Transformation of Filamentous Fungi with Plasmid DNA by Electroporation

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ABSTRACT

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We investigated the use of high-voltage electric pulse to transform *Fusarium solani* and *Aspergillus nidulans* with DNA. This method involved exposure of fungal protoplasts, in the presence of vector DNA, to a high-amplitude exponential-decay electric pulse of short duration (up to 123 msec) delivered from a Gene Pulser apparatus. The effects of pulse amplitude and duration on transformation frequency and cell viability were evaluated. The optimal transformation is rapid, simple to perform, and avoids the use of toxic chemicals. It may also provide an alternative method of introducing foreign DNA into fungi that cannot be transformed by traditional methods.

Additional keywords: Aspergillus nidulans, Fusarium solani.

Genetic analysis of various filamentous fungi, especially agriculturally and industrially important species, is often impaired by a lack of methods for molecular transformation. Although, in recent years, several fungal plant pathogens have been successfully transformed (14,18,19), genetic manipulations in others face major difficulties.

Electroporation has been used to transfer DNA into animal cells (16), plant protoplasts (15), yeast (8,9), and bacteria (6,13). The technique is based on reversible permeabilization of biomembranes by short-duration, high-amplitude electric fields. The membrane changes during the electric pulse allow the uptake of the recombinant DNA, which, in turn, can result in molecular transformation.

Usually, transformation procedures involve the treatment of protoplasts with polyethylene glycol (20) or spores with alkali cations (4). Interest in the development of alternative methods for the transformation of filamentous fungi led to the decision to test the use of electroporation on two fungal species, for which chemically induced transformation systems have already been established (11,20). In the experiments presented below, the filamentous fungi used in these studies were transformed by electroporation. The approximation of optimal conditions by using a Gene Pulser apparatus is also presented.

MATERIALS AND METHODS

Fungal strains. *Fusarium solani* (Mart.) Sacc. f. sp. *phaseoli* (Burkholder), isolate FB1-S4, was described earlier (3). *Aspergillus nidulans* (Eidam) Wint., strain UCD1 (*pabaA1, yA2, biA1, argB2, metG, trpC 801*) (17), and *A. nidulans*, strain PolArgB (*metG, argB2, biA1*) (1), were obtained from W. E. Timberlake.

Plasmids. Plasmid pETM2 contains a bacterial aminoglycoside-3′-phosphotransferase structural gene fused to the cauliflower mosaic virus 35S promoter. This plasmid confers resistance to the antibiotic geneticin in *F. solani* FB1-S4 upon transformation (11). Plasmid pKBY2 contains the cloned *A. nidulans trpC* gene (21) and confers tryptophan prototrophy to *A. nidulans* UCD1 upon transformation. Plasmid pDC1 contains the *argB* gene of *A. nidulans* (1) cloned into pIC-2OR (12) and confers arginine prototrophy to *A. nidulans* PolArgB upon transformation. Plasmids pKBY2 and pDC1 were obtained from W. Timberlake.

Plasmid DNAs were isolated by alkaline or sodium dodecyl sulfate lysis (10) and purified by ethidium bromide-cesium chloride density gradient centrifugation (10).

Electroporation apparatus. Exponential-decay high-voltage electric pulses were delivered by a Gene Pulser apparatus (Bio-Rad) capable of generating pulses of up to 2,500 V from 0.25–25 μF capacitors. For experiments requiring higher capacitance, the Capacitance Extender (Bio-Rad) with 125–960 μF capacitors was connected to the Gene Pulser. The maximum voltage setting was reduced to 450 V when the Capacitance Extender was used. To prevent arcing, which may occur during delivery of high-voltage pulses into the highly resistant media used in fungal electroporation, a 5-Ω resistor (Bio-Rad) was connected in series with the sample cuvette. The duration of the pulse was determined by total resistance, capacitance, and the initial field strength. It was displayed by the Gene Pulser as a "time constant"—the time needed for the pulse to decline 37% from its initial setting. Electro-

poration vials were made by lining the parallel walls of disposable plastic cuvettes with strips of aluminum foil. The inter-electrode distance was 4 mm and the sample volume was 0.8 ml.

