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Transformation and Mutagenesis of the Nematode-trapping Fungus *Monacrosporium sphaeroides* by Restriction Enzyme-mediated Integration (REMI)

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In this study, the nematode-trapping fungus, *Monacrosporium sphaeroides,* was transformed with a plasmid harboring the hygromycin B phosphotransferase gene, via restriction enzyme-mediated integration (REMI). Frequencies of up to 94 transformants μg^{-1} per linearized plasmid DNA were obtained by optimizing the PEG concentration, as well as the category and quantity of the added restriction enzyme. 90% of the transformants were determined to be stable for drug resistance when 20 randomly selected transformants were tested. Southern analyses revealed that the transforming DNA was integrated into the *M. sphaeroides* genome either with or without rearrangement. Five mitotic stable mutant strains were obtained using this approach, all of which had been altered with regard to sporulation capacity and pathogenicity toward nematodes. Southern blot analyses of the five mutants revealed that foreign plasmid DNA had integrated into the genome. Three of the mutants, Tms2316, Tms3583 and Tms1536, exhibited integration at a single location, whereas the remaining two, Tms32 and Tms1913, manifested integration at double or multiple locations. Our results suggest that the transformation of *M. sphaeroides* via REMI will facilitate insertional mutagenesis, the functional analysis of a variety of genes, and the tagging or cloning of genes of interest.

Key words: restriction enzyme-mediated integration, nematode-trapping fungus, *Monacrosporium sphaeroides,* insertional mutagenesis, hygromycin resistance

Each year, plant-parasitic nematodes inflict a great deal of damage on a host of world crops. Nematode-trapping fungi, which infect nematodes via the formation of an adhesive or non-adhesive trapping-device, are currently attracting major worldwide interest, with regard to their potential application as biological control agents against plant and animal parasitic nematodes (Jansson *et al*., 1997). Although biological control of plant-parasitic nematodes has been effected using these fungi for decades, only a very few effective commercial formulations are available for practical application (Stirling and Mani, 1995; Stirling *et al*., 1998). The nematode-trapping fungi capture, infect, and digest their prey using predacious structures (trapping-device), with the participation of virulence factors, most notably protease (Tunlid and Jansson, 1991; Zhao *et al*., 2004). As was reported by Åhman *et al.* (1996) and Rosén *et al*. (1997), successful parasitic nematode control using nematode-trapping fungi remains, to some degree, limited by our understanding of the infection process and virulence factors exploited by these organisms. Thus, by cloning genes relative to the trapping-device and conidia formations, we should be able to glean insight into the infection process, as well as the relationship of the trapping-device with the virulence factors, and the proliferation strategies of the nematode-trapping fungi. Furthermore, the cloning of these genes will provide molecular proof as to the appropriate taxonomy for the nematode-trapping fungi, as the trapping-device and conidia constitute important taxonomic characteristics (Scholler *et al*., 1999).

The screening of insertional mutants for the loss of the trapping-device or conidia is an effective method, not only for the investigation of the molecular genetic basis of the trapping-device and conidia formation, but also for the tagging and cloning of genes that relate to their formation. In order to conduct these molecular studies without any known genetic background for the trapping-device and conidia, we first considered the restriction enzyme-mediated integration (REMI) technique. REMI was first used with *Saccharomyces cerevisiae* (Schiestl and Petes, 1991), and was later applied to *Dictyostelium discoideum* (Kuspa and Loomis, 1992). More recently, this method has been successfully used in the isolation of virulence genes from several fungal phytopathogens (Lu *et al*., 1994; Bölker *et*

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al., 1995; Shi *et al*., 1995; Sweigard *et al*., 1998; Tanaka *et al*., 1999). The major advantage of REMI is that it provides a method by which genes can be randomly disrupted via plasmid insertion, and also allows for the subsequent cloning of these genes via plasmid rescue in *Escherichia coli* (Sánchez *et al*., 1997). Moreover, in many cases, this technique has been associated with increases in transformation frequencies. In the nematode-trapping fungi, REMI was initially used by Tunlid *et al.*, to transform *Arthrobotrys oligospora* (1999). Here, we report the transformation of the nematode-trapping fungus, *M. sphaeroides* to a hygromycin B resistant strain, using REMI methodology, with special reference to the associated increase in transformation frequency, as well as the generation of morphological mutants.

