Co-transformation of the sclerotial mycoparasite *Coniothyrium minitans* with hygromycin B resistance and β-glucuronidase markers

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Coniothyrium minitans was successfully co-transformed with the *uidA* (β-glucuronidase) and the hygromycin-resistance (*hph*) genes. Both were under the control of the glyceraldehyde-3-phosphate promoter from *Aspergillus nidulans*. Hygromycin resistance was used as a selectable marker for transformation. In successive transformation experiments, transformation frequencies of up to 1000 transformants μg^{-1} of plasmid DNA were obtained for isolate A69. Of the ten monospore hygromycin-resistant cultures tested, nine also expressed the *uidA* gene. Expression of *hph* and *uidA* was stable in all transformants after several months of successive subculturing on non-selective medium, and after passage through a sclerotium of *Sclerotinia sclerotiorum*. Southern hybridization analyses showed all transformants carried multiple copies of each marker gene. When grown on PDA, the culture morphology of three of the transformants of (T × 2, T × 3 and T × 4) was similar to the wild type. Four of the five transformants (T × 3, T × 4, T × 21 and T × 24) grew as well as the wild type on different media, and responded to changes in water potential in a similar manner to the wild type. All five transformants were equally parasitic on sclerotia of *S. sclerotiorum* compared with the wild type. Transformants T × 3 and T × 4 were the most similar to the wild type in biological characteristics and will be used in future studies. The results indicate that *hph*- and *uidA*-transformed strains of *C. minitans* will be useful for ecological studies on its survival and dissemination.

Sclerotinia sclerotiorum (Lib.) de Bary is a soil-borne fungal pathogen which attacks a wide range of fruit and vegetable crops (Purdy, 1979). The sclerotia of *S. sclerotiorum* are crucial for the pathogen's survival, serving as a primary inoculum source and remain viable in soils for many years (Merriman, 1976). Mature sclerotia germinate either myceliogenically to produce mycelium which infects host plants directly (Huang & Dueck, 1980), or carpogenically to produce apothecia which discharge wind-borne ascospores which subsequently infect the host (Huang & Kokko, 1992).

Control of *Sclerotinia* spp. has been obtained by fungicide application, in particular the use of members of the dicarboximide group, but enhanced degradation of the fungicides in soils by other microorganisms has led to reduced efficacy of control in Europe (Martin *et al*., 1990). Enhanced degradation of dicarboximides has been reported in onion paddocks in New Zealand (Slade *et al*., 1992) and is likely to occur in other vegetable paddocks where these chemicals are being used to control *Sclerotinia* diseases. Soil sterilisation by stem or methyl bromide is effective but costly. Further, environmental concerns over the use of methyl bromide mean that it is likely to be deregistered in the near future. Consequently, there is an urgent need to identify additional and more sustainable methods of control of *S. sclerotiorum*.

Fungal antagonists including the obligate sclerotial mycoparasite *Coniothyrium minitans* W. A. Campb. have shown potential for biological control of *S. sclerotiorum*. For example, *C. minitans* has been shown to be strongly parasitic toward *S. sclerotiorum*, *S. minor*, *Sclerotium cepivorum* and *Sclerotium rolfsii* in laboratory and glasshouse trials in New Zealand (Stewart & Harrison, 1988; Alexander, 1992). A wheat-bran formulation of *C. minitans*, applied as a soil amendment, has given significant control of *Sclerotinia* rot of lettuce in small scale field trails (A. Stewart, unpublished data). Similar activity for *C. minitans* isolates has been demonstrated in both glasshouse and field trials elsewhere (Budge & Whipps, 1991; McLaren *et al*., 1994; McQuilken *et al*., 1995) although control was less effective than that resulting from routine fungicide application as disease levels increased. Little is known about the mode of action involved in mycoparasitism of *S. sclerotiorum* by *C. minitans* or of the population biology of *C. minitans*. This lack of knowledge has been considered to be a major constraint to the development of the fungus as a commercial biological control agent (Whipps & Gerlagh, 1992).

The mycoparasite can survive in soils for periods of 2–3 years (McLaren *et al*., 1994) and there is evidence to suggest that it can be disseminated to a limited extent (McQuilken *et*

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al. 1995). Phenotypic differences in colony morphology have been described within *C. minitans* with at least seven classes recognized based on morphological characteristics (Sandys-Winsch *et al*., 1993). Differences in the mycoparasitic ability of *C. minitans* isolates toward *S. sclerotiorum*, *in vitro*, have also been detected (E. Jones, unpublished data). Successful biological control may depend, among other factors, on selection of an appropriate isolate for particular conditions. Given the limited morphological variation between isolates, however, it is difficult to monitor the survival and spread of any single *C. minitans* isolate in soil as this species occurs naturally in most soils (Sandys-Winsch *et al*., 1993). A key requirement, therefore, in extending to natural soils any study on the potential of individual isolates to effectively control *S. sclerotiorum* will be the ability to unambiguously detect and monitor the specific isolate or isolates applied to soils from those naturally occurring in the soils. Constitutive expression of a selectable antibiotic resistance gene or other reporter gene provides one such means of detection/monitoring. The utility of chimeric constructs (Punt *et al*., 1987; Roberts *et al*., 1989) of the hygromycin phosphotransferase gene (*hph*) as a selectable antibiotic resistance gene and the *E. coli uidA* gene, coding for β-glucuronidase (Jefferson, Burgess & Hirch, 1986; Jefferson, 1987), as a reporter gene have been demonstrated in numerous filamentous fungi. The aim of this study was to co-transform *C. minitans* with constructs encoding the *hph* and *uidA* genes and to investigate the effect of constitutive expression of these genes on colony growth under different environmental conditions and on mycoparasitic ability towards sclerotia of *S. sclerotiorum*.

MATERIALS AND METHODS

Fungal cultures, bacterial strains and bacterial transformation

C. minitans isolate A69 was isolated from sclerotia of *Sclerotium cepivorum* obtained from Pukekohe, New Zealand. *S. sclerotiorum* isolate G18 was obtained from I. Harvey (AgResearch, Christchurch, New Zealand) and was originally isolated from diseased carrot. Single spore cultures of *C. minitans* A69 and transformants of A69 were stored as conidial suspensions dried on filter paper disks at −20 °C. Fungal isolates were periodically cultured at 20° on potato dextrose agar (PDA).

