Trichoderma Transformation Methods

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3.1 General Methods

Most of the transformation techniques currently available for bacteria, e.g., electroporation (Schuster et al. 2012), biolistic transformation (Lorito et al. 1993; Te'o et al. 2002), and transformation by the use of shock waves (Magaña-Ortíz et al. 2013), also have been reported for *Trichoderma*. However, currently the most widely used and optimized procedures for *Trichoderma* strains are based on protoplasts and the *Agrobacterium*-mediated transformation (Cardoza et al. 2006).

Thus, in the methods described below, transformations of *Trichoderma* strains by protoplasts and mediated by *Agrobacterium* are explained in detail. Eventually, both procedures could be used to transform a particular strain. However, usually one strain can be transformed more efficiently using one of these two methods. The explanation for this phenomenon is still unknown, but the structure and composition of the cell wall as well as some properties like growth rate in a particular culture medium or the nutrient requirements would be important points to take in consideration in order to transform a particular strain.

Also, other alternative techniques are briefly described.

3.2 Detailed Procedure Description

3.2.1 Transformation of *Trichoderma* Mediated by Protoplasts

These procedures based on the isolation of *Trichoderma* protoplasts have been developed by the improvement of several methods previously described for *Trichoderma reesei* (Penttila et al. 1987; Gruber et al. 1990) or *Trichoderma* spp. (Sivan et al. 1992; Cardoza et al. 2006).

One of the limiting steps in this transformation technique is obtaining the protoplasts. For this reason, both the growth conditions and composition of the culture media have to be optimized for each strain. Other factors, as incubation temperature, time of growth, viscosity of the selection media, concentration of the lytic enzymes, and composition of solutions for release and purification of protoplasts, are critical factors that have to be observed.

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M.A. van den Berg and K. Maruthachalam (eds.), *Genetic Transformation Systems in Fungi, Volume 1*, Fungal Biology, DOI 10.1007/978-3-319-10142-2_3,
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3.2.1.1 Growth Conditions and Protoplasts Formation

1. Inoculate plates of a complex media, usually PDA or PPG media, with 10^6–10^7 conidia per plate. Incubate at 28–30 °C during 3–6 days, depending on the strain (Table 3.1).
2. Collect conidia from one plate and inoculate in 100 mL of CM medium (approximately 10^6–10^7 conidia/mL) in a rotary shaker at 250 rpm and 28 °C during 13–16 h (Table 3.1). Be sure that most of the conidia have germinated and avoid the formation of closed pellets. In case of pellet formation, time and/or temperature of incubation, composition of the medium or speed of the shaker have to be optimized. Under these standardized conditions, between 1 and 2.5 g of mycelia will be recovered from 100 mL of culture.

   For *T. reesei* and *T. parareesei*, conidia will be spread on MA plates (about 5×10^6 conidia per plate) over a sterile cellophane membrane covering the plate surface. Incubate for 16–22 h (depending of the strain) at 30 °C. About five plates will be needed to get enough amount of protoplasts.
3. Filter the mycelia through a sterile nylon filter (25–30 μm of pore diameter) and wash once with 0.9 % NaCl and once more with TLT (Washing buffer: 10 mM sodium phosphate buffer, pH 5.8; 0.6 M $MgSO_4$). Then, resuspend 0.5 g of mycelia in 50 mL of TPT (protoplasts buffer: 10 mM sodium phosphate buffer, pH 5.8; 0.8 M $MgSO_4$) with or without the addition of dithiothreitol (DTT) (see Note 1). Incubate the suspended mycelia at 30 °C in a rotary shaker at 250 rpm for 2 h (Table 3.1).
4. Collect the mycelia by centrifugation at $7{,}500 \times g$ for 5 min.
5. Wash the DTT-treated mycelia with TPT to remove the DTT and recover the mycelia as in step 4.
6. Resuspend the mycelium in 20 mL of TPT containing lytic enzymes (Lysing enzymes, catalog # L-142, Sigma, USA) at concentrations between 5 and 15 mg/mL (Table 3.1). Incubate the mixture (mycelium + lytic enzymes) at 30 °C for 0.5–2 h at low speed (80–100 rpm), to allow for release of the protoplasts. Previously to this step, the optimal magnesium sulphate concentration in TPT and TLT has to be determined to get the highest number of protoplast. In the case of *T. arundinaceum*, 0.7 M NaCl was used instead of magnesium sulphate (Table 3.1).
7. Check the protoplast formation at each hour using a light microscope (see Note 2). Once the protoplasts have been released, collect them by filtration (through filters with 25–30 μm pore diameter) and dilute 1:5 with ST buffer (10 mM Tris–HCl, pH 7.5; 1 M sorbitol) (see Note 3). Pellet the protoplasts by 10 min centrifugation at $3{,}000 \times g$.
8. Wash the protoplasts twice with ST and then once with STC (ST containing 20 mM $CaCl_2$). Proceed to pellet the protoplasts by centrifugation as in step 7.
9. Resuspend the protoplasts in STC at a concentration from 5×10^7 to 1×10^8 protoplasts/mL. Add 1/10 of volume of PTC (10 mM Tris–HCl, pH 7.5; 20 mM $CaCl_2$; 60 % polyethylene glycol 6000).

