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Use of molecular markers for monitoring fungi involved in stalk rot of corn

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Summary. Separate genes conferring antibiotic drug resistance have been inserted into *Fusarium graminearum* and *Fusarium moniliforme*. These organisms are associated with stalk rot of corn, a disease of uncertain cause. Antibiotic resistant fungi were obtained by developing a gene transfer system using whole cells as recipients for DNA. Hygromycin B and benomyl-resistant colonies were isolated by treating fungal tissue with lithium acetate and adding plasmid vectors containing the respective genes which give drug resistance. The DNA was stably integrated into the fungal chromosome. Following plant inoculation, disease symptoms developed and the isolates were recovered on selective medium. In each case, these fungi retained the transformed phenotype, although extensive rearrangements and/or deletions occurred. Specific molecular tagging allows detailed studies of this interaction and should be of general use in situations involving complex multiple pathogen diseases.

Key words: *Fusarium* spp. – Benomyl – Gene transfer – Hygromycin B – Molecular tagging

Introduction

Stalk rot of corn is a disease of uncertain etiology. *Fusarium moniliforme* and *Fusarium graminearum* have been consistently associated with the disease and as such have been putatively accepted as having causal roles in symptom expression in the corn plant. Both of these organisms, as well as others, may be isolated from apparently healthy plants during the growing season and are also present at maturity when, under appropriate (stress) conditions, symptoms are expressed. However, despite extensive studies (Christensen and Wilcoxson 1966; Kommedahl et al. 1978; Kommedahl and Windels 1981; Lengkeek 1979; Marshal and Partridge 1981a, b), evidence is far from conclusive. The ubiquity of these two species compounds the difficulty in proving that the specific organism inoculated is the same as that recovered, and the precise role of these Fusaria in this complex disease process remains unknown. If Fusaria with specific distinguishing markers were available, the fate of inoculated species could be more easily monitored.

Towards this end, we have introduced selectable markers into *Fusarium moniliforme* and *Fusarium graminearum*. Specifically, separate antibiotic drug resistance genes, hygromycin B phosphotransferase (Kaster et al. 1983) and the β-tubulin gene from *N. crassa* (Orbach et al. 1986) have been introduced into these stalk rot fungi to serve as molecular "tags". Hygromycin phosphotransferase confers resistance to the broad spectrum aminoglycoside antibiotic hygromycin B and has been successfully transformed into a number of fungi and other eukaryotes (Kaster et al. 1984; Rodriguez and Yoder 1987; Turgeon et al. 1987; Dickman 1988; Wang et al. 1988). The β-tubulin gene gives resistance to the anti-microtubule fungicide, benomyl (Vollmer and Yanofsky 1986). Transformation was accomplished using whole fungal cells and lithium acetate. This technique originally developed for yeast (Ito et al. 1983), and later *Neurospora* (Dhawale et al. 1984), has been recently extended to fungal phytopathogens (Dickman and Kolattukudy 1987; Dickman 1988). We report successful transformation of these Fusaria and the stable integration of drug resistance markers. Furthermore, we have looked at the stabilities of the transformed phenotypes in the plant host. Finally, we demonstrate that these transformed fungi can be recovered and separated from the inoculated, infected host tissue by virtue of their drug resistance.

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Materials and methods

Strains and culture conditions E. coli strain DH5α (Hanahan 1983) was used as recipient for bacterial transformation and routine propagation of plasmids. *Fusarium moniliforme* Sheldon (M-1068) and *Fusarium graminearum* Schwabe (R-4839) were provided by the Fusarium Research Center (Pennsylvania State University). Single-spore isolates were obtained and maintained on 10% V-8 juice agar (VJA) at 28 °C. For liquid culture, fungi were grown on cutin mineral medium (CMM) substituting 2% glucose for the carbon source (Dickman 1988). Cultures were grown at 28 °C at 80 rpm.

Transformation vectors. The cosmid vector pSV50 was obtained from Dr. C. Yanofsky, Stanford University. This 7.9-kb vector harbors a 2.6-kb fragment containing the *N. crassa* β-tubulin gene which confers resistance to benomyl (Vollmer and Yanofsky 1986). The plasmid p1TH, generously provided by Dr. O. C. Yoder, Cornell University, contains a truncated hygromycin resistance gene translationally fused to promoter elements of *Cochliobolus heterostrophus* (Turgeon et al. 1987). A 2.4-kb *Sal*-1-*Hin*dIII fragment containing the promoter-gene fusion was gel-purified and ligated to a similarly digested phagemid, Bluescript + (Stratagene). The resulting construction yielded a 5.4-kb plasmid designated pBHM-1.