Escherichia coli strain DH5α (Life Technologies Inc., Gaithersburg, MD) was used for propagation of plasmids. Preparation of electroporation competent cells and transformation were performed according to Smith *et al*. (1990). Plasmid DNA was isolated using Wizard Maxipreps DNA Purification Systems (Promega Corporation, Madison, WI).

Preparation of protoplasts

A modification of a previously published procedure (Crowhurst *et al*., 1992) was used. Growing medium (GM, Crowhurst *et al*., 1991) was inoculated with spores scraped form 7–14 d old PDA cultures and shaker incubated at 25°, 200 rpm, for 24 h. Spores were harvested by centrifugation at 4300 ***g*** (Sorvall GS3 rotor) at 4° and suspended at a concentration of 100 mg ml^{-1} in filter-sterilised OM buffer (1·2 M $MgSO_4 . 7H_2O$, 10 mM sodium phosphate, pH 5·8) containing 5 mg ml^{-1} filter-sterilised lysing enzyme (Sigma L2265). The digest was incubated at 29°, 80 rpm, for 3 h in a Grant Model SS40-2 shaking water bath (Grant Instruments, Cambridge, U.K.), overlaid with 4 ml of ST buffer (0·8 M sorbitol, 100 mM $CaCl_2$, 100 mM TrisHCl, pH 7·5) and centrifuged in a Sorvall HB-4 rotor at 1500 ***g*** for 20 min at 4°. Protoplasts were recovered from the interface using a wide bore pipette, transferred to a fresh tube, diluted five fold with ST buffer and pelleted by centrifugation at 1000 ***g*** for 5 min at 4°. The protoplasts were suspended in 20 ml of ST buffer and the centrifugation repeated. The protoplasts were washed three times in 20 ml volumes of ice-cold STC buffer (0·8 M sorbitol, 50 mM, $CaCl_2$, 50 mM TrisHCl, pH 7·5) and finally suspended in four parts of STC/one part of 40% PTC (40% PEG 4000, 50 mM $CaCl_2$, 50 mM TrisHCl, pH 7·5) at a final concentration of $1–2 \times 10^8$ protoplasts ml^{-1}. Protoplasts were used immediately for transformation.

Transformation of C. minitans

Vector DNA (2 μg each of pAN7-1 and pNOM102) in a volume of 10 μl STC was mixed with 100 μl of protoplast suspension, incubated on ice for 30 min and then 900 μl of PTCS buffer (40% PEG 4000, 0·8 M sorbitol, 50 mM $CaCl_2$, 50 mM TrisHCl, pH 7·5) was added and incubation continued at room temperature for 30 min. Aliquots (100 μl) were mixed with 10 ml of molten (45°) top-agarose (GM containing 0·8 M sucrose and 0·8% Ultrapure low melting point agarose, Life Technologies, Inc) and overlaid onto 20 ml of selective medium (PDA containing 120 μg ml^{-1} of hygromycin B, Boehringer Mannheim Biochemicals, Mannheim, Germany). Plates were incubated at 20°. Transformants were transferred to selection medium (PDA containing 80 μg ml^{-1} of hygromycin B). Single spore progeny colonies were recovered for each transformant and mitotic stability assessed by multiple passages on PDA prior to exposure to selection medium.

DNA isolation and Southern analysis of transformants

Wild type A69 and selected transformants were inoculated into GM and incubated at 24° for 5 d. Mycelium was harvested by filtration through miracloth, snap frozen in liquid nitrogen and lyophilized. Genomic DNA was extracted from the lyophilized mycelium according to previously described methods (Crowhurst *et al*., 1991). Aliquots (5 μg) of genomic DNA were digested with *Bgl* II, *Bam*H I, *Cla* I, *Eco*R V, or *Nco* I, singly, or with *Sst* I and *Xho* I together then size fractionated by electrophoresis in 1% TAE agarose and Southern blotted using standard procedures (Sambrook, Fritsch & Maniatis, 1989). Amplification of the internal fragment of 765 bp from the *hph* gene using pAN71 as a template was carried out with the IAN7F (5′-ATGCCTGAACTCACCG-CGAC-3′) and IAN7R (5′-TGCAAGCTCCGGATGCCTCC-3′) primers. Similarly, amplification of the internal fragment of 1037 bp from the *uidA* gene using pNOM102 as a template was carried out with the INOMF (5′-TGGTCCGTCCTGT-AGAAACC-3′) and INOMR (5′-GATGCCATGTTCATCT-GCCC-3′) primers. Amplifications were performed in 50 μl

reaction volumes (100 nM of each dNTP, 2–200 pg of plasmid template, 1 U of Taq DNA polymerase (Boehringer Mannheim), 100 nM of each primer, 50 mM KCl, 100 mM Tris-HCl (pH 8·3), 20 mM $MgCl_2$) in a Twinblock System Easy Cycler (Ericomp) programmed for an initial denaturation step at 94° for 3 min followed by 35 cycles of denaturation at 94° for 30 s, annealing at 55° for 2 min, and extension at 72° for 2 min. A final extension step at 72° was for 7 min. The PCR products were gel purified and labelled with α-^{32}P-dCTP using a RadPrime Labelling System (Life Technologies, Inc.) and hybridization performed in 0·5 M sodium phosphate (pH 7·2), 7% SDS at 60° overnight. Filters were washed four times in 50–100 ml volumes of 1 × SSC, 1% SDS at 60°, 20 min per wash. The washed filters were exposed to Curix X-ray film (Agfa) for 24 h at −70°.

In situ *colony colour assay of* β*-glucuronidase activity*

To test for β-glucuronidase activity, isolates were grown in potato dextrose broth (PDB) and transferred to 96-welled micro-titre plates containing minimal medium for 2 d. To each well, 100 µl of 0·1% 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) (0·5% Triton X-100, 50 mM sodium phosphate buffer, pH 7·5, 5% *N,N*-dimethylformamide) was added and incubated for 24–72 h at 24°.