Materials and Methods

Fungal strain and transformation vector

The nematode-trapping fungus, *M. sphaeroides* Castaner (YMF1.00539), was used in this study. This fungal strain was originally isolated from soil, and stored in the Fungal Store Centre of our laboratory. Two different plasmids harboring the hygromycin B phosphotransferase (*hph*) gene from *Escherichia coli* were used, under the control of different heterologous fungal promoters. The pBChygro plasmid (Silar, 1995) harbors the *cpc-1* promoter from *Neurospora crassa* and the *trpC* terminator from *Aspergillus nidulans*. The pCB1003 plasmid was used under the control of the *A. nidulans trpC* promoter and terminator.

Transformation protocol

Protoplasts were prepared according to the methods described by Liang *et al*. (1981) and Tunlid *et al*. (1999), with some modifications. In brief, the mycelia and conidia from 5-day-old cultures (60 mm plate) on TGA medium (10 g tryptone, 10 g glucose, 1 liter water, 18 g agar) were collected, and inoculated in 250 ml Erlenmeyer flasks containing 50 ml of TG medium (TGA minus agar). After 36 h of incubation at 165 rpm, and 28°C, the mycelia were harvested and washed with 50 ml of MN buffer (0.3 mol/liter $MgSO_4$, 0.3 mol/liter NaCl). Approximately 0.5 g of wet mycelia were suspended in 5 ml of MN buffer containing 5 mg/ml snailase (Beijing Jingke Company, China), and 5 mg/ml cellulase (Yakult Honsha Company, Japan). After 5 h of digestion at 200 rpm, and 32°C, the protoplasts were filtered through four layers of lens polishing tissue (Hangzhou Xinghua Company, China), which had been fitted into a funnel in order to remove undigested mycelia, and then precipitated via 10 min of centrifugation at 5000 rpm. The pelleted protoplasts were then washed in 5 ml of KTC buffer (1.2 mol/liter KCl, 10 mmol/liter Tris-HCl, pH 7.5, 50 mmol/liter $CaCl_2$), and resuspended in 1 ml of KTC for immediate transformation.

The transformation was conducted according to the methods described by Lu *et al*. (1994) and Tunlid *et al*. (1999), with some modifications. In brief, 150 μl of protoplasts (suspended in KTC, circa 8.0×10^7/ml) were mixed with 60 U *Hind*III and 10 μg linear pBChygro DNA (linearized by 20 U *Hind*III in 150 μl of 1×restriction enzyme buffer just before transformation) in a 5 ml centrifuge tube. After 40 min of incubation on ice, 600 μl of PTC (50% polyethylene glycol 6000, PEG 6000, 20 mmol/liter Tris-HCl, pH 7.5, 50 mmol/liter $CaCl_2$) was added into the mixture and mixed gently. The mixed suspension was then incubated for 30 min at 28°C, and was plated with 10 ml PDAS medium (potato dextrose broth agar medium, PDA, supplemented with 10 g/liter molasses, 0.6 mol/liter saccharose, 0.3 g/liter yeast extract, 0.3 g/liter tryptone and 0.3 g/liter casein peptone) containing 200 μg/ml of hygromycin B (Yunke Bioproduct Company, China). The transformant colonies were isolated after 6-9 days of incubation at 28°C.

Mitotic stability and Southern Blot analysis

The stability of the hygromycin B resistance of the transformants was tested in accordance with the methods established by Tunlid *et al*. (1999). In brief, Mycelial discs (diameter 4 mm) acquired from the edges of transformant colonies were serially transferred five times (one week intervals) to PDA medium, with or without 150 μg/ml of hygromycin B.

Genomic DNA was extracted from liquid-grown mycelia according to the method described by Persson *et al*. (1996). Probe labeling and hybridization were conducted with Digoxigenin-dUTP Kits (Boehringer Mannheim, USA) and Nylon membranes (positively charged, Cat. No. 1209299, Roche, Germany), according to the manufacturer's instructions.