3.2.1.2 Protoplasts Transformation

1. Mix 100 μL of the protoplast suspension with 10 μg of plasmid (see Note 4).
2. Maintain the mixture on ice for 20 min and add 500 μL of PTC. Mix gently and incubate at room temperature for 20 min.
3. Dilute the mixture with 600 μL of STC and then mix aliquots of the final reaction with 5 mL of the appropriate regeneration medium (see Note 5). Spread as overlays on plates containing 5 mL of the same medium.
4. Maintain the plates at room temperature during 5–10 min to solidify the medium. Incubate at 28–30 °C during 4–6 days, to allow for regeneration of protoplasts and growth of the colonies (see Notes 6–8; Table 3.2).
5. Check the mitotic stability of the transformants: transfer them to a new Petri dish containing twice the concentration of the antibiotic used to select them. Allow them to grow and transfer the colonies to a fresh medium without antibiotic and, finally, to a fresh medium plus antibiotic. At this point those transformants are considered mitotically stable.

Table 3.1 Optimal conditions used to obtain protoplasts in different *Trichoderma* strains

Strain	Growth in solid medium (28 °C)	Growth in CM (spores/mL, h)[a]	$MgSO_4$ in TPT	DTT treatment	Lytic enzyme concn (mg/mL)[c]	Protoplast yield (protoplasts/mL)	Transformants/μg DNA[b]
T. harzianum T34	PPG, 3 d	10^7, 17	0.8 M	25 mM 2 h/30 °C	5 (2 h)	$1–2.5 \times 10^8$	60/30
T. atroviride B11	PDA, 3 d	5×10^6, 15	1.0 M	–	12 (3–4 h)	$1–5 \times 10^6$	40/15
T. longibrachiatum T52	PDA, 5–6 d	10^7, 17	0.8 M	50 mM 2 h/30 °C	7.5 (2 h)	$1.2–1.6 \times 10^8$	70/30
T. asperellum T53	PDA, 4–5 d	5×10^6, 15	1.0 M	–	10 (3–4 h)	$5 \times 10^6–1 \times 10^7$	45/10
T. parareesei T6	PPG, 4–5 d	5×10^6, 16–22*	1.2 M	–	10 (1–2 h)	$1–5 \times 10^7$	nd/5
T. reesei	PPG, 4–5 d	5×10^6, 20–22*	1.2 M	–	10 (0.5–1 h)	$1–2.5 \times 10^8$	600–800
T. arundinaceum IBT 40837	PPG, 5–7 d	10^9, 24	NaCl 0.7 M	–	5 (14 h) 25.0 driselase 0.05 chitinase	2×10^7	20/nd
T. brevicompactum IBT 40841	PPG, 5–7 d	10^7, 24	1 M	–	12 (3 h)	$<10^4$	0/nd

[a]Growth in CM medium was performed at 28 °C and 250 rpm. *Indicates growth in the surface of Petri dishes containing MA medium covered with a cellophane membrane
[b]These data refer to the efficiency observed when using hygromycin B/phleomycin. For *T. reesei*, the efficiency range was obtained when the *pyrG* marker was used in the transformation
[c]Lysing enzymes from Sigma catalog # L-142 (Sigma, USA) were used in all the strains used except for *T. arundinacerum*

Table 3.2 Procedures and markers used to transform different *Trichoderma* strains

