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Biolistic co-transformation of *Metarhizium anisopliae* var. *acridum* strain CG423 with green fluorescent protein and resistance to glufosinate ammonium

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Abstract

Metarhizium anisopliae var. *acridum* (syn. *M. flavoviride*) is recognized as a highly specific and virulent mycopathogen of locusts and grasshoppers and is currently being developed as a biological control agent for this group of insects in Brazil. Intact conidia of *M. anisopliae* var. *acridum* strain CG423 were transformed using microparticle bombardment. Plasmids used were: (1) pBARKS1 carrying the *bar* gene of *Streptomyces hygroscopicus* fused to the *Aspergillus nidulans trpC* promoter, encoding resistance to glufosinate ammonium (or phosphinothricin) and modified by addition of the telomeric repeat $(TTAGGG)_{18}$ of *Fusarium oxysporum* and 2.pEGFP/gpd/tel carrying a red-shifted variant gene for *Aequorea victoria* green fluorescent protein (EGFP) which we have fused to the *A. nidulans gpd* promoter and *trpC* terminator. Highly fluorescent co-transformants were selected on solid minimal medium containing 100 μg ml^{-1} glufosinate ammonium using an inverted microscope with 450–490 nm excitation/510 nm emission filter set. Southern blot analysis of co-transformants revealed varying multiple chromosomal integrations of both *bar* and *egfp* genes at both telomeric and non-telomeric loci. Transformants retained pathogenicity in bioassays against *Rhammatocerus schistocercoides* and showed unaltered lack of pathogenicity against larvae of the non-target insect *Anticarsia gemmatalis*. One co-transformant from four tested, however, showed a significant, but non-dose-dependent, elevation in virulence against *Tenebrio molitor*.

Keywords: Biological control; Entomopathogenic fungus; Transformation; *gfp*

1. Introduction

Metarhizium anisopliae var. *acridum* is of considerable interest for its potential as a specific biocontrol agent for locusts and grasshoppers. Following an extensive development phase an isolate, IMI 330189, has been mass produced and commercialized under the product name 'Green Muscle®' [1]. This product has been shown to be a good alternative to organophosphate pesticides and carries the benefits of specificity to acridid insects, compatibility with conventional ultra low volume spray application and long term effect and persistence [2,3]. Another *M. anisopliae* var. *acridum* strain, CG 423, has been isolated and developed in Brazil for grasshopper control [4].

Monitoring and quality control of these mycoinsecticide products demands that the fungus should be correctly identifiable and distinguishable from other field isolates which may co-exist in the sites of artificial application. Several studies have applied molecular approaches to the characterization of *Metarhizium*. *M. flavoviride* was distinguished from isolates of *M. anisopliae* by restriction fragment length polymorphisms (RFLP) and isoenzyme patterns [5] and recently, sequence analysis of ribosomal DNA was used in a taxonomic revision of *Metarhizium* [6]. At the strain differentiation level, RAPD PCR analysis showed high genetic homogeneity among five Brazilian *M. anisopliae* var. *acridum* isolates [7]. The same set of isolates, however, were found to be highly distinguishable by RFLP using a telomeric probe [8].

Genetic marking with heterologous genes is an alternative to molecular screening of numerous environmental isolates, where introduction of genes for fungicide resistance allows direct selection. Using this approach, *M. anisopliae* var. *acridum* strain CG 423 was transformed using a mutant form of β-tubulin from *Neurospora crassa*, causing benomyl resistance [9]. Development of transformation

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systems also allows construction of transgenic strains with possible improved characteristics. Here, a strain of *M. anisopliae* was engineered to constitutively overexpress a subtilisin-like protease, significantly improving biocontrol effectiveness [10]. Secondary phenotypic markers such as GUS expression [11] permit verification of the applied fungus at the microscopic level. More recently, green fluorescent protein (GFP) technology has been developed which allows non-invasive monitoring of transformed cells and has been applied to biocontrol fungi [12,13]. The current work describes the co-transformation of *M. anisopliae* var. *acridum* strain CG 423 with the *bar* gene, conferring resistance to the herbicides bialaphos and glufosinate ammonium (GA) and expression of the *egfp* gene as an in vivo detectable marker.

