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T. L. Buhr · S. Oved · G. M. Truesdell · C. Huang
O. Yarden · M. B. Dickman

A kinase-encoding gene from *Colletotrichum trifolii* complements a colonial growth mutant of *Neurospora crassa*

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Abstract *Colletotrichum trifolii* is a fungal pathogen which is responsible for anthracnose disease of alfalfa. To initiate research on molecular communication in this fungus, a kinase-encoding gene (*TB3*) and the corresponding cDNA were cloned and sequenced. The deduced amino acid sequence of *TB3* closely resembles that of a *Neurospora crassa* serine/threonine protein kinase, COT1, required for hyphal elongation and branching. The C-terminal catalytic domains of TB3 and COT1 are highly conserved but the N-terminal regions are divergent, particularly in the homopolymeric glutamine repeats of TB3. Northern analysis indicated that *TB3* expression was highest 1 h after inducing conidial germination and 1 h before germ tubes were first observed. Expression of TB3 transcripts returned to constitutive levels by 4 h after induction of germination. *TB3* complemented the *cot-1* mutant of *Neurospora crassa*, demonstrating the functional conservation of this kinase between a pathogenic and a saprophytic fungus.

Key words Protein kinase · Germination · Fungal pathogen

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T. L. Buhr · G. M. Truesdell · C. Huang · M. B. Dickman
406 Plant Sciences Hall, Department of Plant Pathology,
University of Nebraska-Lincoln, Lincoln, NE 68583-0722, USA

S. Oved · O. Yarden
Department of Plant Pathology and Microbiology
and the Otto Warburg Center for Agricultural Biotechnology,
The Faculty of Agriculture, The Hebrew University of Jerusalem,
Rehovot 76100, Israel

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Introduction

Pathogenicity of *Colletotrichum trifolii*, the causal agent of alfalfa anthracnose (Barnes et al. 1969), depends on cellular growth and differentiation (Dickman et al. 1995). In fungal species like *C. trifolii* mechanisms exist that regulate quiescence in spores and subsequent germination, proliferation and the establishment of a pathogenic relationship.

Hyphae appear following conidial germination and elongate from the tip. Hyphae account, in part, for the success of fungi both as saprophytes and pathogens, since the exploratory ends can forge through solid substrates (including plant tissue), spread and assimilate nutrients.

Hyphal growth is of interest in terms of the processes by which cell polarity is established and maintained. In addition, the behavior of hyphae can be modified by a variety of stimuli, resulting in the production of structures such as spores or appressoria (Emmet and Parberry 1975; Staples and Hoch 1987). Such differentiation processes are governed by gene expression. However, our understanding of genes governing hyphal growth and morphogenesis is rudimentary.

Coordinated control of cell growth and differentiation in eukaryotes is achieved in part through the activation of intracellular communication networks in response to external stimuli. Protein kinases represent integral components of signal transduction pathways (Edelman et al. 1987) in species ranging from mammals to yeast and bacteria (Hanks and Quinn 1991; Hunter 1987). Successful functional interchange of kinase genes among organisms (e.g. King et al. 1990; Neimann 1993) illustrates the conservation of these genes.

To examine molecular signaling in *C. trifolii*, a kinase gene was cloned and characterized. Here we report the cloning, sequence and expression pattern of this

gene during hyphal elongation. The structural and functional conservation of this kinase between the saprophyte *Neurospora crassa* and the phytopathogen *C. trifolii* is also demonstrated.

Materials and methods

Strains and plasmids

The wild-type strains of *C. trifolii*, race 1 and race 2, used in this work were isolated from alfalfa cultivars saranac and arc, respectively. *C. gloeosporioides* forma specialis (f. sp.) *aeschynomene* strain 3.1.3 was isolated from Northern jointvetch and was provided by Dave TeBeest, University of Arkansas. *N. crassa* strains used included wild-type 74-ORS-1A (FGSC 987) or 74-ORS-6a (FGSC 4200) and *cot-1* (FGSC 4065). DNA was subcloned in *Escherichia coli* strain XL-1 with the plasmid vectors pBluescript KS+ and KS−. Single-stranded DNA was generated with helper phage VCS-M13.

Media and culture conditions

YpSs (Tuite 1969) agar plates were inoculated with spore suspensions of *Colletotrichum* strains stored at −70° C. Cultures were grown at 24° C with a 12-h photoperiod using white fluorescent light. For DNA isolation, 250 ml of YpSs liquid media was inoculated with approximately 0.5 cm^2 plugs of mycelia from YpSs agar plates. Cultures were grown for several days at room temperature on a rotary platform at 100 rpm. To obtain vegetative mycelia, cultures were grown without agitation for 1–2 weeks.

N. crassa growth studies and crosses were performed as described by Davis and de Serres (1970). Cultures were maintained on 1.5% agar slants containing Vogel's minimal medium N (Vogel 1956). When appropriate, the medium was supplemented with hygromycin B (Calbiochem or Boehringer Mannheim) at 100 μg/ml. DNA-mediated transformation of *N. crassa* was carried out as described by Orbach et al. (1986).

E. coli cultures were grown on Luria-Bertani agar or 2 × YT media Sambrook et al. 1989). Selective medium contained 100 μg carbenicillin or ampicillin/ml, 10 μg tetracycline/ml or 25 μg kanamycin/ml as necessary.

