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Transformation of the plant pathogenic fungus, *Rhynchosporium secalis*

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Abstract The barley leaf scald fungus, *Rhynchosporium secalis*, was transformed to hygromycin-B and phleomycin resistance using the *hph* gene from *E. coli* and the *ble* gene from *Streptoalloteichus hindustanus* under the control of *Aspergillus nidulans* promoter and terminator sequences. Plasmid DNA was introduced into fungal protoplasts by PEG/$CaCl_2$ treatment. Transformation frequencies varied from 59 to 493 transformants per 10 μg of DNA and 5×10^7 protoplasts. The antibiotic-resistant phenotype appeared to be stable under selective, as well as under non-selective, conditions for several generations. Co-transformation using the *E. coli uidA* gene under the control of *A. nidulans* promoter and terminator sequences on a non-selectable plasmid occurred at frequencies of up to 66%.

Key words Hygromycin · Phleomycin · GUS

Introduction

The imperfect filamentous fungus *Rhynchosporium secalis* (Oudem.) J. J. Davis causes leaf scald of barley (Shipton et al. 1974). The interaction of *R. secalis* with its host plant complies with the gene-for-gene hypothesis (Flor 1971), explaining the incompatibility of the plant/pathogen interaction as the consequence of a parasite avirulence gene and a complementary plant resistance gene. Due to the lack of a sexual stage, the functional analysis of *R. secalis* genes involved in the interaction with the host requires transformation-based techniques.

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Here we report on the transformation of *R. secalis* to hygromycin-B as well as to phleomycin resistance by using the plasmids pAN7-1 (Punt et al. 1987) and pAN8-1 (Mattern et al. 1988), respectively. These vectors, originally constructed for the transformation of *Aspergillus* spp., have been successfully used for the transformation of various filamentous fungi (van den Hondel and Punt 1991). In addition, co-transformation studies were carried out using the *E. coli uidA* gene encoding β-glucuronidase (GUS) on a second non-selected plasmid.

Materials and methods

Fungal culture. The origin of the *R. secalis* races, spore preparation and the inoculation of plants were as previously described (Lehnackers and Knogge 1990). The fungus was grown on solid Lima bean agar (2.3%) or in liquid culture in Fries medium No. 3 supplemented per l with 7.5 g of sucrose and 1 g of yeast extract. Alternatively, "CZV8CM" solid and liquid media were used (Newton 1988).

Transformation vectors. Plasmids pAN7-1 and pAN8-1 contain the hygromycin-B phosphotransferase (*hph*) gene from *E. coli* and the bleomycin resistance-encoding gene (*ble*) from *Streptoalloteichus hindustanus*, respectively, under the control of promoter (*gpdA*) and terminator sequences (*trpC*) from *Aspergillus nidulans* genes (Punt et al. 1987; Mattern et al. 1988). Both plasmids were obtained from P. J. Punt (Rijkswijk, NL). Plasmid pTUBA-GUS, carrying the *uidA* (β-glucuronidase, GUS) gene from *E. coli* under the control of the β-tubulin-A promoter from *Stagonospora* (*Septoria*) *nodorum* along with the *hph* expression cassette, was obtained from P. Bowyer (Norwich, UK). Plasmid pMR-GUS was constructed by cloning the 2.3-kb *Hin*dIII fragment from plasmid pTUBA-GUS, containing the *uidA* expression cassette, into the *Hin*dIII site of plasmid pBluescript II KS- (Stratagene, Heidelberg, Germany).

