Curr Genet (1990) 17:169–1174

Current Genetics

Transformation of *Trichoderma harzianum* by high-voltage electric pulse

G. H. Goldman, M. Van Montagu, and A. Herrera-Estrella

Laboratorium voor Genetica, Rijksuniversiteit Gent, K. L. Ledeganckstraat 35, B-9000 Gent, Belgium

Received September 4/November 13, 1989

Summary. We have developed conditions for an efficient method of genetic transformation in *Trichoderma harzianum*, using high-voltage electroporation. Transformation was obtained with a plasmid carrying the *Escherichia coli*, hygromycin B phosphotransferase gene as a dominant selectable marker, and the *gpd* promoter and *trpC* terminator from *Aspergillus nidulans*. The transformation frequency is up to 400 transformants per μg of plasmid DNA. The transformants were phenotypically 100% stable; they were also mitotically stable. Hybridization experiments suggest that the transforming DNA might be integrated at the same position in the *T. harzianum* genome. This report opens possibilities for improving transformation systems that have already been described for fungi, or else for transforming filamentous fungi where the use of polyethylene glycol is not efficient.

Key words: *Trichoderma* – Electroporation – Transformation – Biocontrol

Introduction

Fungal diseases significantly reduce the yield, quality and preservation of many economically important crops. Despite extensive efforts to find sources of resistance through classical breeding programs, no solution has been found for some fungal diseases such as *Sclerotina*. *Trichoderma harzianum* is a fungal species which has proven to be a good biological control agent against a range of economically important aerial and soil-borne plant pathogens (Chet 1987; Dubos 1987; Papavizas 1985). However, the little knowledge accumulated on the physiology, and classical and molecular genetics of *Trichoderma* spp. has limited the understanding of the mechanisms of action involved in this biocontrol system.

Offprint requests to: M. Van Montagu

Considering that the sexual stage (*Hypocrea*) is rare and that there is no information about a parasexual cycle in *Trichoderma* spp, genetic manipulation using transformation and gene cloning provides the most targeted approach to dissect, and eventually alter, the physiology of these important fungi. Recently, transformation systems for biocontrol strains of *Trichoderma* (Herrera-Estrella et al. 1990) and a cellulolytic strain of *Trichoderma reesei* (Penttilä et al. 1987) have been described using the traditional PEG and calcium chloride approach to direct DNA transformation.

Virtually all fungal transformation protocols call for the addition of a high concentration of polyethylene glycol (PEG) following the initial period of exposure to DNA (Fincham 1989). Other approaches, such as transformation procedures involving particle bombardment (Klein et al. 1987), partial cell breakage by blending with glass beads (Costanzo and Fox 1988), or electroporation (Shigekawa and Dower 1988), could also be useful to introduce genes into filamentous fungi (for review, see Timberlake and Marshall 1989). Most recently electroporation has become a valuable technique for transfering nucleic acids into both eukaryotic and prokaryotic cells (Miller et al. 1988; Föster and Neumann 1989). In this paper, we have used high-voltage-mediated transformation as an efficient method for genetic transformation of *T. harzianum* with plasmid DNA. To our knowledge, this is the first successful report of electroporation in filamentous fungi.

Materials and methods

Strains and Plasmid. T. harzianum strain IMI206040 was used as the transformation host. Plasmid pAN7-1, a derivative of pUC19 containing the *E. coli* hygromycin B phosphotransferase gene, as a dominant selectable marker, and the *gpd* promoter and trpC terminator signals form *Aspergillus nidulans*, was kindly provided by Dr. P. Punt (Punt et al. 1987). Plasmid DNA was isolated from *E. coli* MC1061 by standard procedures (Maniatis et al. 1982), purified for its use in electroporation by equilibrium centrifugation in cesium chloride/ethidium bromide density gradients and dialyzed with distilled water prior to use.

