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TECHNICAL NOTE

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Agrobacterium tumefaciens-mediated genetic transformation of the phytopathogenic ascomycete *Calonectria morganii*

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Abstract Conidia of the phytopathogenic fungus *Calonectria morganii* were transformed to hygromycin B resistance using the *hph* gene of *Escherichia coli* as the selective trait, governed by a heterologous fungal promoter and the *Aspergillus nidulans trpC* terminator. *Agrobacterium tumefaciens*-mediated transformation yielded stable hygromycin B-resistant clones (average number 10^6 per 10^7 conidia). Putative transformants appeared to be mitotically and meiotically stable. The presence of the *hph* gene was checked by PCR. In four randomly chosen transformants, single-copy integrations of the marker gene at different chromosomal sites were proven by Southern analysis.

Keywords Genetic transformation · Mitotic and meiotic stability · Marker integration · Phytopathogenicity

Introduction

Several representatives of the genus *Calonectria* are the causative agents of a number of plant diseases (Crous and Wingfield 1994). As necrotrophic pathogens, they grow abundantly on a panoply of hosts belonging to genera as diverse as *Rhododendron*, *Eucalyptus*, *Pinus* and *Rosa* (Alfieri et al. 1972; Waipara et al. 1996). In Europe, much attention has been paid to *Calonectria morganii*, because this species causes stem and root rot in economically important ornamental plants, such as *Calluna vulgaris*, *Erica gracilis* and *Rhododendron simsii* (Neubauer and Zinkernagel 1995; Overmeyer et al. 1996; Litterick and McQuicken 1998). The fungus affects predominantly young seedlings, cuttings, or otherwise impaired plants. Thus, losses are particularly high in nurseries specialised in the culture and marketing of ericaceous plants. The main sources for the spread of infection are the conidiospores and microsclerotia, which remain viable in the soil for years (Hunter and Barnett 1976).

Since the fungus is highly resistant to commonly applied fungicides, the use of healthy seedlings, cuttings and plants is at present the only way to control the disease (Peerally 1991; Neubauer and Zinkernagel 1995). Genetic variability among isolates and sexual offspring of the fungus was recently investigated (Overmeyer et al. 1996; Schoch et al. 2000), but essentially nothing is known about the genetic and physiological basis of its phytopathogenicity.

Approximately 30 years ago, cyclic tetrapeptides (Cyl 1 and Cyl 2) were reported as potential phytotoxic compounds for *Cylindrocladium scoparium*, the imperfect state of *Calonectria morganii* (Hirota et al. 1973a, b, c). Candidate genes encoding putative peptide synthetases have been identified only recently (von Wallbrunn and Meinhardt 1999; von Wallbrunn 2000). In a search for bioactive compounds among European strains, we have isolated and identified two new phytotoxic substances from isolate CS5 (Overmeyer et al. 1996), which is under investigation here. These were identified as chaetoglobosin A and 19-0-acetylchaetoglobosin A, C-18 polyketides linked to the amino acid tryptophan (von Wallbrunn et al. 2001) and originally known from *Chaetomium globosum* (Sekita et al. 1973).

The development of a suitable transformation protocol for *Calonectria morganii* is an essential prerequisite for understanding the role of such bioactive compounds in phytopathogenicity and to facilitate the use of genetic manipulation for disease control.

Here, we describe procedures for the genetic transformation of conidia from *C. morganii* by applying an *Agrobacterium tumefaciens*-mediated transformation system. This is the first report of the genetic transformation of *C. morganii* and provides a powerful means for the genetic manipulation of this phytopathogenic fungus.

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Materials and methods

C. morganii strain CS5 (Overmeyer et al. 1996; DSMZ No. 12673) and compatible strain *C. morganii* E71 (Neubauer and Zinkernagel, 1995; DSMZ No. 12674) were routinely grown on Czapek Dox agar (CD; Difco, Detroit, Mich., USA) at 25–28 °C to obtain conidia. Liquid cultures were grown in 150 ml malt extract medium (malt extract 2%, glucose 2%, pH 6.3) in Fernbach flasks; and for the selection of transformants, the fungus was cultivated on malt extract agar (2%) at 25–28 °C. *A. tumefaciens* strain LBA1100 was routinely grown on LC agar (sodium chloride 0.8%, tryptone from casein 1%, yeast extract 0.5%, pH 7.0) with kanamycin (100 μg/ml) and spectinomycin (250 μg/ml) to maintain plasmid pTAS5 for transformation experiments.

