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Research Article

Co-transformation of a tropical maize endophytic isolate of *Fusarium verticillioides* (synonym *F. moniliforme*) with *gus*A and *nia* genes

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Abstract

A tropical endophytic isolate of the fungus *Fusarium verticillioides* (synonym *Fusarium moniliforme*) obtained from *Zea mays* was co-transformed with plasmid pNH24 containing the *Fusarium oxysporum* nitrate reductase *nia* gene and plasmid pNOM 102 carrying the *Escherichia coli* β-glucuronidase *gus*A gene. Transformation frequency for the *nia* marker was 75 transformants μg^{-1} vector DNA and introduction of the *gus*A gene by co-transformation was 57.2% as indicated by the presence of the GUS^+ phenotype on plates. Southern analyses confirmed the integration of both plasmids into the genome of ten GUS^+ transformants. All co-transformants showed mitotic stability in respect of the GUS^+ phenotype. To assess the potential of transformed endophytic fungi as vectors for introducing desirable characteristics into host tropical plants of biotechnological and agricultural importance we successfully infected maize roots and detected GUS^+ phenotype".

Key words: co-transformation, gus, nia, *Fusarium verticillioides*, endophyte.

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Introduction

The fungus *Fusarium verticillioides* (synonym *F. moniliforme*) is often found in maize, and constitutes an important source of inoculum in soil. *Fusarium* spp., associated with assymptomatic plants living within their host, may be considered an endophyte. According to Azevedo *et al*. (2000), much of the research on endophytes has been conducted using hosts from temperate climates in the Northern Hemisphere and from New Zealand, with data on endophytes from tropical climates being scarce because only a few studies have been carried out on fungus-host interactions in tropical regions. This may be important because endophyte-plant interactions become more complex in regions having a wide variety of organisms, including many different plant-attacking insects. Fungal endophytes isolated from tropical plants could be used in genetic manipulation experiments (especially those involving genetic transformations with morphological markers) which would result in a better understanding of the interactions occurring between endophytic fungi and plants in the tropics. Furthermore, genetically manipulated fungal endophytes have the potential to introduce important biotechnological characteristics (*e.g.* insect-resistance) into the host plants.

The β-D-glucuronidase (GUS) gene (*gus*) is widely used as a histochemical marker in genetic transformation experiments, the *Escherichia coli* GUS gene fusion system (Jefferson, 1989) being a powerful tool for both the assessment of gene activity in transgenic plants and the development of molecular genetic analysis systems. In fungi, GUS transformation has been performed on *Fusarium moniliforme* (Yates *et al.* 1999) and *Fusarium culmorum* (Doohan *et al.* 1998), albeit with very low transformation frequencies (≈1 transformant μg^{-1} DNA), while Couteaudier *et al*. (1993) obtained very high co-transformation frequencies of up to 75% in *Fusarium oxysporum* using the plasmid vectors pNOM102 (containing the *E. coli gus*A gene) and pAN301 (carrying the *Aspergillus nidulans nia*D gene).

The aim of our research was to use the co-transformation procedure employed by Couteaudier *et al*. (1993) to obtain an increased transformation frequency in a tropical endophytic strain of *F. verticillioides* using the *F. oxysporum* nitrate reductase *nia* gene instead of the *A. nidulans nia*D gene as the main transformation marker.

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Material and Methods

Fungal strains and culture media

In our experiments we used *Fusarium verticillioides* strain S68 (Pamphile *et al*., 1996; Hua-Van *et al*., 2001), a *nia*$^-$ mutant derived from the endophytic *F. verticillioides* strain L7 which had been isolated from surface disinfected seeds collected from asymptomatic maize growing in Brazil (Pamphile and Azevedo, 2002). Strain S68 was grown on the complete medium (CM) and minimal medium (MM) described by Pontecorvo *et al.* (1953) or potato-dextrose agar (PDA) (Smith and Onions, 1983). As a positive control for β-D-glucuronidase (GUS) activity we used *F. oxysporum* strain MT5 which was grown on the same media as strain S68. For protoplast preparation and DNA analysis the strains were grown in PD broth (PDA without agar) supplemented with 1% (w/v) each of yeast extract and casein (PD-YC).