Nucleic acid isolation

Colletotrichum genomic DNA was isolated as described (Panaccione et al. 1988), except that total DNA was purified in a single cesium chloride gradient. Total RNA from vegetative mycelia was purified according to published procedures (Cathala et al. 1983). RNA for Northern blots were purified with Trizol (Gibco BRL) according to the manufacturer's instructions. Plasmids and phage DNA were isolated as described (Sambrook et al. 1989).

Two-day-old mycelial cultures, grown in 25 ml Vogel's N medium and collected by filtration on Whatman No.1 filter paper in a Buchner funnel, were the source for *N. crassa* genomic DNA. Samples were frozen in liquid nitrogen and lyophilized. Dried samples were powdered by grinding and suspended in an equal volume of lysis buffer (0.50 mM TRIS-HCl pH 8.0, 50 mM EDTA, 2% SDS, 1% 2-mercaptoethanol) containing 25 μg/ml RNase A. Following a 30-min incubation at 37° C, 100 μg/ml proteinase K (Boehringer Mannheim) was added to the solution and incubation was continued for 1 h at 65° C. Two phenol/chloroform (1 : 1) extractions were followed by a chloroform extraction, isopropanol precipitation and a 75% ethanol wash. The DNA pellet was dried and dissolved in TE buffer (Sambrook et al. 1989).

Construction of libraries, isolation of clones, and sequencing

A genomic library of *C. trifolii* race 1 was constructed in the vector EMBL3 (Frischauf et al. 1983). Genomic DNA was partially digested with *Sau*3A and size-fractionated on sucrose gradients. The 15- to 20-kb fragments were ligated with *Bam*HI-digested EMBL3 arms and the ligation products were packaged in vitro by incubation with bacteriophage lambda packaging extracts (Stratagene). The resulting library was transfected into *E. coli* strain P2392. Plaque hybridization was performed by the method of Benton and Davis (1977). Two degenerate oligonucleotides specific to conserved catalytic domains of serine/threonine protein kinases were obtained from Mike Lawton (Rutgers University, Piscataway, N.J.). The oligonucleotides were GGYTTNAGRTCNC(G/T)RT and TCNGGNGC(T/R)ATRTARTC encoding the (H/Y)RDLKP and DYIAPE peptide motifs, respectively. Plaque filters were sequentially screened with each end-labeled oligonucleotide as a probe, essentially as described by Hanks (1987). Subcloning involved standard procedures (Sambrook et al. 1989). Deletions were constructed by restriction endonuclease digestion and Klenow end-repair, followed by ligation and transformation. DNA was sequenced using the dideoxy chain-termination method (Sanger et al. 1977) using three-lane sequencing as described by Nelson et al. (1992). Oligonucleotides were synthesized on an Applied Biosystems nucleic acid synthesizer to sequence DNA in gaps. All enzymes were used according to manufacturer's specifications.

Construction of a cDNA library began with mRNA isolation from *C. trifolii* race 1 vegetative mycelia. PolyA RNA was isolated with Dynabeads (Dynal) according to the manufacturer's directions. cDNA was synthesized with random hexanucleotide and oligo-dT primers using the cDNA synthesis system (Gibco BRL). Oligonucleotide adapters containing *Eco*RI, *Not*I and *Sal*I restriction sites were ligated to both cDNA ends. *Eco*RI-digested cDNA fragments were ligated into *Eco*RI-digested λgt11 arms and packaged in vitro as previously described. Plaque hybridizations were performed using random primer-labeled (Feinberg and Vogelstein 1983) pTB3 as a probe.

Digested *Colletotrichum* DNA samples (0.75 μg/lane) were electrophoresed on 0.8% agarose gels in 0.5 × TBE buffer (Sambrook et al. 1989) and transferred to nylon membranes with 0.4 M sodium hydroxide by the method of Southern as described by Sambrook et al. (1989). A *TB3* probe was labeled with [α^{32}P]dGTP using the random priming method of Feinberg and Vogelstein (1983). Hybridization was carried out using 25 ng probe in 10 ml of hybridization solution [5 × SSPE, 5 × Denhardt's (Sambrook et al. 1989), 0.5% SDS, 20 μg single-stranded salmon sperm DNA/ml] at 65° C for 20 h. Filters were washed at high stringency including two washes in 0.2 × SSPE, 0.1% SDS at 65° C for 20 min each. Filters were exposed to Kodak X-omat film at −70° C using intensifying screens.

Northern hybridization

Aliquots (10 μg) of RNA were mixed with 0.48 μg ethidium bromide, 1 μl formamide and 1 μl 10 × MOPS buffer [0.2 M 3-(N-morpholino) propanesulfonic acid, 80 mM sodium acetate pH 7, 10 mM EDTA, 3 M formaldehyde) in 10 μl volumes. Samples were heated at 75° C for 5 min prior to adding 1.1 μl 10 × loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficoll, 10 mM EDTA) and loading on 1% agarose gels in 1 × MOPS buffer. Gels were run at 3 V/cm for 3.5 h in 1 × MOPS buffer. Equivalent sample loading was confirmed by visually comparing ethidium bromide staining between samples. Gels were soaked twice in 2 × SSPE for 15 min each. RNA was blotted onto MagnaGraph nylon filters (MSI) with 20 × SSPE for 16 h and subsequently fixed by ultraviolet irradiation. Blots were hybridized in 0.25 M dibasic sodium phosphate pH 7.4, 7% SDS, 2% blocking reagent (Boehringer Mannheim) and 1 mM EDTA. A *TB3* probe was labeled with digoxigenin-dUTP (Boehringer Mannheim) using the polymerase

