Curr Genet (1989) 15:421–428

Current Genetics

Molecular transformation of *Fusarium solani* with an antibiotic resistance marker having no fungal DNA homology*

Elzbieta T. Marek, Christopher L. Schardl, and David A. Smith
Department of Plant Pathology, University of Kentucky, Lexington, KY 40546-0091, USA

Summary. A vector was constructed for transformation of the plant pathogenic fungus *Fusarium solani.* The promoter *35Sp,* from cauliflower mosaic virus, was fused to the bacterial gene *APH(3')II,* which confers resistance to the aminoglycoside antibiotic G418. Two transformation procedures were developed: one using isolated fungal protoplasts, the other using germinated fungal spores. A transformation frequency of 3.3 G418-resistant colonies were obtained per microgram DNA. Of 14 colonies analyzed, 12 had vector sequences integrated into their high molecular weight DNA, and 2 were untransformed. Integration was sometimes accompanied by rearrangements of both the vector and flanking fungal DNAs. Primer-extension analysis of the mRNA from one transformant revealed two putative transcription initiation sites in the chimeric *APH(3')II* gene. Both sites differed from the normal initiation site in plants. This vector will be useful in transformation systems in which integration by non-homologous recombination is desired.

Key words: *Fusarium* transformation – Cauliflower mosaic virus promoter

Introduction

Fusarium solani (Mart.) Sacc. is a fungal species that includes a number of important plant pathogens, animal pathogens, and producers of trichothecene mycotoxins (Mirocha et al. 1977). *F. solani* is ubiquitous and, in some places, represents up to 20% of the microflora in soil (Nelson et al. 1981). Among the plant diseases involving *F. solani* is et al. 1982 stem rot of French bean (*Phaseolus vulgaris*), caused by *F. solani* f. sp. *phaseoli* (Smith and Banks 1986). There is substantial circumstantial evidence that this pathogen is capable of overcoming the defensive phytoalexin response of its host by modifying isoflavonoids produced by *P. vulgaris* (Smith and Banks 1986).

Many form species of *F. solani* have no known sexual stage and are not amenable to classical genetic analysis. It is necessary, therefore, to develop protocols for DNA-mediated transformation to facilitate molecular genetic studies of this important fungal species. Transformation systems for several other filamentous *Ascomycetes* have been described (Case et al. 1979; Hynes 1986; Punt et al. 1987; Turgeon et al. 1987; Yelton et al. 1984; Wang et al. 1988), including *F. oxysporum* (Kistler and Benny 1988). These procedures employ selectable markers that either complement auxotrophic mutations or confer antibiotic resistance. The *Escherichia coli* genes encoding aminoglycoside-3′-phosphotransferase and hygromycin B phosphotransferase confer resistance to G418 (Geneticin), and to hygromycin B, respectively. They can act as selectable markers in *Saccharomyces* (Gritz and Davies 1983; Jimenez and Davies 1980), plants (Rogers et al. 1986) and in mammalian cells (Gorman et al. 1983; Santerre et al. 1984) when fused with an appropriate eukaryotic promoter. Recently, the hygromycin B phosphotransferase gene, fused to fungal promoters, has been used as a selectable marker in the transformation of filamentous fungi (Kistler and Benny 1988; Punt et al. 1987; Turgeon et al. 1987; Wang et al. 1988)

This paper describes the construction of a G418-resistance gene and its use as a dominant selectable marker for transformation of *F. solani.* The gene contained no fungal DNA, but was a chimera of cauliflower mosaic virus (CaMV) DNA, including the promoter of the 35S transcript, linked to the amino-

* The investigation reported in this paper (No. 89-11-42) is in connection with a project of the Kentucky Agricultural Experiment Station and is published with approval of the director

Offprint requests to: C. L. Schardl

glycoside-3′-phosphotransferase gene (*APH (3′)II)* encoded by the bacterial transposon *Tn5*.

Materials and methods

Strains, media. Fusarium solani f. sp. *phaseoli,* isolate FB1-S, has been described earlier (Kuhn and Smith 1978). It is a wild-type laboratory strain, a pathogen of *P. vulgaris* causing progressive hypocotyl lesions. Strain FB1-S4 (Choi et al. 1987) was recovered following chemical mutagenesis of FB1-S with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. This fungus causes insignificant lesion development on French bean. Both isolates were grown on modified Fries agar (Smith et al. 1982) or potato dextrose agar (PDA), as previously described (Kuhn and Smith 1978).

Construction of the plasmid, pETM2. The construction of the transformation vector is described in Fig. 1. The plasmid, pNEO, (Pharmacia, LKB Biotechnology, Piscataway, NJ) is comprised of pBR322 sequences, ligated to the aminoglycoside 3′-phosphotransferase II (kanamycin kinase, E.C. 2.7.1.05) gene (*APH(3′)II*) from bacterial transposon *Tn5* (Beck et al. 1982). A segment of cauliflower mosaic virus (CaMV) DNA, including the 35S promoter (*35Sp*) (Odell et al. 1985), was removed as an *EcoRI-HindIII* fragment from pKYLX6 (Schardl et al. 1987). This fragment replaced the small *EcoRI-HindIII* fragment of pNEO. The resulting plasmid pETM1 contained the 35S promoter upstream of *APH(3′)II.* Its structure was verified by restriction mapping. In order to remove the bacterial *APH(3′)II* promoter, pETM1 was digested with *HindIII* and *BglII* and the recessed ends were filled using the large fragment of DNA polymerase I (Klenow), then ligated to give the plasmid pETM2 (Fig. 1). The structure of pETM2 was verified by restriction mapping, and the DNA sequence of the junction between the CaMV sequence and *APH(3′)II* was determined (see Fig. 4B).

