Genetic transformation of the phytopathogenic fungus

Pyrenophora graminea

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Pyrenophora graminea was transformed by exposure of protoplasts to vector DNA in the presence of polyethylene glycol and CaCl₂. The phleomycin and G418 resistance genes present in plasmids pAN8-1 and pneoΔ respectively were expressed in P. graminea. Transformants were obtained at similar frequencies for both selectable markers and were analysed by Southern hybridization. Transformation occurred by integration of single or tandemly repeated copies of plasmids into the fungal genome. There was no evidence of autonomous plasmid replication. Transforming DNAs were stably maintained during mitosis on selective and non-selective medium. Phleomycin-resistant transformants were tested for pathogenicity. They were capable of infecting barley seeds, and after recovery from infected tissues retained the pheo® phenotype. It is concluded that transformation may be a useful tool for molecular analysis of genes required by P. graminea to cause barley leaf stripe disease.

The causal agent of leaf stripe disease of barley (Hordeum vulgare L.) is the filamentous fungus Pyrenophora graminea Ito & Kuribayashi [anamorph Drechslera graminea (Rabenh. ex Schlecht.) Shoemaker].

The fungus is a seed-borne pathogen that is widely distributed in most barley growing areas (Richardson, Whittle & Jacks, 1976; Delogu, Porta-Puglia & Vannacci, 1989). It has been reported that culture filtrates of P. graminea contain a phytotoxic compound(s) able to reproduce leaf stripe symptoms when infiltrated into the host plant (Aragona et al., 1991). The toxic compound(s) was partially characterized and its purification is in progress in order to define the chemical nature and role in the disease (Haegi et al., unpublished). At present attempts at disease control have focused on the exploitation of natural host resistance (Knudsen, 1985) or the application of fungicides (Khan & Loughman, 1988; Jones, Slade & Birks, 1989). More effective control methods might be developed if the biochemistry and genetics of the toxin(s) are better understood. One approach to identifying pathogenicity genes requires the development of a genetic transformation system.

We have recently obtained mutants from P. graminea protoplasts (Vannacci et al., 1991) which are currently being tested for toxin(s) production. The availability of strains altered in the synthesis of toxin(s), together with a transformation system, may allow the cloning and analysis of genes involved in toxin synthesis. Cloned toxin biosynthesis genes would allow the role of toxin production in pathogenicity to be definitively established. In addition, the rapid expansion of knowledge of the molecular biology and genetics of barley will make P. graminea an important organism for molecular studies of host–parasite interactions.

Transformation systems are now available for a number of filamentous fungi (Hynes, 1986; Fincham, 1989; Royer et al., 1991; Upchurch et al., 1991). The major obstacle in the development of such systems is the lack in many fungi of appropriate auxotrophic mutants which can be complemented by the transformation vector. For P. graminea, which lacks an amenable sexual system for genetic manipulation, it is not easy to obtain the appropriate auxotrophic recipient strains. The use of dominant selectable markers, such as antibiotic resistance, eliminates the need for induced mutation in recipient strains. Here we describe the development of a transformation system for P. graminea based on resistance to phleomycin and G418, two antibiotics which have been successfully utilized as selective agents in other fungi (Randall, Rao & Reddy, 1989; Pilgeram & Henson, 1990). The resulting transformants were characterized for mitotic and pathogenic stability of the transformed phenotype and genotype.

MATERIALS AND METHODS

Strains and storage conditions

Escherichia coli ED8767 (Murray, Branmar & Murray, 1977) was used for propagation of vector DNAs.

P. graminea strain DG2, used throughout this study, was isolated in Italy in 1982. It was cultured on potato dextrose agar (PDA) plates. The wild-type strain and transformants were stored in 33% glycerol at -80 °C and were freshly recovered for each experiment.

Plasmids

Plasmids p3SR2, pAN7-1 and pAN8-1 were kindly provided by Dr K. Johnstone (University of Cambridge, U.K.). Plasmid
pneoΔ was supplied by Dr MacDonald (University of Cambridge, U.K.). Plasmid p3SR2 carries amds5, the acetamidase-coding region of A. nidulans (Hynes, Corrck & King, 1983). pAN7-1 and pAN8-1 contain the E. coli hygromycin B phosphotransferase gene (bph) and the Streptococus hindustanicus phleomycin resistance gene respectively. Both genes are under the control of A. nidulans expression signals (Punt et al., 1987; Mattern et al., 1988). Plasmid pneoΔ is composed of the E. coli Neo gene, conferring resistance to the antibiotic G418, fused to nopaline synthase sequences which control expression of the Neo gene in plant cells (constructed by Dr M. W. Bevan).