Strain	Transformation procedure	Marker gene	Phenotype of transformants	References
T. reesei	AMT	*hph*	Hygromycin B^R	de Groot et al. (1998)
				Zhong et al. (2007)
T. atroviride	"	*hph*	"	de Groot et al. (1998)
				Cardoza et al. (2006)
T. harzianum	"	*hph*	"	Cardoza et al. (2006)
				Yang et al. (2011)
T. longibrachiatum	"	*hph*	"	Cardoza et al. (2006)
T. asperellum	"	*hph*	"	Cardoza et al. (2006)
T. brevicompactum	"	*hph*	"	Tijerino et al. (2011a)
T. arundinaceum	"	*ble*	$Phleomycin^R$	Malmierca et al. (2013)
T. longibrachiatum	"	*ble*	"	Cardoza et al. (2006)
T. asperellum	"	*ble*	"	Cardoza et al. (2006)
T. reesei	Protoplast	*hph*	Hygromycin B^R	Penttila et al. (1987)
T. viride	"	*hph*	"	Zhu et al. (2009)
T. harzianum	"	*hph*	"	Cardoza et al. (2006)
T. longibrachiatum	"	*hph*	"	Cardoza et al. (2006)
T. arundinaceum	"	*hph*	"	Malmierca et al. (2012, 2013)
T. harzianum	"	*ble*	$Phleomycin^R$	Cardoza et al. (2006, 2007)
T. atroviride	"	*ble*	"	Cardoza et al. (2006)
T. longibrachiatum	"	*ble*	"	Cardoza et al. (2006)
T. asperellum	"	*ble*	"	Cardoza et al. (2006)
T. parareesei	"	*ble*	"	Gutiérrez S (unpublished data)
T. harzianum	"	*amdS*	Growth in acetamide	Cardoza R.E. (unpublished data)
T. atroviride	"	*nptII*	$Geneticin^R$	Gruber et al. (2012)
T. reesei	"	*pyrG*	Uridine prototrophy	Gruber et al. (1990)
T. reesei	"	*Hxk1*	Growth on mannitol	Guangtao et al. (2010)
T. longibrachiatum	Electroporation	*hph*	Hygromycin B^R	Sánchez-Torres et al. (1994)
T. harzianum	Biolistic	*hph*	"	Lorito et al. (1993)
T. reesei	Shock wave	*hph*	Hygromycin B^R	Magaña-Ortíz et al. (2013)

AMT. Agrobacterium mediated transformation

3.2.2 Transformation of *Trichoderma* Mediated by *A. tumefaciens*

1. Electroporate *A. tumefaciens* AGL1 with constructs containing the T-DNA region [e.g., plasmids pUR5750 (de Groot et al. 1998) and pUPRS0 (Cardoza et al. 2006)] according to Mozo and Hooykaas (1991).
2. Grow the transgenic *Agrobacterium* strains overnight at 30 °C on LB plates supplemented with 50 μg/mL kanamycin, 100 μg/mL carbenicillin, or 25 μg/mL rifampicin.
3. Streak out the cells from a single colony on a minimal medium plate containing the appropriate antibiotics. *Agrobacterium* minimal medium (MM) contains per liter: 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 $MgSO_4·7H_2O$, 15 g/L NaCl), 1 mL 1 % $CaCl_2·2H_2O$ (w/v), 10 mL 20 % glucose (w/v), 10 mL 0.01 % $FeSO_4$ (w/v), 5 mL trace elements (100 mg/L $ZnSO_4·7H_2O$, 100 mg/L $CuSO_4·5H_2O$, 100 mg/L H_3BO_3, 100 mg/L $MnSO_4·H_2O$, 100 mg/L $Na_2MoO_4·2H_2O$), 2.5 mL 20 % NH_4NO_3 (w/v), and 15 g/L bacto-agar (Difco, USA) at pH 7.5 (Hooykas et al. 1979).
4. Incubate the plates at 30 °C for 1–2 days. Inoculate several colonies from these plates in

liquid minimal medium containing 50 μg/mL kanamycin and incubate at 30 °C and 250 rpm for 24 h. Collect bacteria by centrifugation and resuspend in induction medium (IM = MM + 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 optical density at 660 nm of 0.5 absorbance units. Then, incubate this bacterial suspension for 6 h at 30 °C in a rotary shaker (250 rpm) to pre-induce the virulence of *A. tumefaciens*.

