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A Comparison of the Phenotypic and Genetic Stability of Recombinant *Trichoderma* spp. Generated by Protoplast- and *Agrobacterium*-Mediated Transformation

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Four different *Trichoderma* strains, *T. harzianum* CECT 2413, *T. asperellum* T53, *T. atroviride* T11 and *T. longibrachiatum* T52, which represent three of the four sections contained in this genus, were transformed by two different techniques: a protocol based on the isolation of protoplasts and a protocol based on *Agrobacterium*-mediated transformation. Both methods were set up using hygromycin B or phleomycin resistance as the selection markers. Using these techniques, we obtained phenotypically stable transformants of these four different strains. The highest transformation efficiencies were obtained with the *T. longibrachiatum* T52 strain: 65-70 transformants/μg DNA when transformed with the plasmid pAN7-1 (hygromycin B resistance) and 280 transformants/10^7 spores when the *Agrobacterium*-mediated transformation was performed with the plasmid pUR5750 (hygromycin B resistance). Overall, the genetic analysis of the transformants showed that some of the strains integrated and maintained the transforming DNA in their genome throughout the entire transformation and selection process. In other cases, the integrated DNA was lost.

Keywords: *Trichoderma* transformation, phenotypic stability, genetic stability

The application of genetic engineering techniques to filamentous fungi has led to great advances in the knowledge of the structure, organization, and genetic regulation of these microorganisms. Integrative transformation systems were first developed in fungi such as *Neurospora crassa* and *Aspergillus nidulans* (Mishra, 1985; Ramboseck and Leach, 1987; Mohr and Esser, 1990). However, in spite of the great importance of these microorganisms in basic research, both have limited biotechnological importance.

However, the transformation systems that were used for these fungi have been used as models to develop suitable methods of transformation for other species with industrial relevance, such as *Aspergillus niger*, *A. oryzae*, *A. awamori, Penicillium chrysogenum,* and *Trichoderma reesei* (Beri and Turner, 1987; Penttilä *et al.*, 1987; Yelton *et al.*, 1987). In parallel, the search for selection markers that yield stable transformants has also been important, in order to allow the design of more flexible transformation methods.

Over the last 70 years, many species within the genus *Trichoderma* have been extensively investigated due to their potential as biological control agents (Harman, 2000; Monte, 2001). Numerous *Trichoderma* strains produce extracellular hydrolytic enzymes and compounds with antifungal activity, and they may also compete with fungal pathogens for space and nutrients through rhizosphere competence (Howell, 2003). *Trichoderma* species can also promote plant growth and induce resistance in plants (Harman *et al.*, 2004).

Recently, the biodiversity of a collection of *Trichoderma* biocontrol strains was explored using molecular techniques that involved the sequencing of the 5.8S rDNA gene internal transcribed spacers, ITS1 and ITS2. The distribution of these spacers in

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the species *T. asperellum* and *T. longibrachiatum,* and the *T. harzianum* and *T. atroviride/T. koningii* complexes was investigated in these studies (Hermosa *et al.*, 2000).

These four *Trichoderma* species/complexes display differences in their biocontrol-related enzyme activities, mainly involving the expression of cell wall-degrading hydrolases (Benítez *et al.*, 1998; Lorito, 1998), as well as antifungal compounds (Vicente *et al.*, 2001; Vizcaíno *et al.*, 2005). Due to the importance of *Trichoderma* as a producer of significant metabolites with various applications (Sivasithamparam and Ghisalberti, 1998), procedures and techniques need to be developed that further our knowledge of both the biocontrol mechanisms and the production of metabolites of industrial interest at a molecular level.

The transformation of fungi, as mediated by protoplasts, was initially described for *Saccharomyces cerevisiae* (Hinnen *et al.*, 1978) and was later adapted to the transformation of filamentous fungi, such as *Neurospora*, *Aspergillus*, *Penicillium*, *Acremonium,* and *Trichoderma* (Balance *et al.*, 1983; Yelton *et al.*, 1984; Queener *et al.*, 1985; Cantoral *et al.*, 1987; Penttilä *et al.*, 1987). Generally, the transforming DNA is randomly integrated into the fungal genome, and the efficiencies of transformation range between 1 and 10^3 transformants/μg DNA when dominant selection markers are used (e.g., resistance to antibiotics), and between 1×10^2 and 1×10^5 transformants/μg DNA when auxotrophic markers are used (e.g., *pyrG, pyr4, niaD, acuD, trpC*). On the other hand, *Agrobacterium*-mediated transformation was first discovered and