Plasmid pTAS5 contains both the hygromycin B (HmB) resistance cassette, based on pAN7.1 (Punt et al. 1987) for the selection of resistant fungal clones (see also Fig. 1), and a kanamycin and spectinomycin resistance gene for selection in *Agrobacterium*. This vector was kindly provided by Drs. A. Ram and C.A.M.J.J. van den Hondel, Leiden, the Netherlands.

The transformation procedure applied is based on the protocol described by Bundock et al. (1995), with the following modifications: *A. tumefaciens* strain LBA1100 carrying plasmid pTAS5 was grown overnight at 28 °C in LC medium with appropriate antibiotics added to ensure maintenance of the plasmid. Conidia of *C. morganii* CS5 were obtained by growing the strain on CD agar plates for 7 days at 25–28 °C, followed by collecting spores in 0.9% standard saline salt solution. Co-cultivations between *A. tumefaciens* and *C. morganii* CS5 were performed as follows: 100 μl containing approx. 10^6 conidia were mixed with 100 μl of the *A. tumefaciens* culture (optical density at 546 nm = 0.5–0.6) and plated onto sterile Hybond-N filters (Amersham Pharmacia Biotech, Freiburg, Germany); and filters were then incubated at 22–25 °C for 3 days on induction medium containing 200 μM acetosyringone (Bundock et al. 1995) for induction of the *vir* genes (Engstrom et al. 1987). Following incubation, the filters were transferred to selection medium containing 100 μg HmB/ml (and 200 μM Cefotaxim to inhibit the growth of *A. tumefaciens*).

C. morganii CS5 bulk DNA was prepared as follows. Mycelia, 7–14 days old, were harvested and ground in a mortar. Then, 20–30 mg of the mycelia were dissolved in 500 μl Cenis lysis solution (Cenis 1992) and 250 μl sodium acetate (3 M, pH 5.2). Cell debris and precipitated proteins were removed by centrifugation. Bulk DNA was purified by phenol/chloroform extraction and precipitated with iso-propanol as described by Sambrook et al. (1989). PCR analysis for detection of the *hph* gene in putative transformants was performed using primer pair *hph*122U (5′-TTCGATGTAGGAGGGCGTGGAT-3′) and *hph*725L (5′-CGCGTCTGCTGCTCCATACAAG-3′), a primer combination taken from Irie et al. (2001). PCR amplification included an initial denaturating step of 4 min at 95 °C (hot start), followed by 35 cycles of 45 s denaturation (94 °C), 1 min annealing (60 °C) and 1.5 min polymerisation (72 °C). Southern Blot analysis was carried out as described by Sambrook et al. (1989) with 5–10 μg DNA in each sample. DNA probes were labelled by PCR (for primers, see above) using the PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany), following the instructions of the manufacturer.

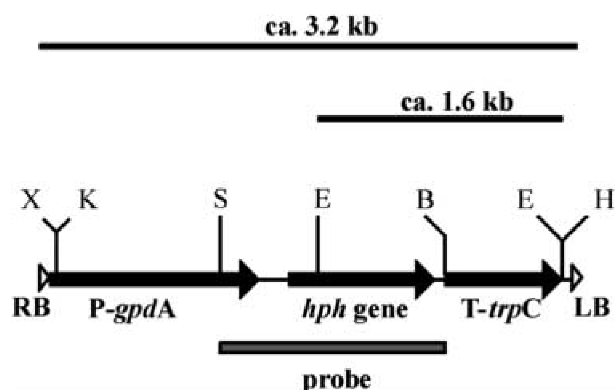

Fig. 1 Physical map of the hygromycin B (HmB)resistance cassette of pTAS5. A selection of restriction sites is given (*X XhoI*, *K KpnI*, *S SalI*, *E EcoRI*, *B BamHI*, *H HindIII*). *LB* Left border, *RB* right border

Results and Discussion

Growth inhibition of *C. morganii* CS5 was tested by plating 10^5 conidia on complete malt agar medium supplemented with HmB in different concentrations, i.e. 0, 50, 100, 150, 200 μg/ml. Growth was totally inhibited on medium containing 100 μg HmB/ml; and thus, this was considered suitable for the selection of resistant colonies in transformation experiments.