Fungal spore isolation. Spores of FB1-S were washed off 3-week-old Fries agar cultures, filtered through Miracloth to remove mycelial debris, pelleted by centrifugation and resuspended in sterile H_2O. Spores of FB1-S4 were isolated from potato dextrose broth liquid cultures grown for 4 days at 30 °C with vigorous rotary shaking. The cultures were filtered through Miracloth. Spores remaining in the filtrate were pelleted by centrifugation, washed once and resuspended in sterile water.

Isolation of fungal protoplasts. Fries medium (100 ml of 0.5×) was inoculated with 10^4 FB1-S4 spores and the culture grown for 16 h at 30 °C with rotary shaking. The mycelium was harvested by filtration on Whatman no. 1 filter paper, washed with 1 M $MgSO_4$, blotted dry and suspended in 10 mM sodium phosphate buffer (pH 5.8) containing 1 M $MgSO_4$ as an osmotic stabilizer and 1 mM fresh L-cysteine. Initial experiments indicated that when thiol reagents such as β-mercaptoethanol were included in the lytic reaction mixture, the yield of protoplasts increased dramatically, but the regeneration frequency of such protoplasts was below 0.1%. By using L-cysteine, the number of protoplasts released was increased without adversely affecting regeneration. A similar effect has been reported by Sreekrishna et al. (1984). To digest the fungal cell walls, 100 mg Novozyme 234 (batch PPM-1786, Novo Industries, Wilton, Conn. and 20,000 U β-glucuronidase H2 (Sigma Chemical, St. Louis, Mo.) were added to 1 g mycelium in 10 ml osmotic medium, and the suspension was incubated at 30 °C with slow rotary shaking (75 rpm). The release of protoplasts usually began after 30 min. After 60–90 min, the released proplasts were separated from mycelial debris by filtration, first through Miracloth, then through a 20 μm nylon screen. At this point, the presence of small pieces of mycelium did not exceed 2% of the protoplast preparation. Protoplasts were then washed twice by centrifugation (700 g, 5 min) in 1.2 M sorbitol containing 10 mM TRIS (hydroxymethyl) aminomethane hydrochloride (TRIS-HCl), pH 7.6, then resuspended in 1.2 M sorbitol, 10 mM TRIS-HCl, pH 7.6, 10 mM $CaCl_2$.

The protoplasts were regenerated by mixing the suspension with 10 ml of molten (47 °C) potato dextrose or Fries 3% agar containing 1.5 M sorbitol, then overlaying it onto the corresponding 1.5% agar plates. After 2 days at 30 °C, regenerating colonies appeared on the plates. This protocol yielded 2×10^7-1×10^8 protoplasts from 1 g mycelium, 8%–15% of which were capable of regeneration.

Transformation of protoplasts by polyethylene glycol-calcium treatment. The procedure was based on the method of Hinnen et al. (1978). *F. solani* protoplasts (5×10^7-1×10^8 in 1.2 M sorbitol, 10 mM TRIS HCl, pH 7.6, 10 mM $CaCl_2$) were mixed with 10-20 μg plasmid DNA and 25 μg sonicated salmon sperm DNA. Where indicated, the plasmid was first linearized by *EcoRI* restriction endonuclease cleavage. The mock transformation controls included salmon sperm DNA, but no plasmid. After 30 min incubation at room temperature, samples were treated with a ten-fold volume of 60% polyethylene glycol 4000 (PEG) (Sigma) for 30 min at room temperature. Following removal of the PEG solution by centrifugation and washing with 1.5 M sorbitol, 10 mM TRIS-HCl, pH 7.6, the protoplasts were resuspended in the wash solution and plated in molten agar (47 °C). After 5–14 h incubation at 30 °C, each plate was overlayed with 10 ml of molten agar containing 225 μg ml^{-1} G418. Resistant colonies emerged on the surface after 3–4 days.

Transformation of germinated spores by lithium acetate treatment. This procedure was performed according to the method developed by Ito et al. (1978) and modified by Dhwale et al. (1984). *F. solani* spores (3×10^8 in 500 ml 0.5× Fries medium) were germinated for 3 h at 30 °C with rotary shaking. They were then harvested by centrifugation, washed and incubated in 0.1 M lithium acetate at 30 °C. The mixture was shaken slowly (70 rpm). Treated spores were centrifuged and resuspended in 300 μl 0.1 M lithium acetate. Supercoiled or linearized plasmid DNA (20–40 μg as indicated) was incubated with 100 μl of the spore suspension for 30 min at 30 °C. The suspension was then treated with a 20-fold volume of 40% PEG 4000, and incubated for 1 h at 30 °C, then 5 min at 37 °C. The PEG solution was removed by centrifugation. The spores were washed once and resuspended in H_2O, then spread onto PDA plates containing 25 μg ml^{-1} G418. Resistant colonies were observed after 4–5 days.