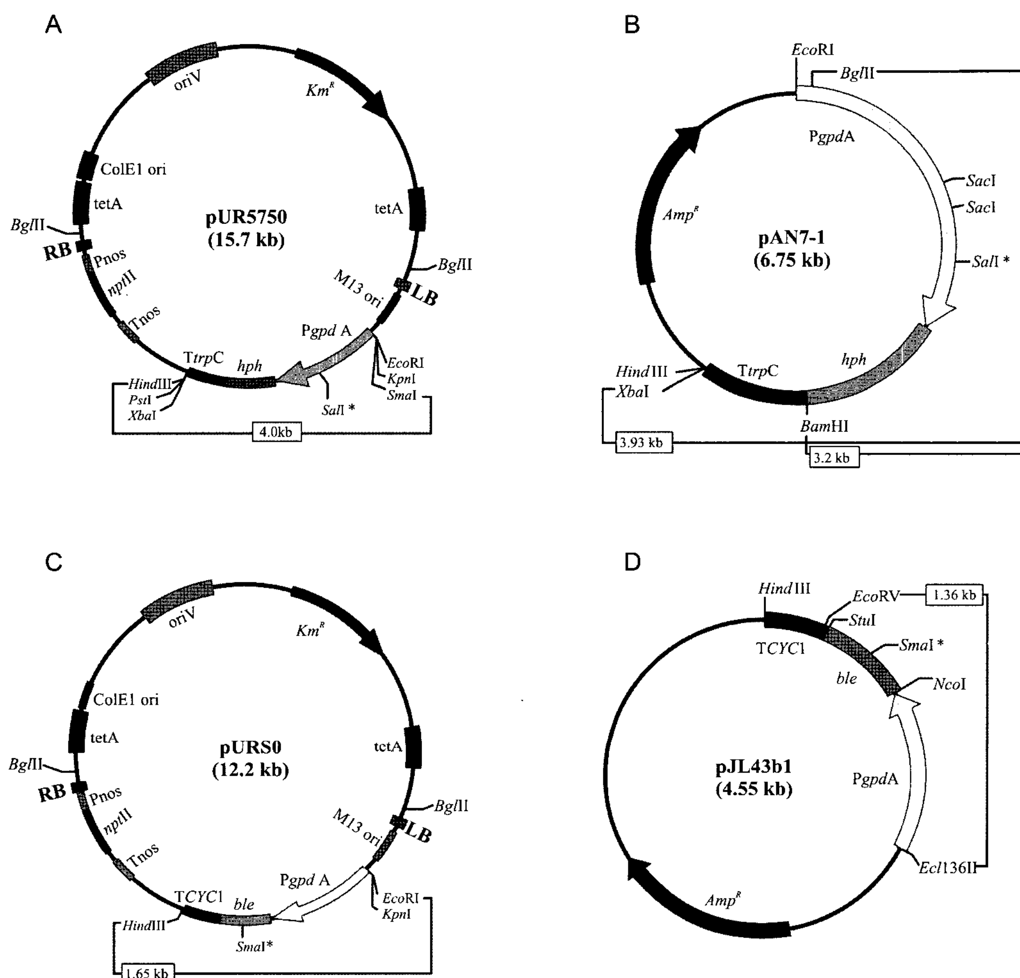

Fig. 1. A, C. – Plasmids used for the *Agrobacterium*-mediated transformation of the *Trichoderma* strains. B, D. – Plasmids used for *Trichoderma* protoplast transformation. The sizes of the expected hybridization bands are indicated in kb. *hph*.- Hygromycin B resistance gene from *E. coli*; *ble*.- phleomycin resistance gene from *S. hindustanus;* Km^R.- kanamycin resistance gene; Amp^R.- Ampicillin resistance gene; P*gpd*A.- Glyceraldehyde 3′- phosphate dehydrogenase gene promoter from *A. nidulans*; *TCYC1*.- Cytochrome 1 oxidase transcriptional terminator; LB and RB.- Left and Right borders of the transference DNA (T-DNA); Pnos.- nitric oxide synthase gene promoter; *nptII*.- neomycin phosphotransferase II gene; Tnos.- nitric oxide synthase transcriptional terminator.

applied in plants, and has since been used to transform filamentous fungi as well (De Groot *et al.*, 1998; Zeilinger, 2004). In *Agrobacterium*-mediated transformation, only a fraction of the plasmid DNA, the region called T-DNA, is transferred. The T-DNA integrates in the fungal genome at a random position by illegitimate recombination. This method confers 100- to 1000-fold higher transformation efficiencies than conventional methods (De Groot *et al.*, 1998). Furthermore, this procedure does not require the use of protoplasts since *A. tumefaciens* introduces DNA directly into the fungal spores.

In this study, we compared and optimized transformation procedures in the four representative *Trichoderma* strains mentioned previously that have high mycoparasitic activity, using both protoplast- and *A. tumefaciens*-mediated transformation with resistance to either hygromycin B or phleomycin as the selection marker. Thus, sixteen different transformation permutations were studied in order to determine the best procedure for use with each of the four *Trichoderma* strains. Overall, the general aim was to facilitate gene expression studies and high-throughput functional genomics analyses in these strains, due to their known biocontrol abilities.

Materials and Methods

Microorganisms

T. harzianum CECT 2413 (Spanish Type Culture Collection, Spain) (referred to as *T. harzianum* T34), *T. longibrachiatum* T52 (NBT52, Newbiotechnic S.A., Spain), *T atroviride* T11 (IMI 35941, International Mycological Institute, UK), and *T. asperellum* T53 (IMI 20268) were the recipients in the transformation experiments and the sources of genomic DNA for hybridization analyses. *Escherichia coli* DH5α was used for bacterial cloning and plasmid amplification. Finally, the strain *A. tumefaciens* AGL1 (Lazo *et al.*, 1991) was used for the *Agrobacterium*-mediated transformation of the *Trichoderma* strains.

Plasmids

pUR5750 (De Groot *et al.*, 1998) (Fig. 1A), pAN7-1 (Punt *et al.*, 1987) (Fig. 1B), pUPRS0 (constructed for this work, see below) (Fig. 1C) and pJL43b1 (Gutiérrez *et al.*, 1997) (Fig. 1D) were used for the *Trichoderma* transformation. pUPRS0 was constructed from pUR5750 by replacing a 4 kb *Eco*RI-*Hind*III fragment in pUR5750 that contained the hygromycin B resistance gene with a 1.55 kb *Ecl*136II/*Hind*III fragment from pJL43b1 that contained the bleomycin/phleomycin-resistance gene (*ble*) from *Streptoalloteichus hindustanus*.

Media used to grow the Trichoderma strains

Trichoderma strains were routinely maintained on PDA medium (Difco Becton Dickinson, USA), with the exception of *T. harzianum* T34, which was maintained on PPG medium (2% mashed potatoes, 2% glucose, 2% agar). *Trichoderma* seed cultures were inoculated on CM medium (0.5% malt extract, 0.5% yeast extract, 0.5% glucose). TSASb medium (Trypticase Soy Agar containing 1 M sorbitol and 1.5% Bacto-Agar [Difco]) was used as the regeneration medium in protoplast-mediated transformation experiments.

Protoplast-mediated transformation of Trichoderma

Transformation procedures based on the isolation of protoplasts were developed through the improvement

Table 1. Summary of the optimal conditions used to obtain protoplasts in the four strains used in the present work

Strain	Growth in solid medium (28°C)	Growth in CM medium[a]	$MgSO_4$[b]	DTT treatment[c]	Lytic enzyme concentration (mg/ml)[d]	Protoplast yield[e]
T. harzianum T34	PPG 3 days	10^7 spores/ml, 13-14 h	0.8 M	25 mM 2 h/30°C	5	1-2.5 × 10^8
T. atroviride T11	PDA 3 days	5 × 10^6 spores/ml, 15-16 h	1.0 M	–	12	1-5 × 10^6
T. longibrachiatum T52	PDA 5-6 days	10^7 spores/ml, 12-14 h	0.8 M	50 mM 2 h/30°C	7.5	1.2-1.6 × 10^8
T. asperellum T53	PDA 4-5 days	5 × 10^6 spores/ml, 15-16 h	1.0 M	–	10	5 × 10^6-1 × 10^7

[a] Growth on CM medium was performed at 28°C and 250 rpm
[b] $MgSO_4$ concentration in the protoplast buffer (TPT, pH 5.8)
[c] The DTT treatment was omitted for *T. atroviride* T11 and *T. asperellum* T53.
[d] The final ratio of grams of mycelia *versus* volume of protoplast buffer was for all the strains 0.5 g : 20 ml.
[e] The protoplast yield is expressed in protoplasts/ml in a final volume of 0.5 ml.

of several previously described methods for other strains, such as *T. reesei* (Penttilä *et al.*, 1987) or *Trichoderma* spp. (Sivan *et al.*, 1992). The conditions used in the protoplast-mediated transformations are summarized in Table 1.