Isolation and analysis of mutants

Mycelial disks from the wild-type and transformant strains were transferred to water agar (WA) plates, and incubated for 5 days at 28°C. The mycelial growth rate and morphological features of the colonies, mycelia, conidia, and conidiophores were examined in order to screen for morphologically altered mutants. Then, in order to screen the mutants exhibiting trapping-device deficiencies, the transformant colonies on the WA plates were challenged with free-living nematodes (*Panagrellus redivivus*). In brief, a 2 cm^2 piece of agar in the center of the plate was removed from a 5-day-old culture, creating an open space. About 200 nematodes were introduced into this free space after the mycelia had emerged from the cut margin. Trapping-device formation potential was assessed in this sample after another 3 days of incubation at 28°C. Those transformants which did not produce trapping-devices were selected for further analyses.

The predation rates were then used to evaluate the

degree to which the pathogenicity toward nematodes of the selected mutants had been altered. The mutants and the wild-type strain were both inoculated into WA medium. After 1 week of incubation at 28°C, about 200 nematodes (*P. redivivus*) were introduced to each of these plates. After 3 more days of incubation at 28°C, the predation rate of each of the strains was calculated, by recording the proportion of nematodes trapped by the trapping-devices. This experiment was repeated once, with five replicates for each isolate. The predation rate of a strain was calculated according to the following formula:

$$\text{Predation rate} = \frac{\text{Number of nematodes trapped by trapping - device}}{\text{Total number of nematodes observed}} \times 100$$

Results

Transformation

The protoplast yield of *M. sphaeroides* was 1.0 ~ 1.9×10^7/ml, and these protoplasts were observed to regenerate at a ratio of 34 ~ 72% on PDAS medium. When these protoplasts were incubated in PEG buffer (10 ~ 50%, MW6000) and handled according to REMI protocol, without adding DNA or restriction enzyme, the regeneration frequency was reduced, to approximately 7 ~ 41%. The growth of the wild-type *M. sphaeroides* strain on the PDA medium, and the regeneration of its protoplasts in PDAS medium, were inhibited completely via the addition of 150 μg/ml of hygromycin B into the media.

The transformants became visible after 6 ~ 9 days of incubation at 28°C. The transformation frequency was usually correlated with changes in a host of factors (Tunlid *et al*., 1999) and the most dramatic effects were observed when varying the PEG concentrations, the category and quantity of the added restriction enzymes, as well as the use of non-use of a linearizing plasmid. The transformation frequencies observed using PTC at volumes of 200, 400, 600 and 800 μl per transformation treatment (using linearized pBChygro DNA and 40 U *Hind*III) were 13.2, 19.2, 36.7 and 21.2 transformants/μg DNA (repeated twice with similar results). When varying the concentration of *Hind*III to 0, 20, 40, 60 and 80 units per transformation treatment (using linearized pBChygro DNA and 500 μl PTC), the frequencies were 3.7, 16.2, 37.6, 58.6 and 31.2 transformants, respectively (repeated twice). Under optimized conditions, the use of linearized pBChygro DNA resulted in an increase in the transformation frequency by about 5 fold over that observed with circular DNA treatment (which yielded 9~18 transformants/μg DNA).

Our REMI protocol, as compared with the protocols established by Lu *et al*. (1994) and Tunlid *et al*. (1999), was somewhat improved. In brief, during the final step of transformation, the reaction solution was mixed with the medium and hygromycin B prior to being poured into a petri dish, rather than being mixed only with medium, and then an upper layer medium containing hygromycin B was overlaid after an overnight incubation. This measure resulted in a 50% reduction in the amount of hygromycin B required, and also simplified the protocol, while still maintaining the required transformation frequency.

Under optimized conditions, the transformation frequencies varied, between 46 ~ 94 transformants per μg^{-1} DNA, when using pBChygro and *Hind*III in the transformations. When *Sma*I was used to replace *Hind*III, the transformation frequency decreased, to 27 ~ 53 transformants per μg^{-1} DNA. When pCB1003 and *Sac*I were used in the manipulation, the transformation frequencies ranged between 32 and 68 transformants per μg^{-1} DNA (lower than that seen when using pBChygro and *Hind*III). However, in the absence of restriction enzymes, the transformation frequencies were noted to decrease to 1 ~ 5 transformants/μg DNA, despite the use of either pBChygro or pCB1003.

Using this protocol, we also successfully transformed two other species of nematode-trapping fungi, *M. candidum* (YMF1.00543) and *A. vermicola* (YMF1.00534).

