Pathogenicity and growth of *Metarhizium anisopliae* stably transformed to benomyl resistance

Mark S. Goettel1,*, Raymond J. S. Leger1, Srirama Bhairi1, M. Katherine Jung2, Berl R. Oakley2, Donald W. Roberts1, and Richard C. Staples1

1 Boyce Thompson Institute, Tower Road, Cornell University, Ithaca, NY 14853, USA
2 Department of Molecular Genetics, The Ohio State University, 484 West Twelfth Avenue, Columbus, OH 43210-1292, USA

Received June 20/October 10, 1989

Summary. The insect pathogenic hyphomycete *Metarhizium anisopliae* was transformed to benomyl resistance using pBENA3, a plasmid containing the *benA3* allele from *Aspergillus nidulans*. The transformation rate was 9 transformants/50 μg DNA/2 × 10^6 viable protoplasts. Southern hybridization analyses indicated that the plasmid integrated by nonhomologous recombination at multiple loci. The sites of integration differed among transformants. There was no evidence for autonomous plasmid replication in the transformants. Transformants grew at benomyl concentrations up to 10 times that which inhibits wild type, and they were mitotically stable on either selective or non-selective medium or insect tissue. The transformants were pathogenic to the hornworm, *Manduca sexta*, producing both appressoria and the cuticle-degrading enzyme, chymoelastase, in the presence of 50 μg/ml of benomyl. These studies demonstrate the potential of using transgenic strains of entomopathogenic fungi along with other components of pest control such as fungicides.

Key words: *Metarhizium anisopliae* – Benomyl-resistance – Transformation – Entomopathogen

Introduction

The insect pathogenic hyphomycete *Metarhizium anisopliae* is currently used for the biological control of insects (Wraight and Roberts 1987). Recent studies demonstrate that *M. anisopliae* produces several cuticle-degrading enzymes during penetration of host cuticle, and these enzymes appear to be associated with pathogenesis by virtue of their early production in high levels during infection and their ability to degrade cuticle (St. Leger et al. 1987).

One strategy for the biocontrol of insects is to develop strains of fungal pathogens resistant to common fungicides. The transgenic strains could then be used to control insect pests at the same time that fungicides were applied to control fungal pathogens of plants. To develop transgenic strains of *M. anisopliae*, we have adapted transformation protocols applied to other Ascomycetes (Case et al. 1979; Yelton et al. 1984; Parsons et al. 1987; Turgeon et al. 1987; Huang et al. 1989), using *benA3*, a gene from *Aspergillus nidulans* (Sheir-Neiss et al. 1978) which confers resistance to benomyl (Orbach et al. 1986). Gene transfer systems have not previously been reported for an entomopathogenic fungus.

A dominant selective marker that was recently developed for transformation of filamentous fungi uses resistance to the fungicide benomyl (Orbach et al. 1986). Benomyl binds to β-tubulin and inhibits formation of microtubules, and mutations in the *benA3* gene of *Aspergillus nidulans*, which encodes the major β-tubulin expressed in hypha, can confer resistance to benomyl (Sheir-Neiss et al. 1978). Using a plasmid, pBENA3, containing *benA3*, a benomyl resistant *benA* allele, we have genetically transformed *M. anisopliae* to benomyl resistance so that it can continue to be used for insect control while the fungicide is used for the control of plant pathogens.

Materials and methods

Plasmid. The plasmid pBENA3 was constructed by K. Jung and B. Oakley (unpublished), it carries the *benA3* allele of *A. nidulans*, pUC19 sequences, and the *pyr4* gene of *Neurospora crassa*. It is identical to plasmid AlpGM4 (May et al. 1985) except that it carries the benomyl-resistant *benA3* allele, instead of the wild type *benA* allele, and lacks a 250 bp *XbaI* fragment 3′ to the *benA* gene. *XbaI* cleaves the plasmid once and *EcoRI* cleaves it at two sites. The plasmid was amplified in *Escherichia coli*, strain DH5α.