Vectors

The vectors used were plasmid pNOM 102 (Roberts *et al*., 1989) containing the *E. coli* β-glucuronidase gene (*gus*A) flanked by a glyceraldehyde 3-phosphate promoter (*gpd*) from *Aspergillus nidulans* upstream and the *A. nidulans trp*C transcription termination signal downstream and plasmid pNH24 (Diolez *et al*., 1993) carrying the *F. oxysporum* nitrate reductase gene (*nia*) subcloned into plasmid pUC19. The pUC plasmid (without the *F. oxysporum nia* gene) was used as a negative control to test for strain stability.

Protoplast preparation

Protoplasts were isolated from germinated conidia. Conidial suspensions from 1 week-old cultures were incubated (1 x 10^8 conidia/ 10 mL PDA + 1% yeast extract and 1% caseine) and shaken (150 revs min^{-1}) at 26 °C for 17 h. After incubation, the mycelia were collected by centrifugation, washed and re-suspended in 10 mL of 1.2 M $MgSO_4$ containing 10 mM Na_2HPO_4 (pH 5.8) and 50 mg mL^{-1} of Glucanex (Novo Nordisk Ferment, Dittingen, Switzerland) and incubated at 26 °C for 2 h until protoplasts formed. Following incubation, 10 mL of MS solution (10 mM MOPS plus 0.6 M Sorbitol, pH 6.3) was added to separate the cell debris from the protoplasts (which collected at the interface) and the protoplasts transferred to 30% sucrose solution and centrifuged at 3000 g, the protoplasts being collected and washed three times in TMS solution (1 M Sorbitol plus 10 mM MOPS, pH 6.3) and then re-suspended in 200 µL TMSC solution (40 mM $CaCl_2$ in TMS).

Transformation procedure

We transformed the *F. verticillioides* protoplasts using calcium chloride/polyethylene glycol (PEG) according to a modified Malardier *et al*. (1989) procedure. For transformation, the number of protoplasts was adjusted to 4 x 10^7 per 100 µL of TMSC and the suspension kept on ice for 20 min before adding plasmids pNOM102 and pNH24 (5 µg each in 60 µL of a solution containing 10 mM Tris, 1 mM EDTA and 40 mM $CaCl_2$, pH7,5) and allowing the mixture to stand on ice for a further 20 min and then adding 160 µL of MS solution containing 60% PEG 4000 (Koch-Light) and incubating at room temperature for 15 min after which 1 mL of TMSC was added and the protoplasts pelleted by centrifugation at 4,500 g for 5 min, re-suspended in 200 µL of TMSC and mixed with 3 mL of MM supplemented with 20% sucrose, 0.4 % (w/v) Oxoid agar and 100 µL of 100 mM glutamine solution. To test the efficiency of transformation a positive control was made under the same conditions except that only plasmid pNH24 was used. The pUC plasmid was used as a negative control to test for strain stability. Transformants exhibiting the NIA^+ phenotype conferred by pNH24 were selected on MM with nitrate as the sole nitrogen source and purified by isolation of uninucleated conidia according to standard techniques.