Mitotic stability and Southern Blot analysis of transformants

A high percentage (90%) of transformants was determined to be mitotically stable when 20 randomly selected transformants (obtained using pBChygro and *Hind*III) were tested. These transformants retained their level of resistance to hygromycin B, even after 5 transfers between selective and non-selective media.

15 randomly-selected transformants (lane 3 ~ 17 in Fig. 1; obtained using pBChygro and *Hind*III) were analyzed via Southern blotting, in order to confirm the integration of the vector DNA into the genome. The digested genomic DNA (digested with *Hind*III, 10 ~ 15 μg loaded into each lane) of 15 transformants was hybridized to the probe (pBChygro labelled with Digoxigenin-dUTP), and exhibited 1 to 2 hybridizing bands. The probe hybridized to the linearized pBChygro at a size of 6.8 kb (Fig. 1, lane 1) and did not hybridize to genomic DNA from the untransformed wild-type *M. sphaeroides* strain (YMF1.00539) (Fig. 1, lane 2).

Isolation and analysis of mutants

We successfully utilized this transformation procedure to introduce random mutations into *M. sphaeroides.* Five mitotically stable mutants (Tms32, Tms1536, Tms1913, Tms2316 and Tms3583), all of which exhibited significant alterations in colony morphology, growth rate, sporulation, and pathogenicity toward nematodes, were selected from over 5000 transformants. These five mutants were

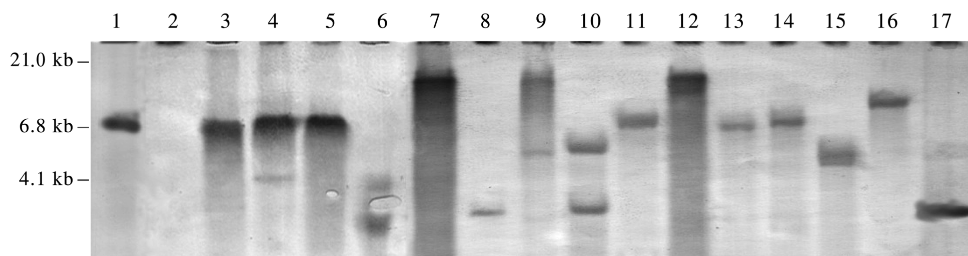

Fig. 1. Southern analysis of randomly-selected transformants. DNA digested with *Hind*III and hybridized with Digoxigenin-dUTP labled pBChygro. Lane 1 contains pBChygro. Lane 2 contains genomic DNA from wild-type *M. sphaeroides*. Lanes 3~17 contain genomic DNA from 15 randomly-selected transformants. Molecular mass markers (kb) are indicated on the left.

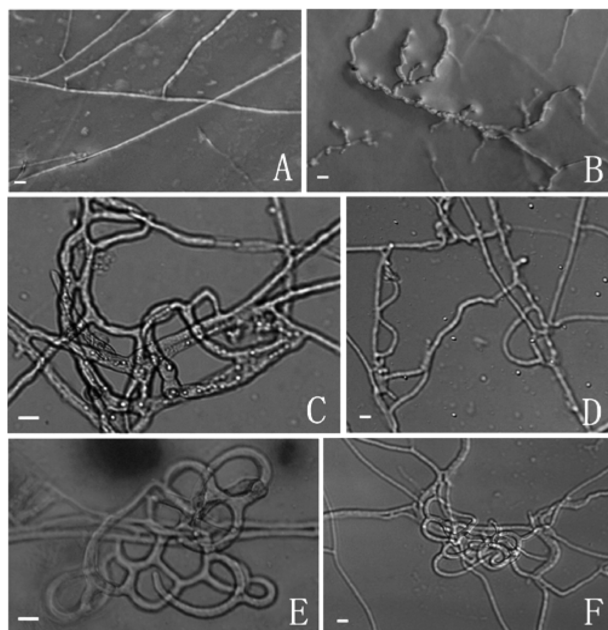

Fig. 2. A~B, the natural mycelia of wild type *M. sphaeroides* on CMA medium (A) and the malformed mycelia of Tms1536 (B). C~F, the atypical trapping-device of Tms1913 on WA media (C and D) and the typical trapping-device of the wild-type strain (E and F). Bars = 20 μm.

