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cAMP signalling is involved in growth, germination, mycoparasitism and secondary metabolism in *Trichoderma virens*

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An adenylate-cyclase-encoding gene, *tac1*, of *Trichoderma virens*, a soil fungus used in the biocontrol of plant pathogens, has been cloned and sequenced. The *tac1* ORF spanned 7032 bp, encoding a protein of 2153 aa, which shared an identity of 65 % with the adenylate cyclase of *Colletotrichum lagenarium*. Deletion of *tac1*, through double-crossover homologous recombination, lowered the intracellular cAMP levels to below the detection limit. The mutants showed only 5–6 % of the wild-type growth rate on agar, but grew normally in shake culture. The mutants did not sporulate in darkness, and the spores failed to germinate in water. In the confrontation assay, the mutants did not overgrow the test plant pathogens *Sclerotium rolfsii*, *Rhizoctonia solani* and *Pythium* sp. Against *Pythium* sp., the mutants produced a clear zone of inhibition in the confrontation assay. HPLC analysis and bioassay showed reduced secondary metabolite production in the mutants. Using suppression subtractive hybridization (SSH), the genes that were underexpressed in the mutants were identified. Based on an array of 53 SSH library clones, 11 clones were identified as strongly downregulated in the Δ*tac1* mutants; of these 11 clones, nine sequences were homologous to secondary metabolism-related gene sequences. Therefore, cAMP signalling positively regulates secondary metabolism in *T. virens*. This is believed to be the first direct genetic study on the role of cAMP signalling in a *Trichoderma* sp. Tac1 is also believed to be the first regulatory protein to be identified in *T. virens* that is involved in growth, germination, mycoparasitism and secondary metabolism.

INTRODUCTION

cAMP, produced from ATP by adenylate cyclase, regulates many of the biological activities of prokaryotic and eukaryotic cells (Pastan & Perlman, 1970; Robinson & Sutherland, 1971). Although cAMP is an important regulator of growth, development and pathogenicity in filamentous fungi, genes encoding adenylate cyclase have been studied in a few species only (Adachi & Hamer, 1998; Choi & Dean, 1997; Fillinger *et al.*, 2002; Ivey *et al.*, 2002; Klimpel *et al.*, 2002; Liebmann *et al.*, 2003). The *crisp* mutants of *Neurospora crassa* which lack a functional copy of the adenylate cyclase are characterized by a colonial growth habit, with impairment in elongation of aerial hyphae, and a premature accumulation of conidia (Rosenberg & Pall, 1979; Terenzi *et al.*, 1974, 1976). Evidence from the analysis of *Aspergillus nidulans* strains defective for adenylate cyclase (CyaA) or PkaA has shown the involvement of cAMP signalling in conidial germination in response to carbon-source sensing (Fillinger *et al.*, 2002).

Abbreviation: SSH, suppression subtractive hybridization.

The GenBank/EMBL/DDBJ accession no. for the sequence reported in this paper is EF189190.

In PkaA-defective strains, cAMP levels have been shown to increase dramatically by 250-fold, indicating that PkaA regulates intracellular cAMP levels (Fillinger *et al.*, 2002). In the human pathogen *Aspergillus fumigatus*, disruption of the adenylate-cyclase-encoding gene *acyA* results in reduced colony diameter and conidiation; the mutants are less virulent as a result of reduced defence against killing by macrophages (Liebmann *et al.*, 2003). In the rice pathogen *Magnaporthe grisea*, *mac1* disruption results in abolition of appressorium formation, and reduction in vegetative growth, conidiation and conidial germination (Adachi & Hamer, 1998; Choi & Dean, 1997). Δ*magB* (G-protein) and Δ*mac1* (adenylate cyclase) mutants share several phenotypes, indicating that adenylate cyclase is downstream of MagB in *M. grisea* (Liu & Dean, 1997). The G_β subunit MGB1 is involved in cAMP signalling for regulating conidiation, surface recognition and appressorium formation in *M. grisea* (Nishimura *et al.*, 2003). In *Botrytis cinerea*, the adenylate cyclase (BAC) is required for full pathogenicity (Klimpel *et al.*, 2002).

Trichoderma spp. are economically important as sources of many industrial enzymes, and as commercial biofungicides (Harman & Bjorkmann, 1998; Hjeljord & Tronsmo, 1998;