The *A. tumefaciens* transformation system, kindly provided by A. Ram and C.A.M.J.J. van den Hondel (see Fig. 1) was used. The results of transformation experiments are presented in Table 1. In three independent experiments, resistant fungal clones were observed after 7 days on selection medium. For all the tested clones ($n = 52$), no difference in growth on HmB-containing and HmB-free medium was observed; and even with 200 μg/ml, the growth of putative transformants was unaffected (3.9 cm/week) indicating the efficient expression of the gene. PCR analyses targeted at the *hph* gene yielded bands of the expected size in all transformants (see Fig. 2).

To check the fate of the resistance gene after transformation, we performed restriction analysis of bulk DNA isolated from four randomly chosen transformants followed by Southern analysis, using as the probe a *hph* internal gene fragment that was PCR-labelled using primers *hph*122U and *hph*725L. The results of such experiments are presented in Fig. 3.

The *hph* gene probe (see also Fig. 1) has one site for the applied restriction enzyme, *Eco*RI. Hence, in Southern blots, two hybridising bands were expected to occur. As the obtained bands clearly differ in each strain under investigation (see Fig. 3), it becomes evident that

Table 1 *Calonectria morganii*: number of hygromycin B-resistant (HmB-R) fungal clones after *Agrobacterium tumefaciens*-mediated transformation in three different experiments. ± *AS* acetosyringone added or lacking

Experiment	Medium	No. of conidia	No. of HmB-R transformants	No. of HmB-R transformants per 10^7 recipients
1	–AS	1.0×10^6	0	0
	+AS		13	130
2	–AS	2.2×10^6	0	0
	+AS		19	86
3	–AS	2.3×10^6	0	0
	+AS		20	87

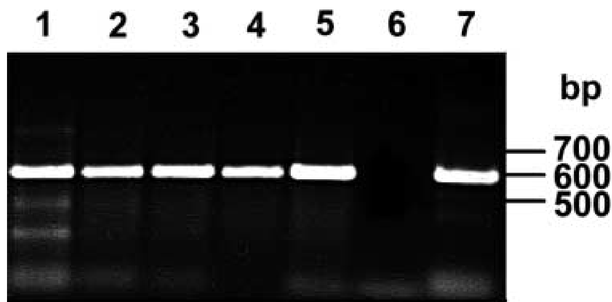

Fig. 2 *Calonectria morganii*: polymerase chain reaction detection of the *hph* gene of randomly chosen HmB-resistant transformants, using bulk DNA as the template and primers *hph*122U and *hph*725L for amplification. *Lanes 1–5 Agrobacterium* transformants AT3–7, *lane 6 C. morganii* wild type, *lane 7* pAN7.1 as control. The molecular weight of marker molecules is given *at the right*

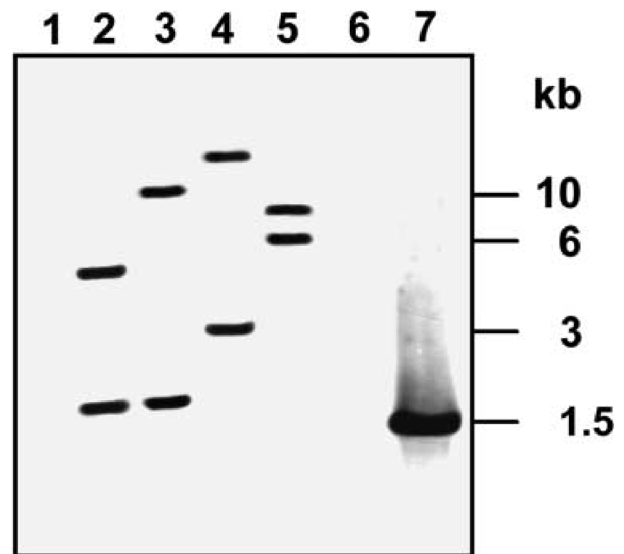

Fig. 3 *C. morganii*: Southern blot analysis of genomic DNA of *A. tumefaciens*-mediated, randomly chosen transformants, using pTAS5 as the vector. Genomic DNA was cut with *Eco*RI and separated on a 0.7% agarose gel. Hybridisation was performed using a pAN7.1-*Bam*HI/*Sal*I fragment containing part of the *hph* gene as probe. The position and size in kilobases are indicated *on the right*. *Lane 1* DNA size marker, *lane 2* AT4, *lane 3* AT5, *lane 4* AT6, *lane 5* AT7, *lane 6* wild type, *lane 7* pAN7.1 digested with *Bam*HI and *Sal*I

integration of the *hph* gene has occurred at different chromosomal sites in each of the transformants.