Transformation protocol. R-4839 and M-1068 thalli were made competent for DNA uptake with lithium acetate. This procedure has recently been described in detail (Dickman 1988). In brief, fungal mycelia and spores were grown in liquid culture and harvested in the log phase of growth. After washing lithium treatment and centrifugation, the tissue was resuspended in a minimal volume of 0.1 *M* lithium acetate and vector DNA (10–20 μg in 10–50 μl TE [10 m*M* TRIS, 1 m*M* EDTA] pH 7.6 containing 4 m*M* spermidine and 1 m*M* spermine) was added. After 30 min at 30 °C, 40% PEG 4,000 was added and the incubation was extended an additional hour. Following a brief heat schock (5 min, 37 °C), the tissue was washed twice and densely plated on VJA plates. Following a 20 h incubation at room temperature, plates were overlaid with 4 ml of 1% agar containing 3 mg of hygromycin B or of carbendazim (MBC). (Final concentrations, 100 μg/ml and 0.75 μg/ml, respectively.) MBC (Dupont) is the active ingredient in benomyl and was kindly provided by Dr. C. P. Woloshuk (North Carolina State University). MBC was dissolved at 1 mg/ml in dimethylsulfoxide (DMSO) prior to use. Colonies appeared in 5–14 days and were transferred to fresh media containing either 100 μg/ml of hygromycin B or 1 μg/ml of MBC.

Mitotic stability and vegetative growth rates. The mitotic stability of the presumptive transformants was assessed by growing hyg^r (hygromycin resistant) and MBC^r (carbendazim resistant) isolates on rich non-selective media (VJA). Following 7 days growth, agar plugs from the colony edges were transferred to the respective antibiotic-containing media. Growth rates for the isolates were evaluated by monitoring radial growth. An agar plug was placed on the edge of a petri dish and mycelial growth was measured at 2-day intervals for 10 days.

DNA Manipulations. DNA was isolated by either of two miniprep procedures previously described (Raeder and Broda 1985; Dickman 1988). DNA was digested and ligated according to manufacturer's recommendations. Large-scale plasmid preparation and minipreps were by the alkaline lysis procedure (Birnboim and Doly 1979). Bacteria were transformed using $CaCl_2$ (Mandel and Higa 1970) with selection on Luria Broth (Maniatis et al. 1982) or 2YT (16 g tryptone, 10 g yeast extract, 5 g NaCl, 1 l dH_2O) plates containing 50 μg/ml carbenicillin. DNA probes were isolated and labeled by nick translation (Rigby et al. 1977) to a specific activity of $5 \times 10^7 - 10^8$ cpm/μg. DNA was transferred to nitrocellulose by the method of Southern (1975) as described by Maniatis et al. (1982). Blots were autoradiographed with Kodak X-OMAT film and Dupont Cronex intensifying screens at −70 °C.

Plant inoculation and fungal isolation. Six transformed isolates each of M-1068, denoted MH1, MH2, etc., and R-4839, denoted RB1, RB2, etc., with resistance to hygromycin or carbendazim, respectively, plus the original non-transformed cultures were grown on toothpicks in liquid medium (clarified V-8) at 30 °C for 2 weeks (Young 1943). By insertion of an infested toothpick, 2-week post-anthesis corn plants (Mo17 × B73) were inoculated in the second internode above the soil. The plants were transplanted, three plants per pot, two of which were inoculated and the third served as a check. Plants were inoculated with a single isolate; mixed infections were not performed. The plants were grown in a BL 2-P approved greenhouse and all plants and debris were autoclaved before disposal.

Isolations were made from the second nodal plate above the inoculation site on the 2nd, 7th, and 21st days after inoculation. Isolations were made to VJA agar plates amended with hygromycin (100 μg/ml) or MBC (75 μg/ml) as appropriate. Control (unwounded, wounded non-inoculated, wounded M-1068, inoculated, and R-4839 inoculated) plants were isolated onto VJA agar with and without the antibiotics.