DNA isolation and manipulation

For both transformed and untransformed isolates, Roux flasks containing 100 mL of PD-YC liquid medium were inoculated with approximately 10^7 spores and incubated for 72-96h, after which the mycelium was collected by filtration using a sterile sintered glass filter, washed with sterile distilled water and lyophilized. To isolate the DNA, Millipore pincers were used to grind the lyophilized mycelia to a powder, 100 mg of which was then suspended in buffer (50 mM EDTA, 0.2% (w/v) SDS, pH 8.5) containing 10 µL mL^{-1} of a 10 mg mL^{-1} proteinase K solution and the mixture incubated for 30 min at 37 °C and 15 min at 70 °C before adding a 1/10 volume of 5 M potassium acetate solution and holding on ice for 30 min. The mixture was centrifuged at 10,000 g for 15 min and the supernatant separated and washed three times with an equal volume of phenol-chloroform-isoamyl alcohol (48-48-4) saturated with 1 M Tris (pH 8.0) and then mixed with phenol-isoamyl alcohol (48-2) and centrifuged at 10,000 g for 5 min after which 1/2 volume of 7.5 M ammonium acetate was added and the mixture left to stand on ice for 15 min and then centrifuged at 10,000 g for 15 min at 4 °C after which the supernatant was mixed with one volume of isopropanol and centrifuged at 12,000 g for 15 min. After centrifugation the supernatant was discarded and the DNA pellet re-suspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8) and 2 volumes of ethanol added and the mixture centrifuged at 10,000 g for 15 min, the supernatant being dried using a speed vac apparatus and re-suspended in 200 µL of TE buffer. Restriction enzyme digestions of fungal genomic DNA and plasmids were performed according to the manufacturer procedures and standard protocols (Sambrook *et al*., 1989).

Southern blot analysis

To determine the fate of plasmid DNA in transformants, Southern blotting and hybridization was carried out according to the method of Daboussi *et al*. (1992). Briefly, Genomic DNA was extracted from the recipient *F. verticillioides* strain (S68) and ten GUS^+ co-transformants and digested to completion with the *Eco*RI restriction enzyme which cuts plasmid pNOM102 once and probed with the 4.5-kb *Hind*III-*Eco*RI plasmid pNOM102 fragment containing the entire GUS construct and the pnH24 plasmid containg the *nia* gene labeled with ^{32}P.

Histochemical localization of β-glucuronidase activity on maize roots infected with *Fusarium verticillioides* GUS^+ transformant

Maize (*Zea mays*) seeds were washed in running tap water with a soft brush and then vigorously washed twice for 15 min in 0.1% Tween 80 solution and then surface disinfected by immersion in 70% ethanol for 1 min, then 3% sodium hypochloride solution for 25 min and then 70% ethanol for 30 s. The disinfected seeds were washed in distilled sterile water, transferred to sterilized petri plates containing moist filter paper and germinated for 32 h at 26 °C under aseptic conditions. For inoculation with fungi the germinating seeds were aseptically immersed into a suspension of *F. verticillioides* GUS^+ conidia (1.6×10^7 conidia mL^{-1}) and immediately transferred to flasks containing Murashige & Skoog (MS) medium (Murashige & Skoog, 1962) and incubated at 26 °C using a 13 h-light and 11 h-dark photoperiod. After 10 days the roots were collected, washed and stained as described by Couteaudier *et al.* (1993), GUS activity being indicated by a blue precipitate in the mycelium of *F. verticillioides* GUS^+ transformants colonizing the maize roots.

Assay of β-glucuronidase activity and assessment of genetic stability

A qualitative β-glucuronidase activity assay was conducted on the NIA^+ transformants using microtiter plates, each well containing 200 µL of MM and 1 mM 4-methylumbelliferyl β-d-Glucuronide (MUG, Sigma) as substrate, fluorescence of GUS^+ co-transformants being observed with a UV transluminator. A fluorometric β-glucuronidase activity assay was also performed on six of the transformants (TG1, TG3, TG4, TG5, TG7, TG8) according to the method of Couteaudier *et al.* (1993) using MUG as substrate, the results being expressed as the concentration (in nanomoles) of 4-methylumbelliferyl (MU) produced min^{-1} mg^{-1} of protein.

The mitotic stability of the GUS^+ transformants was tested by making five serial transfers of each transformant on non-selective PDA medium and testing each using the MUG microtiter assay and a sample with no less than 25 colonies, an isolate being considered stable if all of the colonies showed the GUS^+ phenotype.