Growth conditions and protoplast formation

Plates containing PDA or PPG medium were inoculated with 1×10^7 spores and incubated at 28°C for 3 to 6 days, depending on the strain (Table 1). The spores collected from each plate were used to inoculate 100 ml of CM medium and incubated in an orbital shaker at 250 rpm and 28°C for 13 and 16 h (Table 1). Most of the spores had germinated after this incubation period, giving rise to a mycelium without closed pellets. In these conditions and depending on the strain, between 1 and 2.3 g of mycelia were obtained per 100 ml of culture.

The mycelia were then filtered through nytal (30 μM pore diameter) and washed twice with 0.9% NaCl and once with TLT (Washing buffer: 10 mM sodium phosphate buffer; pH 5.8, 0.6 M magnesium sulphate). Then, 0.5 g of mycelia were resuspended in 50 ml of TPT (Protoplast buffer: 10 mM sodium phosphate buffer; pH 5.8, 0.8 M magnesium sulphate) with or without dithiothreitol (DTT). The mycelia were incubated in TPT with DTT at 30°C on an orbital shaker at 250 rpm for 2 h (this step was omitted for *T. atroviride* T11 and *T. asperellum* T53) (Table 1). The mycelia were then collected by centrifugation in Sorvall tubes at 7000 rpm for 5 min, washed once with TPT to remove the DTT, and resuspended in 20 ml of TPT containing lytic enzymes (Lysing enzymes, catalog # L-1412, Sigma, USA) at concentrations ranging between 5 mg/ml for *T. harzianum* T34 and 12 mg/ml for *T atroviride* T11 (see Table 1). The mycelia were then incubated at 30°C for 2 h with shaking at 80 rpm to allow the release of the protoplasts. The optimal magnesium sulphate concentration in this step to obtain a higher yield of protoplasts was previously determined as 0.8 M for *T. harzianum* T34 and *T. longibrachiatum* T52 and 1.0 M for *T. atroviride* T11 and *T. asperellum* T53.

Protoplast formation was monitored every hour. Once the protoplasts were released, they were collected by filtration through nytal filters (30 μm pore diameter) and diluted 1:5 with ST buffer (10 mM Tris HCl; pH 7.5, 1 M sorbitol). This protoplast suspension was centrifuged for 10 min at 4000 rpm, and the protoplasts were washed twice with ST and then once more with STC (ST containing 20 mM $CaCl_2$). Finally, the protoplasts were resuspended in STC plus 1/10 of the final volume of PTC (10 mM Tris HCl; pH 7.5, 20 mM $CaCl_2$, 60% polyethylene glycol 6000) and counted in a Thoma camera.

Protoplast transformation

One-hundred μl of the protoplast suspension, obtained as previously described, containing between 5×10^7 and 1×10^8 protoplasts/ml, were mixed with 10 μg of linearized plasmid. The plasmids pAN7-1 (containing the hygromycin B resistance gene) and pJL43b1 (containing the phleomycin resistance gene) (Fig. 1B, D) were used to transform *Trichoderma* by the protoplast method. Both plasmids were previously linearized with the enzyme *Hind*III to facilitate the integration of the vector into the fungal genome. The plasmid and the protoplast suspension were mixed and maintained on ice for 20 min. Then, 500 μl of PTC were added and the mixture was incubated at room temperature for another 20 min. Finally, the mixture was diluted with 600 μl of STC and poured as an overlay on regeneration TSASb plates containing the appropriate antibiotic (50 μg/ml hygromycin B or 75 or 100 μg/ml phleomycin). The plates were maintained at room temperature for 5-10 min until the medium had solidified, and subsequently incubated at 28°C for 4-6 days, in order to allow the regeneration of the protoplasts and growth of the colonies.

Agrobacterium tumefaciens-mediated transformation of Trichoderma

A. tumefaciens AGL1 was electroporated with the constructs, pUR5750 and pUPRSO (Fig. 1A, C respectively), according to Mozo and Hooykaas (1991). The *Agrobacterium* strains containing these plasmids were grown at 30°C overnight on LB plates supplemented with 50 μg/ml kanamycin, 100 μg/ml carbenicillin, and 25 μg/ml rifampicin.

Cells from a single colony were spread on a minimal-medium plate containing the appropriate antibiotics. Minimal medium (MM) contains per litre: 10 ml potassium-buffer pH 7.0 (200 g/L K_2HPO_4, 145 g/L KH_2PO_4), 20 ml magnesium-sodium solution (30 g/L $MgSO_4{\cdot}7H_2O$, 15 g/L NaCl), 1 ml 1% $CaCl_2{\cdot}2H_2O$ (w/v), 10 ml 20% glucose (w/v), 10 ml 0.01% $FeSO_4$ (w/v), 5 ml trace elements (100 mg/L $ZnSO_4{\cdot}7H_2O$, 100 mg/L $CuSO_4{\cdot}5H_2O$, 100 mg/L H_3BO_3, 100 mg/L $MnSO_4{\cdot}H_2O$, 100 mg/L $Na_2MoO_4{\cdot}2H_2O$), 2.5 ml 20% NH_4NO_3 (w/v), and 15 g/L bacto-agar at pH 7.5 (Hooykaas *et al.*, 1979). The plates were incubated at 30°C for 1 or 2 days. Several colonies from these plates were inoculated in liquid minimal medium containing 50 μg/ml kanamycin and incubated at 30°C with shaking at 250 rpm for 24 h. Bacteria were collected by centrifugation and resuspended in induction medium (IM = MM plus 10 mM glucose) containing 40 mM MES pH 5.3, 0.5% glycerol (w/v), and 200 μM acetosyringone (AS) (Mozo and Hooykaas, 1991) to an obtical density of 0.5 absorbance units at 660 nm. This bacterial suspension was then incubated for

6 h at 30°C in an orbital shaker (250 rpm) to pre-induce the virulence of *A. tumefaciens*.

Conidia from the four different *Trichoderma* strains were diluted in physiological salt solution to a concentration of 10^7 conidia/ml. Subsequently, 50 µl of this suspension were mixed with 50 µl of *Agrobacterium* cells previously grown and induced with AS, as described above. In order to determine if the transformation of the fungal conidia by *Agrobacterium* is dependent on T-DNA transfer, a negative control was included in which the virulence inducer AS was omitted. The mixtures were then plated onto nitrocellulose filters (Millipore 47-mm diameter black nitrocellulose filters with 0.8 µm pore diameter) placed on IM plates (1.5% bacto-agar) containing 5 mM glucose and 200 µM AS. The plates were incubated at room temperature or 18-20°C for at least 40 h. After this time, the filters were transferred to TSA plates (1.5% bacto-agar) containing 300 µg/ml cefotaxim, to inhibit the *Agrobacterium* growth, and hygromycin or phleomycin to select the *Trichoderma* transformants. Finally, these plates were incubated at 28°C for 5-6 days.