**Materials**

β-glucuronidase, cellulase and all antibiotics were purchased from Sigma (St Louis, U.S.A.), Novozym 234 was from Novo Biolabs ( Bagsvaerd, Denmark) and restriction enzymes were obtained from Bio-Rad or Boehringer.

**Preparation of protoplasts**

The following procedure was modified from that previously developed for P. graminea (Vannacci et al., 1991). Mycelial plugs (5 mm diam.) were obtained from fungal colonies actively growing on PDA plates and fragmented with a tissue homogenizer in sterile distilled water. Two ml of this suspension were used to inoculate 500 ml flasks containing 100 ml of the following complete medium (PM; conc. in g l⁻¹: NaN0₃, 3; KCl, 0.5; MgSO₄, 7H₂O, 0.5; FeSO₄, 7H₂O, 0.01; KH₂PO₄, 1; Na₂HPO₄, 2H₂O, 0.36; glucose, 40; yeast extract, 2). Cultures were incubated on a rotary shaker (200 rpm) at 22–25° for 20 h. Young mycelium was harvested by centrifugation (10 min at room temperature and 700 g, rₑₜ 6-98 cm), washed with 0.9% NaCl and resuspended at 0.1 g ml⁻¹ in filter-sterilized solution containing Novozym 234 (10 mg ml⁻¹), β-glucuronidase (10 000 U ml⁻¹), cellulase (20 mg ml⁻¹), and 1.2 M-NaCl as the osmotic stabilizer. The suspension was incubated at 30° with gentle shaking for 3–4 h.

Protoplasts were separated from mycelial debris by passing the suspension first through a 100 μm mesh followed by a 45 μm mesh. Protoplasts were recovered by centrifugation at 700 g (rₑₜ 6-98 cm) for 5 min at room temperature, washed twice with 1.2 M-NaCl and finally suspended in 1 ml of the same solution. Protoplasts were subsequently washed with NTC (1:2 M-NaCl, 10 mM Tris/HCl pH 7-5, 10 mM-CaCl₂) prior to transformation. The viability of protoplasts was determined by plating in regeneration medium (PM containing 0.6 M sucrose and 2% agar) and colonies appeared after 3–4 d incubation at 22°.

The number of nuclei per protoplast was estimated after staining with DAPI (6-diamidine-2 phenylindole dihydrochloride). Protoplasts suspended in 100 μl NTC were fixed with glutaraldehyde (25% v/v in 0.1 M Tris/HCl pH 7.0) at final concentration of 5%, for up to 30 min at room temperature. A drop of fixed protoplast suspension was then mixed with 3 μl of DAPI solution (100 μg ml⁻¹) on a microscope slide and protoplasts were observed under a fluorescence microscope with an excitation wavelength of 365 nm and a barrier filter of 400 nm.

**Transformation of P. graminea**

The following procedure is based on the method of Yelton, Hamer & Timberlake (1984). Protoplasts (10⁻⁴–10⁰ in 100 μl NTC) were mixed with 25 μl NTC containing 50 μg vector DNA (or without DNA as a control). The suspension was incubated for 20 min at room temperature and volumes of 200, 200 and 800 μl of polyethylene glycol (PEG) 4000 (60% w/v in TC: 10 mM Tris/HCl pH 7.5, 10 mM-CaCl₂) were sequentially added. After mixing, the suspension was incubated for 20 min at room temperature. The protoplasts were recovered by centrifugation at 2800 g (rₑₜ 6.98 cm) for 5 min, washed with NTC and finally suspended in 1 ml NTC. Protoplasts transformed with p3SR2 were plated on minimal medium (PM without NaN0₃, glucose and yeast extract, containing 30 g sucrose 1⁻¹) supplemented with 10 mM acetamide. When pAN7-1, pAN8-1 or pneoΔ were used as transformation vectors, protoplasts were plated on complete regeneration medium (PDA plus 0.6 M sucrose). After 24 h incubation at 25°, plates were overlaid with 5 ml soft-agar to give final plate concentrations of 50 μg ml⁻¹ hygromycin (pAN7-1), 20 μg ml⁻¹ phleomycin (pAN8-1) or 100 μg ml⁻¹ G418 (pneoΔ).

Various modifications of this procedure were performed in order to improve the method. These included incorporating heparin (5 mg ml⁻¹ in NTC buffer) and spermidine (50 mM) with plasmid DNA before addition of the protoplasts; the use of different amounts of DNA (1–50 μg); the use of circular and linearized plasmids; the use of heat-shock treatment of protoplasts (48° for 5 min) before adding DNA; and the recovery of protoplasts in liquid medium (0.5% yeast extract, 2% glucose, 1:2 M sorbitol) for 3 h on a rotary shaker (150 rpm), before plating.