Results

Primary selection in the co-transformation experiment with the pNOM 102 and pNH24 plasmids was performed using MM plates which allowed only *F. verticillioides* colonies harboring the pNH24 plasmid (*nia*$^+$) to grow, this being shown by the fact that when the recipient strain (*F. verticillioides nia*$^-$) was transformed with empty pUC vector (the negative control) no transformants were recovered on MM plates, thus confirming the stability of the recipient strain. We recorded 75 NIA^+ transformants per µg of vector DNA, the MUG microtiter plate β-glucuronidase (GUS) assay showing that 57.20% of these transformants had the GUS^+ phenotype as the result of co-transformation with the *gus*A gene. Ten co-transformants exhibiting the GUS^+ phenotype on MUG microtiter plates were tested for mitotic stability and analyzed by Southern blotting. The fluorometric assay results for the six *F. verticillioides* co-transformants (TG1, TG3, TG4, TG5, TG7, TG8), *F. oxysporum* strain MT5 (the positive control) and the *F. oxysporum* S68 parent strain are given in Table 1.

The mitotic stability of individual transformants tested in the MUG microtiter assay indicated that all the transformants analyzed retained the GUS activity of the original transformant after five serial transfers on non-selective medium. Although the *F. verticillioides* recipient strain (S68) showed basal GUS activity, both histochemical staining and the MUG microtiter assay were negative. All GUS^+ transformants were positive in histochemical staining and, as shown in Figure 1, it was possible to localize β-glucuronidase activity on maize roots infected with a GUS^+ *F. verticillioides* transformant.

Southern hybridization, performed to determine the fate of plasmid DNA in the transformants, detected sequences homologous with those of the pNOM102 plasmid in all of the *F. verticillioides* GUS^+ transformants (Figure 2A). The GUS^+ transformant TG1 showed a DNA fragment corresponding to a linearized copy of plasmid pNOM102, a pattern which may be explained by the integration of repeated and unmodified copies of the plasmids in tandem. Most of the GUS^+ transformants (TG2, TG3, TG4, TG5, TG6 and TG9) showed multiple plasmid integration, with transformant TG5 presenting multiple in tandem integration. Transformant TG7 showed two plasmid DNA bands, neither of which was the same size as the plasmid, indicating a simple copy integration into the chromosome by either a rearrangement of the GUS sequence construct or by integration of two single copies. The hybridization pattern observed in transformant TG8 may indicate simple integration. Transformant TG10 possessed a DNA fragment the same size as the original plasmid plus two other plasmid DNA fragments suggesting the integration of multiple copies of the plasmid in tandem plus integration at other sites or rearrangements.

Table 1 - Morphological, biochemical and molecular characteristics of transformant and recipient *Fusarium* sp. strains.

Strain[a]	GUS phenotype[b]	Mitotic stability[c]	MU Production[d]	Copy number of the pNOM102 plasmid[e]
TG1	GUS^+ transformant	Stable	1631.90	>1
TG2	GUS^+ transformant	Stable	nd	>1
TG3	GUS^+ transformant	Stable	1167.82	>1
TG4	GUS^+ transformant	Stable	754.03	>1
TG5	GUS^+ transformant	Stable	998.35	>1
TG6	GUS^+ transformant	Stable	nd	>1
TG7	GUS^+ transformant	Stable	279.33	1 or 2
TG8	GUS^+ transformant	Stable	1427.41	1
TG9	GUS^+ transformant	Stable	nd	>1
TG10	GUS^+ transformant	Stable	nd	>1
MT5	GUS^+ positive control	Stable	935.28	
S68	GUS^- recipient		115.55	0

[a]*F. verticillioides* GUS co-transformants are designated as TG1 to TG10; MT5 is the *F. oxysporum* GUS^+ transformant obtained by Couteaudier *et al.* (1993) which was used as the positive control; Strain S68 is *Fusarium verticillioides* NIA^- recipient strain.
[b]GUS = β-D-glucuronidase, this phenotype being determined on 4-methylumbelliferyl β-d-Glucuronide (MUG) medium microtiter plates using UV illumination. GUS^+ = fluorescent colonies GUS^- = non-fluorescent colonies.
[c]Mitotic stability was determined using a sample with no less than 25 colonies in a microtiter assay in which MUG was the substrate. Stable, *i.e.* all of the colonies had the GUS^+ phenotype.
[d]MU = 4-methylumbelliferyl concentration expressed as nanomoles of MU produced min^{-1} mg^{-1} of protein. nd = not-determined.
[e]Determined by Southern blotting.