Mach & Zeilinger, 2003). These fungi inhabit soil and the rhizosphere, and inhibit other fungi through antibiosis and mycoparasitism (Chet *et al.*, 1998; Sivasithamparam & Ghisalberti, 1998). They are also good plant growth promoters, and they induce resistance in plants against invading pathogens through limited colonization of plant roots (Harman *et al.*, 2004; Viterbo *et al.*, 2005). Two species (*Trichoderma longibrachiatum* and *Trichoderma citrinoviride*) are opportunistic human pathogens (Kuhls *et al.*, 1999). *Trichoderma virens* IMI 304061 is an aggressive mycoparasite on the plant pathogens *Sclerotium rolfsii* and *Rhizoctonia solani* (Mukherjee *et al.*, 1995). To understand the role of signalling pathways in mycoparasitism and development, we previously isolated loss-of-function mutants for two G_α proteins (TgaA and TgaB) and an MAPK (TmkA) (Mukherjee *et al.*, 2003b; 2004). However, there has been no direct evidence reported in the literature for a role of cAMP signalling in any *Trichoderma* species. Antisense-mediated silencing of the G_α protein Tga1 results in reduced cAMP level in *Trichoderma atroviride* IMI 206040 (Rocha-Ramirez *et al.*, 2002). The mutants are hypersporulating, and have reduced mycoparasitic coiling. Interestingly, deletion of Tga1 in *T. atroviride* ATCC 78058 results in elevated internal cAMP levels (Reithner *et al.*, 2005). These mutants also show hypersporulation, a total loss of mycoparasitism, and increased antibiosis against *R. solani*, even though the production of an antifungal metabolite, 6-pentyl-α-pyrone, is reduced. The deletion of another G_α protein, Tga3, results in reduced intracellular cAMP levels in *T. atroviride*, and is associated with reduced growth rate and germination, light-independent conidiation, and loss of mycoparasitism (Zeilinger *et al.*, 2005). It is interesting that the phenotypes associated with the deletion of Tga1 or Tga3 are similar, although the cAMP level is elevated in Tga1 mutants and reduced in Tga3 mutants. Recently, Casas-Flores *et al.* (2006) studied the role of a protein kinase A regulatory subunit in blue-light-induced conidiation of *T. atroviride*. Expression of an antisense copy of this gene results in a non-sporulating phenotype, whereas overexpression results in light-independent conidiation, indicating the involvement of cAMP signalling in conidiation in *T. atroviride*. In contrast to most of the fungi studied, including the closely related *T. atroviride*, the deletion of the G_α protein TgaA has no effect on growth, sporulation or spore germination frequency in *T. virens* (Mukherjee *et al.*, 2004). This raised the question of whether the functioning of the G-protein–cAMP pathway in *T. virens* differs from other ascomycetes. Also, what are the physiological roles that this pathway plays in this fungus? Since there has been no direct evidence on the role of cAMP signalling through the deletion of adenylate cyclase in any *Trichoderma* sp., we cloned the adenylate-cyclase-encoding gene *tac1* of *T. virens*, which is an important biocontrol fungus, and studied the role of the cAMP signalling by gene knockout.

METHODS

Fungal strains and growth conditions. *T. virens* (IMI 304061), *S. rolfsii* (MTCC 6052), *R. solani* (ITCC 4110) and *Pythium* sp. have been used in our previous studies (Mukherjee *et al.*, 2006a). Routinely, the fungi were grown on potato glucose agar (PDA; Difco) at ambient temperature (26–28 °C), and stored as glycerol stocks at –80 °C for long-term storage and maintenance of genetic stability. For growth on dialysis membranes, 10^6 conidia were spread on PDA plates overlaid with a sterile dialysis membrane (MWCO 12 000–14 000; Spectra/Por), and incubated for 2 days at ambient temperature. The mycelial mat was harvested with a sterile spatula, frozen in liquid nitrogen, and stored at –80 °C until further use. For analysis of the secondary metabolites, the agar was extracted in solvent as described below.

Cloning of the full-length gene. Using the *M. grisea mac1* sequence, two primers, ACFor (CCATATGACTGGAATTGGAA) and ACRev (ACCATGAAGGCGTCACCT), were designed from the region showing high homology with other fungal adenylate cyclase gene sequences, and part of the gene (1.4 kb) was amplified from *T. virens* genomic DNA by PCR. This fragment was cloned, and used as a probe for screening a *T. virens* cosmid library described previously (Mukherjee *et al.*, 2003b). The full-length gene was subcloned from the cosmid clone by a three-step inverse PCR using the primer pairs INV1 (GCCGCCTCATGCTGTCTG) and INV2 (GGAGATGTCC-TCGACTCTTC), INV2 and INV3 (GAATATGCGAATAAAGTAA-GG), and INV4 (GTTCGGGAGGCTGCTCTCG) and INV5 (GCCTGCGATCCACGAATAG). The 3′ end of the gene was cloned as a *DraI* fragment amplified with the gene-specific primer Xin1.2-2GW1 (GATCGGAAGCCTCTTGCGAAGATGAG) by using the Clontech GenomeWalker Universal kit (BD Biosciences). All the clones were sequenced, assembled and translated by using Gene Runner software, and the putative ORF and introns were identified. Homology with adenylate cyclase from other fungi was studied by BLASTP on the NCBI server.

Construction of the gene-deletion construct, protoplast transformation, and selection and purification of transformants. A double-crossover construct, pACDC, was made by replacing 5087 bp of the ORF with a hygromycin-resistance cassette consisting of *hph* (hygromycin phosphotransferase), TrpC promoter and TrpC terminator, taken from the plasmid pAT-BS (Mukherjee *et al.* 2003a). The linear construct (Fig. 1a), consisting of the left flank (2441 bp), the marker (2.2 kb), and the right flank (2010 bp), was amplified by the primer pair AC5′2s (GAGATGCGAGACTGTCAGCG) and ACRFNot (CAATGATATGAGAGCGGCCGCCATTC), and was used for transforming *T. virens* protoplasts, as described previously (Mukherjee *et al.*, 2003b). The transformants were selected using 200 mg hygromycin B l^{-1} (Roche), and transferred to PDA plates containing 100 mg hygromycin B l^{-1}. The transformants that showed non-wild-type phenotypes were purified by repeated single-spore isolation, and three putative knockout mutants were selected based on atypical colony growth and morphology.

