J. Gen. Appl. Microbiol., **45**, 63–67 (1999)

Transformation with green fluorescent protein of *Trichoderma harzianum* 1051, a strain with biocontrol activity against *Crinipellis perniciosa*, the agent of witches'-broom disease of cocoa

Peter W. Inglis,* Paulo R. Queiroz, and M. Cléria Valadares-Inglis

Microbial Molecular Genetics Laboratory (LGM), EMBRAPA Recursos Genéticos e Biotecnologia (CENARGEN), C.P. 02372, Brasília, D.F., CEP 70770–900, Brazil

(Received January 27, 1999; Accepted April 13, 1999)

A plasmid vector for fungal expression of an enhanced, red-shifted variant of the *Aequoria victoriae* green fluorescent protein was constructed by fusion of the EGFP gene to the highly expressed *Aspergillus nidulans gpd* promoter and the *A. nidulans trpC* terminator. This construction was introduced by cotransformation, using benomyl selection, into *Trichoderma harzianum* strain 1051, a strain being evaluated for the biological control of witches'-broom disease of cocoa caused by *Crinipellis perniciosa*. Epifluorescence microscopy was used to monitor germination and attachment of stable transformant conidia on the surface of *C. perniciosa* hyphae.

Key Words——biological control; cocoa; cotransformation; *Crinipellis perniciosa*; green fluorescent protein; *Trichoderma harzianum*; witches'-broom disease

The transformation of *Trichoderma* strains by using dominant selectable markers, such as resistance to benomyl or hygromycin-B, is an established technology (Sivan et al., 1992). Cotransformation using the *E. coli* β-glucuronidase (GUS) gene has been applied in ecological studies, where the marker has been used for the histochemical detection of the transformed strain following in situ staining (Thrane et al., 1995). The disadvantage of this approach is that the staining process is difficult to apply to in vivo studies and requires the use of expensive colorimetric or fluorimetric derivatives of β-glucuronide.

The value of the jellyfish, *Aequoria victoriae*, green fluorescent protein (GFP) as a heterologous marker in biological systems is becoming well recognized (Chalfie et al., 1994). GFP technology has also recently been applied in the fungi, *Aureobasidium pullulans* (Fernandez-Abalos et al., 1998) and *Aspergillus nidulans* (van den Wymelenberg et al., 1997). GFP fluoresces independently of any other cofactors and may be monitored in real time in living cells. The marker gene has also been subjected to mutagenesis, yielding variants with different excitation/emission maxima and improved levels of heterologous expression (Crameri et al., 1996). EGFP (Cormack et al., 1996) is an example of the red-shifted variants of GFP and has an excitation maximum of 488 nm and an emission maximum of 507 nm. This is a great advantage for visualizing EGFP fluorescence in living cells by using blue light excitation, since exposure to damaging ultraviolet radiation is thus avoided.

We are currently investigating potential antagonists of *Crinipellis perniciosa*, the cause of the highly destructive witches'-broom disease of cocoa (*Theobroma cacao*) (Barreto and Evans, 1996; Thorold, 1975). Strains of *Trichoderma* spp. have been shown to suppress basidiocarp formation in infected cacao brooms (Bastos and Dias, 1992) and are thus of great interest as biocontrol agents. The aim of this study was to cotransform *Trichoderma harzianum* strain 1051 by using the EGFP gene as an in vivo detectable marker, utilizing benomyl resistance as the dominant selectable comarker. The marked fungus may then be applied in future investigations of possible biocontrol competence and ecological impact.

Materials and Methods

Fungal strain. *T. harzianum* strain 1051 was isolated in 1996 from a cocoa plantation in Bahia State, Brazil, and was obtained from the collection of CE-

*Address reprint requests to: Dr. Peter W. Inglis, Microbial Molecular Genetics Laboratory (LGM), EMBRAPA Recursos Genéticos e Biotecnologia (CENARGEN), C.P. 02372, Brasília, D.F., CEP 70770–900, Brazil. E-mail: peter@cenargen.embrapa.br