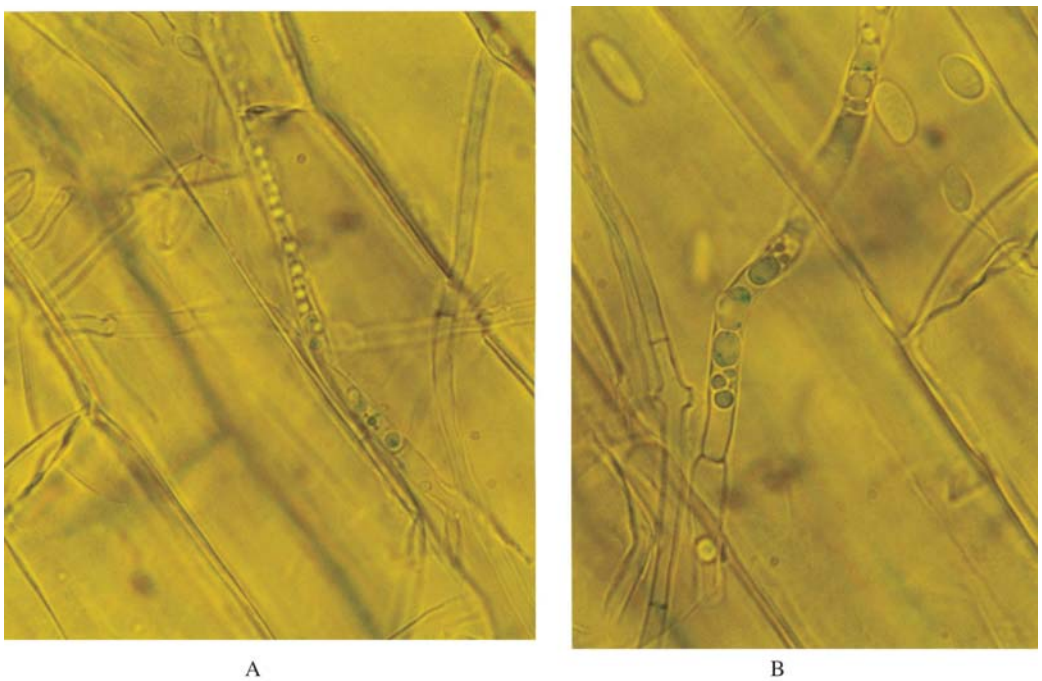

Figure 1 - Histochemical analysis of β-D-glucuronidase (GUS) activity in mycelia of *Fusarium verticillioides* co-transformant TG3 in maize roots. Histochemical analysis at (A) 400x and (B) 1000x magnification. Intracellular GUS activity is visible as discrete blue rounded bodies.

The hybridization pattern using the *nia* probe (Figure 2B) showed integration of the pnH24 plasmid at several sites. A DNA fragment of about 5 kb, which did not correspond to the pUC vector (data not shown), was observed in