Protoplast preparation. *Metarhizium anisopliae*, strain ME1, was grown in shake cultures of Sabouraud Dextrose Broth (SDB) for
40 h at 27°C. Mycelium was collected by centrifugation, resuspended in 0.8% Novozym 234 (Novo Biolabs) in digestion medium (DM, 1.2 M sorbitol, 10 mM Tris, pH 7.0) and shaken (5 rpm) for 3 h at room temperature. Protoplasts were separated from cellular debris by filtration through cheesecloth and a 20 μm plastic screen. The protoplasts were collected by centrifugation (2,000 g, 10 min) washed once each in DM and transformation media (TM, 1.0M sorbitol, 10 mM Tris, pH 7.5, 20 mM CaCl2) and resuspended in TM (Huang et al. 1989).

Transformation. Transformation protocols for M. anisopliae were modified from those of Yelton et al. (1984). Protoplasts (2 × 10⁷) in 200 μl TM were mixed with circular pBENA3 (50 μg in 100 μl Tris EDTA) and incubated on ice for 30 min. Fifty microliters of 60% polyethylene glycol 3000 (PEG – 60% w/v in TM) was added, and protoplasts were incubated on ice for an additional 10 min; 1 ml of the PEG was then added, and after 10 min incubation at room temperature the protoplasts were diluted in 4 ml of regeneration medium (RM, 1.2 M sorbitol, 10 mM Tris, pH 7.5, 0.1% K₂HPO₄, 0.05% MgSO₄, 0.3% NaNO₃). Aliquots containing 2 × 10⁵ protoplasts were mixed in Petri plates with 10 ml of molten (45-48°C) regeneration agar (RA, unbuffered RM with 1.2% Noble agar) and incubated for 18 h at 27°C. The plates were then overlaid with 10 ml of RA containing 10 μg/ml benomyl. A stock solution of benomyl (10 mg/ml of Benlate, a 50% wettable powder or benomyl in dimethylsulfoxide) was kept at −20°C and filter-sterilized aliquots were added after the medium was autoclaved. The plates were incubated at 27°C and benomyl-resistant colonies appeared in 12–23 days. These were then subcultured onto Czapek Dox agar (CDA) containing 2.5 μg/ml benomyl. Three putative transformants, designated as T-2, 4 and 7, were randomly selected for detailed study.

DNA isolation and molecular analyses. DNA was isolated from mycelia grown in shake culture for 3 days at 27°C in SDB with or without 2.5 μg/ml benomyl as required. Standard recombinant DNA techniques were as described by Maniatis et al. (1982), ³²P-labelled DNA was produced by the primer-extension procedure of Feinberg and Vogelstein (1983, 1984).

Mitotic stability, growth, infection structures, and enzyme production. Mitotic stability was tested by transferring single-spore isolates serially to SDA as a nonselective medium. After every fifth transfer, single-spore isolates were tested for benomyl resistance by growth on CDA/benomyl. The effect of benomyl on differentiation of appressoria and secretion of chymoelastase was determined by germinating conidia (10⁶ conidia per 5 ml 0.0125% yeast extract) in Petri plates containing different concentrations of benomyl. The plates were examined for presence of appressoria after incubation for 19 h at 27°C. Chymoelastase secretion after 48 h was assayed using succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide as a substrate (St. Leger et al. 1986).

Pathogenicity. To determine if the transformants remained pathogenic, groups of five 4th-instar Tobacco hornworm (Manduca sexta) larvae were each inoculated with either one of the transformants or the original wild type by dipping in a suspension of conidia (10⁷ ml⁻¹), and incubating at 27°C. Dead larvae were held at 100% humidity until a sporulating layer developed on the cadaver. The stability of the integrated gene after infection was tested by scraping conidia from the cadavers and plating onto benomyl-containing CDA.