Colony morphology characteristics

The gross colony morphologies of the wild type and transformants were examined after incubation for 2 wk in the dark on PDA at 20°. Cultures were categorized according to colony colour and distribution of pycnidia into one of seven groups as described by Sandys-Winsch *et al*. (1993).

Physiological tests of transformants

To ensure that multiple integration of the introduced genetic markers into A69 did not result in reduced growth or mycoparasitic ability of the transformants as compared to wild type, several phenotypic characters of both wild type and the five selected monospore cultures derived from the original transformants were assessed. This included assaying the effect of growth medium, temperature, matric and osmotic potentials on colony extension rates as well as parasitism of sclerotia in both sand and soil.

The growth rates of transformed strains of *C. minitans* A69 were compared to the wild type A69 on three different agar media: PDA, Czapek Dox Agar (CDA) and sterile distilled water agar (SDWA) (10 g Difco Bacto-Agar l^{-1} distilled water). Agar discs (8 mm) were taken from the margins of 14-d old colonies grown on SDWA, placed centrally on plates (five plates per isolate) of each medium and incubated at 20° in the dark. Colony diameters were recorded daily for 7 d and hyphal extension rates were calculated for growth between 3 and 6 d.

The effect of temperature on hyphal extension rate was studied by incubating five plates of inoculated PDA for each transformant at 4, 10, 14, 20, 25 and 30°. Hyphal extension rates were calculated from growth between 3 and 6 d. Hyphal extension rate data on different media and at different temperatures was analysed using Two Way Analysis of Variance, with the treatment means compared using the least significant difference (L.S.D.) test at a probability of 5%.

The effect of osmotic and matric potentials on hyphal extension rates was determined using a modification of the method of Douglas & Deacon (1994). Prior to autoclaving, KCl was added to PDA at concentrations of 0, 10·06, 17·9, 28·44, 36·5 and 47·56 g l^{-1} to produce osmotic potentials of −0·1 (control), −1·1, −1·9, −3·0, −3·9 and −5·0 MPa, respectively. Similarly, polyethylene glycol (PEG) 8000 (Sigma) was added to PDB at concentrations of 0, 300, 414, 530, 595 and 696 g l^{-1} to give matric potentials of −0·1 (control), −1·1, −1·9, −3·0, −3·9 and −5·0 MPa, respectively. PEG was used in a liquid medium because at the concentrations used it prevented gelling of agar. Agar discs (8 mm diam.) were taken from the margins of 14-d old cultures of *C. minitans* on SDWA and placed centrally into sterile Petri dishes containing either PDA supplemented with KCl or PDB (15 ml) supplemented with PEG. Dishes were incubated at 20° in darkness with the minimum of disturbance and colony diam. recorded daily for 7 d. Five plates for each osmotic potential and four plates for each matric potential were inoculated for the wild type isolate and each of the five transformants tested. Hyphal extension rates were calculated between 3 and 6 d and expressed as a proportion of growth in the control (−0·1 MPa). The data conformed to a test of normality and showed equal variance between treatments and therefore were not transformed prior to analysis. The results at each potential were subject to One Way Analysis of Variance with the treatment means compared using the L.S.D. test at a probability of 5%.

Sclerotial parasitism in sand. Sclerotia of *S. sclerotiorum* were produced using the method of Mylchreest & Wheeler (1987) whereby wheat seeds (25 g; Australian Standard White, Champion Flourmills, Christchurch, NZ) were placed in 250 ml flasks containing 60 ml water and autoclaved. Once cooled, each flask was inoculated with three PDA discs (10 mm diam.) taken from the colony margin of 2–3 d old cultures of *S. sclerotiorum*. Flasks were incubated at 20° in darkness. Each flask was shaken after one week to facilitate mixing of the inoculum. After 3 wk, the contents of each flask were washed with SDW and air dried overnight in a laminar flow hood. Sclerotia were separated from the wheat grains, stored at room temperature in the dark and used within 4 wk.

A modification of the method of Whipps & Budge (1990) was used to compare the sclerotial parasitism of the transformants to the wild type A69. Spores from 10–14 d old *C. minitans* PDA cultures were suspended in a solution containing 1 drop Tween 80 100 ml^{-1} distilled water and adjusted to 1×10^6 spores ml^{-1}. For the wild type *C. minitans* and each of the five transformants tested, 60 sclerotia (2–4 mm diam.) were placed in 10 ml of spore suspension in Universal bottles and shaken gently using a wrist action shaker for 10 min. After leaving to stand for a further 10 min the sclerotia were removed and 20 placed in each of three Petri dishes containing 50 g sterile acid washed silver sand (pre-moistened to 50% saturation with SDW), the sclerotia

being lightly pressed into the surface of the sand. Sclerotia treated with SDW were used as a control. The Petri dishes were then incubated at 20° in darkness. After one week incubation the water content of each dish was re-adjusted to 50% saturation using SDW. After two weeks incubation, sclerotia were harvested and surface sterilised by agitation in 20 ml of a solution containing equal parts of 13–15% sodium hypochlorite (Wilsons Chemicals Ltd, NZ) and absolute ethanol for 3 min. Sclerotia were rinsed by three successive transfers into 15 ml of SDW for 3 min.

The sclerotia were then bisected, with each half being placed on a 14 mm diam. disc of PDA (supplemented with 20 μg ml^{-1} aureomycin, containing 93·5% chlorotetracycline hydrochloride; Sigma Chemicals). Inoculated agar discs were incubated at 20° for 10 d and the number of viable sclerotia and *C. minitans*-infected sclerotia then determined. The stability of hygromycin resistance and *uidA* expression after passage through a sclerotium was tested for a number of *C. minitans* colonies recovered from the PDA disc for each of the transformants. Selected colonies were inoculated onto PDA supplemented with hygromycin B (80 μg ml^{-1}) and incubated at 20° for 10 d, after which time the plates were examined for growth. Colonies were also assessed for β-glucuronidase activity in a modified method from that previously described. Isolates were grown for 48 h on 1·5% SDWA in 96 well microtitre plates. To each well was added 25 μl of 2 mM X-gluc in 100 mM sodium phosphate buffer (pH 7) and the reactions incubated in the dark at 20° for 24–72 h prior to assessment of colour development.