chain reaction to produce a cDNA probe encompassing the 5′ region from nucleotides 43–970. A 1.4-kb *Bam*HI-*Eco*RI fragment encoding 17S ribosomal DNA from *N. crassa* (Free et al. 1979) was labeled with digoxigenin-dUTP by random priming (Feinberg and Vogelstein 1983). Hybridizations included 25 ng probe in 10 ml solution at 65° C for 20 h. Filters were washed at high stringency including two washes in 0.2 × SSPE, 0.1% SDS at 65° C for 20 min each. Procedures for digoxigenin detection with the chemiluminescent substrate Lumi-Phos 530 were according to the manufacturer's (Boehringer Mannheim) instructions. Filters were exposed to Kodak X-omat film 14 h after adding the chemiluminescent substrate. Exposure was at room temperature for 90 min with the *TB3* probe and 5 min with the 17S rDNA probe. *TB3* RNA signals were normalized relative to ribosomal RNA signals after computer imaging and analysis using Collage software by Fotodyne.

Sequence analysis

Programs from the University of Wisconsin Genetics Group (Devereux et al. 1984) and MacVector (International Biotechnologies) were used for nucleotide and amino acid sequence analysis. The GenBank accession number for *TB3* is U14989.

Results

Cloning and sequence of *TB3*

A genomic phage library of *C. trifolii* race 1 was sequentially screened with two degenerate oligonucleotide probes designed from serine/threonine kinase-specific motifs. Sequential probing of plaque filters with two oligonucleotides, instead of the use of a single oligonucleotide probe, greatly reduced the number of false positives. Several plaques hybridized to both probes and one strongly hybridizing plaque was isolated. DNA purified from this clone was digested with several restriction enzymes. Subsequent Southern hybridizations revealed hybridizing fragments of 4.8 kb after digestion with *Pst*I and 3.3 kb after digestion with *Mlu*I/*Pst*I. The 4.8-kb fragment was subcloned into *Pst*I-digested pBluescript KS+. This clone was digested with *Bam*HI/*Mlu*I, end-repaired with Klenow, and religated to produce pTB3.

Both strands of the 3.3-kb *Mlu*I-*Pst*I fragment were sequenced (Fig. 1). Several open reading frames (ORFs) were identified including one beginning with ATG and flanked by consensus sequences for fungal translation initiation (Ballance 1986). A *C. trifolii* cDNA library was screened using pTB3 as a probe. A single hybridizing plaque was isolated. The cDNA insert was subcloned into pBluescript KS+ and KS− at the *Eco*RI site. One strand of the cDNA clone was completely sequenced to verify intron positions and identify the 3′ end of *TB3*.

The genomic and cDNA sequences for *TB3* are combined in Fig. 1. The cDNA sequence for *TB3* extends from nucleotide −20 to nucleotide 2627. *TB3* contains three small introns, each with internal guide sequences and intron/exon borders (Fig. 1) conserved among fungal introns (Ballance 1986). Splicing of the intervening sequences produces a single 1995-nucleotide ORF. TB3 encodes a 665-amino acid polypeptide with a predicted molecular weight of 76000 and an estimated pI of 8.9.

TB3 is a single-copy gene

Restriction endonuclease-digested genomic DNA from *C. trifolii* race 1 and race 2 and *C. gloeosporioides* f. sp. *aeschynomene* was separated on agarose gels, transferred to a nylon membrane, and probed with radiolabeled pB2S (Fig. 2), which contains the entire catalyic domain (nucleotides 436–2873, Fig. 1) of *TB3*. As expected, a single *Pst*I fragment of 4.8 kb and a *Bam*HI fragment of 18 kb from *C. trifolii* race 1 and race 2 DNA hybridized at high stringency (Fig. 2). Hybridization with DNA from the related fungus *C. gloeosporioides* f. sp. *aeschynomene* produced a single, hybridizing, polymorphic *Pst*I fragment under high stringency conditions.

TB3 is a kinase-encoding gene, similar to *N. crassa cot-1*

A database search showed that the deduced amino acid sequence of TB3 is markedly similar to that of a *N. crassa* protein kinase, COT1 (Yarden et al. 1992). (Fig. 3). Alignment of TB3 and COT1 showed that the C-terminal catalytic domains are highly similar but the N-terminal domains are divergent. The two domains are delimited by a specific arginine residue (amino acid 237) shown in Fig. 3. Interestingly, intron 1 of *TB3* separates the nucleotide sequences coding for the N-terminal and C-terminal domains at arginine residue 237 (Figs. 1 and 3). Both proteins contain conserved serine/threonine kinase motifs (Hanks et al. 1988), including subdomain I (amino acids 288–296), involved in nucleotide binding, subdomain II (amino acids 309–313), a phosphotransferase site, subdomains VI (amino acids 403–411) and VIII (amino acids 487–496), which predict serine/threonine specificity, subdomain VII (amino acids 423–425), involved in ATP binding, and subdomain IX (amino acids 507–513), which has an unknown role (Fig. 3).

Comparison of *TB3* and *cot-1* showed 65.6% nucleotide identity. Comparing the two genes beginning at arginine codon 237 showed 79.6% nucleotide identity. A comparison of the deduced amino acid sequences showed 70.4% identity in the overall sequence between TB3 and COT1 proteins (Fig. 3). There is 90.4% amino acid identity between the domains beginning at arginine residue 237.

TB3 possesses two glutamine stretches in the N-terminal domain that are absent in COT1 (Fig. 3). This 5′ region contains tracts of 10 and 14 consecutive glutamines; overall 32 of 43 amino acid residues are glutamine.