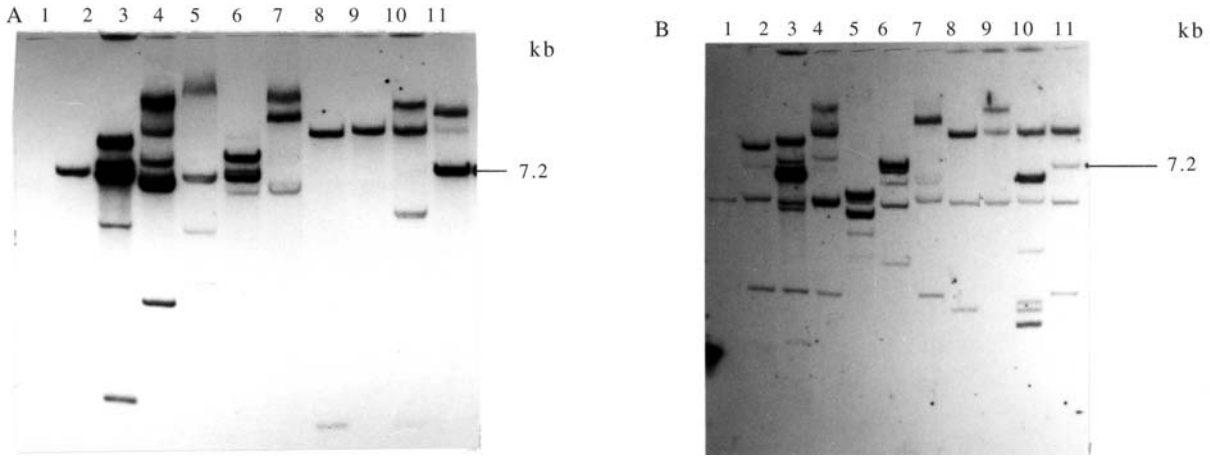

Figure 2 - Southern blot analysis of *Fusarium verticillioides* transformants. DNA was extracted from recipient and co-transformed strains, digested to completion with EcoRI and probed with (A) the 4.5-Kb Hind III-EcoRI pNOM102 fragment containing the entire GUS construct and (B) the pNH24 plasmid linearized with EcoRI and labelled with ^{32}P. Lane 1, *F. verticillioides* recipient strain; lanes 2 to 11, *F. verticillioides* co-transformant TG1, TG2, TG3, TG4, TG5, TG6, TG7, TG8, TG9 and TG10.

both the S68 recipient strain an all transformants and may correspond to a resident copy of the nitrate reductase gene of *F. verticillioides*.

A blue precipitate was clearly observable in the mycelium of *F. verticillioides* GUS$^+$ transformant in the maize roots (Figure 1).

Discussion

Our co-transformation procedure resulted in a transformation efficiency some 75 times higher than that obtained by Yates *et al.* (1999) who performed GUS transformation of *F. moniliforme* using a pHPG plasmid containing the *gus*A reporter gene and the selectable marker gene *hph* encoding for hygromycin-resistance (*hyg*^{r}), while our procedure was about 750 times of that described by Doohan *et al.* (1998) who transformed *Fusarium culmorum* using a plasmid harboring the *E. coli gusA* gene and the *hph* gene. Taking into account only the GUS transformants, co-transformant efficiency was high (57.20%) in our assays, with transformation efficiency attaining a 43 to 430 fold increase as compared to previous reports. Bao *et al*. (2000) obtained a co-transformation efficiency of 46.30% in *F. oxysporum* using the plasmid vector pCF20 containing the *E. coli gus*A gene and pAN7-2 encompassing *E. coli hph* gene.

Some of our *F. verticillioides* transformants gave higher fluorometric GUS activities than the positive control strain M-T5 (Table 1) which had originally been obtained by Couteaudier *et al*. (1993) and had given the best GUS activity in their assay, the GUS activity of strain M-T5 in our study (Table 1) being similar to that reported by Couteaudier *et al*. (1993).

A blue precipitate was clearly observable in the mycelium of *F. verticillioides* GUS$^+$ transformants colonizing maize roots, indicating that the co-transformant expressed the gene in the plant and demonstrating the effectiveness of endophytic fungi as 'vectors' for the introduction of characteristics of biotechnological or agricultural interest into tropical host plants. The result showing co-transformant expressing the gene in the plant is important because, as shown by Pamphile and Azevedo (2001), endophytic *F. verticillioides* strains may be specific to particular maize populations or lines. Direct histochemical examination of the endophyte *in situ* can aid studies on fungal competition, endophyte colonization of plant tissues, endophyte-plant relationships and investigations involving the relationship of endophytes with other components of the plant-associated microbial community.

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