Filter hybridization of genomic DNA. Mycelium for DNA extraction was grown in modified liquid Fries medium. DNA was isolated according to Garber and Yoder (1983). Restriction digests were transferred to the nitrocellulose filters as described by Southern (1975). In the experiments involving the subsequent use of several different probes, the digests were transferred to a GeneScreen Plus (DuPont, Wilmington, Del.) membrane, and DNA was crosslinked to the membrane by UV irradiation according to the manufacturer's instructions. Radiolabelled DNA probes were prepared by the method of Feinberg and Vogelstein (1983). DNA-DNA hybridizations were carried out at 42 °C in 50% formamide, 6×SSC, 1% Denhardt solution, and 0.1% sodium dodecylsulfate (SDS) (Maniatis et al. 1982). When GeneScreen Plus membrane was used, the hybridization solution contained 0.5% dry nonfat milk and 1% SDS (Johnson et al. 1984). Following hybridization, filters were washed twice with 1×SSC, 1% SDS at room temperature, and then washed twice with 0.1×SSC, 1% SDS at 62 °C for 15 min.

Preparation of RNA. Total RNA was isolated by the method of Chirgwin et al. (1979), as modified by Turpen and Griffith (1986). The integrity of the DNA was determined by denaturing glyoxal agarose gel electrophoresis (Maniatis et al. 1982). The poly(A)$^+$ RNA

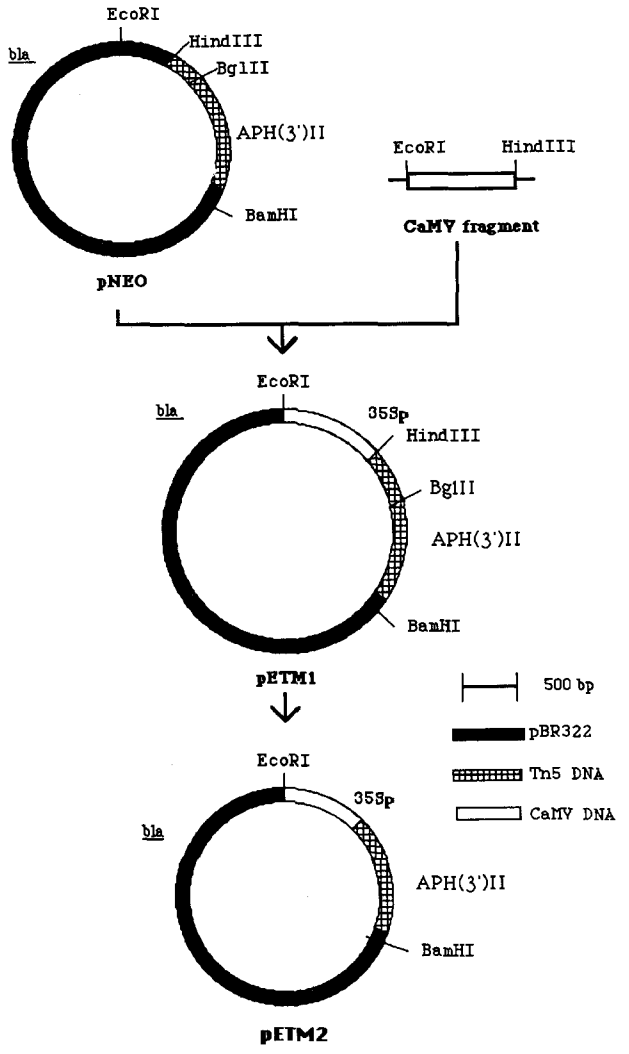


Fig. 1. Construction of transformation vector pETM2 containing a chimeric CaMV-*APH(3')II* marker. A cloned 0.9 kb fragment of CaMV, containing the viral promoter *35Sp*, with a *HindIII* site linker, was subcloned as an *EcoRI-HindIII* fragment into pNEO, so that the 35S promoter was upstream of the *APH(3')II* gene from bacterial transposon *Tn5*. This plasmid, pETM1, was further modified to remove the bacterial promoter, by the sequential action of *HindIII* and *BglII*, the large fragment of DNA polymerase I, and T4 DNA ligase. The resulting plasmid, pETM2, contained the 35S promoter upstream of the APH(3')II coding region. Gene designations are: *bla*, b-lactamase gene; *APH(3')II*, aminoglycoside-3'-phosphotransferase gene; 35Sp, 35S promoter of CaMV. Additional details are provided in Materials and methods

was isolated by chromatography on oligo(dT) cellulose (Pharmacia) (Aviv and Leder 1972).

Mapping of mRNA 5'-ends. Primer-extension analysis was by the method of Calzone et al. (1987). A synthetic 20-nucleotide primer was prepared using a 380 B DNA synthesizer (Applied Biosystems, DuPont) and was labelled at the 5'-terminus with [γ^{32}P]ATP (3,000 Ci/mM, DuPont) and polynucleotide kinase. The labelled primer was purified on a Sep-Pak (Millipore, Bedford, Mass. column. An estimated ten-fold molar excess of the labelled primer was annealed to poly(A)$^+$ RNA in 400 mM NaCl, 10 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.4. The annealing was carried out in sealed glass capillary tubes at 59 °C for 6 h. The primer was extended by the action of Avian Myeloblastoma Virus (AMV) reverse transcriptase (Life Sciences, St. Petersburg, Fla.) and the products analyzed by electrophoresis in 8 M urea-8% polyacrylamide sequencing gels.

Miscellaneous procedures. Escherichia coli strains DH5 (Hanahan 1983) and NM522 (Stratagene, LaJolla, Calif). were transformed according to the method of Hanahan (1983). Small and large scale preparations of plasmid DNAs were obtained from *E. coli* by the alkaline lysis method described by Maniatis et al. (1982), and plasmid DNA was twice purified by isopycnic centrifugation in cesium chloride-ethidium bromide gradients. Supercoiled plasmid DNA was seuquenced according to Chen and Seeburg (1985) using modified T7 DNA polymerase (Sequenase^{tm}) (U.S. Biochemical, Cleveland, Ohio).