2. Materials and methods

2.1. Strains and vectors

M. anisopliae var. *acridum* strain CG423 was originally isolated from the grasshopper, *Schistocerca pallens* in Rio Grande do Norte state, Brazil in 1992. The strain was subsequently maintained as a lyophilized stock and grown on potato dextrose agar (PDA, Difco) at 28°C for production of conidia. The plasmids used for co-transformation were pEGFP/gpd/tel and pBARKS/tel. pEGFP/gpd/tel [13] contains a red-shifted *Aequorea victoria* green fluorescent protein gene, EGFP (GFPmut1; [14]) fused to an *Aspergillus nidulans gpd* promoter and *A. nidulans trpC* terminator and polyadenylation site and also contains a telomeric repeat $(TTAGGG)_{18}$ from *Fusarium oxysporum* [15]. pBARKS/tel was constructed by fusing the *F. oxysporum* telomeric element to pBARKS1, obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, Kansas City, KS, USA [16], carrying the phosphinothricin acetyltransferase (*bar*) gene from *Streptomyces hygroscopicus* under control of the *A. nidulans trpC* promoter and terminator [17]. This gene also confers resistance to the herbicide GA ('Liberty' = 20% w/v GA, Aventis).

2.2. Biolistic co-transformations

The target material for transformation by microparticle bombardment was intact conidia [18]. The two plasmids used for biolistic transformations were used in a 1:1 molar ratio. The bombardment was carried out as previously described [19]. Basically, DNA was bound to 0.2-μm diameter tungsten particles (M5, Sylvania Inc.) by mixing sequentially in a microcentrifuge tube: 50 μl microparticles (60 mg ml^{-1} in 50% glycerol), 4 μl (1 μg μl^{-1}) plasmid DNA, 50 μl $CaCl_2$ (2.5 M) and 20 μl spermidine free-base (100 mM). After 10 min incubation, the DNA-coated microparticles were centrifuged at $15\,000 \times g$ for 10 s and the supernatant removed. The pellet was washed with 150 μl 70% ethanol and then with absolute ethanol. The final pellet was resuspended in 24 μl of absolute ethanol and sonicated for 2 s, just before use. Aliquots of 3 μl were spread onto carrier membranes (Kapton, 2 mil, DuPont) which were allowed to evaporate in a desiccator at 12% relative humidity. A conidial suspension (50 μl) in sterile distilled water containing 1×10^9 conidia ml^{-1} was bombarded with the DNA-coated microparticles utilizing a high pressure helium-driven particle acceleration device built in our laboratory [20].

Following bombardment, conidia were resuspended in 5 ml YG broth (glucose 2.5%, yeast extract 0.5%) and were incubated at 28°C with gentle shaking for 18 h. Conidia were washed and resuspended in 5 ml distilled water and plated (0.5 ml/Petri dish) on a minimal medium (per liter: glucose 10 g, $NaNO_3$ 6.0 g, KCl 0.52 g, $MgSO_4 \cdot 7H_2O$ 0.52 g, KH_2PO_4, pH 6.5) supplemented with 2 ml Hutner's trace elements [21] and 100 μg ml^{-1} GA. Controls consisting of conidia bombarded with microparticles lacking DNA were included.

2.3. Selection and stabilization of co-transformants

Inoculated plates were incubated at 28°C for up to 10 days during which plates were periodically examined directly for EGFP fluorescence using a Zeiss Axiovert inverted microscope equipped with a 450–490-nm excitation/510-nm emission fluorescence filter set. Fluorescent colonies were picked using a sterile needle and transferred to fresh selective medium. Co-transformants were sub-cultured for a further five cycles of mycelial growth and conidiation.