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ACGCGTACTGTTCATTAGTGCCAGCGGGTTGCGAGGAGTGCCATCCCATCCACCCGCCCACCGCAATCTATTCTCGAGATTCCGCCCCGGCGCATCAGCC    -364
CAAATTATCCAACCTCCCGCCTTGACCCTGCCACAACCTGTACACGCCTTAGCTGCTTTTCCCCGCCGTTTACCTACGGTAGCACATTAGCTTCCACCGC    -264
ACAGCGCAGCAGCACCGCACCTCACGACAACCCGACGAACCGTCAACTGTCGATTTGCTCCGCTCGACTTGGCTTGGCTTGCTCGTAGCCACTTCGTCCC    -164
TATTCTGCACTACCTGCGCTCCACGGCTACCTAGCTGCAAGCCCGGCACTGTCAATCAAAGTGCCACTTTGGTAGAGGAGACACCCGCACAGCCCTCACA     -64
ACCCCTTAGGGACTCGCACACCGCGTCCACGGCTCCCCCGAGTCAAGACGAAACCCTTCCACG ATG GAT AAC AAT AAT AAC CGC CTC TAC       27
                                                                 Met Asp Asn Asn Asn Asn Arg Leu Tyr
CTC AAC ATC GGG AAC AAC AAC GAC CGC CTG GGC CCT GGC AGC GAC CGT CAA TAT CCC ACC ACC CCC TCC ACC TTT      102
Leu Asn Ile Gly Asn Asn Asn Asp Arg Leu Gly Pro Gly Ser Asp Arg Gln Tyr Pro Thr Thr Pro Ser Thr Phe
CCC CAG CCC GTC TTC CCT CAT CAA GGC CAG CAG CAG CAG CAG CAG CAG CAG CAG CAA CTG CAT CAC CAA CAA CAG      177
Pro Gln Pro Val Phe Pro His Gln Gly Gln Gln Gln Gln Gln Gln Gln Gln Gln Leu His His Gln Gln Gln
CCC GGT ATG CAG CAC CCT CAG CAG TAC CAG GCC CAG CAG CAG CAG CAA CAG CAG CAA CAG CAG CAG CAG CAG CAG      252
Pro Gly Met Gln His Pro Gln Gln Tyr Gln Ala Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln Gln
CCT TAT CAG ACG GGC TAC GCA CCT TCT GGA TAC TTC AAC CCC AAC CAG CAG GCG GCA CAG TAT CCT CCT CAG GGC      327
Pro Tyr Gln Thr Gly Tyr Ala Pro Ser Gly Tyr Phe Asn Pro Asn Gln Gln Ala Ala Gln Tyr Pro Pro Gln Gly
CAT GGT GAC TAC AAT GCT GCC TAC CAG CCG AGA TCC AAC ACC CCT GGG ACT AAT GAT CCC AAT GTC GGC CTC GCC      402
His Gly Asp Tyr Asn Ala Ala Tyr Gln Pro Arg Ser Asn Thr Pro Gly Thr Asn Asp Pro Asn Val Gly Leu Ala
CAT CAG TTC TCC CAC CAG AAC CTA GGC GGT GCT GCC CGG GCA TCT CCC TAT GGT TCT CGC GGC CCC TCT CCC GGC      477
His Gln Phe Ser His Gln Asn Leu Gly Gly Ala Ala Arg Ala Ser Pro Tyr Gly Ser Arg Gly Pro Ser Pro Gly
CAG CGA CCT CGT ACC GCC GGT GCC TCC GGT CAA CCT CCC TCT GGA TAC GGT CAC TAT GCG ACG CCC CCT CTC CCG      552
Gln Arg Pro Arg Thr Ala Gly Ala Ser Gly Gln Pro Pro Ser Gly Tyr Gly His Tyr Ala Thr Pro Pro Leu Pro
AAC CAG CAG CCC GCC TCC GTC GAT CCC TTC GCT CCG GCC CCC GAG CGC AAT TAC GAA AAA TAC GGC CCC AAT GCC      627
Asn Gln Gln Pro Ala Ser Val Asp Pro Phe Ala Pro Ala Pro Glu Arg Asn Tyr Glu Lys Tyr Gly Pro Asn Ala
AAC GGC AAC CAG AAG AAG TGC ACT CAA TTG GCG TCG GAC TTC TTC AAG GAC AGT GTG AAG CGC GCC CGT GAA AGA      702
Asn Gly Asn Gln Lys Lys Cys Thr Gln Leu Ala Ser Asp Phe Phe Lys Asp Ser Val Lys Arg Ala Arg Glu Arg
AAC CAG AG GTGGGTACACCGACGGCACCTCGTCGTCACGACAATGAGCTAACGCTTCGGCGTGCAG A CAA AGC GAG ATG GAG GCG AAG      790
Asn Gln Ar                                                                 g Gln Ser Glu Met Glu Ala Lys
CTC TCC GAG CCC AAC CAA AGC CAG TCG AGG CGG GAG CAG ATC TGG TCC ACC GCC GGC CGC AAG GAG GGC CAG TAC      865
Leu Ser Glu Pro Asn Gln Ser Gln Ser Arg Arg Glu Gln Ile Trp Ser Thr Ala Gly Arg Lys Glu Gly Gln Tyr
CTG CGC TTC CTG CGC ACC AAG GAC AAG CCC GAG AAC TAC AAT ACC GTC AAG ATC ATC GGA AAG GGA GCT TTC GGT      940
Leu Arg Phe Leu Arg Thr Lys Asp Lys Pro Glu Asn Tyr Asn Thr Val Lys Ile Ile Gly Lys Gly Ala Phe Gly
GAG GTT AAG CTT GTC CAG AAG AAG GGC GAC GGT AAG GTC TAC GCC ATG AAG TCT CTG ATA AAG ACG GAG ATG TTC     1015
Glu Val Lys Leu Val Gln Lys Lys Gly Asp Gly Lys Val Tyr Ala Met Lys Ser Leu Ile Lys Thr Glu Met Phe
AAA AAG GAC CAG CTG GCC CAT GTC CGC TCT GAG CGT GAT ATC CTG GCC GAA TCC GAC AGT CCG TGG GTT GTT AAG     1090
Lys Lys Asp Gln Leu Ala His Val Arg Ser Glu Arg Asp Ile Leu Ala Glu Ser Asp Ser Pro Trp Val Val Lys
CTC TAC ACA ACG TTC CAG GAT TCG TAC TTC CTC TAC ATG TTG ATG GAG TTC TTG CCG GGA GGT GAT CTT ATG ACC     1165