Results

Transformation

Protoplasts not treated with the plasmid only grew in the absence of benomyl, and failed to grow in its presence (Fig. 1). When transformed protoplasts were incubated on RA for 18 h before addition of the benomyl overlay, transformation occurred at an average rate of 9/50 μg DNA/2 × 10⁵ protoplasts. No transformants were observed when protoplasts were directly mixed with, or plated onto, media containing benomyl. In contrast, delaying the addition of the benomyl overlay for 20 h rather than 18 h resulted in significant background growth. Only two transformants were obtained when the DNA-protoplast suspension was incubated at room temperature rather than on ice. Of the three transformants studied in detail, all were highly resistant to benomyl in comparison to the wild type (Fig. 2). Transformant T2 was most resistant with growth occurring on 50 μg/ml benomyl, the highest concentration tested (mean colony diameter of 1.5 ± 0.45 cm after 14 days).

Molecular analyses

Total DNA from a wild-type strain and three putative transformants was analyzed by Southern hybridization using pBENA3 as a probe (Fig. 3). In Southern hybridization to undigested transformant DNA (Fig. 3, lane 8), hybridization occurred only to high molecular weight DNA, while wild type showed no hybridization signals at all. Transformation, thus, has apparently occurred by integration of plasmid sequences into the genome rather than by autonomous replication of the plasmid. Since pBENA3 does not recognize any sequence in the recipient strain DNA, it is unlikely that homologous integration will have occurred. By analogy with other systems (Yelton et al. 1984), heterologous integration most likely involves multiple integration. This is consistent with the complex
Fig. 2. Effects of benomyl on the growth of transformed and wild type *Metarhizium anisopliae*. Colony diameters were taken after 7 days growth at 27°C on Sabouraud Dextrose Agar containing different concentrations of benomyl. Bars indicate standard error of 5 replicates.

Fig. 3. Southern blot hybridization of *Metarhizium anisopliae* DNA transformed with pBENA3. DNA was obtained from mycelia grown for 3 days at 27°C in Sabouraud dextrose broth containing 2.5 μg/ml benomyl for the transformants. Southern transfers were probed with 32P-labelled pBENA3 linearized with XbaI. Plasmid digested with EcoRI (lane 1) and XbaI (lane 2). Wild type (lane 3). Transformants and restriction enzyme treatments: T7, EcoRI (lane 4), XbaI (lane 5); T2, EcoRI (lane 6), XbaI (lane 7), and undigested (lane 8).

Discussion

We have demonstrated that *M. anisopliae* can be transformed to benomyl resistance using the plasmid pBENA3. Since pBENA3 does not hybridize to DNA from untransformed *M. anisopliae*, it is unlikely that the *benA* and *pyr4* genes on the plasmid are sufficiently homologous to the β-tubulin and orotidine-5'-phosphate decarboxylase genes of *M. anisopliae* to direct integration by homologous recombination. Since transformation by homologous recombination is probably not possible with pBENA3, it would not be surprising if the plasmid integrated at more than one site in the genome or if integrative recombination occurred at more than one site in the plasmid.

The frequency of transformation (9 transformants/50 μg/DNA/106 viable protoplasts), while low, is comparable to some other fungal systems (cf. Smith 1985; Turgeon et al. 1985), and may actually be an artifact of the selection method used. Transformed cells required a growth period in the absence of selection, possibly to allow for protoplast regeneration, since transformed protoplasts did not regenerate when mixed directly with selection media. Also, integration and full expression of the resistance gene required much time since transformants appeared in 12–23 days as compared to 2–3 days for controls. Consequently, the addition of benomyl was delayed for 18 h. In such a situation, the timing of selection becomes critical; early addition of the selection drug may result in inhibition of many putative transformants while delayed addition may result in inhibition of transformant growth by growth of non-transformed protoplasts, as occurs in *Neurospora crassa* when the protoplast density is too high (Case et al. 1979). A similar need to delay the application of benomyl to transformant protoplasts has been reported for *N. crassa* (Orbach et al. 1985).