Results

Effects of G418 on spore germination and mycelial growth of F. solani

Both isolates FB1-S and FB1-S4 failed to germinate on PDA medium containing 3 μg ml^{-1} of G418. On Fries agar, the threshhold concentration was 10 μg ml^{-1}. To test the sensitivity of growing mycelia, spores were first germinated without selection for 3 h in liquid medium and then spread onto antibiotic-containing plates. On PDA plates, growth was completely inhibited at 3 μg ml^{-1} G418. On Fries agar, 15 μg ml^{-1} G418 was required to inhibit growth. Spontaneous resistance to G418 occurred at a frequency of 2–3 per 10^7 spores.

Transformation of F. solani

The plasmid pETM2, containing the chimeric CaMV-*APH(3')II* construct (Fig. 1) was used for transformation of isolated protoplasts and germinated spores (see Materials and methods). Putative transformants were selected for G418 resistance. Both procedures yielded comparable results (Table 1). Frequencies of G418-resistant colonies ranged from 0.3 to 2.2 μg DNA for transformation of protoplasts, and 1.1 to 3.3 μg DNA for germinated spores. The lithium acetate procedure for transformation of germinated spores was the favored protocol, being simpler and faster to undertake and yielding more consistent results. The best results were obtained in this procedure when germinated spores were treated with large (40 μg) amounts of vector DNA. Under these conditions a nearly three-fold increase in apparent transformation frequency was obtained by prior linearization of the pETM2 vector DNA at the single *EcoRI* site (see Fig. 1).

Tab. 1. Transformation of *Fusarium solani* FB1-S4[a]

Transformation procedure	Plasmid	Amount of plasmid DNA (μg)	Resistant colonies[b,c]	Colonies per μg DNA[b,c]
Protoplasts	pETM2 supercoiled DNA[d]	10	3– 9	0.3–0.9
	pETM2 linear DNA[d]	10	8– 22	0.8–2.2
	pNEO linear DNA	10	2	0.2
	No vector[e]	0	0– 1	–
Germinated spores	pETM2 supercoiled DNA	20	22	1.1
	pETM2 supercoiled DNA[d]	40	48– 52	1.2–1.3
	pETM2 linear DNA	20	14	0.7
	pETM2 linear DNA[d]	40	117–132	2.9–3.3
	pNEO linear DNA	40	12	0.3
	No vector[e]	0	5– 15	–

[a] Each experiment included 1.1×10^8 protoplasts or germinated spores, plasmid DNA where indicated, and 25 μg sonicated salmon sperm DNA
[b] The total number of colonies arising on antibiotic-selection plates
[c] The range of results is indicated for multiple trials
[d] Results of two trials
[e] Results of three trials

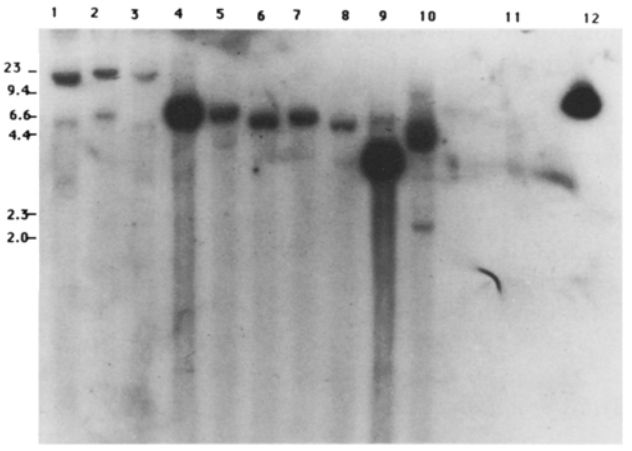


Fig. 2. Identification of plasmid DNA inserts in the genomic DNAs of *F. solani* transformants. Total fungal DNAs (10 μg each) were digested with *HindIII* (*lanes 1–5*) or *EcoRI* (*lanes 6–11*) and electrophoresed in 1% agarose. The DNAs were then blotted onto nitrocellulose paper and probed by hybridization with radiolabelled pETM2, then autoradiographed. Five independent G418-resistant isolates were analyzed. These were designated 4A2 (*lanes 1 and 6*), 1B3 (*lanes 2 and 7*), 3B4 (*lanes 3 and 8*), 2B1 (*lanes 4 and 9*), and L4C-1 (*lanes 5 and 10*). The untransformed parent FB1-S4 (*lane 11*) and 50 pg of *EcoRI*-digested pETM2 (*lane 12*) were included as controls. The positions of the *HindIII* fragments of lambda DNA are indicated as molecular size markers

When pNEO, which contains the *APH(3')II* gene with its normal bacterial promoter, was used in place of pETM2, the frequency of G418-resistant colonies arising was in the range of the normal frequency of spontaneous resistance exhibited by spores of FB1-S4.

After 7–10 days' growth on the original selection plates, G418-resistant colonies from the pETM2 transformations were subcultured onto test plates containing 0–100 μg ml^{-1} G418. All grew on an antibiotic concentration of 25 μg ml^{-1} or higher. The stability of the phenotype was tested by plating conidia from the resistant colonies onto medium without antibiotic, then reculturing on G418-containing plates. Stability of the marker varied greatly. Depending on the resistant isolate tested, 50%–100% of the conidia from non-selective plates gave rise to colonies that retained G418 resistance.