Leu Tyr Thr Thr Phe Gln Asp Ser Tyr Phe Leu Tyr Met Leu Met Glu Phe Leu Pro Gly Gly Asp Leu Met Thr
ATG CTC ATC AAG TAC GAA ATC TTT TCT GAG GAC ATC ACC CGA TTC TAC ATT GCC GAG ATT GTT CTC GCG ATT GAG     1240
Met Leu Ile Lys Tyr Glu Ile Phe Ser Glu Asp Ile Thr Arg Phe Tyr Ile Ala Glu Ile Val Leu Ala Ile Glu
GCG GTC CAT AAG CTG GGC TTC ATC CAT CG GTAGGTGGAACGAAAGCTTCGAATCAACGGGGCCATGCTGACAGACGCAG T GAT ATC     1326
Ala Val His Lys Leu Gly Phe Ile His Ar                                                     g Asp Ile
AAA CCC GAC AAC ATC CTG CTC GAC CGC GGC GGG CAC GTG AAG CTG ACC GAT TTT GGT CTT TCC ACC GGC TTC AAC     1401
Lys Pro Asp Asn Ile Leu Leu Asp Arg Gly Gly His Val Lys Leu Thr Asp Phe Gly Leu Ser Thr Gly Phe Asn
CGC CTG CAT GAC AAC AAC TAC TAC CAA CAG TTG CTT CAG GGT CGG TCT AAC AAA CCA CGA GAT CGC AAC TCA GTC     1476
Arg Leu His Asp Asn Asn Tyr Tyr Gln Gln Leu Leu Gln Gly Arg Ser Asn Lys Pro Arg Asp Arg Asn Ser Val
GCC ATC GAT CAG ATC AAC CTC ACC GTC AGC AAC CGC TCG CAG ATC AAC GAC TGG CGT CGG TCG AGG AGA TTG ATG     1551
Ala Ile Asp Gln Ile Asn Leu Thr Val Ser Asn Arg Ser Gln Ile Asn Asp Trp Arg Arg Ser Arg Arg Leu Met
GCG TAC TCT ACC GTT GGT ACC CCG GAT TAC ATT GCA CCA GAG ATT TTC ACC GGT CAC GGC TAC ACC TTT GAC TGC     1626
Ala Tyr Ser Thr Val Gly Thr Pro Asp Tyr Ile Ala Pro Glu Ile Phe Thr Gly His Gly Tyr Thr Phe Asp Cys
GAT TGG TGG TCG CTG GGA ACC ATC ATG TTC GAG TGC TTG GTC GGA TGG CCT CCC TTC TGC GCC GAG GAC AGC CAC     1701
Asp Trp Trp Ser Leu Gly Thr Ile Met Phe Glu Cys Leu Val Gly Trp Pro Pro Phe Cys Ala Glu Asp Ser His
GAC ACG TAT CGC AAG ATT GTC AAC TGG AGG CAG ACG CTG TAC TTC CCC GAT GAC ATC CAG CTC GGT GTC GAG GCC     1776
Asp Thr Tyr Arg Lys Ile Val Asn Trp Arg Gln Thr Leu Tyr Phe Pro Asp Asp Ile Gln Leu Gly Val Glu Ala
GAG AAC CTG ATT CGC AG GCAAGTTTGACGTCTCGTTTGGCCTAGGACTGCCCAAGCTAACAGTAGCAG C CTT ATC TGC AAC ACC GAG     1863
Glu Asn Leu Ile Arg Se                                                         r Leu Ile Cys Asn Thr Glu
AAC CGT CTT GGC CGC AGT GGC GCC CAC GAG ATC AAG GCT CAC TCC TTC TTC CGC GGC GTC GAA TTT GAC AGC CTG     1938
Asn Arg Leu Gly Arg Ser Gly Ala His Glu Ile Lys Ala His Ser Phe Phe Arg Gly Val Glu Phe Asp Ser Leu
CGC CGC ATC AGA GCT CCT TTC GAG CCG CGC CTC ACT TCT GCC ATC GAT ACC ACG TAC TTC CCC ACC GAC GAG ATC     2013
Arg Arg Ile Arg Ala Pro Phe Glu Pro Arg Leu Thr Ser Ala Ile Asp Thr Thr Tyr Phe Pro Thr Asp Glu Ile
GAT CAG ACC GAC AAC GCC ACC GTG CTC AAG GCA CAG GCT ATC CAA CAG GCG CGC TCT GGC ATC CCC CAG GTG GAG     2088
Asp Gln Thr Asp Asn Ala Thr Val Leu Lys Ala Gln Ala Ile Gln Gln Ala Arg Ser Gly Ile Pro Gln Val Glu
GAG TCG CCC GAG ATG AGC TTG CCC TTC ATC GGC TAC ACA TTC AAG CGT TTC GAC AAC AAC TTC CGT TGA TGTGCTGG     2165
Glu Ser Pro Glu Met Ser Leu Pro Phe Ile Gly Tyr Thr Phe Lys Arg Phe Asp Asn Asn Phe Arg End
GGCCGCGCTGGTGGCGTGCGTATTGGCCTGGAGTCGATCGCGCCGAAACGATGGCCGGGTCGCCACCGACTTGGTACTCGATTTTGCTTACCGCCAACCG     2265
AACCAGGTTCATGTTACCAAGACTTGCATTTCGTTTTTGTTTTCACCAACCACTGCATAAAGGGCGTTGACTCGCATCAAGACACAAACAAAATCGCACT     2365
ATCCGACACGTAGATGGAAAGACAGGCTGAGTCGTCGCCTGGAGCCCTGGTCATAGGTTGATGGGTGATGGACGGCTAGAGGTTCATTACATTCTCACGA     2465
GCAGCATGGTGGGCCATAGTGGAAGAGGAAGAGGTGGTCAGGATCTAGTCGTAGTGCTGGATCTAGGTCGTGGATGCAGATGTAAAATTCAGGAGACACG     2565
AGGACTGAAGCGGCAGTTGGGCGATAGGATGCACTCAGATAATGCATTGAGACCAAGTCAAATCTGGTATCATTGATGTGCCGTCAAATCCTATGTGATT     2665
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CTTGCCACCCTCCCATGATTTTATATTGTCTATGCCTCACCTGCGGGAGCTCAAGCCCGGCGCGGCCACGTACCGCTCTTCTCTCTCTGACATGAACATA     2865
ACCTGCAG                                                                                                  2873
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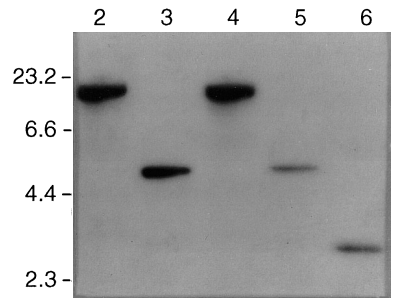