Sclerotial parasitism in soil. The method of Whipps & Budge (1990) was used to assess parasitism of sclerotia in non-sterile field soil. *C. minitans* A69 wild type and transformants were grown in 250 ml flasks containing 100 ml of autoclaved kibbled maize:perlite (15:85% v/v) moistened with 20% distilled water. Each flask was inoculated with 5 ml of 1×10^6 spores ml^{-1} of the appropriate spore suspension and incubated at 20° in the dark for 4 wk. The flasks were shaken once a week. For the wild type *C. minitans* and each transformant, 5 ml of the colonized maize:perlite was ground using a mortar and pestle and mixed thoroughly into 495 ml of dry soil (Wakanui silt loam, sieved to 4 mm mesh size). The mixture (50 g) was placed in each of three Petri dishes (9 cm diam. 1 cm deep) and moistened to −0·1 MPa (9 ml H_2O 50 g^{-1} soil) with SDW. For the wild type *C. minitans* and each transformant, 60 sclerotia (2–4 mm diam.), produced as previously described, were pressed lightly into the surface of the inoculated soil, 20 per dish, and incubated at 20° in darkness. The water content of plates was adjusted to −0·1 MPa weekly. Sclerotia placed on non-inoculated soil were used as a comparative control. After two weeks, all sclerotia per treatment were harvested and then surface sterilized as described previously. The sclerotia were bisected and placed on pairs of PDA discs (14 mm diam.), one supplemented with 20 μg ml^{-1} aureomycin, the other with 20 μg ml^{-1} aureomycin and 80 μg ml^{-1} hygromycin B. Discs were incubated at 20° and the bisected sclerotia were assessed for viability and infection by *C. minitans* after 7 and 14 d. The inoculum level of *C. minitans* contained within the maize:perlite was estimated using standard dilution plating techniques (Whipps, Budge & Ebben, 1989) except that dilutions were plated on three plates of both CDA supplemented with aureomycin (20 μg ml^{-1}) and Triton X-100 (2 ml l^{-1}) and CDA supplemented with aureomycin (20 μg ml^{-1}) and hygromycin B (80 μg ml^{-1}). The amount of antagonist inoculum in the soil bioassays at the start of the assay and after the 2 wk incubation was estimated using a soil dilution technique. A 10 ml soil sample for each treatment was diluted with 90 ml sterile 0·01% agar, and treated as described previously (Whipps *et al.*, 1989) except that dilutions were plated on CDA supplemented with aureomycin and either Triton X-100 or hygromycin B as described above. The colony forming unit (cfu) counts for the wild type and five transformants were subjected to One Way Analysis of Variance, with the treatment means compared using the L.S.D. at a probability of 5%. Paired *t*-tests were used to compare cfu counts on CDA amended with aureomycin and Triton X-100 to those on CDA amended with aureomycin and hygromycin B.

Parasitism of sclerotia was also assessed using spore suspensions for the wild type *C. minitans* and each of the five transformants. Nine ml of diluted spore suspension was added to each of three Petri dishes containing 50 g of air-dried soil (Wakanui silt loam sieved 4 mm) and mixed thoroughly, to give a final concentration of 1×10^6 spores g^{-1} soil and soil water potential of −0·1 MPa. For each treatment, 20 sclerotia of *S. sclerotiorum* were pressed gently into the surface of the soil in each dish. The Petri dishes were incubated at 20° in the dark, with the plates being watered to their original content using SDW after a week. Control plates were inoculated with SDW. After two weeks, all sclerotia were harvested and assessed for viability and infected by *C. minitans* as previously described. The cfu counts of the wild type *C. minitans* and each of the 5 transformants in the soil after 2 wk incubation was estimated using the soil dilution technique as described previously.

RESULTS

Transformation of C. minitans

Mycelium and spores of A69 grew on PDA with either 5, 10 or 25 μg ml^{-1} hygromycin B; at 50 μg ml^{-1} spores germinated but failed to continue growth and no hyphal growth was detected when mycelial agar plugs were used as inoculum. No germination of spores or growth of mycelium from agar plugs was observed when hygromycin B was $\geqslant$ 80 μg ml^{-1}.

Isolate A69 was co-transformed with pAN7-1 and pNOM102. Successive transformations experiments using 2 μg each of pAN7-1 and pNOM102 into A69 resulted in frequencies of transformation ranging between 0 and 1000 transformants μg^{-1} of plasmid DNA or PDA containing 80 μg ml^{-1} of hygromycin B. To select for stable transformants, single spores from 104 hygromycin B resistant colonies were passaged three times on PDA without antibiotic selection followed by a fourth passage onto PDA plates containing 100 μg ml^{-1} of hygromycin B. Two monosporic cultures for each of five independent stable transformants selected from the 104 transformants were qualitatively assayed for *uidA*