Preparation of protoplasts. Protoplasts were prepared essentially as described by Cooley et al. (1988) and Brückner et al. (1992). *R. secalis* was grown in liquid medium for 2–3 weeks. Eight grams of fungal mycelia were transferred to 50-ml Falcon tubes and centrifuged for 5 min at 3500 g. The mycelial pellet was then re-suspended in 20 ml of protoplast buffer I (10 mM Tris/HCl, 0.7 M KCl, 50 mM $CaCl_2$, pH 7.5). After centrifugation and re-suspension in the same volume of protoplast buffer I, to completely remove the growth medium, mycelia were mechanically dispersed using an ULTRA-TURRAX (Janke and Kunkel, Staufen, Germany) for 5 s on speed

level 5. After another centrifugation step, the pellet was re-suspended in 25 ml of protoplast buffer I containing 5 mg/ml of Novozyme 234 (InterSpex, Foster City, California) and incubated at room temperature for 2 h. Then, mycelial debris was separated from protoplasts by filtering the suspension through sterilized sinterglas filters (G1) covered with a layer of glass wool to prevent plugging of the sinterglass pores. To minimize loss of protoplasts, filters were subsequently washed again with 25 ml of protoplast buffer I. The protoplasts were sedimented by centrifugation (5 min, 2500 g), carefully re-suspended in 30 ml of protoplast buffer I and again centrifuged to remove the Novozyme 234. The protoplast pellet obtained was resuspended in 1 ml of protoplast buffer I and the titer and purity of the protoplast suspension were determined using a haematocytometer. Finally, the protoplast titer was adjusted to 5×10^7/200 µl.

Transformation protocol. The protoplast suspension (200 µl) was transferred to a 15-ml Falcon tube to which 10 µg of linearized plasmid DNA were added. The sample was incubated at room temperature for 20 min. Then, aliquots of 200 µl, 200 µl and 1000 µl of 50% polyethylene glycol (PEG) 6000 in protoplast buffer I were added stepwise and each time gently mixed with the protoplast suspension before the next aliquot was added. The sample was again incubated 20 min on ice and then 10 ml of protoplast buffer I (without PEG) were added to facilitate sedimentation of protoplasts by centrifugation (5 min, 2500 g, 4 °C). The completely remove PEG, the protoplasts were re-suspended in 10 ml of protoplast buffer II (10 mM Tris/HCl, 1 M sorbitol, 50 mM $CaCl_2$, pH 7.5) and again centrifuged. The supernatant was discarded and protoplasts were carefully re-suspended in 1 ml of protoplast buffer II and 100 µl of 10× growth medium. To allow regeneration, protoplast suspensions were incubated at 17 °C for 3 days before aliquots were plated on selective medium (2.3% Lima bean agar, 0.6 M sucrose, supplemented with 100 µg/ml of hygromycin B or 20 µg/ml of phleomycin).

β-glucuronidase assay. GUS activity was determined as previously described (Roberts et al. 1989) with the exception that crushing of the mycelial material was omitted. One half of the fungal colonies (∅ approximately 5 mm) was transferred into wells of microtiter plates. One-hundred microliters of extraction buffer (100 mM Tris/HCl, 50 mM NaCl, pH 7.5) and 5 µl of 20 mM 4-methyl umbelliferyl β-D-glucuronide (Sigma, Deisenhofen, Germany) were added to each well and the reaction was incubated for 1 h at 37 °C. Then, 400 µl of Ca_2CO_3 were added and the microtiter plate was analyzed by its fluorescence upon UV irradiation.