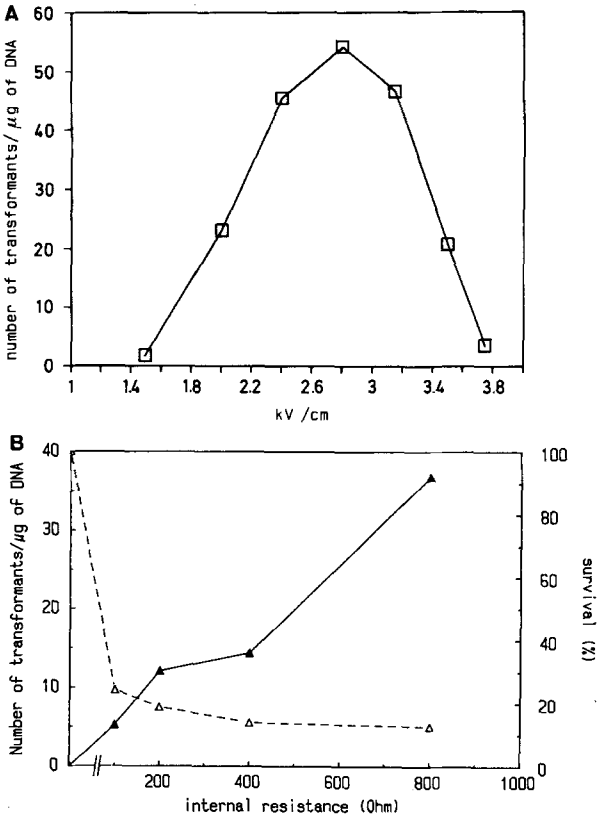

Fig. 1 A, B. Effect of different electrical conditions on the frequency of transformation of *T. harzianum*. **A** effect of the field strength (kV/cm) was tested maintaining constant capacitance (25 µF), internal resistance of the power supply (200 Ω), and 2.0×10^8 osmotically sensitive cells (OSC) per ml. **B** effect of the internal resistance was tested maintaining constant capacitance (25 µF), voltage (2.0 kV/cm), and 2.0×10^8 OSC/ml. The number of transformants/µg of DNA (▲) and the OSC survival (△) are displayed

Electrocompetent cells. Osmotically sensitive cells (OSC) were prepared according to Laurila et al. (1985) with the following modifications. Cellophane sheets placed on PDA plates were inoculated with 5×10^6 spores/ml and incubated for 21 h at 28°C; the germinative tubes from five cellophane sheets were suspended in 15 ml of 1.2 M $MgSO_4$, – 10 mM sodium phosphate buffer, pH 5.8, plus Novozyme-234 (5 mg/ml), in a Petri dish and these plates incubated at 28°C for 30 min with agitation in a rotatory shaker at 150 rpm. The OSC generated with this treatment were then centrifuged in corex tubes at 4,000 g for 5 min and the pellet washed twice with 1.2 M sorbitol in water; after the last wash the OSC were resuspended in 1.2 M sorbitol, at the desired concentration.

Transformation by electroporation. Two micrograms of transforming DNA (unless otherwise indicated) were mixed with 400 µl of OSC suspension and kept on ice. High-voltage pulses were delivered to 400 µl samples in a 1.0 ml disposable cuvette (Bio-Rad Laboratories; inter-electrode distance of 0.2 cm) by using a Gene Pulser apparatus (Bio-Rad Laboratories Richmond, CA, USA). Following delivery of the electrical pulse, OSC were mixed with 1.0 ml of PDB plus 1.2 M sorbitol (PDBS), incubated for 10 min on ice and for 2 h at 28°C. In the experiments where PEG was used, the following modifications were made: before adding the transforming DNA, spheroplasts were centrifuged at 4,000 g for 15 min and the pellet resuspended in 400 µl 1.2 M sorbitol plus 1.0% PEG (6000; Fluka Buchs, Switzerland); after electroporation, OSC were mixed with 5.0 ml PDBS. In both treatments, after the incubation period, aliquots of 100, 200, and 400 µl were plated using an agar overlay on plates containing PDA plus 1.2 M sorbitol and 100 µg/ml of hygromycin B as previously described by Herrera-Estrella et al. (1990). All the sets of experiments were repeated at least three times and no significant variation was found between replicates.