Isolation of genomic DNA from fungi

Genomic DNAs were isolated from mycelia as described by Specht *et al.* (1982). Briefly, 10^8 spores were inoculated on 100 ml of CM medium and grown for 36 h at 28°C in a rotary shaker at 250 rpm. The mycelium was then filtered through nytal filters (30 µm pore diameter) and washed once with 0.9% NaCl and once with grinding buffer (0.2 M Tris-HCl pH 8.0, 0.1 M EDTA). Then, 0.5 g of mycelia in grinding buffer plus 1% SDS were frozen with liquid nitrogen and broken in a mortar. The resultant mixture was then treated with 0.5 ml 25:24:1 v/v/v phenol:chloroform:isoamylic alcohol (phenol-CIA) and incubated for 30 min at 50°C. The phenol-CIA treatment was repeated until the interface was clear. Finally, the DNA was precipitated with 3 M sodium acetate, pH 7.0, and ethanol and resuspended in 50 µl of TE buffer (Sambrook *et al.*, 1989).

Hybridization analysis

Aliquots of genomic DNA (5 µg) were digested with several restriction endonucleases. They were then electrophoresed on 1.0 % agarose gels and transferred onto positively-charged nylon membranes (Roche Diagnostics GmbH, Germany) by capillary transfer. Hybridization was performed using a 3207 bp *Bgl*II-*Bam*HI fragment from pAN7-1 (probe A, Fig. 2) as a probe to hybridize total DNAs from the putative hygromycin B-resistant transformants, and a 1350 bp *Sac*I-*Eco*RV fragment from pJL43b1 (probe B, Fig. 2) for the putative phleomycin-resistant transformants. Finally, a 7954 bp *Bgl*II probe (probe C, Fig. 2) isolated from the plasmid pUR5750 was used for the

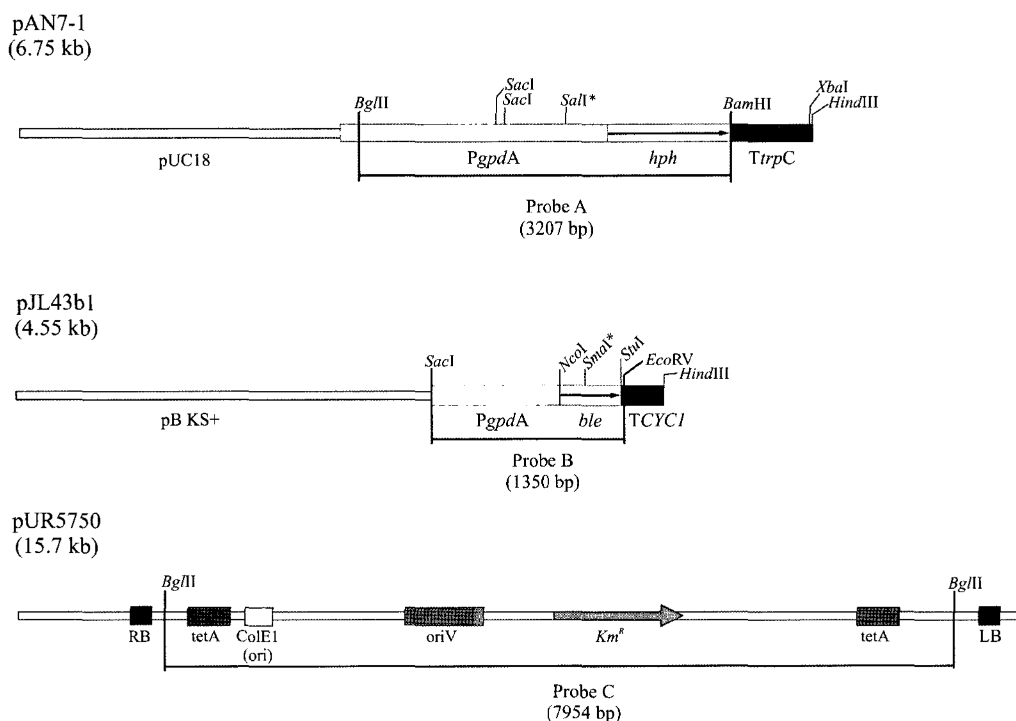

Fig. 2. Probes used in the hybridization experiments. The sites of recognition for the relevant restriction endonucleases, and the sizes of the probes are indicated.

putative *Agrobacterium*-mediated transformants. These probes were labelled with digoxigenin, and the hybridization was performed overnight at 42°C. Prehybridization and hybridization were carried out in the same solution (5 × SSC, 40 % formamide, 0.1 % sarcosine, 0.02 % SDS and 2% blocking reagent [Roche Diagnostics GmbH, Germany]); in the hybridization, the solution contained the labelled probe at a concentration between 25-30 ng/ml. The membranes were washed twice for 15 min at 42°C in 2× SSC containing 0.1 % SDS, then once with 0.1× SSC containing 0.1 % SDS in the same conditions, and finally, once with this same solution at 65°C for 15 min. The filters were developed by chemiluminescence (Roche Diagnostics GmbH, Germany) using CDP-Star as substrate.

Results

Protoplast-mediated transformation

The transformation efficiencies, using the procedures and plasmids described above, are shown in Table 2 and ranged between 9-10 transformants per microgram of DNA for *T. asperellum* T53 with the plasmid pJL43b1 and 65-70 transformants per μg DNA for *T. longibrachiatum* T52 with the plasmid pAN7-1 (Table 2).

Table 2. Efficiencies of transformation mediated by protoplasts

Strain	Transformants/ μg DNA	
	Hygromycin B (50 μg/ml)	Phleomycin (50 μg/ml)
T. harzianum T34	60	20-30*
T. atroviride T11	35-40	15
T longibrachiatum T52	65-70	30
T. asperellum T53	40-45	9-10

* The selection of phleomycin resistant transformants using protoplasts of *T. harzianum* T34 was performed using 100 μg/ml of this antibiotic.

Table 3. Number of transformants obtained by the *Agrobacterium*-mediated transformation

Strain	Transformants/ 10^7 spores	
	Hygromycin B (100 μg/ml)	Phleomycin (50 μg/ml)
T. harzianum T34	260	120*
T. atroviride T11	120	80
T. longibrachiatum T52	280	180
T. asperellum T53	120	40

* The concentration of phleomycin used to select the *T. harzianum* T34 transformants was 100 μg/ml.