Fig. 2 High-stringency Southern hybridization of genomic DNA samples probed with pB2S. pB2S contains a 2.4-kb *Sma*I-*Pst*I fragment subcloned from pTB3. The samples are as follows: *C. trifolii* race 1 DNA digested with *Bam*HI (lane 2) and *Pst*I (lane 3); *C. trifolii* race 2 DNA digested with *Bam*HI (lane 4) and *Pst*I (lane 5); and DNA from *C. gloeosporioides* f. sp. *aeschynomene* digested with *Pst*I (lane 6). Molecular weight markers run in lane 1 are indicated in kb

TB3 complements *cot-1*

The *cot-1* gene of *N. crassa* is necessary for hyphal elongation and branching. A temperature-sensitive mutation in this gene causes a compact morphology due to excess branching and failure of hyphae to elongate. To examine whether *TB3* can functionally restore the wild-type phenotype, *cot-1* protoplasts were transformed with pTB3/2.4, a construct containing *TB3* and a 2.4 kb *Sal*I hygromycin resistance (hyg^R) cassette derived from pHA1.3 (Powell and Kistler 1990). Transformants were plated on Vogel's sucrose medium containing hygromycin at 34° C and incubated for 48 h. At 34° C transformants grew like wild-tytpe hyg^R transformants on medium containing hygromycin. Since rapidly growing hyphae can fuse and form heterokaryons the transformation was repeated on minimal medium supplemented with sorbose. The resulting, more slowly growing, colonies facilitated isolation of individual transformants. Eleven hyg^R colonies were isolated and purified by three successive conidial passages to obtain stable hyg^R homokaryon transformants. Microscopic analysis of transformants grown at the restrictive temperature of 34° C revealed normal hyphal growth from regenerated protoplasts within 10 h after plating (Fig. 4A). The *cot-1* mutant revealed a characteristic, compact, colonial and highly branched phenotype when grown under the same conditions (Fig. 4B).

Southern analysis of hyg^R transformants exhibiting wild-type hyphal growth confirmed the presence of *TB3* (Fig. 5). Each pTB3/2.4 transformant contained at least one integrated copy of *TB3*. Under these standard hybridization conditions there was no evidence for *N. crassa* DNA that hybridized to TB3. To confirm *TB3* complementation of the *cot-1* mutant several transformants were crossed with a wild-type strain of the opposite mating type. Harvested ascospores were germinated on Vogel's sucrose medium and concomitantly incubated at 34° C. Approximately 70% of germinating ascospores exhibited wild-type growth, while 30% displayed the *cot-1* phenotype. Progeny exhibiting colonial growth at 34° C grew normally at 25° C and were hygromycin sensitive. These data show segregation of *TB3* and *cot-1* (which are presumably each present in single copy and unlinked in the transformants) in these crosses.