Detection of DNA. DNA was isolated according to the method described by Raeder and Broda (1985) from *T. harzianum* mycelia grown in liquid cultures in PDB medium plus 20 µg/ml of hygromycin B (Calbiochem San Diego CA, USA). DNAs from transformants and from the untransformed *T. harzianum* control strain were digested with *Bam*HI, *Eco*RI, *Hind*III, or *Eco*RV. DNA was separated by electrophoresis in a 1% agarose gel by standard procedures (Maniatis et al. 1982), transferred to Hybond-N membranes (Amersham UK) and hybridized according to the manufacturer's instructions. The plasmid pAN7-1 was nick-translated for its use as a probe in Southern analysis using ^{32}P-dCTP (Amersham UK). The filters were exposed on Kodak X-OMAT AR film at −70° C using intensifying screens.

Results

Electrical parameters

Intact cells, as well as cells treated with wall-degrading enzymes, have been used to obtain transformants with electroporation (Fromm et al. 1985; Dower et al. 1988; Shigekawa and Dower 1988). As electroporation of germinative tubes or mycelia of *T. harzianum* did not give any transformant, osmotically sensitive cells (OSC) were tested. A method for protoplast preparation and regeneration from *T. reesei* has been described (Laurila et al. 1985). However, it is not clear if protoplast could resist severe stress conditions like electroporation. Therefore, the influence of time of incubation of the germinative tubes with the cell wall-degrading enzymes on the frequency of transformation by electroporation was tested. Periods of time of 15, 30, and 45 min were used. An incubation period shorter than 30 min did not give any transformants while 45 min gave 50% less transformants than 30 min (data not shown). Since different batches of Novozyme-234 were found to influence the production of competent cells for electroporation in the current set of experiments, it is necessary to test the time of incubation for each particular batch of enzyme.

It is evident that the strength of the electrical field and pulse height are critical factors in determining the efficiency of DNA introduction into cells and that the optimum varies greatly from organism to organism. Different combinations of voltages and capacitances were chosen, always using an internal resistance of 200 ohms and 2.0×10^8 osmotically sensitive cells (OSC) per ml (data not shown). The electroporation medium used was the simplest possible, consisting of distilled water and sorbitol as osmotic protectant. These preliminary results indicated the transformation could be obtained in the range of 2.0 kV/cm with a capacitance of 25 µF. Figure 1 A shows that this approximation was reasonable since the maximum yield of transformants was found at 2.8 kV/cm. For field strengths between 1.5 kV/cm and 2.4 kV/cm, the efficiency of transformation increased in a linear manner, approximately doubling every 400 V/cm. The opposite effect was observed for field strengths between 3.0 kV/cm and 3.7 kV/cm. The effect of the internal resistance of the