Genomic DNA analysis

Total DNAs from the FB1-S4 parent and from 14 putative transformants, 8 obtained by lithium acetate procedure, 6 by calcium-PEG procedure, were analyzed for the presence of pETM2 sequences. Among these, 12 isolates (6 from lithium acetate procedure , 6 from calcium-PEG procedure) possessed homology to the plasmid sequences in the high molecular weight genomic DNA (data not shown). Restriction analyses of five of the transformants are shown in Fig. 2. The DNAs were digested with *EcoRI* and *HindIII,* electrophoresed in 1% agarose gels, blotted onto nitrocellulose, and probed with ^{32}P-labelled pETM2.

When genomic DNAs were digested with *EcoRI* (Fig. 2, lanes 6–10), which cleaves once within pETM2, two (lanes 6–8) or three (lanes 9 and 10) hybridization bands were identified. The sum of the sizes of the hybridizing fragments in each case was greater than the

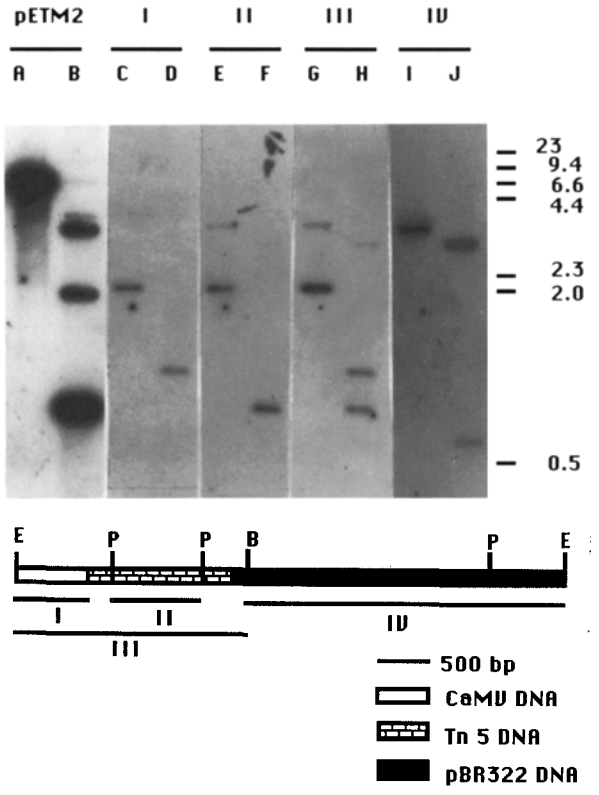


Fig. 3. Mapping plasmid sequences inserted into the genome of *F. solani* transformant L4C-12. Total DNA (4 µg) isolated from the transformant was digested with *EcoRI* (*lanes C, E, G, and I*) and *PstI* (*lanes D, F, H, and J*), then electrophoresed in 1% agarose. DNA was blotted onto GeneScreen Plus (DuPont) and hybridized sequentially with radiolabelled probes I, IV, II and III, as indicated. Previous probes were washed off prior to reprobing. The weak signal at 4.2 kb in *lanes E and G* resulted from incomplete removal of the previous probe. *Lanes A and B* are *EcoRI* and *PstI* digests, respectively, of pETM2, probed with pETM2. Molecular size markers, indicated at the *right,* were generated by *HindIII* digestion of lambda phage DNA. The diagram presents schematically linearized pETM2 with the relevant restriction sites as well as the fragments used as probes (I, II, III and IV)

size of the transforming vector (6.5 kb), suggesting that they represented vector sequences together with flanking fungal DNA. There was no hybridization of the pETM2 probe to DNA of the untransformed strain (lane 11).

Digestion of the genomic DNAs with *HindIII,* which does not cleave within pETM2, produced major hybridizing fragments of 15–23 kb, and weak hybridization bands in the 6–6.5 kb region for three of the transformants (Fig. 2, lanes 1–3). DNAs of transformant 2B1 produced one intense band at 6.4 kb (lane 4) and of transformant L4C-1 produced two bands at 6.5 kb and 4.0 kg (lane 5). The presence of more than one hybridization band in *HindIII*-digested DNA (lanes 1, 2, 3 and 5) suggested the possibility that either the integration of the vector DNA was accompanied by occasional rearrangements, or it occurred in more than one locus in the genome. The 6–6.5 kb and >15 kb bands in *HindIII*-digested DNA of transformants 4A2, 1B3 and 3B4 (lanes 1–3) could also represent the autonomously replicating vector, the large molecule being a concatameric, self-replicating form (Kurz et al. 1987). However, all fungal DNAs presented in Fig. 1 failed to produce the ampicilin-resistant bacterial colonies when transformed into competent *E. coli* DH5, under experimental conditions allowing the detection of 0.1 pg of supercoiled pETM2.