Mitotic stability and pathogenicity of transformants

Three primary transformants were resistant to benomyl after growth on SDA without benomyl. After five successive transfers (passages) on benomyl-free media, 1 of 26 single-spore isolates from one transformant lost its ability to grow on CDA/benomyl media. All the remaining single-spore isolates, as well as the single-spore isolates of the other two transformants, remained benomyl resistant when grown for 25 growth cycles on non-selective medium. None of the wild-type cultures grew on the benomyl-supplemented medium.

The three transformants retained their pathogenicity towards *Manduca sexta* larvae after the larvae were dipped in a suspension of conidia (10^7/ml) and incubated at 27°C for 5 days. Single-spore isolates from insect-passaged transformants also retained their resistance to benomyl, indicating that the integrated plasmid sequences are maintained during infection and growth in the insects. Chymoelastase, the cuticle degrading enzyme, was synthesized at similar rates in controls and transformants (ca. 0.7 gmoles min\(^{-1}\)). Activity was reduced by 95% in the wild type in the presence of 9 μg/ml benomyl and by approximately 50% in the transformants in the presence of 50 μg/ml benomyl. No enzyme was produced by transformants at benomyl concentrations greater than 100 μg/ml. Transformants continued to produce appressoria in the presence of up to 100 μg/ml benomyl, while differentiation in the wild type was much reduced at 25 μg/ml and totally absent at 50 μg/ml.

Pattern of bands produced by restriction with the enzymes used. However, we cannot rule out the possibility that some of the bands of homology are due to incomplete digestion of the DNA.
In contrast, Henson et al. (1988) mixed protoplasts of transformed *Gaeumannomyces graminis* directly with regeneration media containing benomyl. In addition, they found that more benomyl was required for selecting resistant transformants than was required for inhibition of subsequent growth. This indicates that the sensitivity of protoplasts to benomyl, and the rate of expression of benomyl resistance, varies among different fungal species.

We apparently selected only transformants with multiple copies of the resistance gene. This is not surprising, since integration occurred by nonhomologous recombination and the wild type benomyl-sensitive β-tubulin gene(s) of *M. anisopliae* was still present in the transformants. Therefore, only transformants with multiple copies of the benA3 allele would have appreciable resistance. It would be expected that by reducing the level of selection pressure, i.e. the amount of benomyl, transformants would be selected which had fewer copies of pBENA3, since such integrations would be expected to produce semi-resistant phenotypes. While difficulties in selection of transformants might have been alleviated by using a dominant gene, strain ME1 is not sensitive to hygromycin B (unpublished information) precluding the use of hph, a hygromycin resistance gene isolated from *E. coli* which has been widely used as a selection marker in fungi (Yoder et al. 1988).

Despite the random and multiple integrations that appear to be associated with most fungal transformation systems, the transformants obtained in this study displayed high mitotic stability, comparable grown to the wild type, and retention of pathogenicity to insects. Further studies will enable the development of transgenic strains with improved virulence to insects and increased compatibility with other components of pest control such as fungicides.

**Acknowledgements.** We thank Clay Weeks for technical assistance and Lucille Laccetti, Pauline Freve, and Dafang Huang for useful discussions. This work was supported by the Natural Sciences and Engineering Research Council of Canada, the USAID Bean/Cowpea Collaborative Research Support Program, the Jesse Smith Noyes Foundation, and the Cornell Biotechnology Program which is sponsored by the New York Science and Technology Foundation, a consortium of industries and the U.S. Army Research Office. The cloning of benA3 and construction of pBENA3 were supported by grant 86-CRCR-l-1963 from the US Department of Agriculture. The transformants are held at the USDA-ARS Collection of Entomopathogenic Fungi, Ithaca N.Y. and have been designated as ARSEF 2602-2612.

**References**

Case ME, Schweizer M, Kushner SR, Giles NH (1979) Proc Natl Acad Sci USA 76:5259–5263


St Leger RJ, Cooper RM, Charnley AK (1987) J Gen Microbiol 133:1371–1382


Communicated by H. Bertrand