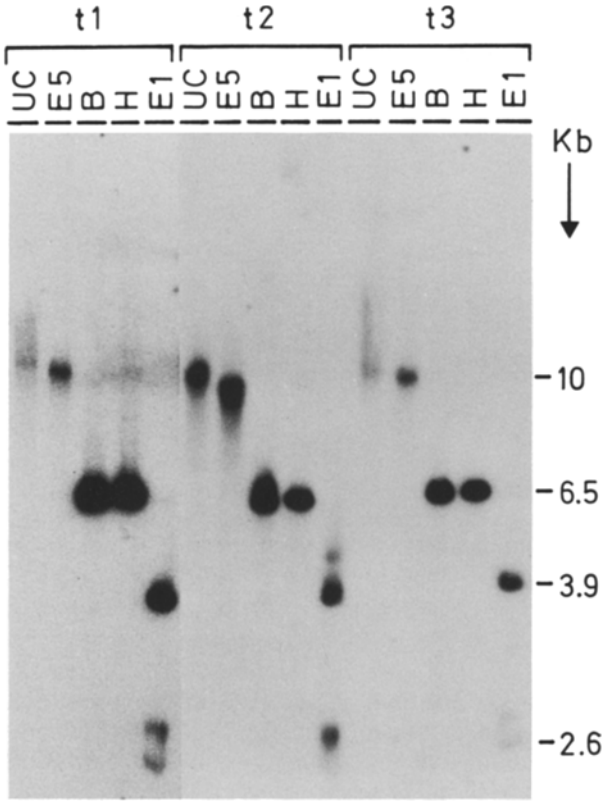

Fig. 2. Southern blot analysis of electrotransformants of *T. harzianum*. Genomic DNA from three transformants (t1, t2, and t3) was digested with *Eco*RV (*E5*), *Bam*HI (*B*), *Hind*III (*H*), and *Eco*RI (*E1*), or undigested (*UC*) and separated by agarose gel electrophoresis and analyzed by the Southern blot technique

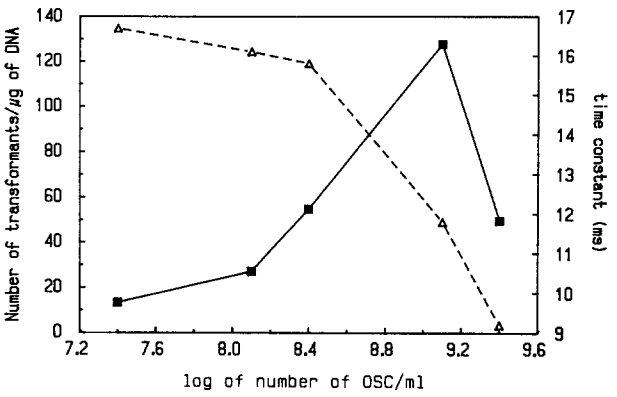

Fig. 3. Effect of the concentration of osmotically sensitive cells (OSC) on the frequency of transformation of *T. harzianum*. OSC ranged from 5.0×10^7 to 2.0×10^9/ml. The electrical conditions were a capacitance of 25 µF, an internal resistance of 800 Ω, and a voltage of 2.8 kV/cm. The number of transformants/µg of DNA (■) and the time constant (△) are displayed

power supply on electroporation efficiency was also examined (Fig. 1B). The best yield was obtained when a voltage of 2.0 kV/cm was delivered using an internal resistance of 800 ohms and a capacitance of 25 µF. The number of transformants doubled from 100 ohms to 200 ohms, remaining practically constant from 200 ohms to 400 ohms and reaching a maximum at 800 ohms. Using these conditions, the survival of OSC decreased sharply at 100 ohms, reaching 20%, and slowly from 200 ohms until 800 ohms reaching about 10.0% at this last point. From these initial experiments, it was possible to conclude that the best electrical conditions we have found for electroporation of *T. harzianum* involved a voltage of 2.8 kV/cm with a capacitance of 25 µF and an internal resistance of 800 ohms. *T. harzianum* OSC subjected to these electrical conditions in the absence of pAN7-1 did not produce any hygromycin-resistant colonies. The conditions used for electroporation of this organism are different from the conditions previously described for *Dictyostelium discoideum, Saccharomyces cerevisiae*, mammalian cells, and plant protoplasts in electroporation experiments (Fromm et al. 1985; Karube et al. 1985; Howard et al. 1988; Shigekawa and Dower 1988).

