Biolistic transformation of *Cercospora caricis*, a specific pathogenic fungus of *Cyperus rotundus*

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Cercospora caricis is being considered as a bioherbicide agent for use against purple nutsedge (*Cyperus rotundus*), one of the world's worst weeds. However, first its efficiency must be improved, for example by genetic transformation. By optimizing physical and biological parameters, the particle gun acceleration method (biolistics) has been adapted to transform mycelial cells of *C. caricis*. Two genes were expressed in *C. caricis* by biolistic transformation. The β-glucuronidase gene (GUS) fused to the *GDP1* promoter of *Cochliobolus hetrostrophus* and the hygromycin B resistance gene under control of the *PtrpC* promoter of *Aspergillus nidulans*. Although the transformation frequency was not high, all transformants were stable when they were propagated on a selective medium after eight subsequent transfers.

INTRODUCTION

Cercospora caricis is a phytopathogenic fungus of purple nutsedge (*Cyperus rotundus*), an economically costly pest weed, especially in tropical and subtropical regions (Holm *et al*. 1977). A survey conducted in Israel between 1986 and 1989 reported that *Cercospora caricis* has a high potential as a biocontrol agent of this weed (Quimby *et al*. 1991). Additionally, its pathogenicity is host-specific: inoculation experiments with *C. caricis* revealed that 17 Israeli isolates caused symptoms only on purple nutsedge that never appeared in any other plant species tested (J. Hershenhorn, pers. comm.).

Spraying plant hormones together with *C. caricis* increased the damage caused to the purple nutsedge plants by 30% (Hershenhorn, unpubl.). Increased expression of the genes encoding for IAA and cytokinins could improve virulence of the pathogen for the control of purple nutsedge.

The transformation of filamentous fungi was first reported for *Neurospora crassa* in 1973 (Fincham 1989) and has been extended to other species, genes, promoters and new technologies (Lu *et al*. 1994; Christiansen & Giese 1995, Durand *et al*. 1997, Forbes *et al*. 1998, Maor *et al*. 1998). Although several methodologies have been used successfully to transform fungi, molecular studies and transformation of *C. caricis* have not been yet reported. Our aim was to determine the physical and cultural conditions necessary to establish a suitable transformation system for this fungus. Genetic modification will be the next step to improve its virulence as a biocontrol agent.

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MATERIALS AND METHODS

Fungal material, media and culture conditions

A 10-d old culture of *Cercospora caricis* grown on Difco potato dextrose agar (PDA) was blended in 50 ml sterile double distilled water in a Waring blender for 2 min at maximum speed. The suspension was spread onto PDA in Petri dishes and held at 24 ± 1 °C under fluorescent light with a 12 h photoperiod. The strain is being deposited in ATCC.

Plasmids

Two plasmids were used: (1) pUCATPH (Lu *et al*. 1994) which contains a bacterial hygromycin B resistance gene (hygB) fused to the regulatory sequences *trpC* promoter and terminated by *TtrpC* from *Aspergillus nidulans*. This plasmid was provided by Olen Yoder (Plant Health, San Diego) and G. B. Turgeon, (Cornell University, NY). (2) The GPD–GUS construct provided by David C. Straney (University of Maryland) which contains the bacterial β-glucuronidase gene (GUS), fused to the *GPD1* promoter of *Cochliobolus hetrostrophus* and terminated by the bacterial *Nos* terminator. This plasmid also contains the hygromycin B resistance gene driven by the *A. niger Glaa* promoter and terminated by *TtrpC* from *A. nidulans*.

Transformation

Biolistic transformation of *Cercospora caricis* mycelium was carried out using the PDS-1000/He device (Bio–Rad® Laboratories) (Kikkert 1993). Tungsten particles M17 (Bio–Rad

Laboratories, Richmond, CA) were coated with the plasmid DNA according to the procedure of Sanford *et al*. (1993). The following parameters of the biolistic system were optimized: the age of target cells (the mycelium was disrupted into small fragments by blending, followed by inoculation onto PDA and 2–5 d growth), helium pressure (350, 1100 and 1350 psi), distance of the target cells from the macrocarrier launch (3, 6 and 12 cm), and frequency of bombardment (2–4 shots).

Twenty-four hours after bombardment, the mycelium was collected, blended in a Waring blender for 2 min and the suspension spread onto selective medium (PDA containing 30 μg ml^{-1} hygromycin B); 6–14 d after incubation at 24 ± 1° under fluorescent light with a 12 h photoperiod, transformants were recovered on the selective medium.

Colourimetric detection of GUS expression

Wild-type and transformed mycelia were rinsed in sterile water and placed in X-Gluc buffer (0.1 M $NaPO_4$, pH 7.0, 0.5% Triton X-100, 0.01 M EDTA) containing 0.5 mg ml^{-1} (5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-Gluc). The blue coloured product was visualized in the fungal hyphae by microscopy after 24 h incubation at room temperature.

DNA manipulations

Bacterial culture, transformation, and DNA manipulations were done using standard methods. The isolation of genomic DNA from *C. caricis* and Southern blot analysis were carried out as described by Upchurch *et al*. (1991).

RESULTS AND DISCUSSION

We began our experiments on the transformation of *Cercospora caricis* using the known standard methodologies and protocols such as protoplasts (Upchurch *et al*. 1991) and electroporation (Becker & Guarente 1991). Since our initial attempts to express different genes using various promoters failed (data not shown), in this study we report for the first time the transformation of the pathogenic filamentous fungi *C. caricis* by means of microprojectile bombardment. We were able to express two genes in *C. caricis*, the β-glucuronidase gene (GUS) under the control of *GPDI* promoter of *Cochliobolus hetrostrophus*, and the hygromycin B resistance gene (hygB) fused to the regulatory sequences of *trpC* promoter from *Aspergillus nidulans*.