Electroporation procedure. Protoplasts of *A. nidulans* and *F. solani* were isolated as described previously (11,20). The sample, in 800 μl of osmotic buffer (1.2 M sorbitol + 10 mM Tris-HCl [pH 7.5] + 10 mM $CaCl_2$ for *A. nidulans,* 1.2 M sorbitol + 10 mM Tris-HCl [pH 7.6] for *F. solani*) contained 3–7 $\times$ 10^7 fungal protoplasts mixed with 1–20 μg of plasmid DNA and 25 μg of sonicated salmon sperm DNA (carrier DNA). Plasmid pETM2 was linearized by cleavage with restriction endonuclease *Eco*RI; pKBY2 and pDC1 were applied in circular form. Control samples had no added DNA or carrier DNA only, as indicated. The suspension was then chilled on ice for 10 min. For electric field treatment, each sample was transferred to an ethanol-sterilized electroporation cuvette. After the electric pulse, the sample was kept on ice for 10–20 min.

To determine the percentage of protoplasts killed by the treatment, aliquots of the pulsed mixture were diluted and plated on nonselective agar. Following dilution with osmotic buffer, protoplasts of *A. nidulans* were plated on minimal selective agar plates containing 1.2 M sorbitol (20) without tryptophan for the strain UCD1 treated with pKBY2 DNA, and without arginine for the strain PolArgB treated with pDC1 DNA. The respective tryptophan and arginine prototrophs were observed on the plates after incubation for 2 days at 37 C.

The protoplasts of *F. solani* were plated in molten (47 C) regeneration potato-dextrose 3% agar containing 1.5 M sorbitol. After 5 hr of incubation at 28 C, they were overlayed with potato-dextrose 1.5% agar containing 225 μg of geneticin per milliliter, as described previously (11). Antibiotic-resistant colonies were observed on the plates after incubation for 4 days at 28 C.

Hybridization analysis of fungal genomic DNA. *A. nidulans* and *F. solani* genomic DNAs were isolated by the method of Garber and Yoder (7).

Total DNA from *A. nidulans* was digested with *Sac*I, size-fractionated in an 0.8% agarose gel, and blotted onto GeneScreen*Plus* according to the manufacturer's instructions. Hybridization was done at 50 C for 16 hr in hybridization mix (1 M NaCl, 10% dextran sulfate, 1% $NaDodSO_4$, and denatured salmon sperm DNA [100 μg ml^{-1}]). Hybridization probes were radioactively labeled with [α-^{32}P]-dCTP (5). A 4.1-kb *Xho*I fragment of pKBY2, containing the *trpC* gene, was used to probe putative UCD1 transformants. A 1.8-kb *Bam*H1-*Sph*I fragment of pDC1, containing the *argB* gene, was used to probe putative PolArgB transformants.

Total DNA isolated from *F. solani* was digested with *Pst*I, electrophoresed in a 1% agarose gel, and blotted onto GeneScreen*Plus* according to the manufacturer's instructions. Hybridizations were done in 50% formamide at 42 C with the chemically modified pETM2 probe, using the Genius DNA detection system (Boehringer Mannheim Biochemicals, Indianapolis, IN), according to the manufacturer's instructions.

TABLE 1. Effect of varying capacitance and voltage during electroporation of fungal protoplasts

Fungus and strain	Capacitance (μF)	Voltage[a] (V)	Time (msec)	Viability[b] (%)	Transformation frequency[c]
Fusarium solani					
FB1-S4	1	1,000	0.3	100	0
		1,500	0.3	100	0
		2,000	0.3	100	0
		2,500	0.3	100	0.1
	3	2,000	0.6	7	2.4
	25	500	—[d]	24	0
		1,500	—[d]	10	0.7
		2,500	—[d]	5	0.7
	125	450	84	20	0.5
	500	450	97	30	1.2
	960	450	114	28	1.3
Aspergillus nidulans					
UCD1	1	1,000	0.3	90	0
		1,500	0.3	86	0
		2,000	0.3	88	1.1
		2,500	0.3	86	0
	3	1,500	0.5	82	1.2
		2,000	0.6	65	8.4
		2,500	0.6	21	0.35
	25	2,000	3.7	5	0.8
		2,500	3.5	5	0
	125	450	22	59	0
	500	450	84	36	0.8
	960	450	123	30	0.8

[a]Voltage applied across electrodes spread 4 mm apart.
[b]Percentage of protoplasts surviving electroporation treatment.
[c]Transformants per 1 μg of DNA.
[d]Data not recorded.