For the examination of mitotic stability, hyphal tips taken from the rim of a petri dish culture were transferred successively to fresh plates either containing or lacking HmB (in triplicate). Transformants retained their resistance through all successive serial subcultivations. Consistent with this observation is the fact that conidiospores from transformants germinated readily (35 colony-forming units per 10^2 plated spores).

Since strain E71 isolated by Neubauer and Zinkernagel (1995) was found to be sexually compatible to CS5 (von Wallbrunn 2000), genetic stability was additionally tested by mating one of the *A. tumefaciens*-mediated transformants with strain E 71. Fruiting bodies were obtained as described by Overmeyer et al. (1996) and random ascospore analysis was performed. A total number of 30 germinated ascospores were grown on HmB medium and 19 of these exhibited HmB resistance. Thus, the transformants are not only mitotically stable but also segregate as a Mendelian trait after karyogamy and meiosis.

Other attempts at obtaining stable transformants were unsuccessful. Lithium acetate treatment of germinated conidiospores, according to the method described by Dhawale et al. (1984), resulted in only a few resistant clones and these – upon subcultivation – readily lost their HmB resistance. As the *hph* gene could not be detected by PCR nor by genomic Southern blots (data not shown), it became evident that those clones constitute abortive transformants at all events. Protoplast-mediated transformation with polyethylene glycol (Hynes 1996) did not result in resistant clones (data not shown).

Hence, transformation protocols involving lithium acetate treatment or protoplasting as the crucial step are probably not suited for obtaining stably transformed *C. morganii* clones.

A. tumefaciens-mediated transformation procedures have been developed for a number of plants (Hooykaas and Schilperoort 1992; Hooykaas and Beijersbergen 1994) but such a protocol for the transformation of yeasts and filamentous fungi was developed only quite recently (Bundock et al. 1995; de Groot et al. 1998). The transformants of *C. morganii* obtained in experiments using *A. tumefaciens* appeared to be mitotically and meiotically stable. The average number of resistant clones obtained was 10^6 per 10^7 conidia and is, thus, in the same range as for other filamentous fungi, e.g. *Agaricus bisporus* and *Aspergillus niger* (5 resistant clones per 10^7 spores) and up to 1,300 transformants per 10^7 spores in *C. gloeosporioides* (de Groot et al. 1998). The successful use of the inducible *A. tumefaciens* DNA transfer system has been reported for a rather limited number of fungi (de Groot et al. 1998; Covert et al. 2001; Mikosch et al. 2001). Thus, our findings support the general applicability of this natural DNA transfer system for obtaining fungal transformants, irrespective of whether conidia, protoplasts or mycelia are used (de Groot et al. 1998; Abuodeh et al. 2000; Covert et al. 2001; Mikosch et al. 2001).

Each of the resistant clones obtained and analysed further had a single copy integration, as judged from Southern analysis. The restriction patterns of transformants AT6 and AT7 differ, however, from what was expected (Fig. 3; lanes 3, 4). In the transferred DNA situated between both borders (RB and LB), there are two *Eco*RI sites, one in the middle of the *hph* gene and the other close to LB (see Fig. 1). The distance between both sites is 1.6 kbp. Accordingly, one would expect a band of such a size in each of the transformants. However, the *Eco*RI site close to LB was evidently lost in strains AT6 and AT7.

A possible explanation might come from the T-DNA transfer machinery itself, as T-DNA transfer is mediated by the Ti-plasmid-encoded Vir proteins; and the VirE2p is suggested to protect ss-T-DNA from exonucleolytic digestion. In plants, it has been shown that insufficient expression of VirE2p may result in deletions at the 3′ end ranging in size between 52 bp and 1.1 kbp (Rossi et al. 1996). However, although mitotic and meiotic stability is quite obvious, it is not to be excluded at present that rearrangements might have occurred.

This is the first report of a successful transformation of *C. morganii*. The transformation procedure turned out to be easily applicable, yielding highly reproducible results. As *A. tumefaciens* transformation resulted in single-copy integrations, genetic manipulation of fungal genes involved in phytopathogenicity is facilitated. Moreover, by multiple rounds of integrative transformation, it should be possible to create a complete collection of insertional mutants which can subsequently be checked phenotypically and genotypically. Thus, our findings are not only of great significance for studying the phytopathogenicity of the fungus, but they also provide a powerful means for investigating its genetics in general.

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