Southern hybridization, PCR and RT-PCR. High-molecular-mass genomic DNA from the wild-type (WT) and the candidate Δ*tac1* mutants (hereafter referred to as 'mutants') was extracted, and subjected to PCR and Southern analysis. PCR was performed by the primer pair INV3 and INV5 for the adenylate cyclase gene, and hphfor (GAGGGCGAAGAATCTCGTGC) and hphrev (CACTGAC-GGTGTCGTCCATC) for the *hph* gene. For genomic Southern analysis, the DNA was digested with *Xba*I, and hybridized with a 1.1 kb fragment amplified from the genomic DNA using the primer pair INV3 and INV5. For analysis of the *tac1* transcript level, total RNA was isolated from the mycelial mat of the WT and mutants grown on the dialysis membrane, and first-strand cDNA was synthesized from 1 μg total RNA using the First Strand cDNA Synthesis kit (Roche) in a 20 μl reaction volume. A 5 μl volume of the first-strand cDNA was used for amplification with *Taq* polymerase (Genei) in a 25 μl volume, using the primer pairs INV3 and INV5 (for

tac1), hphfor and hphrev (for *hph*), and h3for (GCCCGCACCAAGCAGACCG) and h3rev (GGCGGGCGAGCTGGATGTC) (for *histone3*).

Assay for intracellular cAMP concentration. For measuring the intracellular cAMP concentration, the WT strain and the mutants were grown on a dialysis membrane, as described above. Two-day-old mycelial mat was harvested, frozen in liquid nitrogen, ground, homogenized in 10 vols 0.1 M HCl, and centrifuged (600 ***g***). The cAMP level was measured by using the Direct cAMP Enzyme Immunoassay kit (Sigma), as per the manufacturer's instructions. Protein concentration was determined by using the Folin phenol reagent (Lowry method). The intracellular cAMP concentration was expressed as pmol (mg protein)$^{-1}$.

Growth and morphology of the mutants. Mycelial discs were inoculated on PDA plates, and the linear growth measured. For studying the colony development from single spores, PDA plates were spread with diluted conidial suspension. Dry weight (biomass) was measured after 3 days of shake incubation of 100 ml potato glucose broth (PDB) inoculated with 10^5 conidia. For the germination assay, conidia were harvested from 10-day-old cultures grown on PDA plates, and washed thrice in double-distilled water. Approximately 5×10^4 conidia in 50 µl water were incubated on sterile glass slides in a moist chamber. The conidia were observed for germination after 20 h.

Confrontation assay. The ability of the mutants and the WT to overgrow and lyse the mycelia of the test plant pathogens was assessed using a confrontation assay on PDA plates (Mukherjee *et al.*, 2003b). Observation was recorded for overgrowth of *Trichoderma* on the test fungi, and for the lysis of the mycelia.

Antibiosis and HPLC analysis. The ability of the mutants and the WT to inhibit the growth of *Pythium* sp. through the production of diffusible antibiotics was assessed by bioassay and HPLC. Conidial suspension was spread on dialysis membrane, and grown for 2 days, as described above. The dialysis membrane and the mycelial mat were removed, and the agar was blended in 80 % aqueous acetone (1 g agar in 3 ml acetone), and centrifuged at 10 000 r.p.m. (13 000 ***g***) for 10 min. The supernatant was extracted with an equal volume of chloroform. The chloroform was evaporated under N_2 flush, and the metabolites were reconstituted in methanol (1/100 original volume). A 10 µl volume of the reconstituted metabolites was assayed by the agar-well technique, in which the solution was added to a 5 mm well that had been cut at the centre of a PDA plate. After evaporation of the solvent, three mycelial discs of *Pythium* sp. were inoculated near the edge of the plate, and photographed after 2 days incubation. HPLC was performed as described previously (Mukherjee *et al.* 2006a).

Suppression subtractive hybridization (SSH), array and sequencing of the differential clones. Total RNA was isolated from the mycelial mat of the WT and the Δ*tac1* mutant ACM1 grown on a dialysis membrane, as described above. First-strand cDNA from the WT and ACM1 total RNA was synthesized using the BD SMART RACE cDNA amplification kit with 3′-RACE CDS Primer A and BD SMART IIA oligonucleotide (BD Biosciences). The first-strand cDNA was purified by selective precipitation (ammonium acetate precipitation), and the second-strand cDNA was amplified using 2.5 µl first-strand cDNA as the template, the Nested Universal Primer A (BD Biosciences) as the oligonucleotide, and Advantage polymerase (BD Biosciences), in a reaction volume of 100 µl. The cycling parameters were as follows: initial denaturation at 95 °C for 1 min, then 17 cycles (optimized initially by taking samples up to 30 cycles, based on the protocol described in BD Super SMART-PCR cDNA synthesis kit) at 95 °C for 15 s, 65 °C for 30 s, and 68 °C for 6 min. The amplified cDNA was treated with RNaseA (Roche), purified using the High Pure PCR product purification kit (Roche), reconstituted in 20 µl nuclease-free water, and the concentration was determined spectrophotometrically. Amplified cDNA (2 µg) from the WT or the Δ*tac1* mutant ACM1 was subjected to SSH using the Clontech PCR-Select cDNA subtraction kit (BD Biosciences), according to the manufacturer's instructions. The WT cDNA was used as the tester, and the mutant cDNA was used as the driver. After the second (nested) PCR, the products were cloned in the pTZ57R/T vector (Fermentas). The presence of single inserts was confirmed by colony PCR using the Nested PCR primers 1 and 2R, which were supplied with the kit, and 53 clones showing a single insert were arrayed on a positively charged nylon membrane (Roche). Histone *h3* cDNA was included as a control. A 2 µl volume of the heat-denatured PCR product of each clone was blotted on membranes, and hybridized with WT or mutant cDNA, which was labelled with [^{32}P]dCTP (BRIT). After hybridization, the blots were exposed to X-ray films overnight. The clones that were clearly differential were purified, then sequenced with T7 primer using an automated sequencer, and homologous sequences in the database were identified by BLASTX on the NCBI server.