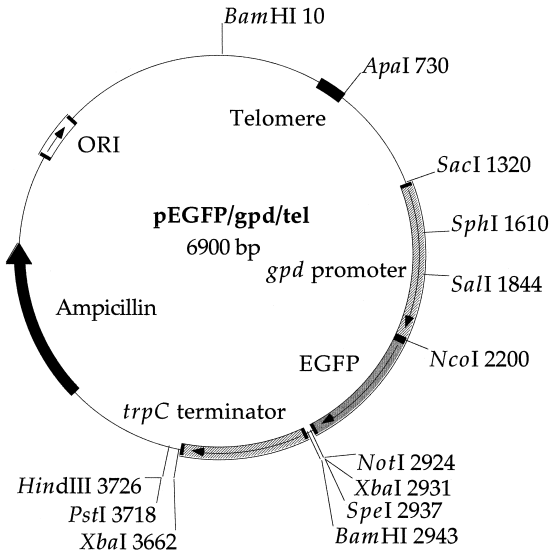

Fig. 1. Physical map of plasmid pEGFP/gpd/tel.

PLAC, Bahia, Brazil. The fungus was subsequently maintained in the laboratory as a liquid nitrogen-stored conidial stock. Cultures for preparation of conidia were seeded onto potato dextrose agar (PDA; Difco) plates and incubated at 28°C for 5 days.

Construction of an EGFP vector for fungal expression. The expression of the EGFP gene in *T. harzianum* was accomplished by fusion to the fungal expression signals present on plasmid pNOM102 (Roberts et al., 1989). This pUC-based construction contains the GUS marker gene fused to the highly expressed *gpd* promoter from *A. nidulans* (Punt et al., 1990, 1991) and the trpC terminator and polyadenylation site, also from *A. nidulans* (Mullaney et al., 1985). The EGFP gene (GFPmut1; Cormack et al., 1996) was excised from pEGFP-N1 (purchased from Clontech), using *Not*I and *Sst*I and ligated to similarly digested pBluescript II SK+ (Stratagene), thereby providing the EGFP gene with a downstream *Bam*HI site. The 743 bp *Nco*I-*Bam*HI fragment from the pBluescript clone was then ligated to the 5,717 bp *Nco*I-*Bam*HI fragment of pNOM102 forming pEGFP/gpd. The redundant 5′ 1,420 bp region of the *gpd* promoter of this construction, bounded by *Eco*RI and *Sst*I sites, was then replaced by DNA containing the telomeric repeat $[TTAGGG]_{18}$ from *Fusarium oxysporum* (Powell and Kistler, 1990), contained on plasmid pMSC1 (Redman and Rodriguez, 1994), following end repair by Klenow polymerase, thus forming plasmid pEGFP/gpd/tel (Fig. 1).

Fungal Cotransformation. Cotransformation of strain 1051 was carried according to a PEG-mediated method (Sivan et al., 1992). Plasmid pBT6, containing the cloned β-tubulin gene from a benomyl-resistant (Ben^r) mutant of *Neurospora crassa* (McClung et al., 1989; Orbach et al., 1986), was previously modified by addition of the same telomeric unit used in the construction of pEGFP/gpd/tel, forming plasmid pBT6tel (P.W. Inglis, unpublished). Five micrograms of plasmid pBT6tel and 5 μg pEGFP/gpd/tel were added to 200 μl protoplast suspension (containing 7.5×10^8 protoplasts ml^{-1}). The transformation mixture was immediately mixed with molten PDA (50°C) containing 1.2 M sorbitol and 10 ml aliquots, then poured into petri dishes. Plates were incubated overnight at 28°C, after which 10 ml PDA containing 1.5 μg ml^{-1} benomyl was overlaid. Plates were then incubated at 28°C for up to 14 days.

Selection and stabilization of transformants. Ben^r transformants started to appear 4 to 10 days after plating. At 10 days, plates/transformants were examined directly for EGFP fluorescence by using a Zeiss Axiovert inverted microscope equipped with a 450–490 nm excitation/510 nm emission fluorescence filter set. Colonies with at least 50% green fluorescing hyphae were cut from the agar and transferred to PDA plates containing 1.5 μg ml^{-1} benomyl. Following incubation at 28°C for about 5 days, conidia were collected, diluted, and plated on fresh PDA/benomyl plates. After 48 h, homogeneously fluorescing colonies were selected as before and subcultured. Monosporic colonies were selected for a further 2 generations, after which conidia were plated on PDA lacking benomyl for 2 successive generations. Conidia collected from this final generation were then plated on PDA/benomyl plates and the proportion of fluorescing germinated conidia assessed microscopically after an overnight incubation.