TB3 expression during hyphal growth

Conidia of *C. trifolii* were suspended in nutrient-rich media. Conidia were then induced to germinate and form germ tubes (hyphae) using a hard surface, and harvested at 1-h intervals for 5 h after inducing germination. Appressoria were not observed during the 5 h time course although appressoria began forming 6 h after including germination. Germ tubes were not visually observed until 2 h after inducing germination. Germ tubes were observed in approximately 15% of conidia 2 h after inducing germination and in greater than 70% of conidia 3, 4 and 5 h after inducing germination (data not shown). RNA was isolated, fractionated on a denaturing agarose gel, transferred to a nylon membrane, and probed with the 5′ region of *TB3* (Fig. 6). *TB3* was most highly expressed 1 h after inducing germination, although large numbers of germ tubes were not observed until 3 h after induction. *TB3* expression was at basal levels in conidia and had fallen slightly below this level 4–5 h after induction. *TB3* transcripts formed a band at approximately 2.8 kb. Furthermore, transcripts of approximately 3.2 kb were present in ungerminated conidia and 1 h after inducing germination. The banding of these transcripts was somewhat obscured since they comigrated with the large ribosomal RNA. A fainter band of approximately 7.0 kb was also visible in the 1- and 2-h samples (Fig. 6) and was present in all samples after longer exposure times (data not shown).

Discussion

A kinase-encoding gene (*TB3*) from *C. trifolii* was cloned and characterized. TB3 protein has significant sequence identity to numerous serine/threonine protein kinases, particularly COT1 kinase, which is required for hyphal elongation and hyphal branching in *N. crassa* (Yarden et al. 1992). Importantly, *TB3* complements the *cot-1* mutant to normal hyphal growth. Thus *TB3* may be the *cot-1* homolog in *C. trifolii*. If

Fig. 1 The nucleotide sequence of *TB3* and deduced amino acid sequence. Internal conserved sequences within introns are *underlined*

Fig. 3 Comparison of deduced amino acid sequences for TB3 and COT1 (Yarden et al. 1992) kinases. Identical amino acids are indicated with the *vertical lines*, highly similar amino acids are indicated by *colons*, and similar amino acids are indicated by *dots* between the two amino acid sequences. Gaps are introduced within sequences to maintain maximum amino acid homology. Stretches of glutamine residues in the N-terminal region of TB3 are in *italics*. An arginine residue which separates the N- and C-terminal domains is in *bold*. Conserved kinase motifs are *underlined*

that is the case, TB3 is likely to be important for hyphal elongation in *C. trifolii* in a manner similar to that found in *N. crassa*.

Southern analysis showed that *TB3* is a single-copy gene. Overexposure of Southern blots indicated that *TB3* is a member of a gene family (data not shown) in *C. trifolii*. The related fungus *C. gloeosporioides* f. sp. *aeschynomene* also possesses a *TB3* homolog and gene family. The conservation of the TB3/COT1 kinase among pathogenic and saprophytic fungi indicates that these fungi use similar mechanisms to regulate hyphal growth and branching. Hyphal elongation occurs immediately after germination and precedes appressorium formation in *Colletotrichum*. Hyphal elongation also occurs during plant penetration. Furthermore, hyphal extension and branching are critical as fungi invade plants and assimilate nutrients. Thus, the regulation and function of this kinase during early and late stages of plant colonization by pathogenic fungi is of great interest.

TB3 and COT1 proteins have highly homologous, C-terminal catalytic domains and the DNA sequences encoding these domains have identically positioned introns. The nucleotide sequences encoding the divergent, N-terminal, regulatory domains have differently positioned introns. Divergence of the N-terminal, regulatory domains may reflect differences in the proteins which interact with TB3 and COT1. However, a database search with only the N-terminal domain of TB3 protein showed highest identity to the N-terminal domain of COT1 despite the obvious divergence in these regions.

Two stretches of glutamine residues are found in the N-terminal regulatory domain of TB3, but not COT1. Glutamine tracts can be important for protein-protein interactions. The homopolymeric glutamine stretches of human Sp1 protein bind to TATA box-binding protein and interaction of these proteins correlates with transcriptional activation (Emili et al. 1994). Numerous transcription factors contain homopolymeric tracts of glutamines (Gerber et al. 1994). Furthermore, a stretch of 10 to 30 glutamines fused to the DNA-binding domain of GAL4 can activate transcription in animal cell lines (Gerber et al. 1994). Other kinases also contain glutamine-rich stretches (e.g. Haribabu and Dottin 1991). These data suggest that TB3 kinase may be positioned in a signaling cascade, as a transcription factor.

Northern analysis showed that *TB3* was most highly expressed 1 h after induction of germination in the presence of a hard surface and nutrient-rich media. The