RESULTS

Isolation of *tac1*

Using the primer pair ACFor and ACRev, taken from the *M. grisea mac1* sequence in the region of greatest similarity to other fungal adenylate cyclase genes, a 1.4 kb fragment was cloned and sequenced. BLASTX analysis confirmed the fragment to be part of the adenylate cyclase gene. Using this product as the probe, a cosmid library was screened, and a cosmid clone of approximately 26 kb was confirmed to harbour the adenylate cyclase gene by PCR and Southern hybridization (data not presented). Using inverse PCR, a fragment of approximately 7.3 kb was cloned in three steps, and the missing 3′ end was cloned by genome walking as a 800 bp *DraI* fragment. Together, we cloned a 9623 bp sequence containing the entire coding region (7032 bp), the upstream region (2523 bp), and the 68 bp sequences downstream of the stop codon; the entire 9623 bp sequence has been deposited under GenBank accession no. EF189190. The ORF, encoding a protein of 2153 aa, is interrupted by three introns of 174, 327 and 69 bp. The *T. virens* adenylate cyclase Tac1 is highly homologous to adenylate cyclases from other fungi, such as *Colletotrichum lagenarium*, *M. grisea* and *Podospora anserina* (65, 62 and 59 % identity, respectively, at the amino acid level).

Isolation of Tac1 loss-of-function mutants

Using protoplast-mediated transformation of *T. virens*, with the disruption cassette amplified from the plasmid construct pACDC, several hundred transformants were obtained by selection on 200 mg hygromycin B l^{-1}. One hundred transformants were transferred to fresh PDA containing 100 mg hygromycin B l^{-1}, and three colonies were found to be extremely slow growing, and they did not appear to be similar to the WT colony. These three colonies were purified by repeated single-spore isolations. The

mutants (designated ACM1, ACM2 and ACM3) were confirmed for deletion of the adenylate cyclase gene by genomic PCR, Southern hybridization and RT-PCR. Using the primer pair INV3 and INV5, we obtained a 1.1 kb band in the WT, and this was found to be absent in the three mutants (Fig. 1b). All the mutants were positive for the *hph* gene, which was not detected in the WT (Fig. 1b). Gene deletion was also confirmed by Southern hybridization (data not presented). RT-PCR data (Fig. 1c) indicated the absence of the *tac1* transcript in the mutants, but it was present in the WT. The opposite was true for the *hph* transcript. The intracellular cAMP level in the 2-day-old WT mycelial mat harvested from the dialysis membrane overlying PDA was 6.227 ± 0.415 pmol (mg protein)$^{-1}$. The cAMP levels in the mutants were below the detection limit, although the protein level ranged from 1.19 to 1.57 μg (μl extract)$^{-1}$, compared with 0.59 μg (μl extract)$^{-1}$ in the WT.

Growth, morphology, and conidial germination

The Δ*tac1* mutants, when grown on PDA plates from the disc inoculum, showed a marked reduction in growth rate (1.1 ± 0.3 mm day^{-1} linear growth, compared with WT growth of 19.2 ± 1.4 mm day^{-1}). The WT fully covered the 85 mm plates in 2 days, while the mutants took more than a month to cover the entire plate (Fig. 2a). The mutants had aerial growth, and sporulated initially towards the centre of the plates, but subsequently they showed submerged growth devoid of aerial hyphae and conidiation. A dark-yellow pigment diffusing from the mutant colonies was seen, and the mutant colony growth was very compact. When PDA plates with the WT or mutants were incubated in the dark, the mutants failed to form conidia, but the colonies responded to light by forming green conidia when incubated under constant illumination for 2 days (Fig. 2b). When the plates were inoculated with a diluted conidial suspension, the mutant colonies were visible 2 days after plating, as opposed to the WT colonies, which were visible after 1 day. The mutant colonies from single spores were very small, discrete and compact, and they produced mainly aerial hyphae, in contrast to the typical spreading growth habit of the WT strain (Fig. 2c). On subsequent incubation, the mutant colonies began to sporulate, produce dark-yellow diffusible pigments, and they showed a submerged growth habit. In 3-day-old liquid shake culture, there was no significant difference between the WT and the mutants with respect to dry matter production (672 ± 21 mg for the WT, compared with 630 ± 49, 640 ± 40 and 657 ± 31 mg for the mutants ACM1, ACM2 and ACM3, respectively). The mutants, when transferred from liquid culture to agar medium, grew typically as slow-growing mutant colonies. This shows that no irreversible modification occurred during growth in