Southern blot analysis

Total cellular DNA of several independent transformants was isolated and undigested, *Eco*RV-, *Bam*HI-, *Hind*III-, and *Eco*RI-digested, DNAs were subjected to agarose gel electrophoresis and analyzed from Southern blots using pAN7-1 as a probe. The wild-type *T. harzianum* contained no sequences hybridizing to the vector at the stringency used. Figure 2 shows the pattern of hybridization for three transformants. The size of the plasmid pAN7-1 is about 6.5 kb and it has no sites for *Eco*RV, one site each for *Bam*HI and *Hind*III, and two sites for *Eco*RI (giving two fragments of about 3.9 and 2.6 kb after digestion). Digestion with *Eco*RV produced a hybridizing fragment of high molecular weight (lanes t1, E5; t2, E5; t3, E5). This was the first indication that transforming DNA might be integrated at the same position in the *T. harzianum* genome. Further experiments are being carried out in order to determine the site or sites of integration. *Bam*HI and *Hind*III digestions gave hybridizing fragments with the same size, 6.5 kb (lanes indicated B and H), whereas two hybridizing fragments were detected from digestions with *Eco*RI, one corresponding to 3.9 kb and the other to 2.6 kb (lane E1). All these digestions showed the restriction pattern expected from the restriction map of pAN7-1. The most likely explanation is that each transformant contains tandem repeats of the vector. In all undigested DNAs, hybridization occurred only in the high-molecular genomic band (UC), suggesting that pAN7-1 integrated into the genome and did not replicate autonomously. These data support those previously found by Herrera-Estrella et al. (1990) using the same vector but a different transformation technique.

Effects of DNA concentration and cell density

Once it was demonstrated that all hygromycin-resistant colonies obtained had indeed integrated the transforming plasmid into their genome, we studied the influence of several factors in an attempt to optimize the transform-

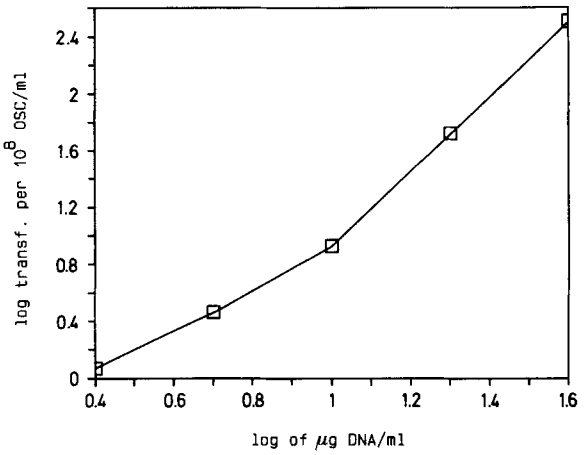

Fig. 4. Effect of DNA concentration on the frequency of transformation of *T. harzianum*. Plasmid pAN7-1, from 5 µg to 40 µg/ml, was added to 0.4 ml of a suspension of osmotically sensitive cells (OSC). The electrical conditions were a capacitance of 25 µF, an internal resistance of 800 Ω, and a voltage of 2.8 kV/cm

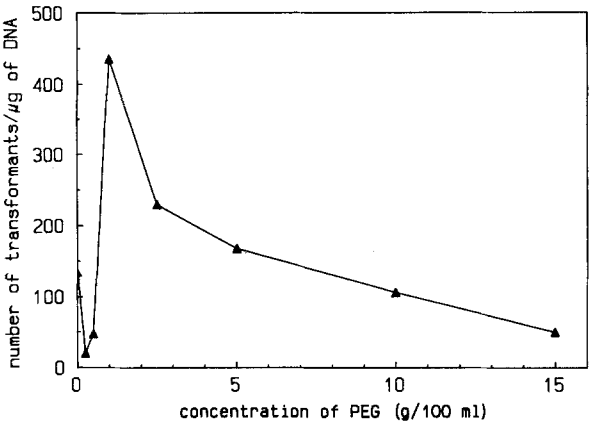

Fig. 5. Effect of the concentration of PEG on the frequency of transformation of *T. harzianum*. PEG ranged from 0.25% to 15.0%. The electrical conditions were a capacitance of 25 µF, an internal resistance of 800 Ω, and a voltage of 2.8 kV/cm