5. Dilute conidia from *Trichoderma* in double distilled water to a final concentration of 10^7 conidia/mL. Then, mix 50 μL of this suspension with 50 μL of the *Agrobacterium* cell suspension from step 4. To confirm if transformation of fungal conidia by *Agrobacterium* is dependent on T-DNA transfer, a negative control has to be included in which AS, the virulence inducer, has been omitted.
6. Subsequently, spread the mixtures onto nitrocellulose filters (47 mm diameter nitrocellulose black filters, 0.8 μm pore diameter) (Millipore, Germany) placed on IM plates (1.5 % bacto-agar) containing 5 mM glucose and 200 μM AS. Incubate the plates at 18–20 °C for at least 40 h. Then, transfer the filters to TSA plates (1.5 % bacto-agar) containing 300 μg/mL cefotaxime to inhibit *Agrobacterium* growth, and the appropriate antibiotic to select the *Trichoderma* transformants (Table 3.2).
7. Incubate at 28 °C during 5–6 days.
8. Check the mitotic stability of the transformants as described for the protoplast mediated transformation.

3.3 Alternative *Trichoderma* Transformation Procedures

3.3.1 Protoplast Electroporation (Sánchez-Torres et al. 1994)

1. Protoplasts were isolated as indicated in Sect. 3.2.1.1. Thus, once the protoplast were released (step 6, Sect. 3.2.1.1), they were pellet by centrifugation at 3,000 × *g* for 10 min and then resuspended in SP solution (1 M sorbitol, 1 % (w/v) PEG 8000) to give a concentration of 1×10^8 protoplast/mL. Mix the protoplasts with the transforming DNA and carrier DNA (salmon sperm DNA). Apply an electric pulse through a Gene Pulse device (Bio-Rad Laboratories, USA) at 25 mF, 800 Ω, and 2.8 kV/cm as electrical parameters.
2. Dilute protoplasts using STC and plate them in the appropriate selective medium and incubate at 28–30 °C during 4–6 days.

3.3.2 Biolistic (Lorito et al. 1993)

1. Sporulate the *Trichoderma* strain by incubation on PDA medium at 28 °C for 7–14 days.
2. Dispose seven portions containing 5×10^7 to 1×10^8 conidia on PDA plates to align with the seven barrels of the Hepta Adaptor (Bio-Rad, USA) and leave to dry. Prepare a mixture of DNA (from 100 to 1,000 ng) precipitated with tungsten particles (0.7 μm mean diameter).
3. Resuspend the DNA mixture in 100 % ethanol, and use an aliquot for bombardment with the Bio-Rad Hepta Adaptor system with seven barrels for particle launch.
4. Incubate the plates for 5–6 h before overlaying with PDA containing the appropriate concentration of antibiotic for selection and incubate 3–5 days more.

3.3.3 Shock Waves (Magaña-Ortíz et al. 2013)

1. Mix 5×10^3 to 5×10^4 conidia and transforming DNA (50 μg/mL) and expose to 50 shock waves, generated by a Piezolith 2300 shock generator (Richard Wolf GmbH, Germany), consisting of a positive pressure peak of 150 MPa with a phase duration of 0.5–3 μs, followed by a decompression pulse of up to 20 MPa and a phase duration of 2–20 μs.
2. Dilute conidia and inoculate on 3 M cellulose filters placed on plates containing minimal medium without selective pressure and incubate for 24 h at room temperature. Transfer the filters to fresh medium with the appropriate antibiotic to select the transformants and incubate at 28–30 °C.