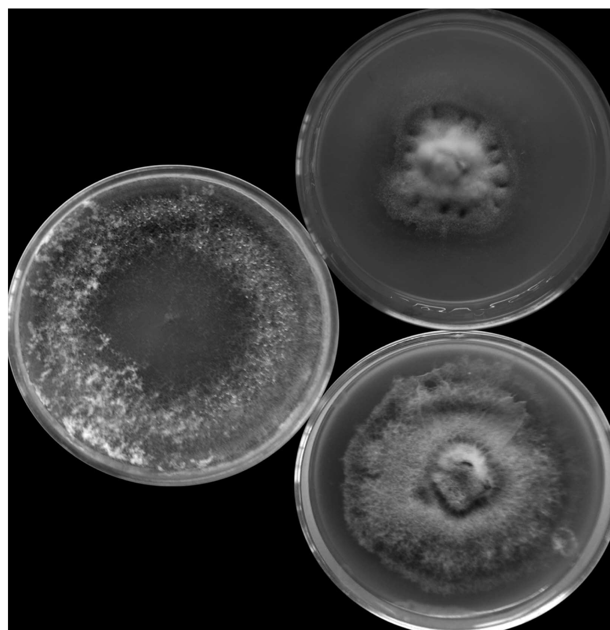

Fig. 3. Colonies of wild-type *M. sphaeroides* (left), and the mutants, Tms1536 (top right) and Tms32 (bottom right), grown for 1 week on TGA medium.

obtained via the transformation of *M. sphaeroides* with pBChygro in the presence of *Hind*III, and all were confirmed to have retained their levels of resistance to hygromycin B and morphological alterations, even after more than 5 transfers between selective and non-selective media. Tms1536 exhibited mycelial malformations (Fig. 2B) on corn meal agar (CMA) medium. Tms32 proved unable to form conidia, and manifested mycelial malformations similar to those observed with Tms1536. Tms1913 formed a trapping-device (Fig. 2C and D), but a morphologically different trapping-device than was observed in the wild-type strain (Fig. 2E and F). Tms3583 proved unable to form the trapping-device, and Tms2316 was unable to form both the trapping-device and the conidia. The mycelial growth rates of Tms1913, Tms2316 and Tms3583 on TGA were similar to that of the wild-type strain, but the mycelial growth rates of Tms32 and Tms1536 were observed to have decreased by more than 50%. Furthermore, the Tms32 and Tms1536 variants exhibited significant folding on the surface and the irregular edges of the colonies growing on TGA, as compared to the wild-type strain (Fig. 3). In addition, the predation rates of Tms32, Tms1536, and Tms1913 decreased to 0, 4% and 12% comparing with the rate of the wild-type strain (34%, average of 10 replicates). Tms2316 and Tms3583 manifested no pathogenicity toward nematodes.

Southern Blot analysis of the mutants

All five of the selected mutants were generated via transformation using pBChygro (6.8kb) in the presence of *Hin*dIII. In order to characterize the vector DNA integration patterns, the genomic DNA of the five mutants was digested with *Hind*III (the enzyme used in REMI, possesses one cut site in pBChygro), *Bgl*II (no cut site in pBChygro) or *Mlu*I (one cut site in pBChygro), followed

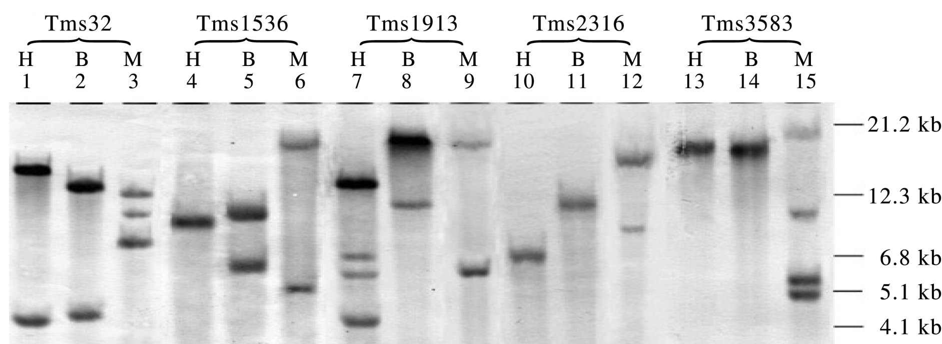

Fig. 4. Southern analysis of the five mutants. Genomic DNA from each of the mutants was digested with *Hind*III, *Bgl*II, or *Mlu*I, and hybridized with Digoxigenin-dUTP labled pBChygro. H = *Hind*III, B = *Bgl*II, M = *Mlu*I. Molecular mass markers (kb) are shown on the right.

by Southern blot analysis using labelled pBChygro as a probe (Fig. 4).