ation system. The effect of the number of electrocompetent cells on the frequency of transformation has already been observed in bacteria, mammalian cells, and plant protoplasts (Fromm et al. 1985; Dower et al. 1988; Miller et al. 1988; Shigekawa and Dower 1988). We also investigated the effect of this factor on the number of transformants in *T. harzianum*. Figure 3 shows the influence of the number of OSC on the frequency of transformation. By increasing the concentration of OSC to about 1.0×10^9/ml, we were able to increase the transformation frequency 2.3-fold from 54 (Fig. 1A) to 128 transformants per µg of DNA. When a concentration of 2.5×10^9 OSC/ml was used, a sharp decrease in the number of transformants/µg of DNA occurred. This low frequency could be correlated with a decrease in the time constant (Fig. 3) caused probably by the high concentrations of OSC providing a higher resistance. A sharp decrease in the frequency of transformation was observed when concentrations of OSC lower than 2.0×10^8/ml were used. The effect of plasmid DNA concentration on the number of transformants obtained by the electroporation of identical quantities of cells was examined. As shown in Fig. 4, the transformation efficiency increased with increasing concentrations of plasmid DNA. The same effects have already been reported for *S. cerevisiae*, bacteria and plant protoplasts (Fromm et al. 1985; Karube et al. 1985; Chu et al. 1987; Dower et al. 1988; Miller et al. 1988; Shigekawa and Dower 1988).

Effects of PEG

To date, all known protocols for transformation of filamentous fungi are based on the utilization of PEG. Therefore, we decided to investigate the influence of PEG on the electroporation of *T. harzianum*. Figure 5 shows the influence of different concentrations of PEG on the frequency of transformation. Using 1.0% of PEG in the electroporation medium it was possible to obtain a frequency of 435 transformants/µg of DNA. This frequency is about four times greater than electroporation without PEG (Fig. 3). A control using only PEG in the same concentration did not show any transformants. Maas and Werr (1989) found that transformants from plant protoplasts could not be obtained using PEG alone. Recently, efficient transformation of *Rhodoccocus fascians* (Desomer et al. 1990) and *Bacillus thuringiensis* (Mahillon et al. 1989) has been obtained by combining PEG and electroporation.

The decreased efficiency of transformation obtained by increasing the concentration of PEG was not correlated with the survival of the OSC (data not shown). Unexpectedly, the number of transformants was lower than in the control treatment without PEG when concentrations of PEG lower than 1% were used; this behaviour was found to be reproducible in at least three independent experiments. The volume of OSC used in the cuvette was also found to be important for the efficiency of transformation. Volumes smaller than 400 µl produced lower numbers of transformants. This result was expected since increasing the inter-electrode distance, or reducing the cross sectional area of the solution at the electrode surface, will increase resistance (Shigekawa and Dower 1988).

Divalent cations have been shown to affect the efficiency of electroporation of eukaryotic cells (Shigekawa and Dower 1988). Low concentrations of Ca^{+2} improved the yield in electrotransfection of carrot protoplasts (Fromm et al. 1985). Since most protocols for transformation in filamentous fungi are based on the use of $CaCl_2$ and PEG, different concentrations of this cation, (5, 10, 20, 30, 40, and 50 mM) were added to the electroporation medium containing 1.0% PEG. Neither positive nor negative effects on the number of transformants/µg of DNA were observed with any of the concentrations of $CaCl_2$ tested (data not shown).