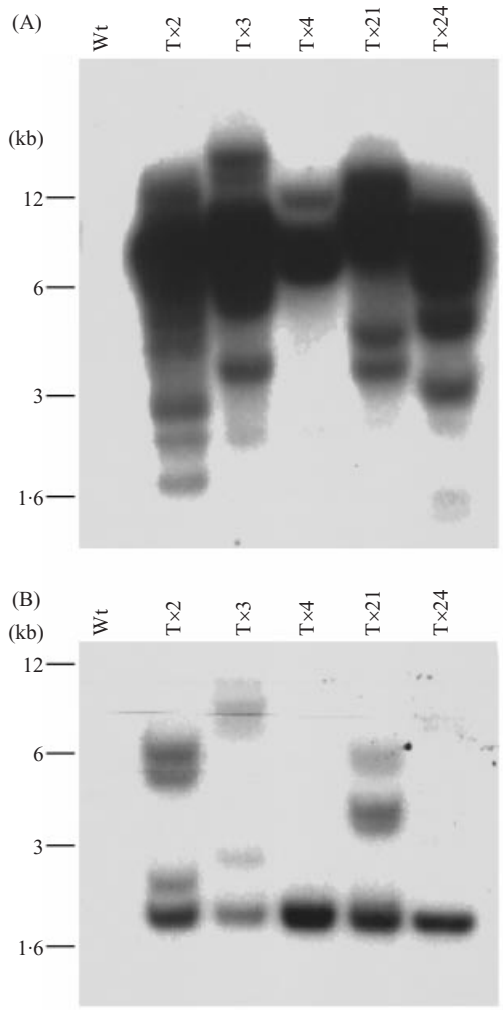

Fig. 1. Southern analysis of integration of plasmids pAN7-1 (A) and pNOM102 (B). DNA (5 μg/lane) from wild type A69 (Wt) and five *Coniothyrium minitans* transformants was digested with *Nco* I and hybridized either with the 1 kb *Aat* II-*BamH* I fragment from the *hph* gene in pAN7-1 (A) or with the 1·9 kb *Nco* I fragment from the *uidA* gene in pNOM102 (B). Selected mol. wt size fragments are indicated.

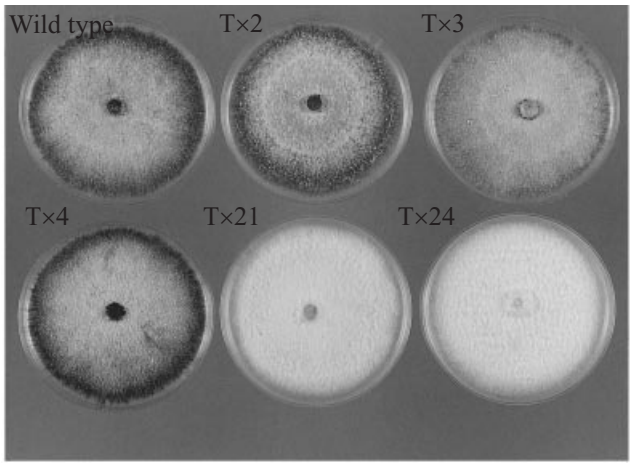

Fig. 2. Colony morphology of *Coniothyrium minitans* A69 wild type and five transformants on PDA after 2 wk incubation at 20° in the dark.

Table 1. Hyphal extension rates of five *uidA* and *hph* expressing transformants of *C. minitans* isolate A69 compared to the wild type isolate on three agar media

	Mean growth rate (mm d^{-1}) ± S.E.		
	PDA	CDA	SDWA
Wild type	4·6 ± 0·04[a]	1·4 ± 0·2	0·6 ± 0·2
T × 2	2·4 ± 0·2	1·4 ± 0·1	0·4 ± 0·1
T × 3	3·7 ± 0·1	0·7 ± 0·1	0·3 ± 0·1
T × 4	3·3 ± 0·2	0·6 ± 0·1	0·3 ± 0·1
T × 21	3·3 ± 0·1	0·8 ± 0·1	0·4 ± 0·1
T × 24	3·3 ± 0·2	0·6 ± 0·04	0·4 ± 0·1
L.S.D. 0·32 (72 d.f.)[b]			

[a] Mean ± S.E. of five replicate plates.

[b] Significant differences in means are given by L.S.D. ($t_v \times$ S.E.D.), where t = critical value ($P = 0{\cdot}05$) of Student's t-distribution for v d.f. and S.E.D. = standard error of the differences between means derived from analysis of variance.

expression using the microtitre plate colour assay. All but one of the 10 monospore hygromycin resistant cultures tested was also found to express the *uidA* gene suggesting that co-transformation is very efficient in *C. minitans*. A single spore colony for each transformant was used for subsequent assays and Southern analysis.

Southern analysis showed that neither the PCR-derived *hph*-gene probe nor the *uidA*-gene probe hybridized to digested DNA from wild type A69 (Fig. 1). Both probes did hybridize to one or more fragments in digested DNA of each of the five selected *hph*- and *uidA*-expressing transformants thus confirming the integration of each plasmid into the genomes of these transformants. Not all integration events, however, may have resulted in functional copies of these integration vectors. As expected, the *uidA*-gene probe hybridized to a single 1878 bp vector fragment in *Nco* I-digested DNA of each transformant. The *uidA*-gene probe, however, also hybridized to at least two additional higher mol. wt fragments in four of the five transformants (Fig. 1B). Since *Nco* I sites occur at the translation start codon and also at the 3′ end of the coding region of the *uidA* gen in pNOM102, it is likely that these patterns of hybridization represent integration events wherein integration of pNOM102 has occurred into the genome of A69 at a site between these two *Nco* I sites in the vector. Such integration would result in disruption of the coding region of the *uidA* gene.

Colony morphology

According to the classification of Sandys-Winsch *et al.* (1993), the wild type A69 was categorized into colony type 3 (Fig. 2), with the colony being moderate yellow or honey in colour from the top and darker in appearance from the bottom. Many black mature pycnidia were visible from both sides of the plate. Transformants T × 2, T × 3, and T × 4 were similar in colony morphology and were also categorized as colony type 3. T × 21 and T × 24 were, however, paler in colour (cream) from both sides of the colony with more aerial mycelium visible from the top (Fig. 2). The pycnidia were sparsely distributed and mainly visible from the reverse as brown patches in these two cultures. This morphological phenotypic pattern fits more the description of colony type 4.