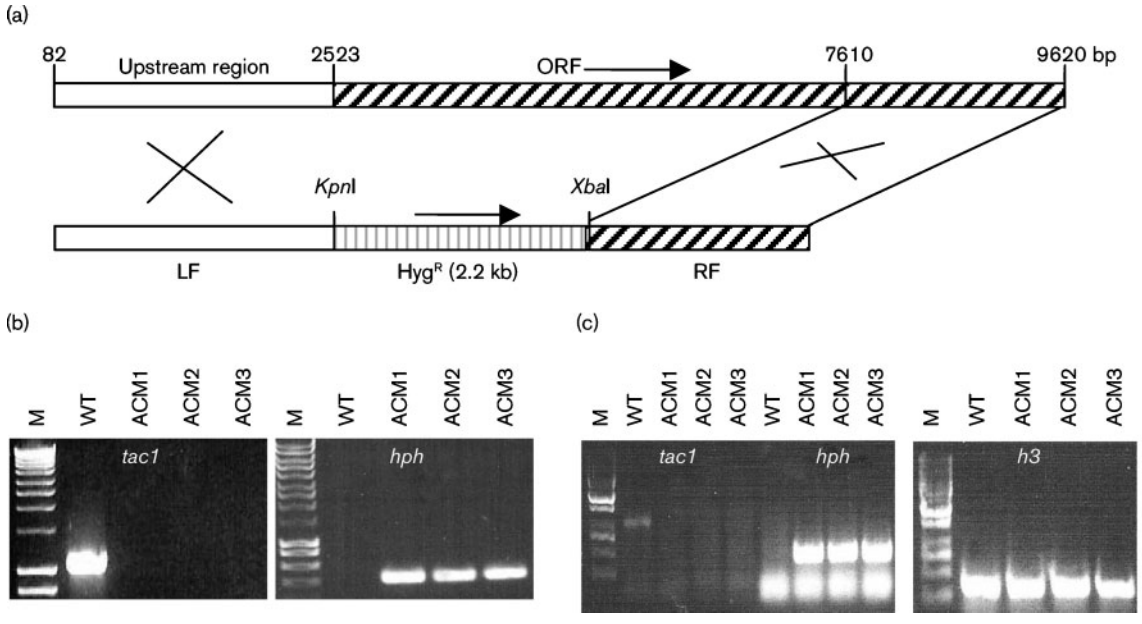

Fig. 1. Disruption of *tac1* by homologous recombination. (a) Strategy for double-crossover homologous integration: a 5087 bp section of the ORF was replaced with a 2.2 kb hygromycin-resistance cassette (Hyg^R), resulting in a construct with a 2441 bp upstream region as the left flank (LF), and a 2010 bp right flank (RF). (b) PCR amplification of *tac1* and *hph* from the genome of the WT and Δ*tac1* mutants (ACM1, ACM2 and ACM3). (c) RT-PCR analysis showing the presence of the *tac1* transcript in the WT, and the absence of the *tac1* transcript in the mutants, and the presence of the *hph* transcript in the mutants, but not in the WT. Histone *h3* was included as a loading control. M, molecular-mass marker (hyperladder, 0.2–10 kb).