Stability of transformants

In contrast with transformants from *Trichoderma* ssp. obtained with the same plasmid using PEG and calcium chloride (Herrera-Estrella et al. 1990), the electrotransformants obtained were phenotypically 100% stable through at least five generations. We cannot provide a reasonable explanation for this phenomenon. In order to show that they were also mitotically stable, condidia from five transformants, randomly chosen from PDA plates, were transferred to PDA plus hygromycin, and found to grow at a slightly lower rate, but showing normal morphology.

Cotransformation provides a convenient means to introduce nonselectable genetic material into many organisms. For this reason cotransformation experiments were also carried out by electroporation. Two different plasmids were introduced into *T. harzianum* spheroplasts together with pAN7-1 in a molar ratio of 1:1 or 2:1. Southern blot anaylsis showed that they were cotransformed with efficiencies of 27 and 100%, respectively (data not shown).

Discussion

To our knowledge, there are no reports in the literature about electroporation of filamentous fungi. In the protocol described above, the preparation of "competent cells" was made by partial digestion of the fungal cell wall (production of OSC) whereas in order to induce the cells to take up the transforming DNA an electric pulse and PEG were used in the electroporation medium. Frequencies of up to 400 transformants/μg of DNA were obtained. These frequencies are comparable to the transformation efficiencies (200–800 transformants per μg of DNA) previously reported for *Trichoderma* spp. (Herrera-Estrella et al. 1990) and *T. reesei* (Penttilä et al. 1987) using PEG and $CaCl_2$ mediated DNA transformation. There are two main advantages of electroporation over the traditional method. The first is simplicity: the OCS do not need to be purified by sorbitol gradients and it is possible to perform many more transformations. The second advantage we have found is that this method is more reproducible than PEG-mediated transformation. An important difference that was observed between the classical method and electroporation was the stability of transformants from *T. harzianum* when transformed with plasmid pAN7-1. A possible explanation for this effect could be the activation of the repair system caused by the stress conditions put upon the OSC by the high-voltage electric pulse.

The electric strength and the internal resistance are important for electroporation and optimal values depend on the fungal species and strain being tested. The best electrical conditions observed for electroporation of *T. harzianum* are different from those observed for other prokaryotic and eukaryotic cells (Shigekawa and Dower 1988). However, these results confirm major differences in the electrical conditions observed between eukaryotic and prokaryotic cells with prokaryotes generally responding only to much higher field strengths (Chassy et al. 1988; Shigekawa and Dower 1988). Higher concentrations of DNA and OSC produced more transformants. The effect of these variables on the frequency of transformation has already been described for bacteria, mammalian cells, and plant protoplasts (Shigekawa and Dower 1988). The efficiency of transformation was increased by addition of 1.0% PEG to the electroporation medium. In the traditional PEG and calcium chloride mediated DNA transformation, the exact mechanism by which $CaCl_2$, and PEG promote DNA uptake is unknown (Rambosek and Leach 1987). Apparently, in this transformation system OSC are induced to fuse by the addition of PEG, and DNA molecules are internalized during fusion, as no transformation occurs when PEG is omitted (Timberlake and Marshall 1989). Using electroporation conditions, transformants were obtained without the addition of PEG. However, the frequency using PEG was about four times greater than electroporation without PEG. It is likely that the same proposed mechanism of DNA uptake is taking place during electroporation but, since no transformants appeared when PEG was added and the electric pulse was not delivered (see Results), some differences may be occurring between the two processes. Recently, it has been shown that relatively high concentrations of PEG, when used in combination with divalent cations during direct DNA transformation, produce particles of precipitated DNA (Mass and Werr 1989) and it was argued that these particles enter the plant protoplast to produce transformants. Since there is neither precipitation of DNA nor transformants when PEG alone is used (Maas and Werr 1989; see also Results) we suggest that, in the case of electroporation, the pores opened in the membrane by the discharge are being stabilized by PEG. The presence of different concentrations of $CaCl_2$ in the electroporation solution plus 1.0% PEG did not increase the number of transformants.