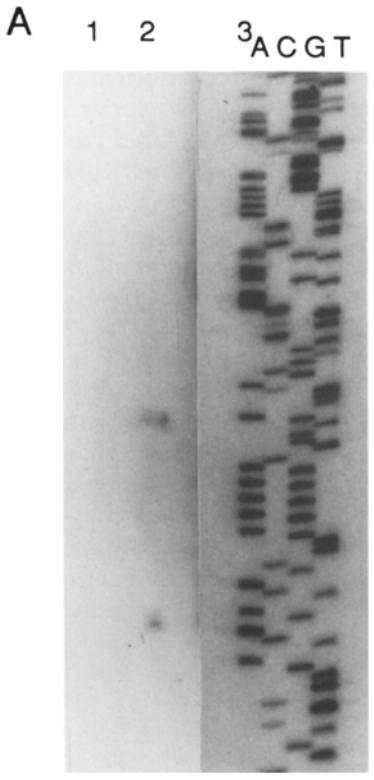
There was no direct correlation between the intensity of the hybridization signal and the level of resistance to G418, although there was considerable variation in the rate of growth of the individual transformants on selective agar. Hybridization signals could be enhanced by spore isolation and growth of the fungus on antibiotic-containing medium prior to DNA isolation (data not shown), suggesting that only some of the cells in the primary colony were actually transformed, or transforming DNA was unstable.

Restriction analysis of transformant L4C-12

Transformant L4C-12 was chosen to analyze the integration pattern of transforming DNA and the transcription of *APH(3')II* gene. This transformant exhibited a stable G418 resistant phenotype; after several transfers on the medium without the antibiotic, the isolate continued to grow vigorously upon transfer on Fries agar containing 50 µg G418/ml. Genomic DNA from the transformant L4C-12 was digested separately with *PstI* and *EcoRI,* electrophoresed in agarose gels, and hybridized with labelled DNA probes derived from different functional regions of the transforming plasmid pETM2 (Fig. 3).

Digestion with *PstI* shows that the 2.1 kb fragment of pETM2, composed of 0.9 kb of CaMV sequences flanked by 0.7 kb of pBR322 DNA and 0.2 kb of *Tn5* DNA (Fig. 3, lane B), has been disrupted, producing two new fragments; a 1.1 kb band hybridized to the probes I and III (lanes D and H) but not to the probe IV (lane J), indicating that it contained CaMV sequences. A separate 0.7 kb band hybridized only to probe IV (lane J), indicating that it contained the pBR322 sequences. The 0.8 kb fragment of pETM2 (lane B), composed entirely of *Tn5* DNA, remained unchanged and hybridized only to probes II and III (lanes F and H), suggesting that the *APH(3')II* gene was not rearranged upon transformation.

The *EcoRI* digest of the fungal DNA revealed that integrated CaMV and *APH(3')II* sequences remained linked on a 2.2 kb fragment, which hybridized to probes I, II and III (lanes C, E and G), but not to probe



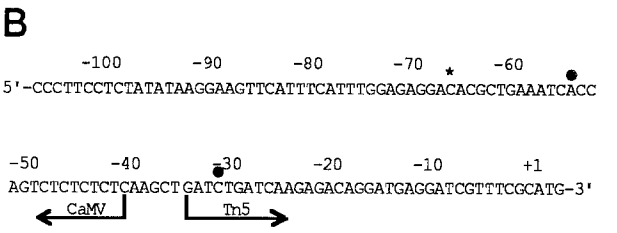


Fig. 4A, B. Mapping mRNA 5′-ends by primer-extension analysis. *Panel A:* An oligonucleotide (5′-GCCGAATAGCCTCTCCACCC-3′) was synthesized, with sequence complementary to the non-coding strand of DNA, at positions +41 to +60 of the *APH(3′)II* reading frame. This oligonucleotide was labelled at the 5′-end with ^{32}P, then 40 fmoles of labelled primer (specific activity = 1,100 dpm fmol^{-1}) was annealed to 10 µg poly(A)$^+$ RNA from FB1-S4 (*lane 1*) and transformant L4C-12 (*lane 2*). The primer was extended by the action of AMV reverse transcriptase, and the products separated on an 8% polyacrylamide-urea gel. The same radiolabelled primer was used to generate a sequence ladder from pETM2 by the method of Chen and Seeburg (1985) (*lanes 3A, 3C, 3G and 3T*). *Panel B:* The sequence of the non-coding strand at the junction of CaMV and *Tn5* sequences is shown, numbered relative to the first codon of the APH(3′)II reading frame. The normal start of the 35S CaMV transcript in plants is indicated by an *asterisk* (*). *Closed circles* (●) indicate the 5′-ends mapped by primer extension of poly(A)$^+$ RNA from transformant L4C-12

IV (lane J). Hybridization to probe IV containing only pBR322 sequences, identified a 4.2 kb *EcoRI* fragment (lane J). Similar results were obtained by *XhoI* analysis (not shown). It is unlikely that both an *EcoRI* site and an *XhoI* site would have been fortuitously generated by minor mutations. Thus, the pBR322 sequence appeared to be separated from the chimeric CaMV-*APH(3′)II* sequence during, or subsequent to, transformation.

Mapping of the 5′-terminus of the APH(3′)II mRNA by primer extension

Transcription initiation sites were mapped (Fig. 4A) by extension of a 20-bp synthetic primer 5′-GCCGAA-TAGCCTCTCCACCC-3′, complementary to nucleotides 41–60 downstream of the initiation codon of *APH(3′)II* (2). The primer was labelled at the 5′-end, hybridized to poly(A)$^+$ RNA from untransformed FB1-S4 (Fig. 4A, lane 1) or transformant L4C-12 (Fig. 4A, lane 2), then extended by the action of AMV reverse transcriptase. Comparison of the sequencing ladder of the plasmid pETM2 (employing the same primer) (lane 3) with the primer extension products mapped the 5′-ends of the mRNAs to two locations (Fig. 4B). One mRNA 5′-end was within the CaMV sequence, approximately 12 nucleotides downstream of the native start of CaMV 35S RNA (Guilley et al. 1982). The second transcript started within the *Tn5* DNA, near the CaMV-HindIII linker-*APH(3′)II* junction. This position is 35 nucleotides downstream of the native CaMV transcription initiation site. There was no indication of initiation at the normal site associated with CaMV expression in plants (Odell et al. 1985). The RNA from the untransformed recipient strain FB1-S4 did not produce any labelled cDNA under the experimental condition employed (Fig. 4A, lane 1).