The effect of several parameters (Christiansen *et al*. 1995), such as the age of target cells, helium pressure, distance of the target cells from the macrocarrier launch, and frequency of bombardment were investigated and determined for *C. caricis*. Transformation frequency with both plasmids (GPD–GUS and pUCATPH), ranged from 0.3 to 2 transformants μg^{-1} of DNA. Optimum results (2 transformants μg^{-1} plasmid DNA) were obtained when the age of the target cells was 5 d after PDA media inoculation, with a helium pressure of 1100 psi, M17 tungsten particles, a frequency of 3 bombardments per Petri dish, and a 6 cm distance between the target cells and the macrocarrier launch. When the distance was shorter (3 cm), injury to the mycelium was observed. Bombardment with a construct containing the GUS gene driven by the *GPDI* promoter (Turgeon *et al*. 1987) resulted in GUS gene expression: blue spots were visible within the hyphae of transformed *C. caricis* cells (Fig. 1B). No blue cells were seen in the wild-type *C. caricis* cells confirming that no endogenous GUS activity was found in this fungus (Fig. 1A).

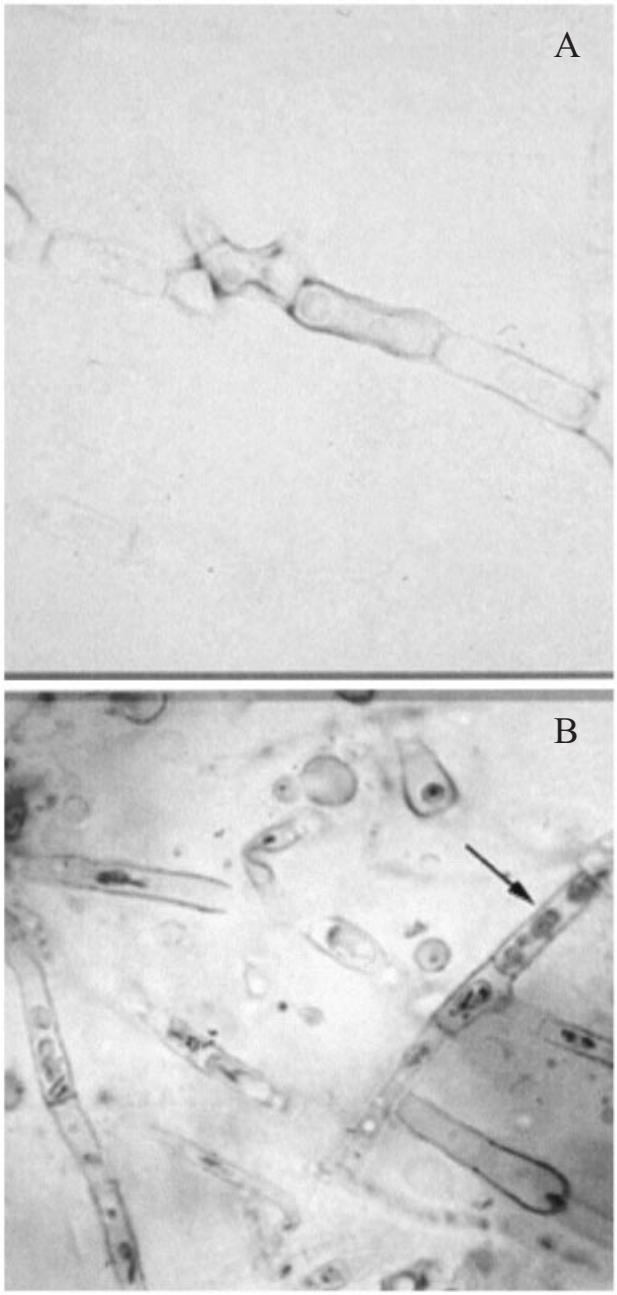

Fig. 1. Expression of β-glucuronidase gene (GUS) in *Cercospora caricis* cells. Transformed cells were detected in the mycelium by histochemical assay (B, arrow). (A), control; represents untransformed mycelium.

We found that 8 μg ml^{-1} hygromycin B was sufficient to impede the growth of *C. caricis* mycelium *in vitro*. Despite the low transformation frequencies, all transformants were stable when propagated on the selective medium (60 μg ml^{-1} hygromycin B) after eight subsequent transfers.

Southern analysis showed integration of the hygromycin B gene introduced into the chromosomal DNA of the fungus. In

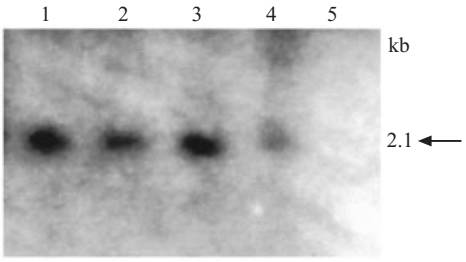

Fig. 2. Southern blot analysis of putative *Cercospora caricis* transformants. Genomic DNA (10-μg lane^{-1}) from transformed lanes (1–4) and untransformed lane (5) isolates was digested with *Xba*I, and electrophorised through a 0.8% agarose gel. The DNA was transferred to a nylon membrane and probed with ^{32}P-labelled 5.08-kb pUCATPH plasmid DNA.

the transformed fungi, Fig. 2 (1–4), the probe detected a 2.1-kb *Xba*I band. In the non-transformed fungi, Fig. 2 (5), such a band was not found. Our results indicate that particle bombardment appears to be a promising method for transforming mycelial cells of *C. caricis*. This development will allow analysis of genes and promoters with potential for the improvement of the biocontrol efficacy of bioherbicide fungi.

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