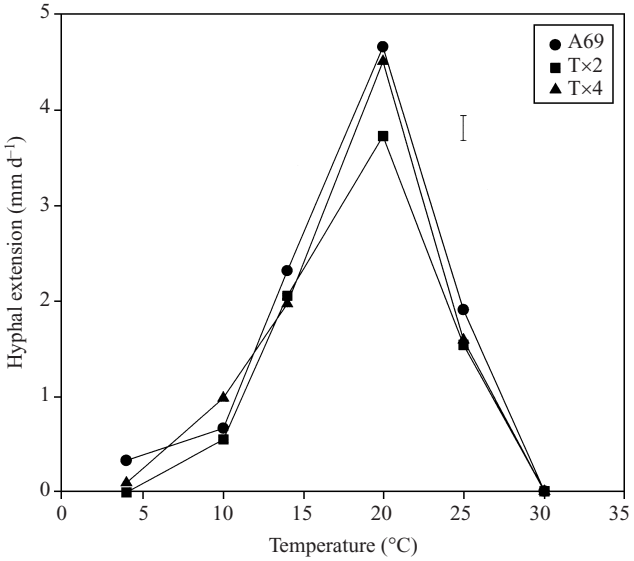

Fig. 3. Effect of temperature on radial hyphal extension between 3 and 6 d on PDA for GUS-hygromycin B transformants compared with the wild type *Coniothyrium minitans* A69; means of five replicates with bar representing L.S.D. at $P < 0{\cdot}05$. T × 3, T × 21 and T × 24 not significantly different from T × 4 (data not presented).

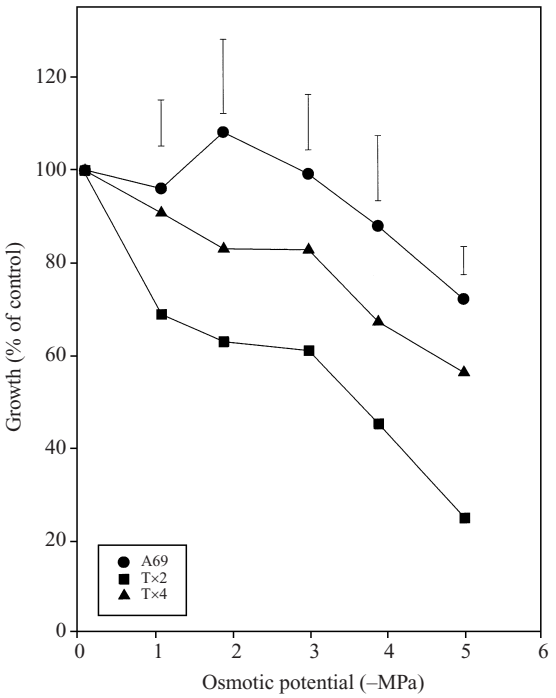

Fig. 4. Effect of osmotic potential on radial extension between 3 and 6 d as a percentage of the control (−0·1 MPa) for GUS-hygromycin B transformants compared with the wild type *Coniothyrium minitans* A69; means of five replicates, with bars representing L.S.D. at $P < 0{\cdot}05$. T × 3, T × 21 and T × 24 not significantly different from T × 4 (data not presented).

Physiological tests of transformants

The hyphal extension rates of all isolates were significantly ($P < 0{\cdot}05$) lower on CDA and SDWA than on PDA (Table 1), with hyphal extension rates on CDA being significantly higher than those recorded on SDWA for wild type, T × 2, T × 3 and T × 21. On PDA, all transformants grew sig-

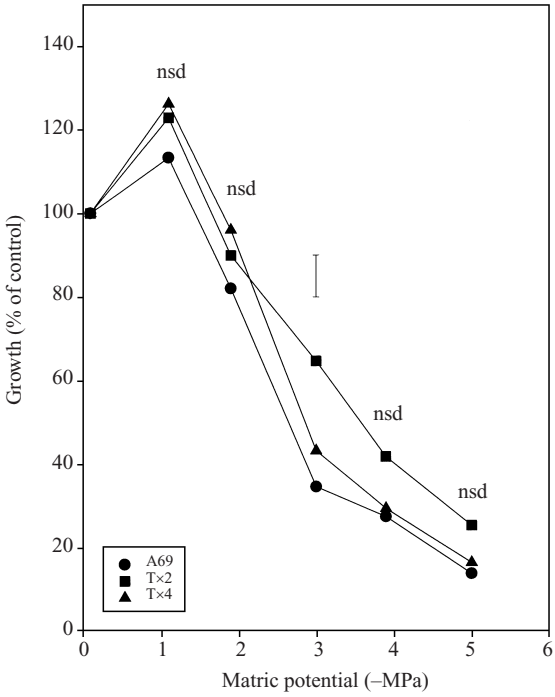

Fig. 5. Effect of matric potential on radial extension between 3 and 6 d as a percentage of the control (−0·1 MPa) for GUS-hygromycin B transformants compared with the wild type *Coniothyrium minitans* A69; means of four replicates, with bar representing L.S.D. at $P < 0{\cdot}05$, nsd = no significant difference. T × 3, T × 21 and T × 24 not significantly different from T × 4 (data not presented).

nificantly slower than the wild type, with transformant T × 2 growing significantly slower than the other four. T × 2 and wild type grew significantly faster on CDA, however, as compared with the other four transformants, with there being no significant difference in the extension rates on SDWA for any of the transformants compared with that of the wild type.

For both the wild type and the five transformants, the optimum temperature for maximal hyphal extension was 20°, with growth taking place between 4 and 25° (Fig. 3). Although there were small, but significant, differences in the hyphal extension rates of the transformants compared with the wild type at different temperatures, the general trend was similar in each case.

To determine the effect of osmotic potential on growth of wild type and transformants, colony extension rates were measured at different water potentials (Fig. 4). Transformant T × 2 was significantly more sensitive to high osmotic potentials than the wild type and the other four transformants. Wild type A69 was observed to be the most tolerant of increased osmotic potential with a hyphal extension rate at −5·0 MPa of 72% of that recorded on the control plate (−0·1 MPa). Transformants T × 3, T × 4, T × 21 and T × 24 were intermediate between wild type and T × 2 in their sensitivity to high osmotic potential and were able to grow at −5·0 MPa at approximately 60% of their growth rate on the control plates (−0·1 MPa).

There was no significant difference (at $P < 0{\cdot}05$) in radial growth rates after 5 d on the control PDB plates (wild type, $3{\cdot}2 \pm 0{\cdot}1$; T × 2, $1{\cdot}9 \pm 0{\cdot}8$; T × 3, $2{\cdot}6 \pm 0{\cdot}3$; T × 4, $2{\cdot}6 \pm 0{\cdot}3$; T × 21, $2{\cdot}4 \pm 0{\cdot}2$ and T × 24, $2{\cdot}6 \pm 0{\cdot}2$ mm $d^{-1} \pm$ S.E., re-

Table 2. The effect of incorporating 1% maize:perlite, colonized by either *C. minitans* isolate A69 or one of five *uidA* and *hph* expressing transformants, into soil on the viability of sclerotia of *Sclerotinia sclerotiorum* after 2 wk incubation at 20°.