Agrobacterium-mediated transformation of T. harzianum T34, T. atroviride T11, T. longibrachiatum T52 and T. asperellum T53

The numbers of transformants for the four different *Trichoderma* strains are summarized in Table 3 and ranged between 40 transformants per 10^7 spores for *T. asperellum* T53, with resistance to phleomycin, and 280 transformants per 10^7 spores for *T. longibrachiatum* T52 with resistance to hygromycin B (Table 3).

Phenotypic stability

To study the phenotypic stability of the isolated *Agrobacterium*-mediated transformants from the four *Trichoderma* strains, twenty transformants from each strain, obtained with the plasmids pUR5750 and pUPRS0, were randomly selected and grown on TSA medium containing 300 μg/ml cefotaxim and 150 μg/ml hygromycin B or 100 μg/ml phleomycin, respectively. These transformants were cultured three consecutive times in this medium for 4 days at 28°C; the stability of the transformants was then determined. Of twenty transformants selected from each strain by hygromycin B resistance, all twenty of the *T. asperellum* T53 transformants kept the antibiotic resistance phenotype after the three consecutive selection steps. However, only 18, 17, and 10 transformants of the strains *T. harzianum* T34, *T. atroviride* T11, and *T. longibrachiatum* T52, respectively, were phenotypically stable over these three selections. Of the twenty transformants selected from each *Trichoderma* strain by phleomycin resistance, all the transformants of *T. longibrachiatum* T52 were phenotypically stable, while only 15, 14, and 6 transformants obtained from *T. harzianum* T34, *T. asperellum* T53, and *T. atroviride* T11, respectively, kept the phleomycin resistance phenotype.

In the same way, ten protoplast-mediated transformants from each strain, obtained with the plasmids pAN7-1 and pJL43b1, as described in Material and Methods, were also analyzed. The transformants were cultured three consecutive times on TSA medium containing 100 μg/ml hygromycin B (125 μg/ml in the case of *T. harzianum* T34) or 75 μg/ml phleomycin (100 μg/ml for *T. harzianum*T34). Accordingly, for the ten transformants selected by hygromycin B resistance, 9, 4, 10, and 8 transformants obtained from *T. harzianum* T34, *T. atroviride* T11, *T. longibrachiatum* T52, and *T. asperellum* T53, respectively, kept the hygromycin B resistance phenotype. When the resistance to phleomycin was analyzed, 8 transformants from both *T. harzianum* T34 and *T.*

atroviride T11 and 10 transformants from both *T. longibrachiatum* T52 and *T. asperellum* T53 kept the resistance phenotype.

Genetic analysis of isolated protoplast-mediated transformants with hygromycin B resistance as the selection marker

Genomic DNAs from two phenotypically stable transformants from each of the four *Trichoderma* strains obtained with the plasmid pAN7-1 (containing the hygromycin B resistance gene) were digested with restriction endonucleases (see Fig. 3). These DNAs were hybridized with probe A (Fig. 2) in order to identify the transformants that had integrated the transformation cassette into their genome. Positive hybridization was observed in the *T. harzianum* T34 and *T. longibrachiatum* T52 transformants. However, no hybridization signal was detected in the *T. atroviride* T11 and *T. asperellum* T53 transformants, even though these strains exhibited the appropriate phenotype. When the hybridization was performed with DNAs from *T. harzianum* T34 digested with *Bgl*II and *Xba*I (Fig. 3, panel A, lanes 3-4) or from *T. longibrachiatum* T52 digested with *Bgl*II and *Bam*HI (Fig. 3, panel C, lanes 3-4), the whole hygromycin resistance cassette was released. Due to

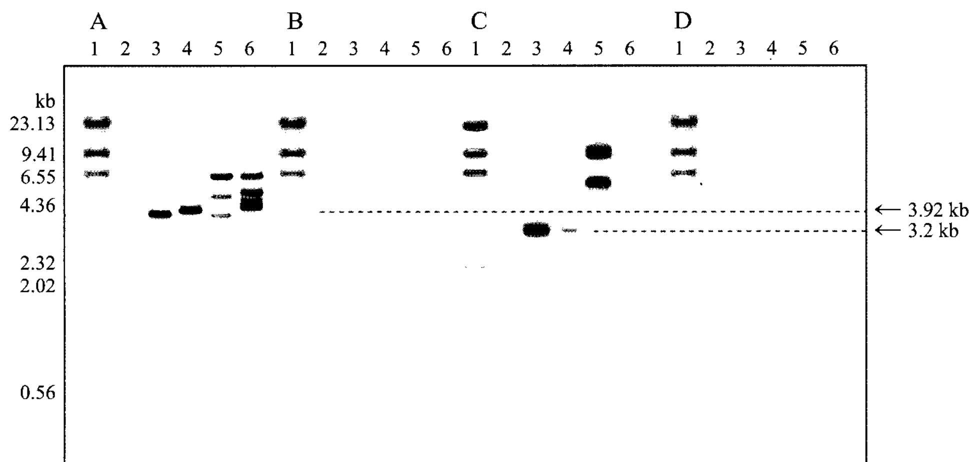

Fig. 3. Southern blot analysis of DNAs isolated from putative hygromycin resistant transformants obtained with the plasmid pAN7-1. A.- *T. harzianum* T34; B.- *T. atroviride* T11; C.- *T. longibrachiatum* T52; D.- *T. asperellum* T53.
Lane 1: Size markers; lane 2: Total DNA from the untransformed strain digested with *Bgl*II and *Xba*I; lanes 3-4: total DNA from two transformants digested with *Bgl*II and *Xba*I (T52 transformants were digested with *Bgl*II and *Bam*HI); lanes 5-6: total DNA from the same transformants digested with *Sal*I.
The 3.93 kb *Bgl*II-*Xba*I and 3.2 kb *Bgl*II-*Bam*HI hybridization bands, containing the *hph* gene, are indicated by the two arrows at the right of the figure.