RESULTS

To optimize the conditions for transformation of fungal protoplasts by electroporation using the Gene Pulser, two parameters were varied. Table 1 shows the effect of the initial field strength and capacitance on the frequency of transformation. Selecting different capacitors resulted in different pulse durations. At the pulse duration of 0.3 msec released from the 1 μF capacitor, only the highest initial field strength applied (6,250 V cm^{-1}) resulted in geneticin-resistant colonies of *F. solani*. Similar results were obtained for *A. nidulans*; only 5,000 V cm^{-1} resulted in tryptophan prototrophs. In both cases, few transformants were obtained.

The reverse conditions, pulses of long duration (up to 123 msec) from the 125- to 960-μF capacitors delivered at the lower field strength of 1,125 V cm^{-1}, resulted in a very low frequency (0.8 per 1 μg of DNA) of *A. nidulans* trp^+ colonies. After this pulse treatment, geneticin-resistant colonies of *F. solani* FB1-S4 were observed with a frequency of up to 1.3 per 1 μg of DNA.

These results showed the importance of both extended pulse duration and high initial field strength for useful electroporation. Consequently, high-voltage pulses delivered from the 3-μF capacitor and from the 25-μF capacitor were tested. The increased electric field strength, as well as the longer pulse, resulted in a decrease in protoplast viability (Table 1). Although some transformants were produced under each of those conditions, the highest frequency of selected colonies appeared at 3 μF, 3.5 msec, 5,000 V cm^{-1} (Table 1). Under the employed electroporation conditions, a level of 10^7 protoplasts per sample was sufficient to obtain maximal transformation frequency. The absence of carrier DNA from the sample decreased the transformation frequency. In the absence of appropriate vector DNA, no colonies of *A. nidulans* appeared on selective plates, and few (6–12 per sample) colonies of *F. solani* appeared on antibiotic-containing plates, regardless of the parameters of electric pulse (data not shown). *A. nidulans*, strain PolArgB, could be transformed with the plasmid pDC1 by an electric pulse treatment employing the

TABLE 2. Best electroporation conditions obtained for transformation of filamentous fungi

	Fungus strains		
	Fusarium solani FB1-S4	*Aspergillus nidulans* UCD1	*A. nidulans* PolArgB
Voltage (V cm^{-1})	5,000	5,000	5,000
Capacitance (μF)	3	3	3
Number of protoplasts	3 $\times$ 10^7	6 $\times$ 10^7	6 $\times$ 10^7
Viability of protoplasts (%)	7	65	25
Time constant (msec)	0.6	0.6	0.6
Transformation frequency (transformants per 1 μg of DNA)	2.4	8.4	20

parameters established for *A. nidulans* UCD1 and *F. solani* (Table 2).

The results in Table 2 represent the highest transformation frequencies obtained. Although the viability and competence (the ability to take up DNA) varied among the different protoplast preparations, these optimized conditions consistently gave the highest transformation frequencies for any one protoplast preparation (data not shown).

Some attempts were also made to test the effect of the conductivity of the electroporation medium on the transformation frequency of *F. solani*. When the osmotic medium described above was changed by the addition of 50 mM NaCl, the survival of the protoplasts during the high-voltage pulse increased. The use of higher-conductivity media also permitted the use of higher voltage settings on the Gene Pulser (2,500 V, 25 μF) without the risk of arcing. The transformation frequencies obtained in salt-containing medium were 0.5 per 1 μg of DNA at 2,500 V, 25 μF and 0.8 per 1 μg of DNA at 2,000 V, 3 μF.

Putative transformants of *A. nidulans* and *F. solani* obtained by electroporation were analyzed by hybridization. One out of six transformants of *A. nidulans*, strain UCD1, contained the plasmid vector pKBY2 incorporated into the genome as indicated by the hybridizing band of 9.9 kb. The other transformants had hybridization patterns identical to that of the recipient strain, UCD1 (Fig. 1). Five out of nine transformants of *A. nidulans*, strain PolArgB (lanes 2, 4, 6–8), contained the plasmid pDC1 incorporated into the genome, as indicated by the hybridizing band of 1 kb. The other transformants had hybridization patterns identical to that of the recipient strain, PolArgB (Fig. 2).