2.4. Molecular analysis of co-transformants

Genomic DNA from selected co-transformants was extracted from mycelium using CTAB [22]. DNA (5 μg) was digested with *Eco*RI and Southern blots prepared according to standard procedures. Blots were hybridized with radiolabelled probes consisting of the 720-bp *Nco*I–*Bam*HI fragment of pEGFP/gpd/tel, carrying the complete EGFP structural gene, a 1-kb *Xba*I–*Bgl*II fragment of pBARKS/tel carrying the *bar* gene and a probe containing the telomeric repeat, $(TTAGGG)_{18}$.

2.5. Virulence testing of co-transformants against host and non-host insects

The virulence of selected transformants was compared with that of wild-type strain CG423 against the grasshopper *Rhammatocerus schistocercoides* (Orthoptera, Acrididae). A suspension of conidia, previously produced by cultivation of the fungus on parboiled rice, was prepared in soya oil at a concentration of 1.7×10^6 spores ml^{-1}. Three microliters of this suspension (± 5000 spores) was

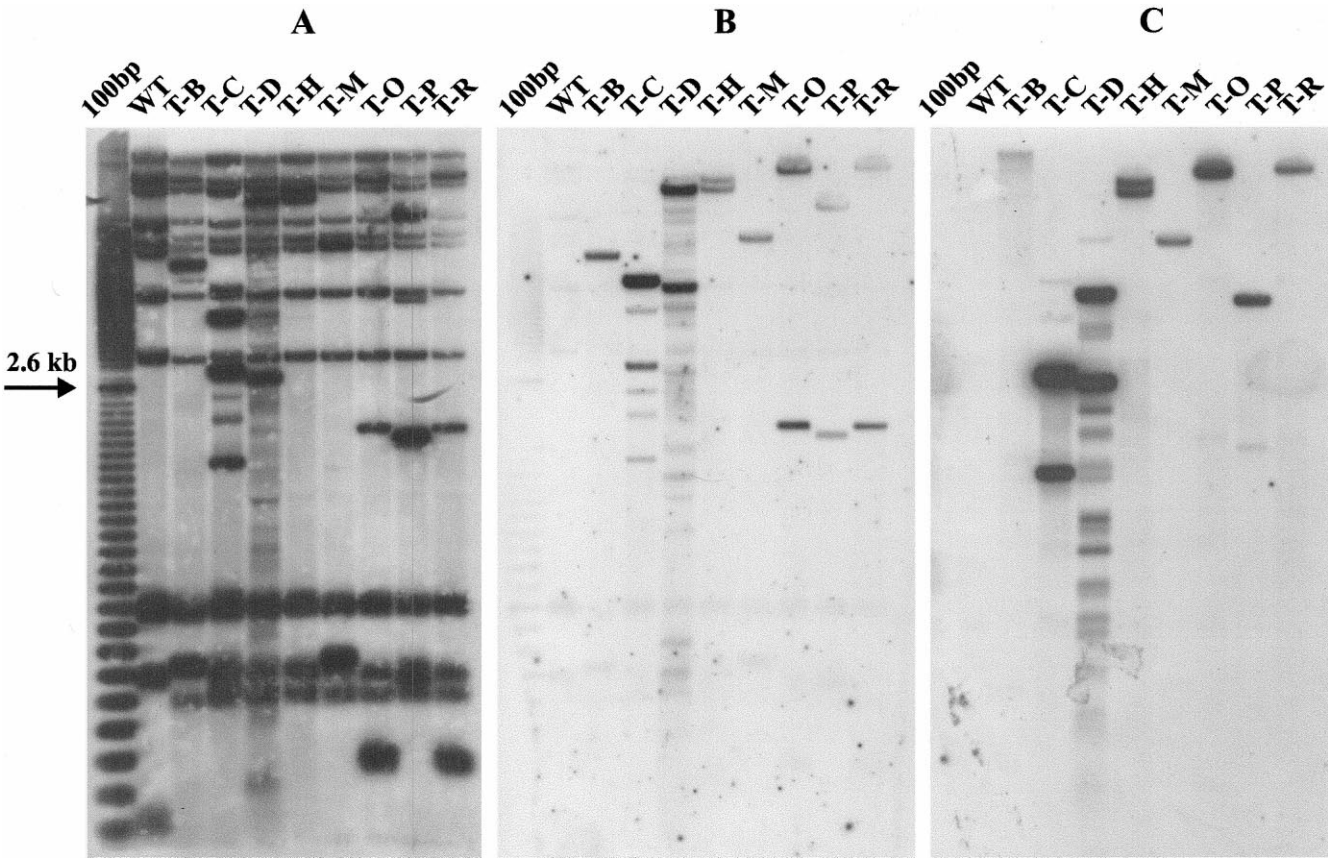

Fig. 1. A: Southern hybridization analysis of *M. anisopliae* var *acridum* strain CG423 pBARKS/tel; pEGFP/gpd/tel co-transformants genomic DNA, digested with *Eco*RI and probed with an approximately 1.2-kb fragment of pUC18 DNA also carrying the telomere sequence from *F. oxysporum*, $(TTAGGG)_{18}$. Lane 1: *M. anisopliae* var. *acridum* strain CG423, wild-type; lanes 2–11: co-transformants T-B, T-C, T-D, T-H, T-M, T-O, T-P, T-R, respectively. B: Same filter reprobed with the *Xba*I–*Bgl*II fragment of pBARKS/tel carrying the *bar* gene. C: Same filter reprobed with the 720-bp *Nco*I–*Bam*HI fragment of pEGFP/gpd/tel carrying the *egfp* gene.