Molecular analysis of fungal transformants. For PCR analysis, small mycelial pieces were transferred to Eppendorf tubes and homogenized using a conical grinder. DNA was isolated as previously described (Cenis 1993). The final DNA pellet was dried and dissolved in 50 µl of H_2O. PCR was carried out in a volume of 50 µl using 0.1 µg of fungal DNA as template and *Pfu* DNA polymerase (Stratagene) according to the manufacturer's protocol. Oligonucleotide primers were synthesized representing nucleotide positions 2326–2344 (5′-GTCGAGAAGTTTCTGATCG-3′) and positions 3285–3264 of the reverse strand (5′-GTTTCCACTATCGGCGAGTACT-3′) of the *hph* gene (Gritz and Davies 1983; numbering is according to the pAN7-1 sequence, GenBank accession No. Z32698). After denaturation of DNA for 5 min at 94 °C, the cycling parameters were: 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min for 40 cycles and a final autoextension step of 72 °C for 10 min. Reaction products were analyzed on agarose gels and visualized by ethidium bromide staining. For Southern analysis, 4 g of fungal mycelia were frozen in liquid nitrogen and ground with mortar and pestle after the addition of quartz sand. The ground mycelia were suspended in 9 ml of 50 mM Tris/HCl, 0.3 M sucrose, 5 mM $MgCl_2$, 2% Triton X-100, pH 8, and filtered through Miracloth. After 10 min on ice, solutions were centrifuged for 20 min at 10 000 g and 4 °C. The supernatants were discarded and the pellets dissolved in 1 ml of 30 mM Tris/HCl, 10 mM EDTA, 1% Sarcosyl, pH 8. Cells were lyzed by treatment with 1 mg/ml of proteinase K for 4 h at 37 °C and cellular debris and polysaccharides were subsequently removed with hexadecyltrimethylammonium bromide (CTAB; Rozman and Komel 1994). DNA was isolated using standard procedures (Sambrook et al. 1989). The final DNA pellets were dissolved in 500 µl of H_2O. Restriction of DNA, eletrophoresis and other standard procedures were carried out as described by Sambrook et al. (1989).

Results and discussion

Initial tests revealed that *R. secalis* is sensitive to the antibiotics hygromycin B (LD_{50} = 3.5 µg/ml) and phleomycin (LF_{50} = 0.4 µg/ml). For transformation of fungal protoplasts, the bacterial hygromycin-B phosphotransferase (*hph*) gene and the bleomycin resistance-encoding (*ble*) gene, both under the control of *A. nidulans* promoter and terminator sequences, were therefore chosen as selectable markers on two different plasmids, pAN7-1 and pAN8-1, respectively (Punt et al. 1987; Mattern et al. 1988). Osmotic stabilizaion of protoplasts during enzymatic release from fungal mycelia was achieved with 0.7 M KCl in protoplast buffer I. During cellular regeneration, 0.6 M sucrose, 1 M sorbitol or 0.7 M KCl were found to be optimal. In the presence of 1 M sorbitol, 50 mM $CaCl_2$ and 10 mM Tris/HCl, pH 7.5, regeneration rates of 3–5% were obtained. When protoplasts were suspended in PEG buffer as used for transformation (50% PEG 6000, 0.7 M KCl, 50 mM $CaCl_2$, 10 mM Tris/HCl, pH 7.5), regeneration rates were reduced by approximately 20% as compared to samples not treated with PEG buffer.

Transformants became visible approximately 2 weeks after plating of the transformation suspension on selective plates containing either 100 µg/ml of hygromycin B or 20 µg/ml of phleomycin. The transformation frequencies of different *R. secalis* races (UK7, AU2, US238.1; see Knogge et al. 1994) were found to be in the same range, regardless of the selective marker gene used (*hph* or *ble*). In contrast, transformation frequencies varied in 30 individual experiments between 59 and 493 transformants per 10 µg of DNA. These numbers are in the range also found for other phytopathogenic filamentous fungi. Antibiotic resistance of the individual transformants appeared to be stable under selective, as well as under non-selective, conditions for at least 4–5 generations as determined by plating of spores and re-testing resistance to hygromycin B or phleomycin (data not shown).

Since *R. secalis* grows extremely slowly in vitro, DNA was extracted from small mycelial pieces of randomly selected fungal colonies for a first PCR-based molecular screening of the pAN7-1 transformants. Using oligonucleotide primers which were synthesized according to the sequences derived from 3′ and 5′ terminal regions of the *hph* gene, amplification products of the expected size of 960 bp were obtained with DNA from all transformants resistant to hygromycin B. In contrast, no such fragment occurred when DNA from wild-type *R. secalis* colonies was used as a template. Integration of the *hph* gene into the *R. secalis* genome was verified by Southern analysis of several randomly chosen PCR-positive transformants (Fig. 1). DNA was digested with *Hin*dIII having one cleavage site in the vector pAN7-1 14 bp downstream from the *trpC* se-