Twelve randomly chosen geneticin-resistant colonies of *F. solani* were analyzed for the presence of pETM2 sequences in the genomic DNAs. In seven such colonies, the vector sequences could be detected in fungal genomic DNA by hybridization with pETM2. Figure 3 shows six positive transformants (lanes 1–6). These contained pETM2 sequences, whereas DNA from untransformed strain FB1-S4 (lane 7) did not hybridize to pETM2.

DISCUSSION

Electroporation as a means of transformation seems to be based on the general ability of biomembranes to produce channels when exposed to an electric field (9,13,15,16). The results show that the protoplasts of filamentous fungi may be transformed by electroporation. The technique is rapid, simple to perform, and avoids the use of chemicals such as polyethylene glycol or lithium acetate, which may be toxic to cells.

The electric field strength and the time constant of the electric pulse are important factors in electroporation. At a small time constant (0.3 msec), only the pulses of an amplitude higher than 5,000 V cm^{-1} produced transformants. Similar conditions were established for bacteria (13) and yeast (9). The high amplitude of the electric pulse was of greater importance than its duration because moderate (30%) increases in voltage caused an

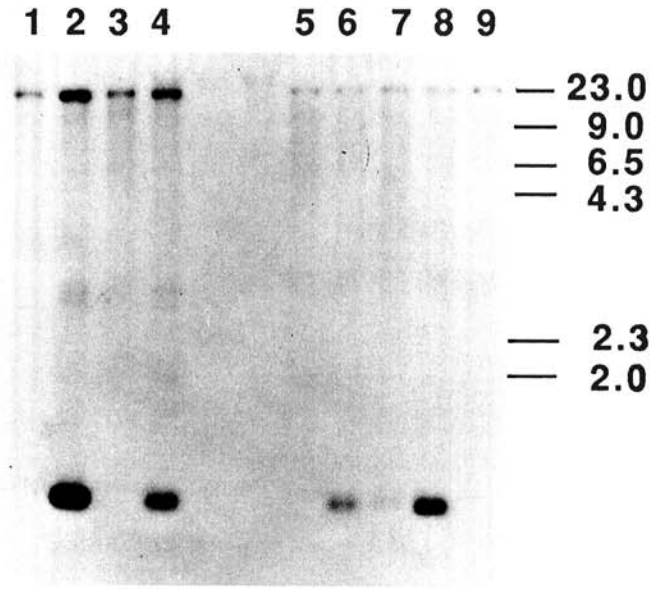

Fig. 2. Hybridization analysis of selected *argB*$^+$ transformants of *Aspergillus nidulans*. The sizes of the molecular weight markers are given in kilobases. Lanes 1–8, Strain PolArgB transformed with pDC1 by electroporation; lane 9, untransformed PolArgB.

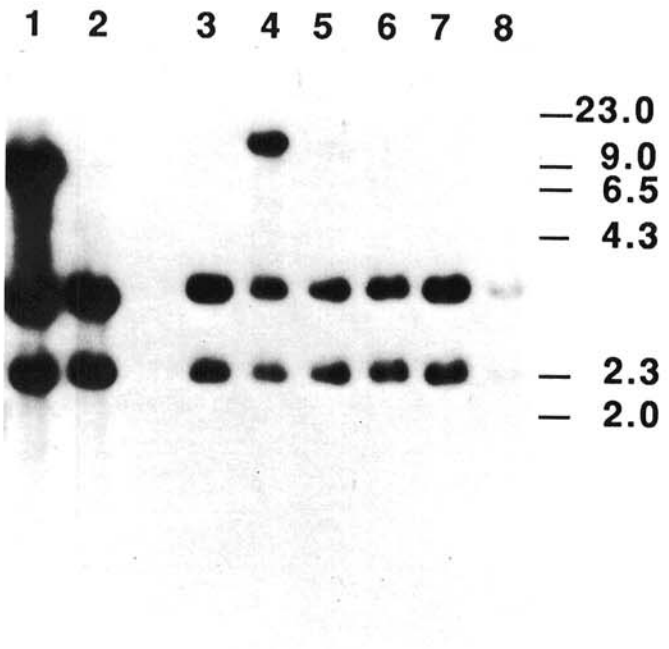

Fig. 1. Hybridization analysis of selected *trpC*$^+$ transformants of *Aspergillus nidulans*. The sizes of the molecular weight markers are given in kilobases. Lane 1, Strain UCD1 transformed with pKBY2 by the method of Yelton at al (20); lane 2, untransformed UCD1; lanes 3–8, UCD1 transformed with pKBY2 by electroporation.