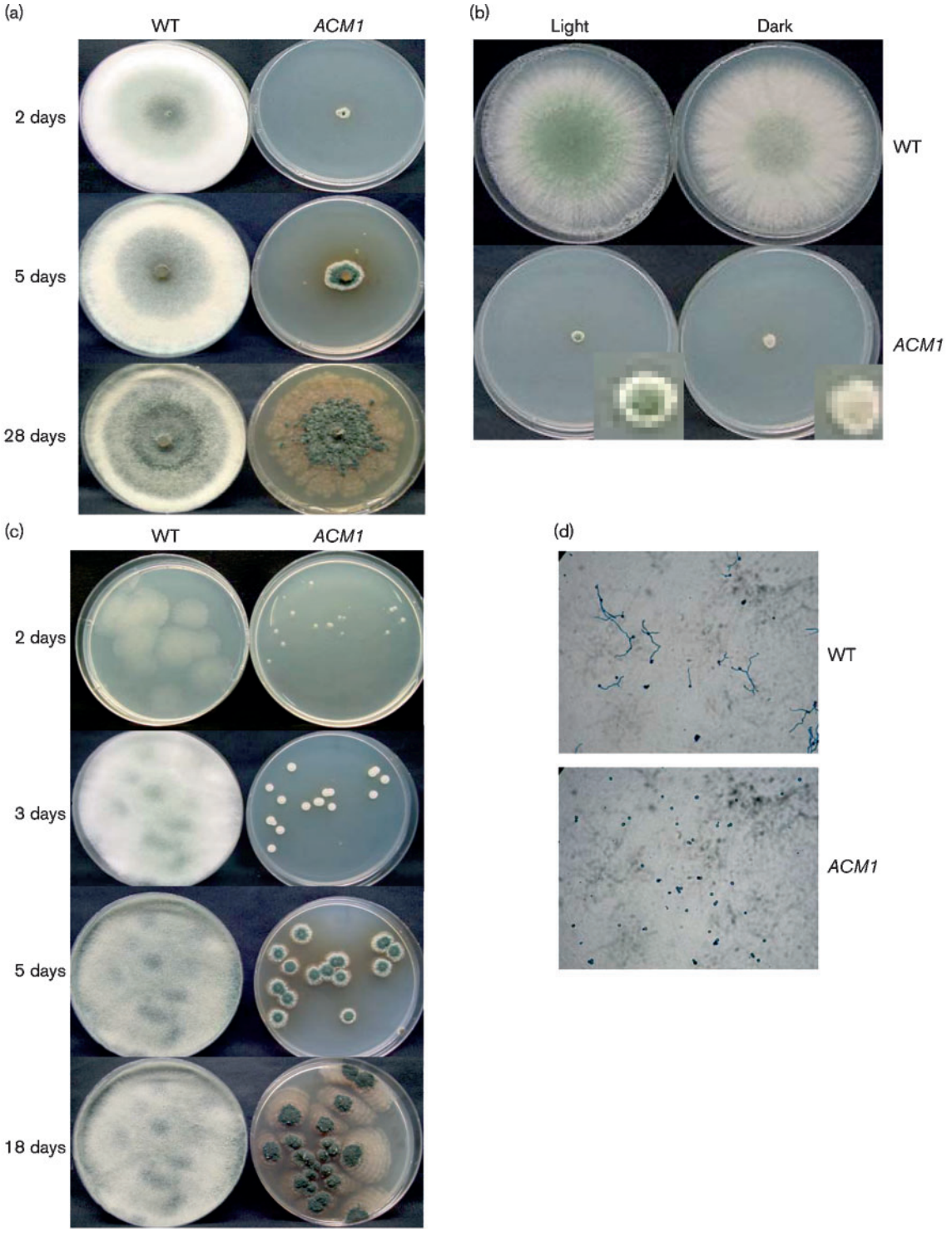

Fig. 2. Growth and morphology of *T. virens* WT and the Δ*tac1* mutant ACM1. (a) Growth on PDA plates from a disc inoculum after 2, 5 and 28 days incubation. (b) Growth and conidiation of the WT and the Δ*tac1* mutant on PDA from disc inoculum after 2 days incubation in the dark and in continuous light. Inset, magnified view of the mutant colonies to show the presence of conidiation in the light, and absence of conidiation in the dark. Note, some conidiation in the WT incubated in the dark was due to nutrient stress as the colony reached the margin of the plate. (c) Growth of the WT and the Δ*tac1* mutant on PDA plates seeded with conidia after 2, 3, 5 and 18 days incubation. (d) Germination of conidia of the WT and Δ*tac1* mutant in water after 20 h incubation.

liquid medium. When incubated in water for 20 h, the conidia from the mutants failed to germinate, while nearly all the conidia of the WT strain germinated through the production of normal germ tubes (Fig. 2d).

Confrontation assay, and secondary metabolite production

In confrontation assays, where the test plant pathogens were inoculated opposite the *Trichoderma* strains, the WT and the test plant pathogens met around the centre of the plate, and, with time, the WT started to overgrow the colonies of the test fungi. The WT fully overgrew and lysed the colonies *R. solani*, *S. rolfsii* and *Pythium* sp. in 5, 10 and 4 days respectively (Fig. 3a). The mutants, however, failed to overgrow any of the test pathogens, even after prolonged incubation. In contrast, *R. solani* and *S. rolfsii* overgrew the mutant colonies, indicating that the mutants could not offer resistance to overgrowth by these plant pathogens. Against *Pythium* sp., a clear zone of inhibition was seen when it was inoculated opposite the mutants. In the bioassay of chloroform-extracted metabolites on *Pythium* sp., the mutants showed reduced inhibition compared with the WT (Fig. 3b). HPLC analysis of the non-polar fractions showed the presence of several unknown metabolites, along with viridiol, which is a reduction product of viridin. The mutants produced lesser concentrations of metabolites in agar, compared with the WT (Fig. 3c).

SSH identification of genes underexpressed in the mutants

Using SSH, we made a library of several hundred clones that were thought to represent genes that were under-expressed in the mutants, since WT cDNA was used as the tester, and mutant cDNA was used as the driver. Of the 53 clones arrayed, 11 (clone nos 2, 4, 9, 10, 12, 19, 21, 25, 40, 41 and 44) were strongly differential (Fig. 4); of these, the most abundant was a cytochrome P450 gene (*vir2*), representing four clones (clone nos 4, 10, 21 and 25). The other clones related to secondary metabolism were *cyp1* (a cytochrome P450 gene, clone no. 9), *vir4* (a cyclase gene, clone no. 12), *vir3* (a cytochrome P450 gene, clone no. 40), *omtB-2* (an *O*-methyl transferase gene, clone no. 41), and a homologue of the GA14-synthase gene of *Fusarium proliferatum* (a cytochrome P450 gene, clone no. 44). Clone no. 2 was a homologue of the stress-response gene *ric-1* of *N. crassa*, while clone no. 19 had no homology in the database.

DISCUSSION

G-protein–cAMP and MAPK signalling play central roles in growth, development and secondary metabolism in filamentous fungi (Lengeler *et al.*, 2000; Calvo *et al.*, 2002; Xu, 2000). We have previously established the role of a *T. virens* MAPK TmkA in repression of conidiation in the