applied by pipette to the head–thorax junction of adult insects. Experiments using groups of 20 insects were performed in triplicate. Following inoculation, insects were kept in cages at a temperature of 25°C and were fed a diet of breakfast cereal and fresh sugar cane leaves. Insects were observed daily for a period of 10 days and any cadavers immediately removed. Cadavers were selected at random on the day of death and observed microscopically for fungal infection and for EGFP fluorescence.

Virulence was also checked against two non-host insect species. A similar inoculation protocol as above was done using the mealworm *Tenebrio molitor* (Coleoptera, Tenebrionidae) as test subject. Thirty insects in each of three treatments were inoculated with 3 μl of an oil suspension containing 1000, 5000 and 10000 conidia respectively. Insects were then observed for 15 days and any cadavers removed to a humid chamber at 28°C daily. A second experiment was set up using the velvetbean caterpillar *Anticarsia gemmatalis* (Lepidoptera, Noctuidae) third instar larvae as test subjects. Here, 30 larvae were dipped five times for a duration of 2 s in a conidial suspension in 0.1% Tween 20 solution containing either 1×10^7 or 1×10^8 conidia ml^{-1}. Larvae were placed in covered individual plastic pots containing approximately 10 g artificial diet. Insects were incubated at 28°C for 18 days and were again observed daily with any cadavers being removed.

3. Results and discussion

Colonies began to appear 4 days after biolistic transformation and plating of conidia on selective minimal medium containing 100 μg ml^{-1} GA. Results of the co-transformation are presented in Table 1, where a wide but low range of transformation frequencies were obtained. Co-

Table 1
Recovery of co-transformed *M. anisopliae* var. *acridum* following conidial bombardment, plating on selective medium (100 μg ml^{-1} GA) and screening for EGFP fluorescence