Molecular analysis of transformants. The total DNA from transformants and wild-type strain 1051 was extracted from liquid-grown mycelium (48 h, 28°C in 200 ml *Aspergillus* complete medium [Pontecorvo et al., 1953]), using CTAB (Rogers and Bendich, 1988). Southern blots were prepared by using Zeta-probe nylon membrane (Bio-Rad, Hercules, CA), using approximately 20 μg *Hin*dIII or *Eco*RV digested fungal genomic DNA in each lane. Probes were labeled by use of a random primer kit (RediPrime; Amersham, Little Chalfont) and ^{32}P-dCTP; following hybridization via standard procedures, blots were exposed to X-ray film. Blots were hybridized with the 720 bp *Nco*I-*Bam*HI fragment of pEGFP/gpd/tel, carrying the complete EGFP gene.

In vitro interaction of transformants with C. perniciosa hyphae. Hyphal fragments of *C. perniciosa* were inoculated into liquid YG medium (g L^{-1}: yeast extract 5 g, glucose 20 g) and incubated at 28°C for 5 days with shaking at 150 r.p.m. Mycelium was aseptically transferred to 25 ml sterile 50 mM phosphate buffer, pH 7.2 in 100 ml flasks and 1×10^6 *Trichoderma*

conidia added. Separate flasks were set up for transformants TE10, TE16, and TE41 and wild-type *T. harzianum* 1051. Flasks were incubated for 24 h at 28°C with shaking at 150 r.p.m. Hyphal fragments were then removed for epifluorescence microscopy.

Results and Discussion

Wild-type *T. harzianum* strain 1051 was found to have a benomyl minimum inhibitory concentrations (MIC) of 1 μg ml^{-1}. With selection on 1.5 μg ml^{-1} benomyl, transformants appeared 3 to 10 days following selective overlay at a frequency of 240 transformants per μg plasmid pBT6tel. This level of transformation efficiency is much higher than that obtained in other published reports of *T. harzianum* transformation to benomyl resistance by using plasmid pBT6. For example, Peterbauer et al. (1992) failed to recover transformants and Ulhoa et al. (1992) obtained from 1.3 to 61.7 transformants per μg pBT6. The higher efficiency recorded in the present work is probably due to the incorporation of telomeric DNA in the transforming plasmid, pBT6tel, possibly conferring stability while the benomyl-resistance allele is sufficiently expressed. This phenomenon was previously observed in *Nectria haematococca* (Kistler and Benny, 1992) and *Colletotrichum* spp. (Redman and Rodriguez, 1994), using the hygromycin B resistance marker.

Brightly green fluorescing cotransformants could be visualized from 72 h in plates by epifluorescence microscopy. Such initial cotransformants usually became characterized by variability in the distribution of the fluorescent phenotype in different branches of the developing mycelium, where some parts could be bright, some faint, and some nonfluorescent (not shown). This is direct in vivo evidence that these cotransformants were probably heterokaryotic and derived from multinucleate protoplasts. The cotransformation frequency was estimated to be at least 30%. Ten cotransformants were selected for further study and subjected to the stabilization procedure. These EGFP expressing cotransformants were designated, TE3, TE10, TE11, TE13, TE16, TE20, TE22, TE38, and TE41. A final transformant, TE42, which lost the fluorescent phenotype at the second subculture, was also selected for molecular analysis.

Benomyl MIC's of the stabilized cotransformants varied from 2.0 to 8.0 μg ml^{-1} (Table 1). However, all cotransformants were found to be able to grow with wild-type morphology (but at variable growth rates) and to be able to sporulate on PDA containing subinhibitory concentrations of benomyl. Growth rates of most cotransformants, in the absence of benomyl, were essentially similar to the untransformed strain. Cotransformants TE11 and TE20, however, exhibited a small reduction in radial growth rate, which could be due to insertional interference with one or more essential genes.