Tms2316 manifested a single hybridization band at the size of pBChygro (6.8 kb) when its genomic DNA was digested with *Hind*III (Lane 10 in Fig. 4), one band (circa 10kb) when digested with *Bgl*II (Lane 11 in Fig. 4), and two bands when digested with *Mlu*I (Lane 12 in Fig. 4). Tms3583 displayed a hybridization fragment larger than 6.8 kb in size when digested with *Hind*III or *Bgl*II (Lane 13 and 14 in Fig. 4), and four hybridization bands when digested with *Mlu*I (Lane 15 in Fig. 4). Tms1536 exhibited a single hybridization band (a little larger than 6.8kb) when digested with *Hind*III (Lane 4 in Fig. 4), two bands (circa 5.2 kb and 18 kb) when digested with *Mlu*I (Lane 6 in Fig. 4), and two hybridization bands when digested with *Bgl*II (Lane 5 in Fig. 4). Tms32 contained two bands (circa 4.2 kb and 14 kb) when digested with *Hind*III (Lane 1 in Fig. 4), two bands (circa 4.4 kb and 10.0 kb) when digested with *Bgl*II (Lane 2 in Fig. 4), and three bands when digested with *Mlu*I (Lane 3 in Fig. 4). Tms1913 exhibited four bands when digested with *Hin-d*III (Lane 7 in Fig. 4), two bands when digested with *Bgl*II (Lane 8 in Fig. 4), and two bands when digested with *Mlu*I (Lane 9 in Fig. 4).

Discussion

In this study, the REMI method was shown to increase transformation frequencies from 1 ~ 5 transformants/μg DNA (in the absence of restriction enzyme) to 46 ~ 94 transformants/μg DNA (in the presence of the enzyme). Similar results were reported in experiments involving fungal REMI transformation. Tunlid *et al.* (1999) reported an increase in frequency, from 2 to 5 transformants/μg DNA, when transforming *A. oligospora*. Black *et al.* (1995) determined that *Bam*HI increased the number of transformants by 2 ~ 5-fold over the control without enzyme addition, and *Not*I was even more effective with regard to the enhancement of transformation (29 ~ 46-times). The mechanism by which REMI augments transformation frequency has been explained as follows: the restriction enzyme cuts the genomic DNA, providing sites for the integration of the vector DNA. Additionally, we achieved frequency increases by optimizing the following factors: the PEG concentration, and the category and quantity of the added restriction enzymes. This indicates that the REMI method may still hold the potential to increase transformation frequency under further optimized conditions.

Although we successfully obtained five morphological mutants from over 5000 transformants, it appears that the probability of mutant generation attributable to REMI is actually quite low. In order to screen more target mutants via the REMI method, a high transformation frequency is key. The morphological deficiency in the five mutants, including malformations of the mycelia or trapping-device and the absence of conidia or (and) trapping-device, suggests that REMI is, however, a useful method for the construction of a variety of mutants which can be used in morphological and genetic fungi studies.

The results of Southern blot analysis of randomly selected transformants (Fig. 1) showed that the vector DNA had been successfully inserted into the fungal genome, and the differences in the size and the number of hybridization bands indicated the different integration patterns. A single band, identical in size to the pBChygro vector (Fig. 1, lane 3, 5, 11, 13 and 14) can be expected when the vector is inserted into a genomic *Hind*III site. A smaller, single band (Fig. 1, lane 8, 15 and 17) indicates that the integrated DNA has been rearranged. The other transformants were determined to contain larger, single bands (Fig. 1, lane 7, 12 and 16); or more than one band (Fig. 1, lane 4, 6, 9 and 10), suggesting that the vector had been integrated into non-*Hind*III sites, or that the restriction site was not maintained during insertion.