Discussion

This paper describes the construction of a chimeric aminoglycoside-3′-phosphotransferase gene for selection of *F. solani* transformants. The construct contained neither sequences homologous to the *F. solani* genome nor any fungal DNA sequences to promote the transcription of the structural gene. Instead, the selectable marker was a chimera containing the 35S promoter of CaMV and the *APH(3′)II* coding sequence from bacterial transposon *Tn5*. Although the level of expression measured by mRNA accumulation was apparently low, it was sufficient to provide a positive selection for transformants and for the development and comparison of two transformation protocols.

Two transformation procedures were evaluated for frequency and viability of transformants. The first

protocol entailed the isolation of protoplasts, followed by treatment with $CaCl_2$, DNA, and polyethylene glycol. The second involved treatment of germinated conidia with lithium acetate and DNA, followed by polyethylene glycol. On a cell-for-cell basis, protoplasts exhibited a higher transformation frequency. However, since germinated spores exhibited a greater viability than protoplasts, more transformants were obtained, per microgram DNA, by the lithium acetate method. Both procedures were certainly adequate, but the transformation frequencies obtained were in the lower range of those reported for other fungal systems (Dhwale et al. 1984; Hynes 1986; Parsons et al. 1987; Punt et al. 1987; Turgeon et al. 1987; Yelton et al. 1984). This was more likely due to the nature of the transforming vector and the type of applied selection rather than to the protocol. Selection based upon antibiotic resistance may be less effective than complementation of auxotrophy. Although this aspect of transformation has not been extensively studied in filamentous fungi, it has been shown that the frequency of yeast transformation is up to 90-fold lower for G418 selection than for selection of prototrophs, even when both markers are contained in the same transforming vector (Sreekrishna et al. 1984; Webster and Dickson 1983).

The FB1-S4 transformants analyzed contained donor plasmid DNA in the high-molecular-weight fungal DNA. Restriction analysis (see Figs. 2, 3) suggested that most or all contained integrated vector. Integration of the pETM2 DNA into the genome of *F. solani* necessarily entailed recombination at sites with little or no vector DNA homology. The construct contained no fungal DNA, and no homology to the transformation vector was detected in the genome of the untransformed parent (see Fig. 2, lane 11). Nonhomologous integration and DNA rearrangements are often associated with fungal transformation (Case et al. 1979; Hynes 1986; Parsons et al. 1987; Yelton et al. 1984). Even when a segment of homologous parental DNA is included on the transformation vector, a substantial proportion of *A. nidulans* and *N. crassa* transformants exhibit integration of the vector DNA at nonhomologous loci (Case et al. 1979; Hynes 1986; Yelton et al. 1984). It is interesting to note that, at least in the case of *F. solani* transformant L4C-12 (see Fig. 3) and in spite of a lack of positive selection for the pBR322-derived sequences, the pBR322 DNA sequence appeared to have integrated at a separate locus from the selection marker DNA (CaMV-*APH(3')II*). This was evident from both *EcoRI* and *XhoI,* analysis, which identified distinct fragments with homology to these two regions of pETM2. In another instance, a transformant lost the antibiotic-resistance phenotype and the *APH(3')II* sequences in successive subcultures, while retaining the pBR322 sequence from the vector (data not shown). Thus, *F. solani* transformants exibited a propensity for DNA rearrangements and mitotic instability similar a that observed in *A. nidulans* and *N. crassa* transformants (Hynes 1986; Yelton et al. 1984).

In order to assess the role of the CaMV sequences in expression of the *APH(3')II* marker, transformant L4C-12 was analyzed in detail. Although some rearrangement of the transformation vector had occurred during or subsequent to transformation, the CaMV sequence remained linked to the phosphotransferase coding sequence. Analysis of the 5'-ends of the mature *APH(3')II* mRNAs from the transformant was conducted by primer extension, under the assumption that no processing of the primary RNA transcript had occurred between mRNA 5'-ends, and the location of the primer (position +41). Two putative transcription starts were identified, both downstream of the normal transcription start in plants. Thus the function of the 35S promoter in *F. solani* was not identical to its normal function in plants.

It is common to identify more than one transcription initiation start in the mRNA-encoding genes of filamentous fungi (Kinnaird and Fincham 1983; Mullaney et al. 1985). A number of motifs have been identified in fungal DNAs in the regions associated with transcriptional starts. These include pyrimidine-rich sequences in the non-coding DNA strand, upstream of the transcription start (Boel et al. 1984; Mullaney et al. 1985; Turgeon et al. 1987). The CaMV sequence used in construction of pETM2 contained several pyrimidine-rich tracts upstream of the normal start in plants and those documented in the transformant described. Other sequence motifs associated with fungal gene transcription are GAAC, TAAG and, most commonly, CAAG in the immediate vicinity of the transcription start points (Mullaney et al. 1985). In the CaMV-*APH(3')II* junction, the sequence CAAG occurred twice. Questions of whether this motif is important in *F. solani,* as well as whether regulatory sequences in the CaMV DNA affect expression in the fungus, will require further analysis.