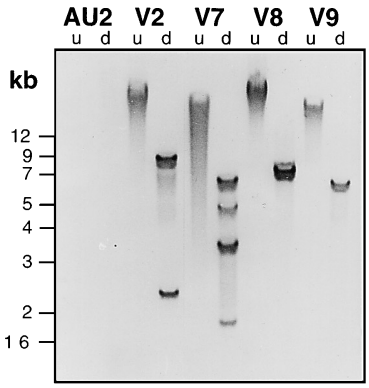

Fig. 1 Southern analysis of pAN7-1 transformants. DNA from an *R. secalis* wild-type race (AU2) and from randomly chosen hygromycin B-resistant transformants (V2, V7, V8, V9) was separated undigested (u, 5 μg) or digested (d, 10 μg) with *Hin*dIII. After transfer onto Hybond N, hybridization was performed using a vector fragment containing the *hph* expression cassette

quence (Punt et al. 1987), and hybridized to a 2.4-kb *Sph*I fragment containing 590 bp of the *gpdA* promoter, the *hph* gene and the *trpC* sequence from the transformation vector pAN7-1. There was no detectable homology between DNA from wild-type race AU2 and the probe. In contrast, DNA from all four transformants hybridized to the probe. Integration of the *hph* gene into the fungal genome was confirmed by hybridization of the probe only to high-molecular-weight DNA in undigested samples. The single bands of different size in the *Hin*dIII-digested DNA from transformants V8 and V9 indicated single integration sites at different locations in the genome. DNA from transformant V2 gave two major hybridizing *Hin*dIII fragments. This could be interpreted as a result of integration at two sites of the fungal genome, the short 2.3-kb fragment arising from an integration within the *gpdA* sequences of the *hph* cassette close to an internal *Hin*dIII site. Alternatively, rearrangements may have occurred during transformation. In contrast, the pattern found with DNA from transformant V7 was more complex. Four *Hin*dIII fragments were found indicating different plasmid-integration sites. The smaller fragments could again be interpreted by an integration within the *gpdA* promoter or by rearrangements. Since a 6.7-kb fragment, representing the entire plasmid in addition to border sequences, was not obtained and since signal intensity was very similar for all detected fragments, multiple tandem integration is unlikely to have occurred.

Since all available *R. secalis* races showed endogenous β-galactosidase activity (data not shown), the *E. coli* β-glucuronidase (GUS, *uidA*) gene was used as a reporter in co-transformation studies. The vector pMR-GUS was constructed by cloning the *uidA* gene under the control of fungal regulatory sequences from the selectable marker pTUBA-GUS into the pBluescript II KS-vector. *R. secalis* races US238.1 and UK7 were transformed with this non-selectable plasmid and one of the selectable vectors, pAN7-1 and pAN8-1. From each co-transformation experiment, 96 transformants were isolated and transferred to fresh agar plates supplemented with hygromycin B and phleomycin, respectively. For subsequent analysis, mycelial material from individual colonies was transferred into the wells of microtiter plates for GUS assays. GUS activity was found in 40–44% of the antibiotic-resistant fungal colonies when the selectable and the non-selectable vector were used in an equimolar ratio. With a molar ratio of 1:2, the number of GUS-positive colonies was raised to 66%. In contrast, no GUS activity was detected in colonies after transformation with the vectors pAN7-1 or pAN8-1 alone. These results suggest that co-transformation is a suitable method to introduce foreign genes into the *R. secalis* genome.

In conclusion, a gene transfer system based on transformation to antibiotic resistance was developed for the barley pathogenic fungus *R. secalis*. Integration of the *hph* transgene occurred at one or a few sites. Since the antibiotic-resistant phenotype was retained after several sub-cultures on non-selective medium, the integration was stable during mitosis even in the absence of selective pressure. While genetic complementation through transformation of a deficient *R. secalis* race with a gene encoding a race-specific elicitor has already proved to be successful (Rohe et al. 1995), replacement of this gene with a disrupted non-functional gene through homologous recombination is currently being pursued. In the future, transformation-based manipulation of *R. secalis* will allow the analysis of other fungal genes involved in fungal pathogenicity or virulence.

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