Table 1. Growth and benomyl MIC of *T. harzianum* strain 1051 EGFP/Ben^r cotransformants.

Strain	Colonial diameter (cm)	Benomyl MIC (μg ml^{-1})
1051, wild-type	3.2	1.0
TE3	3.4	2.0
TE10	3.0	7.5
TE11	2.4	6.0
TE13	3.4	4.5
TE16	3.4	8.0
TE20	2.6	6.0
TE22	3.4	7.5
TE38	3.6	5.5
TE41	3.5	7.0
TE42 (nonfluorescent)	3.7	5.0

The results are the mean of triplicate determinations with standard deviations of less than 10%. Colonial diameters were recorded on PDA (nonselective) after 48 h incubation at 28°C. Benomyl MIC were determined on Benlate (50% w/v benomyl; Du Pont, Boston, MA) supplemented PDA plates following 4 days of incubation at 28°C.

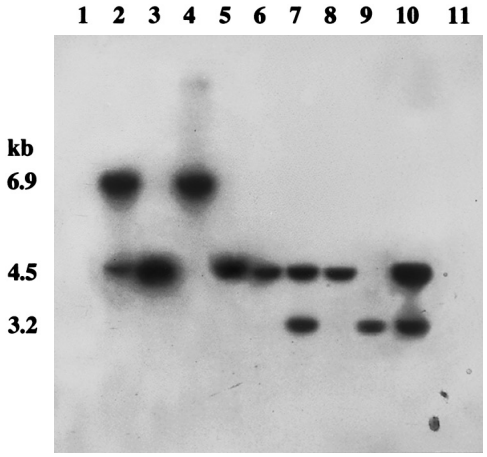

Fig. 2. Southern hybridization analysis of *T. harzianum* strain 1051 pBT6tel/pEGFP/gpd/tel cotransformants genomic DNA, digested with *Hin*dIII and probed with the 720 bp *Nco*I-*Bam*HI fragment of pEGFP/gpd/tel.

Lane 1: *T. harzianum* strain 1051, wild-type; lanes 2–11: transformants TE3, TE10, TE11, TE13, TE16, TE20, TE22, TE38, TE41, and TE42 (nonfluorescent), respectively.

Southern blot analysis (Fig. 2) detected no homology to the EGFP gene in the wild-type *T. harzianum* genome. Neither was a hybridization signal detected in abortive cotransformant TE42, suggesting that the EGFP marker was lost, probably by nuclear segregation, during subculture. Positive hybridization signals, however, were detected in all fluorescent cotransformants. Digestion with *Eco*RV, which does not cut

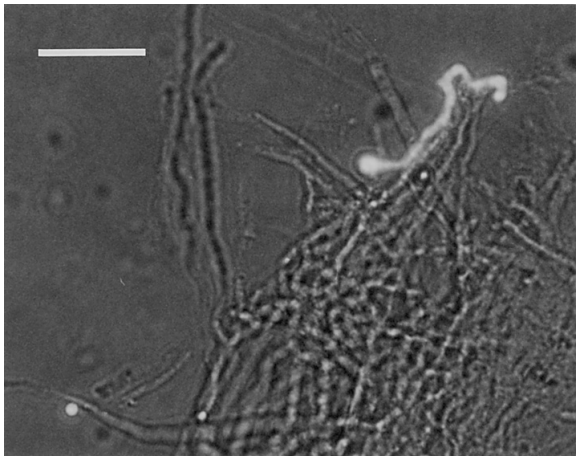

Fig. 3. Epifluorescence visualization of EGFP transformant TE10 germinating and adhering to the surface of *C. perniciosa* mycelium.