The results presented here show that OSC from *T. harzianum* can be efficiently transformed by electroporation. Electroporation is rapid, easy to perform, and requires minimal sample preparation. Therefore, it may be a general method that will be useful for introducing DNA into many fungal species in addition to *T. harzianum*, *S. cerevisiae* (Hashimoto et al. 1985; Karube et al. 1985), and *D. discoideum* (Howard et al. 1988). This report opens the possibility of improving those transformation systems that have already been described or else of transforming other filamentous fungi where PEG-mediated transformation has not been achieved.

Acknowledgements. The authors wish to thank Dr. Allan Caplan and Jan Desomer for critical reading of the manuscript, Martine De Cock for typing it and Karel Spruyt, Vera Vermaercke, Stefaan Van Gijsegem and Baldewin Meireson for drawings and photographs. G. H. G. and A. H.-E. are indebted to the CAPES-BRAZIL and the Commission of the European Communities for a predoctoral fellowship and a training grant, respectively.

References

Chet I (1987) *Trichoderma* – application, mode of action, and potential as a biocontrol agent of soilborne plant pathogenic fungi. In: Chet I (ed) Innovative approaches to plant disease control. Wiley, New York, pp 137–160

Costanzo MC, Fox TD (1988) Proc Natl Acad Sci USA 85:2677–2681
Chassy BM, Mercenier A, Flickinger J (1988) Trends Biotechnol 6:303–309
Chu G, Hayakawa H, Berg P (1987) Nucleic Acids Res 15:1311–1326
Desomer J, Dhaese P, Van Montagu M (1990) Appl Environm Microbiol (submitted)
Dower WJ, Miller JF, Ragsdale CW (1988) Nucleic Acids Res 16:6127–6145
Dubos B (1987) Fungal antagonism in aerial agrobiocenoses. In: Chet I (ed) Innovative approaches to plant disease control. Wiley, New York, pp 107–136
Fincham JRS (1989) Microbiol Rev 53:148–170
Föster W, Neumann E (1989) Gene transfer by electroporation. A practical guide. In: Neumann E, Sowers EA, Jordan CA (eds) Electroporation and electrofusion in cell biology. Plenum Press, New York, pp 299–318
Fromm M, Taylor LP, Walbot V (1985) Proc Natl Acad Sci USA 82:5824–5828
Hashimoto H, Morikawa H, Yamada Y, Kimura A (1985) Appl Microbiol Biotechnol 21:336–339
Herrera-Estrella A, Goldman GH, Van Montagu M (1990) Mol Microbiol (in press)
Howard PK, Ahern AK, Firtel RA (1988) Nucleic Acids Res 16:2613–2623
Karube I, Tamiya E, Matsuoka H (1985) FEBS Lett 182:90–94
Klein TM, Wolf ED, Wu R, Sanford JC (1987) Nature 327:70–73
Laurila HO, Nevalainen H, Mäkinen V (1985) Appl Microbiol Biotechnol 21:210–212
Maas C, Werr W (1989) Plant Cell Reports 8:148–151
Mahillon J, Chungjatupornchai W, Decock J, Dierickx S, Michiels F, Peferoen M, Joos H (1989) FEMS Microbiol Lett 60:205–210
Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
Miller JF, Dower WJ, Tompkins LS (1988) Proc Natl Acad Sci USA 85:856–860
Papavizas GC (1985) Annu Rev Phytopathol 23:23–54
Penttilä M, Nevalainen H, Rättö M, Salminen E, Knowles J (1987) Gene 61:155–164
Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, van den Hondel CAMJJ (1987) Gene 56:117–124
Raeder U, Broda P (1985) Lett Appl Microbiol 1:17–20
Rambosek J, Leach J (1987) Crit Rev Biotechnol 6:357–393
Shigekawa K, Dower WJ (1988) BioTechniques 6:742–751
Timberlake WE, Marshall MA (1989) Science 244:1313–1317

Communicated by C. J. Leaver