**Isolation and analysis of DNA**

Genomic DNA was extracted from lyophilized mycelium as described by Raeder & Broda (1985). For mycelium production, transformants were grown in liquid selective medium containing the appropriate antibiotic at a concentration 20 fold lower than that used for growth selection on agar plates, i.e. 1 μg ml⁻¹ phleomycin or 5 μg ml⁻¹ G418.

Isolation of bacterial plasmid DNA by alkaline lysis and precipitation with PEG, restriction enzyme digestion, transfer of DNA from gels to nylon filters and DNA hybridization were performed according to Sambrook, Fritsch & Maniatis (1989). Radioactive probes were labelled with [α³²P]dATP (Amersham) to greater than 10⁶ cpm μg⁻¹ DNA using a random-primed DNA labelling kit (Boehringer).

**Plant inoculation with transformant strains**

Seeds of barley cultivar Etrusco, highly susceptible to infection by P. graminea strain DG2, were surface sterilized in 2% NaOCl for a few seconds and washed with sterile distilled...
water. For each transformant and wild-type strain DG2, 30 seeds were placed on an overgrown mycelial culture on PDA in Petri dishes, incubated in the dark at 22°. A similar mycelial colony of the fungus was aseptically removed and inverted over kernels. The sandwich was incubated in the dark at 8–10° for 1 month, then the emerged seedlings were transplanted into plastic pots and grown in the greenhouse. Disease symptoms were recorded after 3–4 wk.

*P. graminea* was re-isolated by excising infected leaves and placing them onto non-selective PDA medium followed by transfer to selective PDA medium containing 20 μg phleomycin ml⁻¹ or 100 μg G418 ml⁻¹.

**RESULTS AND DISCUSSION**

**Sensitivity of *P. graminea* to different antibiotics**

The sensitivity of *P. graminea* to hygromycin, phleomycin and G418 was determined. Growth was completely inhibited on agar plates containing complete medium with the addition of 40 μg hygromycin, 10 μg phleomycin or 100 μg G418 ml⁻¹. Another potential method of selection was based on the observation that *P. graminea* is unable to grow on minimal medium containing acetamide as the sole nitrogen source. The acetamidasen gene allows wild-type strains to use acetamide as a sole nitrogen source (Hynes et al., 1983) and was used as a selectable marker.

Successful transformation with pAN8-1 and pneoΔ6 suggested that promoters from *A. nidulans* and plant cells are recognized by *P. graminea*. Since pAN7-1 and p3SR2 failed to transform the fungus to hygromycin resistance and acetamide utilization respectively, we conclude that the selective agents themselves play an important role for the success of the transformation experiments.

**Protoplast production**

Approximately 10⁷–10⁸ protoplasts were obtained from 4 g (wet weight) of mycelium and the regeneration frequency observed was normally 10%. The concentration of 0.1 g (wet weight) of mycelium per millilitre of enzymatic solution is a critical point in protoplast formation. When the concentration is higher than this value only few protoplasts are produced. The procedure reported here produced a large number of protoplasts having an acceptable regeneration frequency. Both of these factors are important to enhance the probability of success in transformation experiments.

**Transformation and mitotic stability**

Attempts to transform *P. graminea* to hygromycin resistance or acetamide utilization were unsuccessful. Some colonies were visible on medium containing hygromycin or acetamide 3–5 d after plating. These colonies stopped growing soon on selective medium and did not give rise to stable colonies, although they grew in non-selective conditions. The reasons for this abortive growth pattern are presently unknown. Transformation of protoplasts with pAN8-1 and pneoΔ6 produced authentic colonies on selective agar medium (20 μg phleomycin or 100 μg G418 ml⁻¹) after a 3 d incubation at 25°. There were two types of transformant, forming large and small colonies on selective medium. The large colonies grew vigorously when transferred to fresh selective medium, while the small ones grew very slowly. Two types of colony have also been observed in transformation experiments of other filamentous fungi (Turgeon, Garber & Yoder, 1985; Tsuge, Nishimura & Kobayashi, 1990; Upchurch et al., 1991). The transformation frequency with both pAN8-1 and pneoΔ6 ranged from 5 to 10 transformants per experiment. The use of different amounts of transformant DNA as well as linearization of plasmids, treatment with heparin and spermidine, heat shock or the recovery of protoplasts in liquid medium did not significantly influence the transformation frequency.

After plating, the transformed protoplasts were allowed to regenerate for 24 h without antibiotic selection. This procedure resulted in maximum rates of protoplast regeneration, which averaged 5%, and allowed expression of the resistance genes.