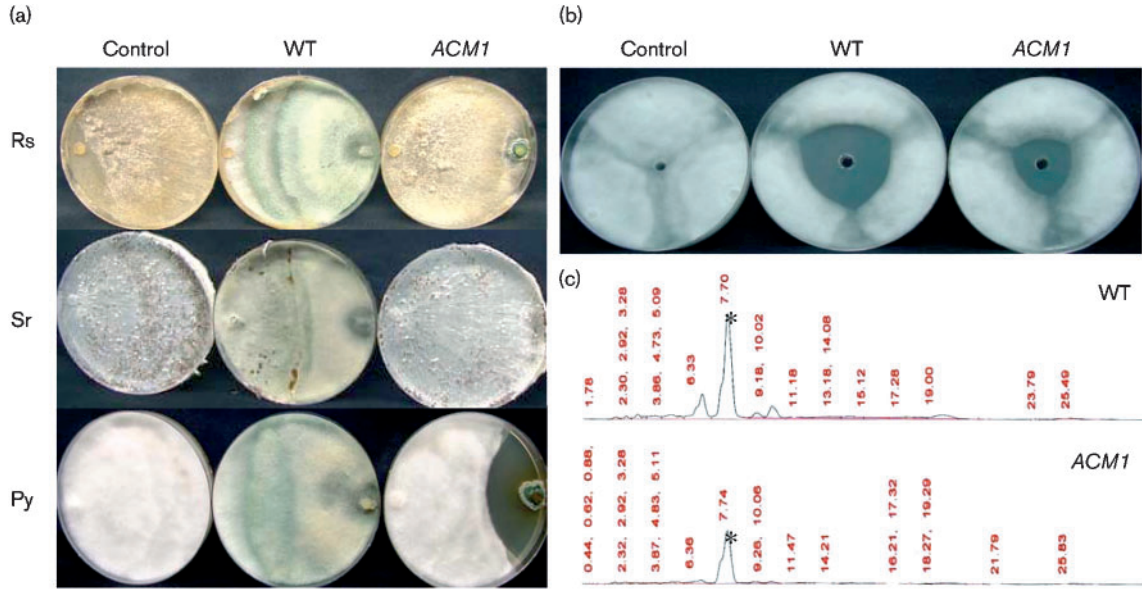

Fig. 3. Antagonism of *T. virens* WT and Δ*tac1* mutant (ACM1) on *R. solani* (Rs), *S. rolfsii* (Sr) and *Pythium* sp. (Py). (a) Confrontation assay. Plates (except control plates, which were inoculated with the test plant pathogen only) were co-inoculated with the test pathogen (left margin) and *Trichoderma* (right margin), and incubated at ambient temperature. Photographs were taken after 5, 10 and 4 days co-inoculation with *R. solani*, *S. rolfsii* and *Pythium* sp., respectively. Note the inability of the mutant to overgrow any of the three plant pathogens, and a clear zone of inhibition against *Pythium* sp. (b) Inhibition of *Pythium* growth by chloroform-soluble metabolites of the WT and the Δ*tac1* mutant ACM1. (c) HPLC profile of the same metabolites; the viridiol peak is marked with an asterisk.

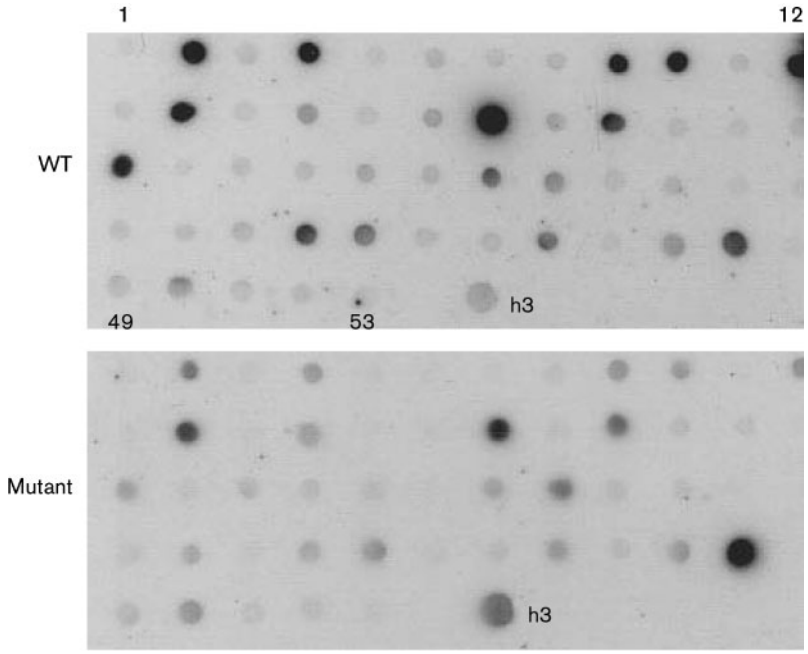

Fig. 4. Array of SSH-derived clones. A total of 53 clones were spotted onto each membrane. h3, histone *h3* cDNA. The upper membrane was hybridized with radiolabelled cDNA of the WT, and the lower blot with cDNA from the adenylate cyclase knockout mutant ACM1. Clone nos 2, 4, 9, 10, 12, 19, 21, 25, 40, 41 and 44 were strongly differential, and were analysed further.