	Number of sclerotial halves giving rise to colonies of *S. sclerotiorum* and *C. minitans* on PDA discs ± hygromycin ($n = 60$)			
	−hyg		+hyg	
	C. minitans	*S. sclerotiorum*	*C. minitans*	*S. sclerotiorum*
Control[a]	6	55	0	0
C. minitans A69	51	15	0	0
C. minitans T × 2	47	21	52	0
C. minitans T × 3	56	13	55	0
C. minitans T × 4	54	20	51	0
C. minitans T × 21	53	19	53	0
C. minitans T × 24	52	18	51	0

[a] Unamended soil.

Table 3. Survival of *C. minitans*, expressed as $\log_{10}$ colony forming units (cfu), in maize:perlite amended soil. Mean of three replicates plates ± S.E.M.

	$\log_{10}$ cfu ml^{-1}					
	Maize:perlite inoculum		Soil 0 wk		Soil 2 wk	
Test isolates	−hyg[a]	+hyg[b]	−hyg	+hyg	−hyg	+hyg
Control	NA[c]	NA	/[d]	/	/	/
C. minitans A69	8·03 ± 0·03	/	5·80 ± 0·04	/	5·23 ± 0·10	/
C. minitans T × 2	7·93 ± 0·06	8·10 ± 0·05	5·76 ± 0·02	5·94 ± 0·09	4·73 ± 0·06	4·83 ± 0·07
C. minitans T × 3	8·10 ± 0·11	8·20 ± 0·08	6·03 ± 0·08	6·11 ± 0·08	4·41 ± 0·10	5·43 ± 0·09
C. minitans T × 4	7·92 ± 0·09	7·96 ± 0·04	5·78 ± 0·05	5·85 ± 0·09	4·80 ± 0·02	5·42 ± 0·06
C. minitans T × 21	8·10 ± 0·07	8·11 ± 0·05	6·00 ± 0·05	6·06 ± 0·07	5·68 ± 0·05	5·65 ± 0·03
C. minitans T × 24	7·83 ± 0·13	7·90 ± 0·07	5·71 ± 0·05	5·80 ± 0·05	4·87 ± 0·09	5·56 ± 0·05

[a] CDA with Triton X and aureomycin.
[b] CDA with hygromycin B and aureomycin.
[c] Not applicable.
[d] No colonies detected.

spectively). There was also no significant difference (at $P < 0·05$) in the response of the five transformants compared with the wild type at matric potentials of −1·1, −1·9, −3·9, or −5·0 MPa (Fig. 5). At a matric potential of −3·0 MPa, however, T × 2 was more tolerant, with its hyphal extension rate being significantly higher than that of the other transformants. The hyphal extension rates of the other transformants were, in turn, significantly higher than that of the wild type A69. All five transformants and wild type grew significantly faster at −1·1 MPa than at −0·1 MPa with the growth relative to the control being between 113 and 136%.

Sclerotial parasitism in sand. All transformants reduced sclerotial viability by 91·7–96·7% ($n = 60$). This reduction was comparable with that observed with A69 (96·7%). Further, the colonies recovered following plating of the sclerotial halves were consistently found to be hygromycin resistant and expressed *uidA*.

Sclerotial parasitism in soil. The viability and infected by *C. minitans* of sclerotia after incubation in *C. minitans* amended soils is shown in Table 2. When applied as solid substrate amendments to the soils, all transformants reduced the viability of sclerotia comparable with that achieved with wild type A69. Cultures of *C. minitans* were consistently recovered from the sclerotia (Table 2) when hygromycin was not included in the selective medium used for assessing viability of sclerotial halves. A low level of recovery of *C. minitans* from control treatments suggests that, not unexpectedly, this organism was present in the natural soils used for these experiments.

After incubation for 4 wk in maize:perlite, the wild type and all transformants gave rise to a minimum of $0·5 \times 10^8$ cfu ml^{-1} substrate, with there being no significant difference (at $P < 0·05$) in colony number either on CDA amended with Triton X-100 and aureomycin (CAT plates) or CDA amended with hygromycin B and aureomycin (CAH plates). Further, for wild type and all five transformants, incorporation of infested maize:perlite based inoculum into soil resulted in recovery of 5×10^5 cfu g^{-1} soil or greater immediately after incorporation. Inoculum viability decreased, however, over the 2 wk incubation following inoculation as indicated by a 2–37 fold decrease in colonies recovered on CAT plates and a 2–13 fold decrease when CAH plates were used (Table 3).

A reduction of between 93·3 and 100% in sclerotial viability was achieved by incorporating spores of the transformants into soil and was comparable to that obtained with the wild type A69 (96·7%). Isolation of the transformants from sclerotia on hygromycin B-amended PDA (98·3–100%) was comparable to that achieved on unamended PDA

Table 4. Survival of *C. minitans* in spore amended soil. Mean of three replicate plates ± S.E.M.

	Log_{10} cfu ml^{-1} soil after 2 wk	
	−hyg[a]	+hyg
Control	/[c]	/
C. minitans A69	4·87 ± 0·06	/
C. minitans T × 2	5·03 ± 0·09	4·97 ± 0·07
C. minitans T × 3	5·07 ± 0·03	5·02 ± 0·01
C. minitans T × 4	4·65 ± 0·13	4·84 ± 0·11
C. minitans T × 21	4·83 ± 0·11	4·89 ± 0·06
C. minitans T × 24	5·07 ± 0·03	5·01 ± 0·08

[a] CDA with Triton X and aureomycin.
[b] CDA with hygromycin B and aureomycin.
[c] No colonies detected.