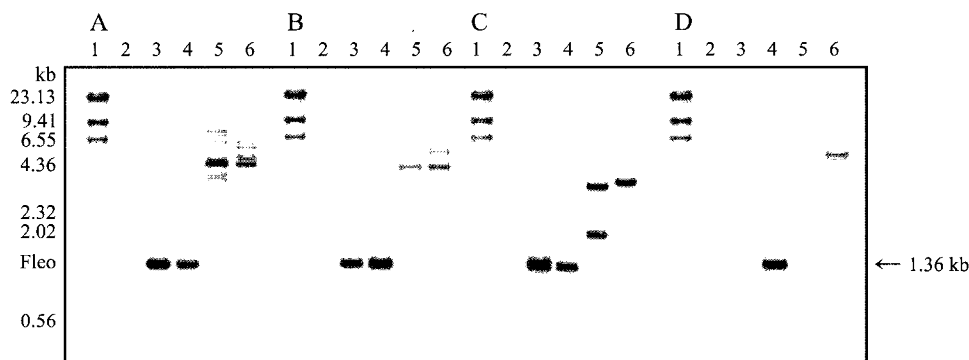

Fig. 4. Southern Blot analysis of DNAs isolated from putative phleomycin-resistant transformants obtained with the plasmid pJL43b1. A.- *T. harzianum* T34; B.- *T. atroviride* T11; C.- *T. longibrachiatum* T52; D.- *T. asperellum* T53.
Lane 1: Size markers; ane 2: Total DNA from the untransformed strain digested with *Sac*I and *Eco*RV: lanes 3-4: total DNA from two transformants digested with the same enzymes; lanes 5-6: total DNA from the same transformants digested with *Sma*I.
The 1.36 kb hybridization band corresponding to the complete phleomycin-resistance cassette is indicated by the arrow at the right of the figure.

the different endonucleases used for these strains, one unique band, of a different size in each strain, was observed,. In the second digestion (panels A-D, lanes 5-6), the DNAs were digested with *Sal*I, which cuts in just one site in the region of the *gpd*A promoter; more than two bands were observed (Fig. 3, panel A, lanes 5-6) in the *T. harzianum* T34 transformants, possibly due to integration at multiple sites. However, in the *T. longibrachiatum* T52 transformants, only two bands were observed (Fig. 3, panel C, lanes 5-6), but with slightly different sizes for each transformant. This could be explained by integration into one unique site in the genome of each transformant, but a different site in each case.

Genetic analysis of isolated protoplast-mediated transformants with phleomycin resistance as the selection marker

Total DNA was extracted from two separate transformants isolated from each of the *Trichoderma* strains by transformation with the plasmid pJL43b1, which contains the phleomycin resistance cassette (Fig. 1). These transformants were hybridized with probe B (Fig. 2), and all of the transformants, with the exception of one from *T. asperellum* T53, exhibited positive hybridization signals with this probe. As shown in Fig. 4, when total DNAs from the transformants were digested with the endonucleases *Sac*I and *Eco*RV to remove the whole phleomycin resistance cassette from pJL43b1, and later hybridized with probe B, one unique band of about 1.36 kb was observed in all the transformants (Fig. 4, panels A-D, lanes 3-4). However, when those DNAs were digested with the endonuclease *Sma*I, which cuts inside the *ble* gene, at least two bands were obtained from the *T. longibrachiatum* T52 and *T. asperellum* T53 transformants (Fig. 4, panels C and D, lanes 5-6). More than two bands were observed in the *T. harzianum* T34 and *T. atroviride* T11 transformants (Fig. 4, panels A and B, lanes 5-6), indicating that there are probably multiple integration sites in the genome of these last two strains.

A. tumefaciens-mediated transformation with hygromycin B resistance as the selection marker

Total DNAs from two transformants obtained from each *Trichoderma* strain with the plasmid pUR5750 (containing the hygromycin B resistance gene) were digested with endonucleases (see Fig. 5) and hybridized with probe A (Fig. 2). As shown in Fig. 5, all the transformants had integrated the exogenous DNA into their genome, except for *T. harzianum* T34. In spite of the fact that the *T. harzianum* T34 transformants were phenotypically stable, they lacked a positive hybridization signal, indicating that the resistance cassette had been lost (Fig. 5, panel A). In addition, the transformants obtained from the other three *Trichoderma* strains all displayed a hybridization band of the same size (4.0 kb) when the DNAs were digested with *Sma*I and *Hind*III, which releases a 4.0 kb fragment from the T-DNA region (Fig. 5, panels B, C and D; lanes 3-4). Thus, the entire transformation cassette was integrated intact into the genome of the transformants. Conversely, digestion with the endonuclease *Sal*I, which cuts once inside probe A (Fig. 2), produced two bands as expected, except in one of the *T. asperellum* T53 transformants. However, different band sizes were detected in each transformant, indicating that the T-DNA integrated into different sites in the genomes (Fig. 5, panels B, C, and D; lanes 6-7).

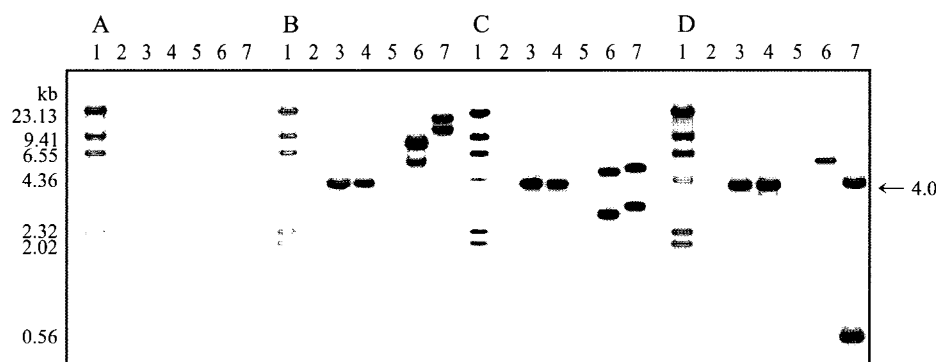

Fig. 5. Southern Blot analysis of total DNAs isolated from putative hygromycin-resistant transformants obtained by the use of the pUR5750 plasmid. A.- *T. harzianum* T34; B.- *T. atroviride* T11; C.- *T. longibrachiatum* T52; D.- *T. asperellum* T53.
Lane 1: Size markers; lane 2: total DNA from the untransformed strain digested with *Sma*I-*Hind*III; lanes 3-4: total DNA from two transformants digested with the same enzymes; lane 5: total DNA from the untransformed strain digested with *Sal*I; lanes 6-7: total DNA from the transformants used in lanes 3-4 digested with *Sal*I.
The 4.0 kb hybridization band corresponding to the complete hygromycin B resistance cassette is indicated by an arrow at the right of the figure.

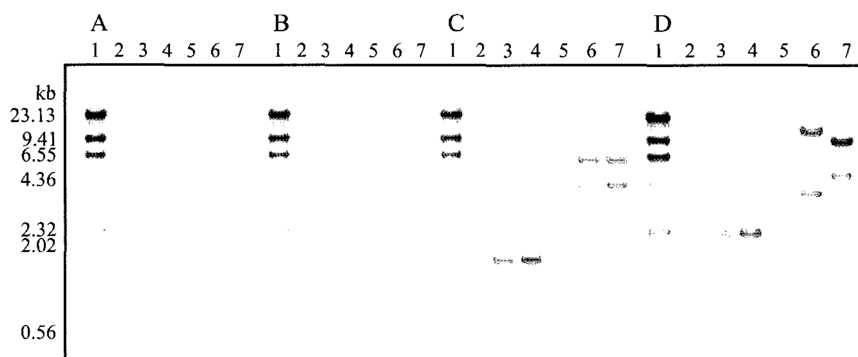

Fig. 6. Southern blot analysis of DNAs isolated from putative phleomycin-resistant transformants obtained with plasmid pUPRS0. A.- *T. harzianum* T34; B.- *T. atroviride* T11; C.- *T. longibrachiatum* T52; D.- *T. asperellum* T53.
Lane 1: size markers; lane 2: total DNA from the untransformed strain digested with *Eco*RI and *Hin*dIII; lanes 3 and 4: total DNA from two transformants digested with the same enzymes; lane 5: total DNA from the untransformed strain digested with *Sma*I; lanes 6 and 7: total DNA from the transformants digested with *Sma*I.