dark, and identified a gene whose expression is repressed by TmkA (Mukherjee *et al.*, 2003b, 2006b). Δ*tmkA* mutants have reduced antagonism against mycelia and sclerotia of some plant pathogens. However, since the deletion of the G-protein TgaA or TgaB does not alter the growth, conidiation or conidial germination frequency of *T. virens* (Mukherjee *et al.*, 2004), the role of cAMP signalling in this fungus has remained elusive. In addition, G-protein-related studies in *T. atroviride* have not established the role of cAMP signalling, since the results have been inconclusive. There is, in general, no direct evidence of cAMP signalling mechanisms in any *Trichoderma* sp., as, to the best of our knowledge, the gene encoding adenylate cyclase has not been cloned and characterized in any member of this genus. We cloned the *T. virens* gene *tac1* encoding adenylate cyclase, and studied its cellular functions. The deletion of the gene through homologous recombination resulted in drastic effects on the colony morphology, and hyphal elongation rate. The linear growth rate was reduced by 94–95 %, which is a very strong reduction compared, for example, with *M. grisea* and *A. fumigatus*, where the deletion of adenylate cyclase reduced the growth rate to 60 and 68 % of the WT, respectively. Though the growth on PDA plates was drastically reduced in the mutants, the growth/biomass production in shake culture was similar to the WT. The normal biomass production in liquid shake culture might be due to a compensatory mechanism through decreased phosphodiesterase activity in submerged culture, as has been suggested for *N. crassa* (Ivey *et al.*, 1999). Alternatively, shake culture may provide a richer environment for growth, as has been suggested for CPG-1 ($G_{\alpha i}$) mutants of *Cryphonectria parasitica* (Segers & Nuss, 2003). Amendment of the medium with 5 mM cAMP did not restore the phenotypes in the mutants (data not presented). The phenotypes of *A. fumigatus* cyaA mutants and *T. atroviride* Tga3 mutants, likewise, are not rescued by the addition of cAMP (Liebmann *et al.*, 2003; Zeilinger *et al.*, 2005).

Unlike the G-protein mutants of *T. atroviride*, and MAP kinase mutants of *T. atroviride* and *T. virens* (Mukherjee *et al.*, 2003b, 2006b; Reithner *et al.*, 2005; Zeilinger, 2004; Zeilinger *et al.*, 2005), the Δ*tac1* mutants did not sporulate constitutively in darkness, and responded to light in the same way as the WT. Thus, the regulation of conidiation in these two closely related species appears to be different: cAMP-dependent regulation in *T. atroviride*, and cAMP-independent regulation in *T. virens*. Also, with respect to the regulation of conidiation, there could be a cross-talk between the Tmk1 and cAMP pathways in *T. atroviride*, but not in *T. virens*.

In the confrontation assay, Δ*tac1* mutants were not able to overgrow and lyse the colonies of the test plant pathogens *S. rolfsii*, *R. solani* and *Pythium* sp. (Fig. 3a). The loss of virulence against the host fungi could be associated with extremely slow growth rate of the mutants. Against *Pythium* sp., however, the mutants produced a prominent zone of inhibition. This visible zone of inhibition could be due to the overproduction of antifungal metabolites that diffuse into agar. However, as measured by both bioassay and HPLC (Fig. 3b, c), secondary metabolite production was decreased in the mutants. This is consistent with the results of SSH, where, out of 11 genes that are under-expressed in the mutants, nine are known to be associated with secondary metabolism in *T. virens* and other fungi (Mukherjee *et al.*, 2006a). *T. virens* strain IMI 304061 shows abundant production of the antifungal viridin and its reduction product viridiol in culture (Mukherjee *et al.*,

2006a). HPLC analysis of the non-polar fraction of the metabolites revealed that the concentration of viridiol was lower in the mutants than in the WT. We did not detect viridin in WT or the mutants, probably because of faster conversion of viridin to viridiol in plate culture compared with liquid shake culture. Nevertheless, the presence of viridiol is an indication of early viridin production. Therefore, it seems that the enhanced antifungal properties of the Δ*tac1* mutants in the confrontation assay are a result of the slow growth of the mutant colonies, rather than of the enhanced production of secondary metabolites. Reducing the growth rate by adding a sublethal dose of fungicides to visualize the zone of inhibition in *Trichoderma*–fungal interactions is a standard practice. For example, Howell (1987) amended PDA with 0.4 μg ml^{-1} benomyl to visualize the zone of inhibition produced as a result of gliotoxin formation by a strain of *T. virens* against *R. solani*. The Δ*tga1* mutants of *T. atroviride* produce a lower amount of the antifungal metabolite 6-pentyl-α-pyrone, although there is a clear zone of inhibition in the confrontation assay against *R. solani* (Reithner *et al.*, 2005). Those authors attributed this enhanced antibiosis to the possible overproduction of some unidentified low-molecular-mass compounds by the mutants. The relationship between G-protein–cAMP signalling, conidiation and secondary metabolism is well documented in *Aspergillus* spp., where increased secondary metabolite production is associated with, or the result of, enhanced conidiation (Calvo *et al.*, 2002; Keller *et al.*, 2005). The deletion of the *Aspergillus* G_α subunit FadA results in premature conidiation, and enhanced sterigmatocystin production (Hicks *et al.*, 1997). The overexpression of PkaA results in reduced sterigmatocystin biosynthesis, while the deletion of this gene results in hypersporulation and aberrant sterigmatocystin production in *A. nidulans* (Shimizu & Keller, 2001). *T. virens* Δ*tac1* mutants did not sporulate constitutively, and had reduced secondary metabolite production. This species, therefore, appears to have a novel regulation of conidiation vis-à-vis secondary metabolism that is different from other fungi studied, for example *T. atroviride* and *Aspergillus* spp. The present findings clearly establish that cAMP signalling is essential for growth, conidial germination and biocontrol properties, including mycoparasitism and secondary metabolism, in *T. virens*. We believe that this is the first report on the cloning and functional analysis of a gene encoding adenylate cyclase in any species of *Trichoderma*. The findings could help to improve the commercial exploitation of *Trichoderma* spp. for industrial and agricultural applications by manipulating the cAMP-signalling pathway, e.g. by overexpression/constitutive activation of one or more of the components to enhance secondary metabolite production.

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