The transformation frequency is low compared to that reported for some other phytopathogenic fungi (Wang, Holden & Leong, 1988; Royer et al., 1991). Low transformation frequencies are not uncommon (Turgeon et al., 1985; Judelson, Tyler & Michelmore, 1991; Upchurch et al., 1991) and may be a consequence of the low regeneration rate of protoplasts. Such a low frequency might be expected for *P. graminea* since its mycelium is coenocytic and therefore many protoplasts are anucleate and not viable; DAPI staining confirmed that a large number of protoplasts (about 30%) are anucleate. Increased efficiencies might be expected by employing a homologous recombination system. Recently the rDNA gene cluster of *P. graminea* has been cloned (Amici & Rollo, 1991). The rDNA sequences are highly repetitive in the genome, so they might be used to construct new vectors able to integrate via homologous recombination (Tsuge et al., 1990).

Mitotic stability of the selectable genes in twenty transformants was assessed. Mycelium plugs of phleo® and G418® transformants were transferred six times in succession on non-selective medium (PDA). After the sixth transfer they were returned to selective medium: all transformants were resistant to phleomycin and G418 at concentrations of 20 μg and 100 μg ml⁻¹ respectively, with the same growth rate and colony morphology as the original transformant cultures.

**Southern analysis of transformant and wild-type DNAs**

Genomic DNA was isolated from five phleo® and five G418® colonies. DNA from untransformed strain DG2 was used as a control. Undigested DNA was probed with pAN8-1 (Fig. 1) and pneoΔ6 (data not shown). Similar results were seen demonstrating the presence of the vector DNA in all the transformant colonies, while no signal was seen when probing DNA from wild-type strain DG2. In all transformants hybridization occurred only at high molecular weight, suggesting that the plasmids integrated into the host genome and did not exist as autonomously replicating molecules. Since equal quantities of DNA (3 μg) were electrophoresed in each lane, the difference in intensity of bands between transformants may be due to heterokaryosis, since mycelium of *P. graminea*
Transformation of Pyrenophora graminea

Figs 1–3. Hybridization of genomic DNA from phleo® transformants (lanes 1–5) and the wild-type strain (w) of P. graminea with pAN8-1 as probe. DNA was isolated from mycelium as described and electrophoresed in 0.7% agarose gel. DNA from the same five transformants is loaded in the same order in all figures. DNA digested with Hind III was used as a size marker and the sizes of fragments are given in kilobases. Fig. 1. Undigested DNA (3 µg per lane). Fig. 2. Approximately 3 µg DNA digested with Hind III. Fig. 3. As Fig. 2 but digested with EcoR I.

is multiciliate. This may suggest a different number of transformed and untransformed nuclei in different transformants.

To characterize the nature of vector integration, high-molecular-weight DNA from five phleo® colonies was digested with Hind III and hybridized with pAN8-1 (Fig. 2). Since Hind III does not digest pAN8-1, the number of hybridizing bands should correspond to the number of sites of vector integration. Some transformants (Fig. 2, lanes 3, 4) had a single hybridizing band larger than the transforming vector (5-9 kb), suggesting that they had a single site of plasmid integration. The different sizes of hybridizing bands indicate that the site of integration varied among the transformants. Other transformants (Fig. 2, lanes 1, 2, 5) showed multiple hybridizing bands, suggesting that they had more than one site of vector integration. Hybridization patterns after digestion with EcoR I, an enzyme which cuts once within pAN8-1, showed an intense band of approximately 5-9 kb for transformants in lanes 1, 2, and 5 (Fig. 3). The size corresponds to that of linearized pAN8-1 and indicates a tandem integration of vector DNA. Transformants 2 and 5 show a complex hybridization pattern with some homologous fragments smaller than transforming vector. Plasmid integration through non-homologous recombination is often accompanied by the formation of tandem arrays with sequence rearrangements, and it has been observed in filamentous fungi (Parsons, Chumley & Valent, 1987; Fincham, 1989; Judelson et al., 1991).

Transformants 3 and 4 appear to have a single integrated copy of pAN8-1 at a unique site in the fungal genome.

In conclusion, transformation events in P. graminea involve stable integration of single or multiple copies of the vectors into chromosomal DNA at random site, since there is no apparent homology between pAN8-1 and pneoΔ6 and the P. graminea genome. If integration is random, it may be possible to use transformation as a method of mutagenesis, showing analogies with bacterial transposon mutagenesis (de Bruijn & Lupski, 1984). In this case cloning of genes mutated by transformation could be facilitated by the presence of vector DNA within those genes.

Pathogenicity of phleo® transformants

Ten transformants were tested for their pathogenicity on barley plants. They appeared to be as pathogenic as their wild-type parent DG2, inducing typical stripe symptoms. This suggests that the presence of exogenous DNA in their genome had no effect on pathogenicity. Transformants were then re-isolated and tested for resistance to phleomycin: all isolates recovered from infected leaves were antibiotic resistant.

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REFERENCES


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