This paper demonstrates the potential utility of the pETM2 vector in the transformation of *F. solani.* The vector provides a dominant selection trait that does not require the prior isolation of auxotrophic mutants. It can also be used to test the competence of G418-sensitive strains that lack complementable auxotrophic markers. Furthermore, its complete lack of homology to the host chromosomal DNA may make it a preferred selectable marker in gene replacement experiments for the identification of *F. solani* genes that are involved in virulence and phytoalexin detoxification.

Acknowledgements. The authors wish to thank Dr. H. D. Van Etten for helpful discussions at the beginning of this work, A. D. Byrd for

technical support, and R. E. Deaton for preparation of the manuscript. This work was supported by USDA Competitive grant numbers 83-CRCR-1-1203 and 87-CRCR-1-2391. The investigation reported in this paper (89-11-42) is in connection with a project of the Kentucky Agricultural Experiment Station and is published with the approval of the director.

References

Aviv H, Leder P (1972) Proc Natl Acad Sci USA 69:1408–1412
Beck E, Ludwig G, Awerswald EA, Reiss B, Schaller H (1982) Gene 19:327–336
Boel E, Hansen MT, Hjort I, Hoegh I, Fül NP (19844) EMBO J 3:1581–1585
Calzone FJ, Britten RJ, Davidson EH (1987) In: Berger SL, Kimmel AR (eds) Guide to molecular cloning techniques. Academic Press, pp 611–633
Case ME, Schweizer M, Kushner SR, Giles NH (1979) Proc Natl Acad Sci USA 76:5259–5263
Chen EY, Seeburg PH (1985) DNA 4:165–170
Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Biochemistry 18:5294–5299
Choi GH, Richey MG, Turbek CSM, Smith DA (1987) Phys Mol Plant Pathol 30:215–224
Dhwale SS, Paietta JV, Marzluf GA (1984) Curr Genet 8:77–79
Feinberg AP, Vogelstein B (1983) Anal Biochem 132:6-13
Garber RC, Yoder OC (1983) Anal Biochem 135:416–422
Gorman C, Padmanabhan R, Howard BH (1983) Science 221:551–553
Guilley H, Dudley RK, Jonard G, Balazs E, Richards KE (1982) Cell 30:763–773
Gritz L, Davies J (1983) Gene 25:179–188
Hanahan D (1983) J Mol Biol 1670:557–580
Hinnen A, Hicks JB, Fink GR (1978) Proc Natl Acad Sci USA 75:1929–1933
Hynes MJ (1986) Exp Mycol 10:1–8
Ito HY, Kukuda K, Murata K, Kimura A (1978) J Bacteriol 153:163–168
Jimenez A, Davies J (1980) Nature 287:869–871
Johnson DA, Gautsch JW, Sportsman JR, Elder JH (1984) Gene Aust Techn 1:3–8
Kinnaird JH, Fincham JRS (1983) Gene 26:253–260
Kistler HC, Benny UK (1988) Curr Genet 13:145–149
Kuhn PJ, Smith DA (1978) Ann Appl Biol 89:362–370
Kurz MB, Cortelyou MW, Miller SM, Lai M, Kirsch DR (1987) Mol Cell Biol 7:209–217
Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 88–95
Mirocha CJ, Pathre SV, Christensen CM (1977) In: Wyllie TD, Morehouse LG (eds) Marcel Dekker, New York, Basel
Mullaney EJ, Hamer JE, Roberti KA, Yelton MM, Timberlake WE (1985) Mol Gen Genet 199:37–45
Nelson PE, Toussoun TA, Cook RJ (1981) Fusarium: diseases, biology and taxonomy. The Pensylvania State University Press, University Park London
Odell JT, Knowlton S, Lin W, Mauvais CJ (1988) Plant Mol Biol 10:263–272
Odell JT, Nagy F, Chua N (1985) Nature 313:810–812
Parsons KA, Chumley FG, Valent B (1987) Proc Natl Acad Sci USA 84:4161–4165
Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, Van den Hondel CAMJJ (1987) Gene 56:117–124
Rogers SG, Horsch RB, Fraley RT (1986) Methods Enzymol 118:627–640
Santerre RF, Allen NE, Hobbs Jr, JN, Rao RN, Schmidt J (1984) Gene 30:147–156
Schardl CL, Byrd AD, Benzion G, Altschuler MA, Hildebrand DF, Hunt AG (1987) Gene 61:1–11
Smith DA, Banks SW (1986) Phytochemistry 25:979–995
Smith DA, Harrer JM, Cleveland TE (1982) Phytopathology 72:1319–1323
Southern EM (1975) J Mol Biol 98:503–517
Sreekrishna K, Webster TD, Dickson RC (1984) Gene 28:73–81
Turgeon BG, Garber RC, Yoder OC (1987) Mol Cell Biol 7:3297–3305
Turpen TH, Griffith OM (1986) Biotechniques 4:11–15
Wang J, Holden DW, Leong SA (1988) Proc Natl Acad Sci USA 85:865–869
Webster TD, Dickson RC (1983) Gene 26:243–252
Yelton MM, Hamer JE, Timberlake WE (1984) Proc Natl Acad Sci USA 81:1470–1474

Communicated by C. S. Levings III

Received November 21, 1988/March 7, 1989