Repetition	Fluorescent/GA resistant colonies	GA resistant only
1	24	65
2	1	24
3	3	36
Control (no DNA)	0	15

transformation frequencies were 37, 4 and 8% for repetitions 1–3 respectively, with an estimated efficiency of between 4 and 88 co-transformants per μg of each plasmid. However, a significant proportion of the non-fluorescent colonies appearing on extended incubation of up to 10 days were likely to be false-positive transformants as indicated by the colonies developing on control plates. Fluorescent colonies all appeared within 7 days, suggesting that the non-transformed false positives were allowed to grow as a result of breakdown of the selective agent, either naturally or as a result of enzymatic breakdown by transformant colonies appearing earlier. Previously, the gene gun has been used to co-transform *M. anisopliae* germlings with two plasmids carrying genes for benomyl resistance and GUS expression at a 1:3 molar ratio [11]. Here, the transformation efficiencies obtained were up to five transformants per μg DNA, with a co-transformation frequency of 12%.

First generation co-transformants appeared brightly fluorescent under epifluorescence microscopy, but as colonies grew, we observed a segregation to fluorescent and non-fluorescent hyphae within discrete colonies in certain transformants. Of the total number of co-transformants originally obtained, we were not able to maintain EGFP expression in 20. Evidently, at this early stage, instability and loss of the EGFP gene was possible within a population of the nuclei in the developing mycelium. However, following five generations of selection for GA resistance and the fluorescent phenotype, fully mitotically stable co-transformants were obtained. However, at least for the EGFP phenotype, significant variation in intensity of fluorescence was noted among spore populations of certain co-transformants.

Molecular analysis of selected co-transformants revealed a highly variable pattern of integration of the *bar* and *egfp* genes (Fig. 1). Genomic Southern blots of undigested DNA produced hybridization signals only within high molecular mass DNA, indicating chromosomal sites for the integration events and excluding the possibility of maintenance of the transforming genes by autonomous extrachromosomal replication (data not shown). The size of hybridized bands in the co-transformants varied between bands of higher molecular mass than the individual transforming plasmids to bands of much lower molecular mass. Some hybridized bands were also highly intense compared to others using the same probe, indicating multiple tandem duplications of partially deleted or rearranged plasmids during the integration process. Certain co-transformants also possessed bands that apparently hybridized with both *bar* and *egfp* probes suggesting fusion and rearrangement of the transforming plasmids prior to integration. Several of these bands also mapped to telomeric loci, suggesting homologous recombination with the telomeric sequence present on both transforming plasmids.

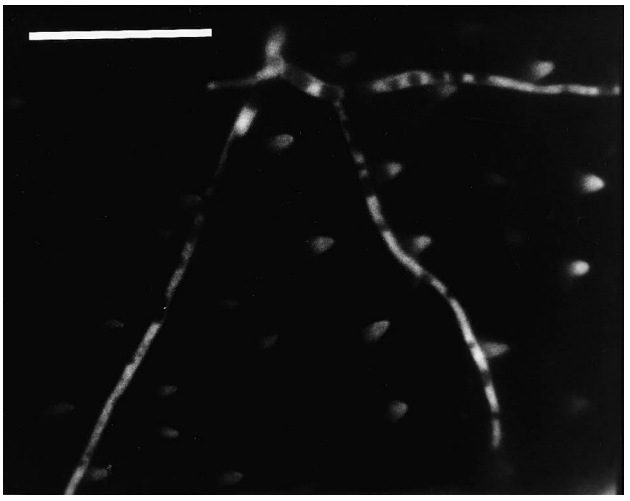

Fig. 2. Epifluorescence visualization of co-transformant T-B germinating and adhering to the surface of *R. schistocercoides* cuticle. Photographed using a Zeiss Axiophot microscope with ×100 objective, 450–490/510-nm fluorescence filter set. Bar = 25 μm.