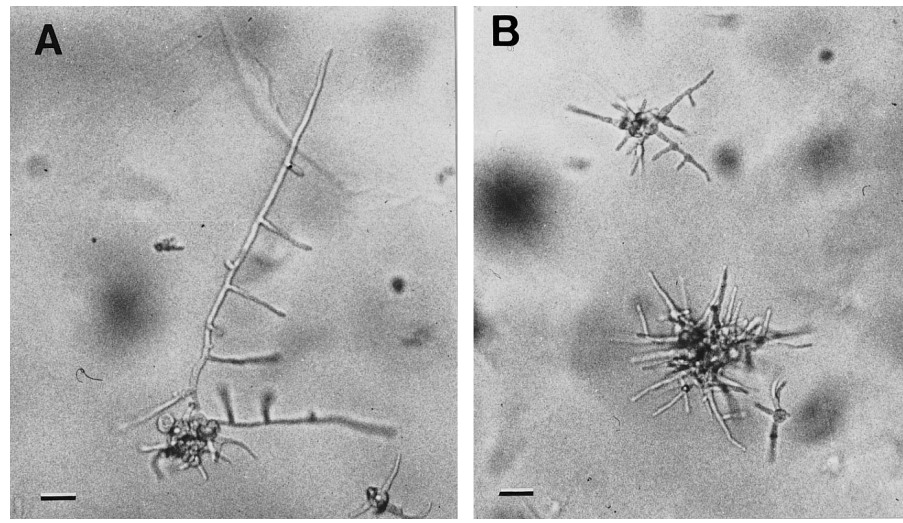

Fig. 4 **A** *N. crassa cot-1* mutant transformed with pTB3/2.4. The *cot-1* mutant transformed with pTB3/2.4 was grown for 10 h at 34° C in Vogel's sucrose mudium supplemented with hygromycin. Complementation is demonstrated by the normally growing hyphae emerging from the *cot-1* protoplast. Branching frequency is much lower than for *cot-1* (see **B**). **B** The *cot-1* mutant was grown in Vogel's sucrose medium for 10 h at 34° C. Two *cot-1* colonies originating from regenerated protoplasts are shown. Restricted, multibranced microcolonies are evident. Bars indicate 100 μM

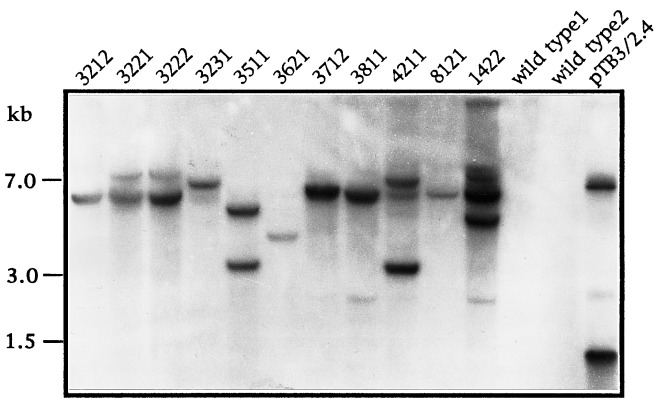

Fig. 5 Southern analysis of eleven hyg^R *cot-1* colonies transformed with pTB3/2.4. Genomic DNA was isolated from transformants and two independent wild-type cultures (wild-type 1 and 2). DNA (2–3 μg) was digested with *Pst*I and the blot was probed with a 1.5-kb *Hin*dIII fragment from pTB3/2.4. pTB3/2.4 digested with *Hin*dIII was included as a positive control

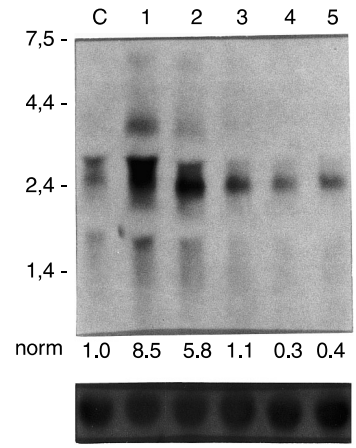

Fig. 6 Northern blot analysis of RNA isolated from *C. trifolii* race 1 and probed with the 5′ cDNA region of *TB3*. Samples are from ungerminated conidia (lane C) and conidia incubated under conditions that induce germination. RNA was harvested 1 h (lane 1), 2 h (lane 2), 3 h (lane 3), 4 h (lane 4) and 5 h (lane 5) after inducing germination and saprophytic growth. RNA markers are shown to the left in kb. Numbers below the lanes indicate relative levels of *TB3* expression (with expression in conidia as a baseline) as assessed using 17S ribosomal RNA expression (shown in the lower panel) as an internal control

high level of *TB3* expression observed after inducing germination and prior to visible germ tubes strongly suggests that TB3 kinase is important during conidial germination or is necessary for germ tube (hyphal) elongation.

Ungerminated conidia and conidia harvested 1 h after inducing germination contained *TB3* transcripts of approximately 3.2 and 2.8 kb in size. *TB3* transcripts from conidia harvested 3–5 h after inducing germination were almost exclusively present in a band at 2.8 kb. Thus the larger 3.2-kb transcripts presumably represent unprocessed transcripts. The low level of the smaller transcript seen 4–5 h after inducing germination shows that *TB3* is expressed during hyphal elongation. Very large 7.0-kb *TB3* transcripts can be observed as a minor band in all samples after longer exposure times. It is possible that these are very large precursor transcripts. Large polycistronic tubulin transcripts have been shown to exist in the eukaryote *Trypanosoma* (Muhich and Boothroyd 1988) and have also been observed in *C. gloeosporioides* (Buhr and Dickman 1994). Another alternative is that these bands may represent transcripts that have paired with other transcripts and migrated slowly in the agarose gel, despite the fact that denaturing conditions were used throughout the Northern analysis.

Genetic evidence from *N. crassa* have shown that *cot-1* is required for hyphal elongation. However, a classical genetic approach is not feasible with the asexual fungus *C. trifolii*. An efficient transformation

system allowed the examination of *TB3* in *N. crassa cot-1*. At present there is no such efficient transformation system for the 'undomesticated' fungus *C. trifolii* to allow functional analysis of genes via domain swapping or gene disruption. Fungal transformation using *TB3* antisense constructs with inducible promoters may be a possible alternative to homologous recombination in *C. trifolii*. Attempts to transform *C. trifolii* with different *TB3* constructs are underway.

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