The results of Southern blot analysis of the 5 mutants (Fig. 1) highlighted differences in the observed integration patterns. The results of Southern blot analysis of Tms2316 (Lane 10, 11, 12 in Fig. 4) revealed that intact pBChygro had been inserted into a single genomic *Hind*III site, and

also revealed the regeneration of the *Hind*III site. Transformants with the integration patterns resembling Tms2316 were previously considered as the ideal materials for cloning the tagged genes via plasmid rescue method (Schiestl and Petes, 1991; Lu *et al*., 1994; Sánchez *et al*., 1997). In Tms3583, a hybridization fragment larger than 6.8 kb, observed in conjunction with both *Hind*III or *Bgl*II digestion (Lane 13 and 14 in Fig. 4), indicated that the vector had been inserted at a single location in the genome, but had been modified. The observation of four hybridization bands when the mutant was digested with *Mlu*I (Lane 15 in Fig. 4) suggested that integrated plasmid rearrangements had occurred. Tanaka *et al.* (1999) obtained a mutant with an integration pattern similar to Tms3583 from the transformation of *Alternaria alternata,* and from this they successfully cloned two genes required for the biosynthesis of the host-specific AK-toxin. Tms1536 exhibited a single hybridization band (a little larger than 6.8 kb) when digested with *Hind*III (Lane 4 in Fig. 4), and exhibited two bands (circa 5.2 kb and 18 kb) when digested with *Mlu*I (Lane 6 in Fig. 4). This suggested that the vector DNA had been inserted at a single location within the genome. However, the two hybridization bands observed in conjunction with *Bgl*II digestion (Lane 5 in Fig. 4) were attributed to the rearrangement of the integrated plasmid. The results of the Southern blots of Tms32 (Lane 1, 2, 3 in Fig. 4) could be explained as the integration of two modified copies of vector DNA into two different genomic sites. The results of Southern blots of Tms1913 (Lane 7, 8, 9 in Fig. 4) verified that the vector DNA had been inserted into the genome, but this pattern was too vague to make any confident conclusions.

Southern blot analysis of the 15 randomly selected transformants and the 5 mutants indicated that 14 of the 20 variants may have harbored rearranged plasmids. These rearrangement and modification phenomena in integrated DNA had also been described in other microorganisms, in other experiments with REMI (Schiestl and Petes, 1991; Lu *et al*., 1994; Sánchez *et al*., 1997; Tanaka *et al*., 1999; Tunlid *et al*., 1999), and the causes for this remain somewhat unclear. However, experiments conducted with other fungi have demonstrated that such integration relies on the restriction enzymes used, and also on whether or not the vector DNA has been linearized prior to integration (Riggle and Kumamoto, 1998).

We have obtained two mutants, Tms2316 and Tms3583, which exhibit integration at a single location, and clear morphological deficiencies. The isolation of these mutants should prove valuable, and may be ideal for the cloning of genes involved with the formation of the trapping-device and the conidia. We are currently using the two mutants to clone the genes involved in trapping-device and conidia formation, and are also attempting to isolate more mutants, using different plasmids/restriction enzymes, for further functional analysis of a variety of genes, as well as for further gene cloning.

In conclusion, we have established an effective REMI protocol for the induction of increased transformation frequencies in *M. sphaeroides,* and have successfully utilized this transformation procedure to introduce random mutations into this fungus. It is worthy of note that we obtained mutants exhibiting significant morphological deficiencies, which will make it possible to clone the genes involved in the formation of the trapping-device and conidia in nematode-trapping fungi.

Acknowledgments

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References

Åhman, J., E.K. Bo, L. Rask, and A. Tunlid. 1996. Sequence analysis and regulation of a cuticle degrading serine protease from the nematophagous fungus *Arthrobotrys oligospora*. *Microbiology* 142, 1605-1616.

Black, M., F. Seeber, D. Soldati, K. Kim, and J.C. Boothroyd. 1995. Restriction enzyme-mediated integrating elevates transformation frequency and enables co-transfection of *Toxoplasma gondii*. *Molecular and Biochemical Parasitology* 74, 55-63.