Photographed by using a Zeiss Axiophot microscope with ×40 objective, 450–490/510 nm filter set, and concurrent partial white-light illumination. Bar=25 μm.

pEGFP/gpd/tel, produced bands of higher molecular weight than the expected 6.9 kb corresponding to the undigested plasmid (not shown), indicating that integrative transformation had taken place in all transformants. *Hin*dIII digestion, which cuts pEGFP/gpd/tel once, produced a range of hybridization patterns limited to 3 different band sizes, 6.9, 4.5, and 3.2 kb. The 6.9 kb band is indicative of tandem, ectopic integration, which is commonly found in higher fungi (Fincham, 1989). The two smaller classes of bands could have been produced by specific deletions in the transforming plasmid before integration and/or tandem duplication. This phenomenon has been previously noted in other transformation systems where the telomere consensus sequence present on the transforming vector was linearized by the fungus (Kistler and Benny, 1992; Powell and Kistler, 1990).

The stabilized cotransformants were readily visible by epifluorescence microscopy in stages from germinated conidia to mature mycelium. In conidial germination tests, 100% of conidia from these strains were fluorescent, confirming the mitotic stability of the EGFP gene following repeated monosporic selection. In germinating conidia and older cultures, fluorescence appeared to be concentrated in granular structures, whereas in young, fast-growing hyphae, fluorescence appeared uniformly distributed. Fluorescence was also more intense in the presence of benomyl, presumably because of a reduction in growth rate, resulting in an elevation of cellular green fluorescent protein levels.

After 24 h incubation, nearly all conidia from transformants TE10, TE16, and TE41 inoculated into phosphate buffer containing *C. perniciosa* were observed to have adhered to and commenced germination on the host mycelium. Few fluorescent conidia could be found in the culture supernatant. By the use of epifluorescence microscopy, it was possible to observe the transformed *T. harzianum* germinating in contact with and growing parallel to *C. perniciosa* hyphae (Fig. 3). All 3 transformants tested were observed to exhibit a similar response. The parental strain, 1051, appeared to have germinated in the same way. However, it was difficult or impossible to microscopically distinguish hyphae of *C. perniciosa* and more mature hyphae of untransformed *T. harzianum*. We have also observed growth and sporulation of *T. harzianum* transformants on mycelial fragments of *C. perniciosa* placed on tap water agar and incubated at 28°C for 5 days (data not shown). The effect in the field, where application of *T. harzianum* strain 1051 is found to suppress basidiocarp formation by *C. perniciosa*, is thus probably a mycoparasitic interaction, since we have demonstrated conidial binding and germination on the potential host surface.

This *T. harzianum*/*C. perniciosa* system amply demonstrates the utility of GFP technology to the in vitro study of biocontrol interactions. The ability to readily distinguish a potential control agent in vivo, from host tissue or from other environmental microorganisms, is especially vital for a clear understanding of the interaction.

This research was supported by grants from Conselho Nacional de Pesquisa Científica e Tecnológica (CNPq/Brazil) and PROMOAGRO/EMBRAPA. We thank C. A. M. J. J. van den Hondel for the gift of pNOM102 and D. Tebeest for the gift of pMSC1. The authors also gratefully acknowledge the excellent technical support of Irene Martins and Helôisa Frazão.

References

Barreto, R. W. and Evans, H. C. (1996) *Crinipellis perniciosa*: a much investigated but little understood fungus. *Mycologist*, **10**, 58–61.

Bastos, C. N. and Dias, J. C. (1992) Reduction in the production of basidiocarps of *Crinipellis perniciosa* by *Trichoderma viride*. *Summa Phytopathol.*, **18**, 235–238.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994) Green fluorescent protein as a marker for gene expression. *Science*, **263**, 802–805.

Cormack, B., Valdivia, R., and Falkow, S. (1996) FACS-optimized mutants of the green fluorescent protein. *Gene*, **173**, 33–38.

Crameri, A., Whitehorn, E. A., Tate, E., and Stemmer, W. P. C. (1996) Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nature Biotechnol.*, **14**, 315–319.

Fernandez-Abalos, J. M., Fox, H., Wells, B., and Doonan, J. H. (1998) Plant-adapted green fluorescent protein is a versatile vital reporter for gene expression, protein localisation and mitosis in the filamentous fungus, *Aspergillus nidulans. Mol. Microbiol.*, **27**, 121–130.

Fincham, J. R. S. (1989) Transformation in fungi. *Microbiol. Rev.*, **53**, 148–170.

Kistler, H. C. and Benny, U. (1992) Autonomously replicating plas-

mids and chromosome rearrangements during transformation of *Nectria haematococca. Gene*, **117**, 81–89.