***A. tumefaciens*-mediated transformation with phleomycin resistance as the selection marker**

In this fourth group of experiments, total DNA was extracted from transformants obtained with the plasmid pUPRS0 that contained the phleomycin resistance cassette (Fig. 1), then digested with restriction endonucleases, as indicated in Fig. 6, and later hybridized with probe B (Fig. 2). Positive hybridization signals were observed in the *T. longibrachiatum* T52 and *T. asperellum* T53 transformants. In contrast, no hybridization signals were detected in the *T. harzianum* T34 and *T. atroviride* T11 transformants, indicating that they had lost the transformation cassette despite their phleomycin resistance phenotype. When total DNAs from the positive transformants were digested with *Eco*RI and *Hind*III to release a 1.65 kb fragment containing the whole phleomycin resistance cassette, one unique band was observed for each transformant. This band was the same size in the transformants from the same strain; thus, it was 1.65 kb in the *T. longibrachiatum* T52 transformants and approximately 2.2 kb in the *T. asperellum* T53 transformants (Fig. 6, panels C and D; lanes 3-4). When total DNAs were digested with the endonuclease *Sma*I, which cuts inside the *ble* gene, two bands were obtained in all of the transformants, as expected. These bands were exactly the same size in the transformants of *T. longibrachiatum* T52, but were different in the *T. asperellum* T53 transformants. These differences in the *T. asperellum* T53 transformants could be explained by integration in the opposite orientation into the fungal genome (Fig. 6, panels C, and D; lanes 6-7).

Fate of the DNA in the transformation of Trichoderma mediated by A. tumefaciens

Total DNA was extracted from one of the transformants obtained from each *Trichoderma* strain by the

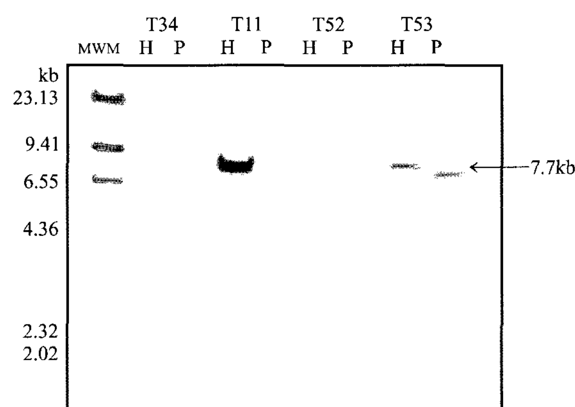

Fig. 7. Southern blot analysis of putative hygromycin B (H) or phleomycin (P) resistance transformants obtained from *Agrobacterium tumefaciens* AGL1-mediated transformation: T34.- *T. harzianum* T34; T11.- *T. atroviride* T11; T52.- *T. longibrachiatum* T52; T53.- *T. asperellum* T53. Total DNAs from each transformant were digested with *Bgl*II and hybridized with a 7.7-kb *Bgl*II fragment (probe C, Fig. 1) isolated from the pUR5750 plasmid. Please note that this fragment does not contain the T-DNA cassette that, hypothetically, is the only portion transferred to the fungal cells.

Agrobacterium-mediated procedure, using both pUR5750 (hygromycin B resistance) and pUPRS0 (phleomycin resistance), and digested with *Bgl*II, an endonuclease that cuts both sides of the T-DNA. This DNA was then hybridized with a 7.7 kb fragment obtained from digestion of pUR5750 with *Bgl*II (probe C, Fig. 2). This probe therefore contains the entire pUR5750 plasmid without the T-DNA fragment.

The aim of this experiment was to determine if any fragment of the plasmid located outside the T-DNA region had been integrated into the genome of any strains that gave a positive signal with a probe corresponding to the T-DNA (Fig. 5 and 6). As shown in

Fig. 7, three transformants gave a positive hybridization signal: *T. atroviride* T11 and *T. asperellum* T53 transformed with pUR5750 (hygromycin B resistance) and *T. asperellum* T53 transformed with pUPRS0 (phleomycin resistance). As expected, the *T. harzianum* T34 transformants, which gave negative hybridization results with a probe located inside the T-DNA (probes A and B, Fig. 2), also gave negative results when hybridized with probe C. For the *T. atroviride* T11 transformants, a positive hybridization signal corresponding to the T-DNA cassette was observed only when the *Agrobacterium*-mediated transformation was performed with hygromycin B resistance as the selection marker. Furthermore, in this case, a probe corresponding to the regions located outside the T-DNA (probe C) also produced a positive hybridization signal. Thus, the whole plasmid was integrated into the fungal genome, not just the T-DNA cassette. On the other hand, all of the *T. longibrachiatum* T52 transformants showed the anticipated results, that is, positive hybridization with the T-DNA probe and no hybridization with probe C. Thus, in this strain only the DNA located between the right and left borders of the T-DNA was transferred to the fungal genome. And finally, both *T. asperellum* T53 transformants exhibited a positive hybridization signal when they were hybridized with the probes corresponding to the T-DNA (probes A and B) and with the probe located outside the T-DNA (probe C). Similar to *T. atroviride* T11, then, in this strain the complete plasmid, and not just the T-DNA, was integrated into the fungal genome (Fig. 7).

Discussion

Transformations mediated by protoplasts or by *A. tumefaciens* are two of the more frequently used methods for introducing exogenous DNA into filamentous fungi (Penttilä *et al.*, 1987; De Groot *et al.*, 1998). Other methods have also been described, such as biolistic transformation, also known as microprojectile bombardment (Lorito *et al.*, 1993; Hazell *et al.*, 2000; Téo *et al.*, 2002).

Different strains of *Trichoderma* have previously been transformed by other research groups, using similar methods to those described in the present work (Herrera-Estrella *et al.*, 1990; Sánchez-Torres *et al.*, 1994; Margollez-Clark *et al.*, 1996; De Groot *et al.*, 1998; Delgado-Jarana *et al.*, 2002). However, the high inter-strain variety within this genus of fungi, together with their unique applications and characteristics, made it especially important to determine the best transformation system that could be applied to transform four of the most representative *Trichoderma* strains.

