of a nitrogen source was detected for the Δtvk24 mutant, whereas for the wild-type strain no signal of the corresponding mRNA was observed (Fig. 4*B*). Thus, Δtvk1 mutants seem to be more sensitive to nitrogen limitation than the wild type in terms of *Tv-prb1* expression. These data suggest that Tvk1 may be involved in nitrogen repression of *Tv-prb1*. Recently, we (6) proposed a positive role for a phosphorylated MAPK similar to either Kss1 or Slt2 on the expression of *prb1* under similar growth conditions in *T. atroviride*. In contrast, in this study we showed that Tvk1 acts as a negative element in the expression of *Tv-prb1*, the *T. virens* homologue of *prb1*, under nitrogen limitation or simulated mycoparasitism. This apparent contradiction may be explained by a differential regulation depending on the state of phosphorylation of the kinase, as in the case of Kss1 in *S. cerevisiae*, which has inhibiting and activating functions depending on the phosphorylation status of the protein (26). On the other hand, expression of the chitinases *Tv-nag1* and *Tv-cht1* driven by the absence of carbon source seems to be more pronounced in Δtvk24. Overall, regulation of MRGs in *T. virens* appears to be complex, but it seems clear that they share common elements including Tvk1.

In contrast with the recently suggested decrease in the production of chitinase and cellulase activity in ΔtmkA mutants (16), chitinase and protease in-gel activities were higher in Δtvk24 than in the wild type, correlating with the elevated transcript levels. Furthermore, even though expression of *Tv-bgn2* was not increased, total β-1,3-glucanase activity did notably increase in Δtvk1 mutants as compared with the wild type. This suggests that additional glucanase genes could be under negative modulation by Tvk1. The observed increase in the production of enzymes correlated with an increase in secretion of proteins in liquid media (seven times more in Δ*tvk1* mutant strains in VM-NR and four times more in VMR).

In direct confrontation assays of *Trichoderma* with a host, expression of *Tv-prb1*, *Tv-nag1*, *Tv-gln2*, and *Tv-cht1* strongly increased compared with *Trichoderma* growing alone in the Δtvk1 mutants (Fig. 4). Surprisingly, in the wild-type strain no induction of MRGs was detected in the presence of *R. solani*. Host-mediated induction in the wild type may be delayed compared to that observed for null mutants. The detectable level of transcription of the genes when Δtvk24 grew alone may be indicative of derepression, driven by carbon and/or nitrogen limitation. The facts that in Δtvk1 mutants all MRGs followed the same pattern of expression in the direct confrontation assays and that this expression reached very high levels suggest that Tvk1 plays a major role in the coordinated regulation of MRGs during the actual *Trichoderma*–host interaction.

Considering the increased production of lytic enzymes observed in the *T. virens* Δtvk1 strains and the relevance of these enzymes in the biocontrol activity of *Trichoderma*, we examined the potential use of the mutants generated as biocontrol agents.

We hypothesized that the robust induction of lytic enzymes in the Δtvk1 strains might lead to a more effective biocontrol. The null mutants showed a greater capacity to control and reduce damage by *R. solani* and *P. ultimum* when compared to the wild-type strain. *T. virens* has been successfully used in combination with several fungicides including metalaxyl (Apron FL) (27). Surprisingly, Δtvk1 strains were more effective than metalaxyl against *P. ultimum* (Fig. 5*B*). This enhanced biocontrol ability appears to be associated with the high level of expression of lytic enzymes observed in direct confrontation assays because both strains formed hooks and coils around *R. solani* and produced antibiotics to the same extent. These results point in the opposite direction of those recently reported by Mukherjee and coworkers (16), who suggested that deletion of *tmk*A reduces the biocontrol efficacy of *T. virens*, based on direct confrontations assays. This apparent contradiction may be explained by the fact that their experiments were carried out on Potato Dextrose Agar medium, where expression of most MRGs is repressed because of the high levels of glucose present in that medium (9). In fact, similar experiments carried out by our group with several phytopathogenic fungi, including *Sclerotium rolfsii* and *R. solani*, indicated varying behavior of the mutant as compared to the wild type, going from slightly decreased overgrowth capacity to increased growth inhibition, depending on the host (Fig. 7, which is published as supporting information on the PNAS web site). These data suggest that *Trichoderma* uses different mechanisms to control different hosts. An additional consideration that must be made is that some *Trichoderma* species are capable of inducing defense responses in plants, as shown for *T. virens* (28). To the best of our knowledge, this is the first report indicating that the deletion of a MAPK gene generates a more aggressive parasite and, consequently, a better biocontrol agent.

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