Bölker, M., H.U. Böhnert, K.H. Braun, H. Görl, H.B. Jansson, A. Tunlid, and B. Nordbring-Hertz. 1997. Biological control: nematodes, p. 38-50. *In* Anke, T., (ed). Fungal Biotechnology. Chapman and Hall, Weinheim, Germany.

Kuspa, A. and W.F. Loomis. 1992. Tagging developmental genes in Dictyostelium by restriction enzyme-mediated integration of plasmid DNA. *Proc. Natl. Acad. Sci. USA* 89, 8803-8807.

Liang, P.Y., H.D. Liu, and X.F. Xiao. 1981. Some factors affecting the formation and regeneration protoplasts in two *Penicilium chrysogenum* auxotrophs. *Acta Phytothysiologia Sinica* 7, 1-10.

Lu, S., L. Lyngholm, G. Yang, C. Bronson, O.C. Yoder, and B.G. Turgeon. 1994. Tagged mutations at the Tox1 locus of *Cochliobolus heterostrophus* by restriction enzyme-mediated integration. *Proc. Natl. Acad. Sci. USA* 91, 12649-12653.

Persson, Y., S. Erland, and H-B. Jansson. 1996. Identification of nematode-trapping fungi using RFLP analysis of the PCR-amplified ITS region of ribosomal DNA. *Mycol. Res.* 5, 531-534.

Riggle, P.J. and C.A. Kumamoto. 1998. Genetic analysis in fungi using restriction-mediated integration. *Curr. Opin. Microbiol.* 1, 395-399.

Rosén, S., K. Sjollema, M. Veenhuis, and A. Tunlid. 1997. A cytoplasmic lectin produced by the fungus *Arthrobotrys oligospora* functions as a storage protein during saprophytic and parasitic growth. *Microbiology* 143, 2593-2604.

Sánchez, O., R.E. Navarro, and J. Aguirre. 1997. Increased trans-

formation frequency and tagging of developmental genes in *Aspergillus nidulans* by restriction enzyme-mediated integration (REMI). *Mol. Gen. Genet.* 258, 89-94.

Schiestl, R.H. and T.D. Petes. 1991. Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 88, 7585-7589.

Scholler, M., G. Hagedorn, and A. Rubner. 1999. A reevaluation of predatory Orbiliaceous fungi. II. A new genetic concept. *Sydowia* 51, 89-113.

Shi, Z., D. Christian, and H. Leung. 1995. Enhanced transformation in *Magnaporthe grisea* by restriction enzyme mediated integration of plasmid DNA. *Phytopathology* 85, 329-333.

Silar, P. 1995. Two new easy to use vectors for transformations. *Fungal Genet. Newsl.* 42, 73.

Stirling, G.R. and A. Mani. 1995. The activity of nematode-trapping fungi following their encapsulation in alginate. *Nematologica* 41, 240-250.

Stirling, G.R., L.J. Smith, K.A. Licastro, and M.E. Lois. 1998. Control of root-knot nematode with formulations of the nematode-trapping fungus *Arthrobotrys dactyloides*. *Biol. Control* 11, 224-230.

Sweigard, J.A., A.M. Carroll, L. Farrall, F.G. Chumley, and B. Valent. 1998. *Magnaporthe grisea* pathogenicity genes obtained through insertional mutagenesis. *Mol. Plant-Microbe Interact.* 11, 404-412.

Tanaka, A., H. Shiotani, M. Yamamoto, and T. Tsuge. 1999. Insertional mutagenesis and cloning of the genes required for biosynthesis of the host-specific AK-Toxin in the Japanese pear pathotype of *Alternaria alternata*. *Mol. Plant-Microbe Interact.* 12, 691-702.

Tunlid, A., J. Ahman, and R.P. Oliver. 1999. Transformation of the nematode-trapping fungus *Arthrobotrys oligospora*. *FEMS Microbiology Letters* 173, 111-116.

Tunlid, A. and S. Jansson. 1991. Proteases and their involvement in the infection and immobilization of nematodes by the nematophagous fungus *Arthrobotrys oligospora*. *Appl. Environ. Microbiol.* **57**, 2868-2872.

Zhao, M.L., M.H. Mo, and K.Q. Zhang. 2004. Characterization of a neutral serine protease and its full-length cDNA from the nematode-trapping fungus *Arthrobotrys oligospora*. *Mycologia* 96, 16-22.