In this work, we tested sixteen different transformation permutations. We obtained phenotypically stable transformants from the different *Trichoderma* strains by both the protoplast method and the *Agrobacterium*-mediated transformation methods that remained stable even when cultured three consecutive times on selection media. However, genetic studies performed to determine if integration of the selection cassette had occurred correctly are harder to explain. In the case of *T. harzianum* T34, transformants with positive hybridization signals were only obtained through protoplast-mediated transformation, and no hybridization was detected following *Agrobacterium*-mediated transformation. In addition, with this second method, no hybridization signal was detected in these transformants when using a probe located outside the T-DNA (probe C, Fig. 2), indicating that neither the T-DNA nor the regions located outside this cassette were integrated into the genome. Since the resistance phenotype exhibited by these transformants was the expected phenotype, one possible explanation of this result is that these transformants are naturally resistant to the antibiotics used in the selection media and are not true transformants. However, the antibiotic concentrations used in the selection media were optimized prior to the transformation experiments to avoid germination of untransformed conidia; in addition, the transformants were cultured three times after the initial selection with a higher antibiotic concentration to avoid growth of non-transformed strains. Thus, at the antibiotic concentrations used, no colony growth was observed in any of the untransformed *Trichoderma* strains (data not shown). Thus, it seems more probable that these transformants lost the integrated fragment during the growth of the fungal cells under no selective pressure, which was carried out in order to obtain mycelia for DNA extraction. In addition, when the transformants of this strain obtained with the pAN7-1 and pJL43b1 plasmids were analyzed, several hybridization bands were observed, indicating that the plasmids had integrated into several *loci* in the genome. Taking all of these results together, it may be possible to explain the behaviour of the transforming DNA in *T. harzianum* T34. In some cases, a high frequency of recombination could result in an increased copy number of the integrated foreign DNA. However, this fact could also promote the loss of the integrated DNAs, as appears to be the case of the DNA integrated in the *Agrobacterium*-mediated transformation. There are no previous studies about Agrobacterium-mediated transformation of *T. harzianum* T34. However, the results obtained with the protoplast isolation method agree with previous reports, which also showed that transformants of *T. harzianum* obtained using the protoplast method and hygromycin B as that selective antibiotic integrated the trans-

forming DNA into their genome (Herrera-Estrella *et al.*, 1990).

In the case of *T. atroviride* T11, positive hybridization signals were found in all of the transformants isolated by the *Agrobacterium*-mediated procedure, but only when hygromycin B resistance was used as the selection marker. Thus, no positive signal was observed when phleomycin was used. In contrast, when transformants of this strain were obtained by the protoplast method, positive hybridization signals were only observed when phleomycin resistance was used as the selection marker. Thus, in *T. atroviride* T11, a very special pattern was found, indicating a high instability of the transforming DNA within the genome of this fungus. This correlates well with the results of the phenotypic stability assays, in which the transformants of this strain with the plasmids pUPRS0 (*ble* gene, *Agrobacterium* method) and pAN7-1 (*hph* gene, protoplast method) had the lowest phenotypic stability detected in the present work. In addition, these results also correlate well with those obtained previously with the same species. In *T. atroviride* T11, all transformants obtained following an *Agrobacterium*-mediated procedure using hygromycin resistance as the selection marker kept the transforming DNA within the genome (Zeilinger, 2004).

The strain *T. longibrachiatum* T52 showed a more stable pattern. In all four cases, by both transformation methods and also with both antibiotic selection markers, positive hybridization signals were found. Even hybridizations that were carried out in order to determine if the transformation cassette was completely integrated (Fig. 3,4,5,6; lanes 3,4; panel C) gave rise to bands of the expected sizes (see Fig. 1 for the sizes of the hybridization bands). Furthermore, when the hybridizations were performed in order to determine if integration occurred in an unique or priority genome *locus* (Fig. 3 and 4, panel C; lanes 5-6, Fig. 5 and 6, panel C; lanes 6-7), only two bands were observed, as expected, indicating that there was only one integration site. Even more remarkable, when this strain was transformed by the *Agrobacterium* method using phleomycin as the selection marker, these two bands were the same size, indicating that, in these transformants, the T-DNA had been integrated into the same *locus*. In addition, these results agree with previous studies (Vizcaíno *et al.*, 2006) in which all of the transformants obtained by the protoplast method with resistance to phleomycin kept the transformation cassette integrated in the genome.

In the *T. asperellum* T53 transformants, the results were highly diverse; the only procedure that did not exhibit positive hybridization signals was protoplast-mediated transformation with plasmid pAN7-1. In the other three experiments, positive hybridization signals were observed, with the exception of one of the pJL43b1 transformants (Fig. 4, panel D). Thus, it seems that the DNAs were randomly integrated in the transformants obtained with plasmids pAN7-1 and pJL43b1. In addition, these transformants were less stable and lost the transforming DNA more easily. On the other hand, the T-DNA that is introduced by *Agrobacterium* mediation seems to have high-priority integration points in the fungal genome.

The transformation efficiencies obtained in this study were similar to or even higher than those reported previously for some of the strains used in this work. The protoplast transformation efficiency for *T. longibrachiatum* T52 has previously been reported to range between 0.5 and 5 transformants/μg DNA when hygromycin B was used as the selection marker (Sánchez-Torres *et al.*, 1994), whereas, using the same selection system, we obtained an efficiency that ranged between 65 and 70 transformants/μg DNA. Previous work with *T. atroviride* T11 reported an *Agrobacterium*-mediated transformation efficiency of 30-50 transformants/10^7 conidia when hygromycin B was used as selection marker (Zeilinger, 2004); in the present work, the efficiency with the same species and transformation system increased to 120 transformants/10^7 conidia.

Obviously, the behavior of each of these four *Trichoderma* strains is different. Furthermore, the behavior of each strain also seems to depend on the transformation procedure, as well as the antibiotic, in most cases. However, with the exception of *T. asperellum* T53, a different behaviour pattern emerges for each strain. First, *T. harzianum* T34 only gave rise to phenotypically and genotypically stable transformants following protoplast-mediated transformation. Second, stable transformants were only obtained from *T. atroviride* T11 when hygromycin B was used as the selection marker, while *T. longibrachiatum* T52 produced stable transformants by all of the methods used. Finally, *T. asperellum* T53 gave rise to stable transformants by the *Agrobacterium*-mediated procedure using both antibiotic selection markers and by the protoplast-mediated method using only phleomycin resistance. However, in the latter case, only 50% of the transformants were analyzed. In summary, we were able to determine the best transformation method for each of these four strains. This work can serve as an example of the genetic diversity of the *Trichoderma* genus, even in strains that are very close phylogenetically.

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