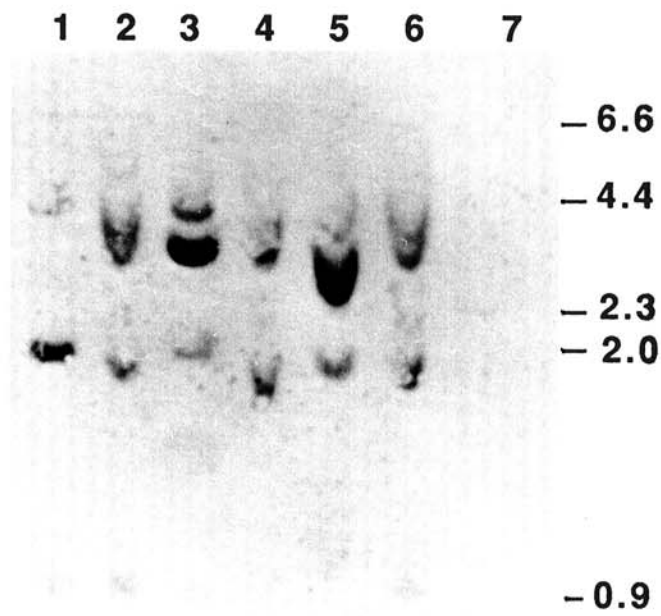

Fig. 3. Hybridization analysis of selected *Fusarium solani* transformants. The molecular weight standards are given in kilobases. Lanes 1–6, FB1-S4 transformed with pETM2 by electroporation; lane 7, untransformed FB1-S4.

approximate 10-fold increase in transformation frequency, whereas a 40-fold increase in the duration of the pulse had a much less pronounced effect (Table 1).

Cells of *A. nidulans* were more resistant to the electric pulse treatment than were cells of *F. solani*. At the conditions most favorable for transformation of both fungi (5,000 V cm^{-1}, 3 μF, 0.6 msec), 93% of cells of *F. solani* were killed, whereas 35% of cells of *A. nidulans* were killed.

The hybridization analyses of the transformants of *A. nidulans* indicated that two types of events had occurred. The transformants that contain the entire introduced plasmid (Fig. 1, lane 4; Fig. 2, lanes 2, 4, 6–8) show integration of the plasmid at the site of the resident gene in the recipient strain. The signal in Figure 2, lane 7, was faint but positive. However, several putative transformants gave hybridization patterns identical to that of the recipient strain (Fig. 1, lanes 2–3, 5–8; Fig. 2, lanes 1, 3, 5). It is unlikely that these are untransformed revertants, since reversion was not observed when pKBY2 or pDC1 was omitted. Rather, these prototrophs may have arisen either from gene conversion, double crossover events at the site of the resident gene or from integration of the entire plasmid followed by an excision of plasmid sequences by homologous recombination. Such events have been observed in transformants of *Neurospora crassa* (2) and *A. nidulans* (20).

The vector pETM2 contains no sequences homologous with fungal DNA (Fig. 3, lane 7); therefore the positive signals in transformants (Fig. 3, lanes 1–6) were a consequence of the integration of pETM2 into the genomic DNA of the recipient strain during electroporation. Five out of six transformants analyzed by hybridization (Fig. 3, lanes 1–4 and 6) had the 0.95-kb *Pst*I fragment of the geneticin-resistance gene from the transforming vector. One colony lacked the 0.95-kb band (Fig. 3, lane 5) but retained other pETM2 sequences. Possibly this isolate underwent vector DNA rearrangement, subsequent to transformation. Some geneticin-resistant colonies arose by mutation rather than by transformation (data not shown). Such mutation to geneticin resistance has been observed previously (11) but was less frequent than transformation.

The transformation frequency under the conditions employed was lower than that obtained by standard transformation methods (>10 transformants per μg of DNA) (19), but it can possibly be improved by further optimization. We expect the procedure to be successfully applied to the transformation of many fungal species, particularly some of agricultural and industrial importance, for which genetic transformation systems are difficult to establish.

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