Virulence tests of selected co-transformants and wild-type *M. anisopliae* var. *acridum* CG423 against *R. schistocercoides* showed that they all caused 100% mortality in the target insects within 10 days (Table 2). Infection by co-transformants was consistently confirmed by conidiation tests and by the observation of EGFP fluorescence in re-isolates. Autofluorescence of internal tissues commonly caused difficulty in the observation of fluorescent hyphae within insect cadavers directly, although well contrasted hyphae were easily observed in germination and binding tests on insect cuticle preparations (Fig. 2). The time to

Table 2
Virulence of wild-type and selected co-transformants of *M. anisopliae* var. *acridum* strain CG423 to *R. schistocercoides*

Day	Control (no fungus)	CG423	T-B	T-C	T-H	T-M	T-P
1	1.6[a]	6.6	1.7	1.6	3.5	3.3	5.0
2	4.8	15.0	1.7	8.4	5.2	3.3	8.3
3	4.8	33.3	8.9	11.8	7.0	5.0	16.6
4	6.4	61.6	8.9	16.9	14.0	8.33	33.3
5	6.4	85.0	25.0	42.3	47.3	33.3	66.6
6	6.4	100	55.3	74.5	85.9	55.0	80.0
8	6.4		80.3	91.5	100	93.3	98.3
9	6.4		92.8	100		100	100
10	6.4		100				

[a]Data are expressed as mortality (%) among a population of 20 insects and are the mean of triplicate tests, with standard errors of less than 10%.

Table 3
Virulence of wild-type and selected co-transformants of *M. anisopliae* var. *acridum* strain CG423 to *A. gemmatalis*

Conidium concentration[a]	Control (no fungus)	Wild-type CG423	Co-transformants			
			T-B	T-C	T-M	T-P
1×10^7	0[b]	3.3	0	0	3.3	0
1×10^8	0	3.3	3.3	6.7	0	6.7

[a]The fungus was inoculated by dipping the insects five times, for 2 s in a conidial suspension.
[b]Data are expressed as mortality (%) among a population of 30 insects.

produce 100% mortality was delayed in the co-transformants by between 1 and 3 days in comparison to the wild-type. This could be a serious disadvantage, since speed-of-kill is considered to be a critical factor in the performance of a mycoinsecticide [10]. Factors affecting this subjective reduction in virulence of the co-transformants could be increased metabolic load caused by constitutive production of foreign proteins, insertional inactivation or modulation of wild-type gene expression or reduction of virulence caused by repeated sub-culture in vitro. Since these co-transformants were sub-cultured at least eight times during the transformation and stabilization processes, the last factor is perhaps the most likely cause, since reduction of virulence by serial in vitro transfer on artificial media and its subsequent restoration by passage through an insect host has been previously reported in *M. anisopliae* [23]. Virulence towards larvae of the non-host insect, *A. gemmatalis* was minimal among wild-type and all co-transformants (Table 3). However, co-transformant T-P was found to cause significantly greater mortality in *T. molitor* in comparison to wild-type CG423 and other tested co-transformants (Table 4), although the observed effect was not dose-dependent. This difference could be due to insertional alteration of gene expression affecting host specificity in *M. anisopliae* var. *acridum* and warrants further investigation.

Given the current renewed interest in the use of entomopathogenic fungi as mycopesticides, genetic manipulation studies of promising strains will attain greater importance. We have herein demonstrated that *M. anisopliae* var. *acridum* strain CG423, which is currently undergoing trials in Brazil for grasshopper control, can be efficiently co-transformed using a selective marker and a second, in vivo detectable marker. Such transformants can now be applied for tracking and infection studies and will serve as a model for the further manipulation and directed improvement of this strain.

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Table 4
Virulence of wild-type and selected co-transformants of *M. anisopliae* var. *acridum* strain CG423 to *T. molitor*

Conidia/insect	Wild-type CG423	Co-transformants			
		T-B	T-C	T-M	T-P
1000	2.2[a]	10.0	1.1	13.3	41.1*
5000	11.1	6.6	5.5	8.9	46.6*
10000	14.5	7.7	14.4	16.6	41.1

Data for co-transformants indicated with an asterisk are significantly different from wild-type results ($P<0.05$) according to a One Way ANOVA/Dunnett's test.
[a]Data are expressed as mortality (%) among a population of 30 insects and are the mean of triplicate tests.

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