McClung, C. R., Phillips, J. D., Orbach, M. J., and Dunlap, J. C. (1989) New cloning vectors using benomyl resistance as a dominant marker for selection in *Neurospora crassa* and in other filamentous fungi. *Exp. Mycol.*, **13**, 299–302.

Mullaney, E. J., Hamer, J. E., Roberti, K. A., Yelton, M. M., and Timberlake, W. E. (1985) Primary structure of the *trpC* gene from *Aspergillus nidulans. Mol. Gen. Genet.*, **199**, 37–45.

Orbach, M. J., Porro, E. B., and Yanofski, C. (1986) Cloning and characterization of the gene for β-tubulin from a benomyl-resistant mutant of *Neurospora crassa* and its use as a dominant selectable marker. *Mol. Cell. Biol.*, **6**, 2452–2461.

Peterbauer, C. K., Heidenreich, E., Baker, R. T., and Kubicek, C. P. (1992) Effect of benomyl and benomyl resistance on cellulase formation by *Trichoderma reesei* and *Trichoderma harzianum*. *Can. J. Microbiol.*, **38**, 1292–1297.

Pontecorvo, G., Roper, J. A., Hemons, L. M., MacDonald, K. D., and Bufton, A. W. J. (1953) The genetics of *Aspergillus nidulans. Adv. Genet.*, **5**, 141–238.

Powell, W. A. and Kistler, H. C. (1990) In vivo rearrangement of foreign DNA by *Fusarium oxysporum* produces linear self-replicating plasmids. *J. Bacteriol.*, **172**, 3163–3171.

Punt, P. J., Dingemanse, M. A., Kuyvenhoven, A., and van den Hondel, C. A. (1990) Functional elements in the promoter region of the *Aspergillus nidulans gpdA* gene encoding glyceraldehyde-3-phosphate dehydrogenase. *Gene*, **93**, 101–109.

Punt, P. J., Zegers, N. D., Busscher, M., Pouwels, P. H., and van den Hondel, C. A. M. J. J. (1991) Intracellular and extracellular production of proteins in *Aspergillus* under the control of the highly expressed *Aspergillus nidulans gpdA gene. J. Biotechnol.*, **17**, 19–34.

Redman, R. S. and Rodriguez, R. J. (1994) Factors which affect efficient transformation of *Colletotrichum* species. *Exp. Mycol.*, **18**, 230–246.

Roberts, R. P., Oliver, R. P., Punt, P. J., and van den Hondel, C. A. M. J. J. (1989) Expression of the *Escherischia coli* β-glucuronidase gene in industrial and phytopathogenic fungi. *Curr. Genet.*, **15**, 177–180.

Rogers, S. O. and Bendich, A. J. (1988) Extraction of DNA from plant tissues. *In* Plant Molecular Biology Manual A6, ed. by Gelvin, S. B., Schilperpoort, R. A., and Verma, D. P. S., Kluwer Acad. Publ., Dordrecht, pp. 1–10.

Sivan, A., Stasz, T. E., Hemmat, M., Hayes, C. K., and Harman, G. E. (1992) Transformation of *Trichoderma* spp. with plasmids conferring hygromycin B resistance. *Mycologia*, **84**, 687–694.

Thorold, C. A. (1975) Diseases of Cocoa, Clarendon Press, Oxford, U.K.

Thrane, C., Lübeck, M., Green, H., Degefu, Y., Allerup, S., Thrane, U., and Funck Jensen, D. (1995) A tool for monitoring *Trichoderma harzianum*: I. Transformation with the GUS gene by protoplast technology. *Phytopathology*, **85**, 1428–1435.

Ulhoa, C. J., Vainstein, M. H., and Peberdy, J. F. (1992) Transformation of *Trichoderma* species with dominant selectable markers. *Curr. Genet.*, **21**, 23–26.

van den Wymelenberg, A. J., Cullen, D., Spear, R. N., Schoenike, B., and Andrews, J. H. (1997) Expression of green fluorescent protein in *Aureobasidium pullulans* and quantification of the fungus on leaf surfaces. *Biotechniques*, **23**, 686–690.