(95–100%). *Coniothyrium minitans* wild type A69 was also isolated at a similar level on unamended PDA (96·7%) but was not isolated on hygromycin B-amended PDA. Again *C. minitans* was isolated at low levels (1·7%) from control treatments, indicating its presence in natural unamended soil. After 2 wk incubation, *C. minitans* cfu counts fell between 8–20 fold for CAT plates and 9–14 fold for CAH plates (Table 4).

DISCUSSION

To facilitate the unambiguous identification of *C. minitans* isolates we have developed a transformation system for this fungus and have produced transformants carrying markers for the reported gene β-glucuronidase (*uidA*) and antibiotic resistance gene hygromycin B (*hph*). A modification of the transformation protocol of Crowhurst *et al.* (1992) was used to co-transform *C. minitans* isolate A69 with plasmids encoding these two marker genes. The rate of co-transformation of the selectable (*hph*) marker gene and the non-selectable (β-glucuronidase) marker gene was relatively high suggesting that co-transformation of supercoiled DNA may be very efficient in this isolate.

All transformants were found to carry multiple copies of each marker gene following co-transformation. Since both the *hph* and β-glucuronidase constructs were under the control of the constitutive promoter from the *Aspergillus nidulans* glyceraldehyde-3-phosphate gene it is possible that this constitutive expression might place an undesirable metabolic burden on a transformant. It is important that constitutive expression of these marker genes does not interfere with expression of other desirable characters, in particular the efficacy of mycoparasitism of *S. sclerotiorum*. We therefore compared transformants with wild type of growth rates in culture, tolerances to different water potentials and ability to parasitize sclerotia of *S. sclerotiorum* both in sand and in non-sterile soil.

Three of the five transformants (T × 2, T × 3, T × 4) were observed to have a similar colony morphology to the wild type on PDA and were assigned to colony type 3 according to the classification of Sandys-Winsch *et al.* (1993). Although derived from the same wild type isolate transformations, however, T × 21 and T × 24 were morphologically more characteristic of colony type 4. Sandys-Winsch *et al.* (1993) reported that many cultures of *C. minitans* changed in morphology whilst growing in pure culture on PDA. Sectors were not only morphologically different, but were often seen to have significantly different hyphal extension rates and pycnidiospore size from the original isolate. The pathogenicity of pycnidiospores, arising from these sectors, towards sclerotia compared with the original culture was not reported, but it would seem that some isolates of *C. minitans* are genetically unstable.

In general, the hyphal extension rates for the wild type were significantly higher than those for the transformants, with the maximum growth occurring on PDA in all cases. The temperature range of 4–25° for hyphal growth for both wild type and the transformants with an optimum at 20° corresponds to that reported by other workers for other isolates of *C. minitans* (McQuilken, Budge & Whipps, 1997).

Wild type A69 was relatively tolerant to increased osmotic potentials, with its hyphal extension rate at −5·0 MPa being 72% of that recorded on the control plates (−0·1 MPa). In comparison Whipps & Magan (1987) reported growth of only 41% of control growth (−0·7 MPa) at −4·2 MPa for their *C. minitans* isolate. In their case, the osmotic potential was achieved by amendment with NaCl and not KCl as in this study, and the two salts may have other physiological effects other than osmotic effects. *C. minitans* isolates may, however, have different inherent tolerances to osmotic potential which is worthy of investigation in itself and may be important in biological control systems. All five transformants, whilst less tolerant of increased osmotic potentials compared with the wild type, displayed the same general trend in response to increased osmotic potential as was observed for the wild type.

This is the first report of the effect of matric potential on *C. minitans*. Matric potential may have an influence on the ability of *C. minitans* to grow through soil and on plant surfaces. All transformants responded in a similar way to the wild type to increased matric potentials and were more sensitive to increased matric potential than increased osmotic potential.

Despite the transformants possessing lower hyphal extension rates on PDA at different temperature and osmotic potentials, with T × 2 being consistently lower in all cases, they were found to be as pathogenic as the wild type in parasitism assays. Since a consistently high proportion of sclerotia treated with the mycoparasite were infected (> 90%), the lack of differences in pathogenicity observed could suggest an inoculum overload. In preliminary work (data not shown), however, no difference in the pathogenicity of the transformants when compared with wild type A69 was detected when the inoculum concentration was reduced to 10^4 or 10^2 spores g^{-1} soil.

In all cases, amendment of soil with spores was seen to be more effective at reducing sclerotial viability compared with amendment with colonized maize:perlite. Since *C. minitans* has been reported to have little ability to grow through soil from an inoculum source (Whipps & Budge, 1990) infection may rely on direct contact between *C. minitans* and the sclerotium. Direct contact is more likely to occur when inoculating with spore suspension than with a solid-substrate-based inoculum, since it is easier to achieve uniform distribution

of *C. minitans* in the soil. Also, a solid-substrate-inoculum is more readily colonized by other soil microorganisms which potentially could lead to displacement of *C. minitans* from the substrate and therefore reduce the inoculum density.

Four of the five transformants examined grew well on a range of culture media, maintained their parasitic activity against sclerotia, and responded in a similar manner to the wild type when grown at different water potentials. This suggests that constitutive expression of the introduced genes did not adversely affect these phenotypic traits in the transformants. Expression of *hph* and *uidA* was found to be stable in all the transformants with both being expressed after passage through a sclerotium. T × 3 and T × 4 appeared to be the most characteristic of wild type in colony morphology, growth and parasitism, however, and it is likely that they will be used in further experiments.

The possession of hygromycin-resistant isolates will allow us to selectively isolate and quantitate isolate A69 from soil samples, thus enabling us to monitor the survival and colonisation of substrates by *C. minitans* over time. The *uidA* gene has already proved useful in other fungal systems to quantify biomass (Oliver *et al.*, 1993), monitor root colonization (Eparvier & Alabouvette, 1994) and infection (Liljeroth, Jansson & Scahfer, 1993). We will use GUS-expressing transformants of *C. minitans* A69 to visualize sclerotial infection *in situ* and determine the potential of *C. minitans* to colonize plant root systems infected by *S. sclerotiorum*.

This research opens the way for the use of genetically marked isolates in answering key ecological questions relating to the survival and dissemination of this mycoparasite.

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