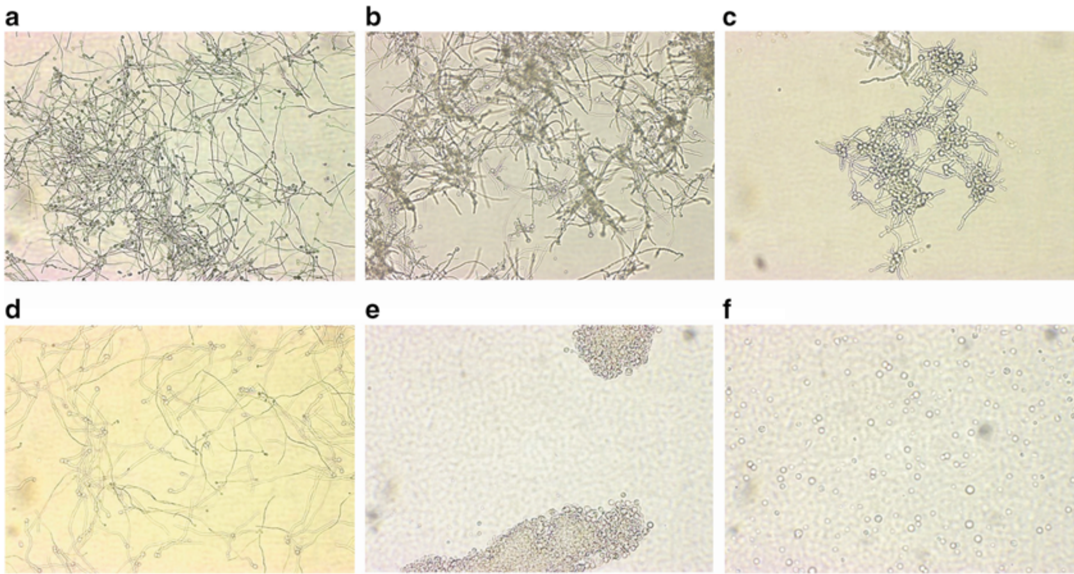

Fig. 3.1 Growth of several *Trichoderma* strains in liquid CM medium. (**a**) *T. harzianum* T34; (**b**) *T. atroviride* T11; (**c**) *T. asperellum* T53; (**d**) mycelia from *T. harzianum* T34 strain treated with 25 mM DTT; (**e**) protoplast formation from *T. harzianum* T34 strain with 1.2 M $MgSO_4$; (**f**) protoplast formation of *T. harzianum* T34 strain with 0.8 M $MgSO_4$

3.4 Notes

Note 1. DTT is used to break disulphide bonds, and is indicated to reduce the time of incubation and the concentration of lytic enzymes employed to get protoplasts. This step is only needed for some *Trichoderma* strains, e.g., *T. harzianum* and *T. longibrachiatum*.

Note 2. The microscopic observation of the mycelium during incubation with the lytic enzymes is the key starting point in the development of fungal transformation procedures (Fig. 3.1).

Note 3. Dilution of protoplasts at this step will help to recover a higher percentage of protoplasts after the first centrifugation step.

Note 4. Linearized plasmids can increase the transformation efficiency, by the generation of DNA ends which show a higher recombinogenic potential than the undigested circular plasmids. Moreover, for some strains, stability of the transformants is higher than that obtained when using undigested plasmids (Cardoza et al. 2006).

Usually, plasmids expressing dominant markers (i.e., antibiotic resistance genes) include strong fungal promoters to allow a suitable expression of the marker genes. These promoters have been isolated from fungi based in their high level of expression, and normally they also drive a high expression level in other fungal species. Examples of some of these promoters are the *gpd* gene promoter from *Aspergillus nidulans* (Punt et al. 1987), the *pki* gene promoter from *T. reesei* (Mach et al. 1994), the *gdh* gene promoter from *Aspergillus awamori* (Cardoza et al. 1998), and the promoter of *tss1* gene from *T. harzianum* (Cardoza et al. 2007).

Note 5. For the selection of transformants when using a dominant marker, complex media are normally chosen (e.g., TSA + Sorbitol, TSA + Sucrose, MA + Sorbitol) containing the appropriate antibiotic (the concentration of the antibiotic has to be optimized for each strain). When an auxotrophic marker is used, strains should be grown on *Trichoderma* minimal medium (Penttila et al. 1987) to select the recombinant strains by complementation of the auxotrophy.

Note 6. When resistance to phleomycin is used as a selection marker, an incubation of the transformation plates at 4 °C for as long as 12 h will increase the selectivity of the antibiotic.

Note 7. When the selection of transformants is based on resistance to an antibiotic, the use of media containing salts as osmotic stabilizers (NaCl, KCl, etc.) can result in a strong increase in background resistance of the WT to the antibiotic. Thus, in these cases, media containing sucrose or sorbitol as osmotic stabilizers are recommended.

Note 8. Another important point to consider is the viscosity of the medium used to select the transformants. A balance has to be established between the agar concentration of the selective medium and the PEG concentration used in the transformation mixture. High concentrations of both agar and PEG will reduce the efficiency of transformation. In addition, other parameters as the water quality and the purity of the culture media and agar have to be considered.

Acknowledgments We thank Dr. Elías R. Olivera for constructive comments and critical reading of the manuscript. Dr. Gutiérrez receives grant-aided support from the Ministry of Science and Innovation of Spain (AGL2012-40041-C02-02) and from the Junta de Castilla y León (LE125A12-2).

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