Transformed isolates were maintained in active culture for 3 months and the above experiment was repeated.

Results

Fusarium moniliforme (M-1068) served as the recipient for transformation with pBHM-1 with selection based on resistance to hygromycin B (hyg B), and *Fusarium graminearum* (R-4839) was transformed with pSV50 DNA and selected on plates containing MBC. The minimum concentration of antibiotic which completely inhibited fungal growth was determined by incorporating serial dilutions of either hygromycin or MBC into VJA. An agar plug of the appropriate species was placed on the edge of the plate and radial growth rate was compared to wild-type parental Fusaria placed in VJA with no antibiotic. From these experiments, it was determined that with 100 μg/ml of hyg B and 0.75 μg/ml of MBC, fungal growth could not be detected so that these concentrations were used for selection and maintenance of the transformed isolates.

Using whole fungal cells and the lithium acetate procedure, resistant colonies were visible after 5–7 days for hyg B and 7–10 days for MBC. Screening and recovery of putative transformants was concluded approximately 2 weeks after the initial colonies were observed. Resistant colonies to either antibiotic were never observed when pUC18 was used as the donor DNA nor did spontaneous mutants exhibiting resistance to either drug occur.

Following the transformation procedure, a lag period was essential, prior to adding antibiotic. As has been previously reported for *Colletotrichum trifolii* (Dickman 1988), a lag period of 20–24 h was optimum.

Transformation frequencies for both species were relatively low. Using 5–25 μg of vector DNA, 0.4–5 stable

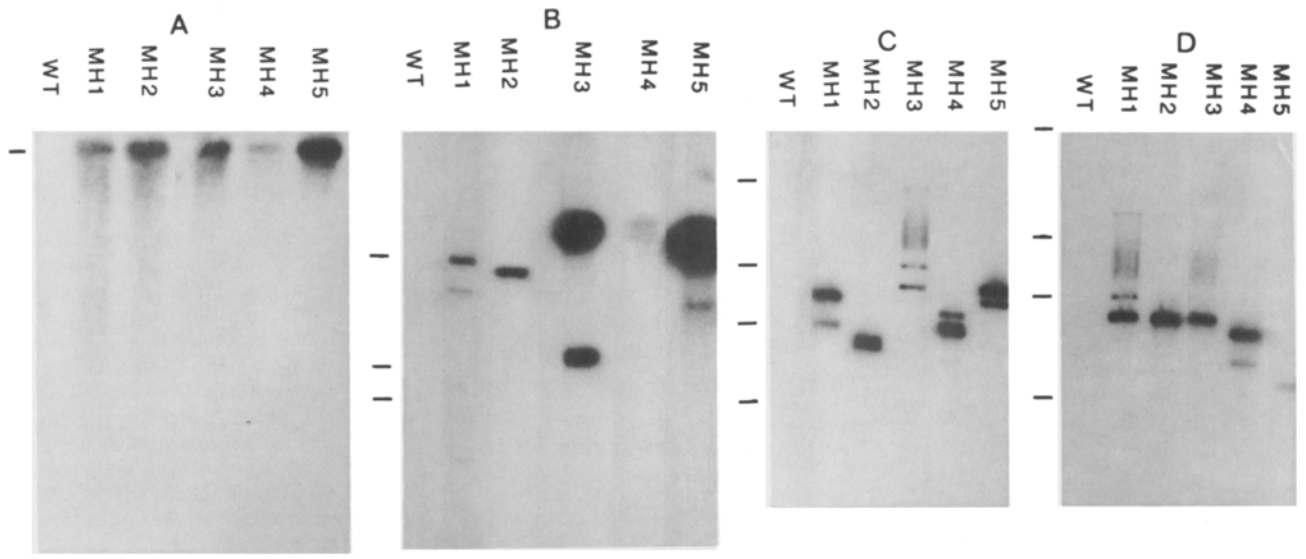

Fig. 1 A–D. Southern blot analysis of *Fusarium moniliforme* wild type (WT) and selected hygromycin B-resistant transformants. Between 5 and 10 μg of undigested total genomic DNA **A**, or DNA digested with *Sty*-1 **B** or *Sal*-1 **C** and **D** was electrophoresed in 0.7% agarose gels. Panel **D** is of reisolated fungal transformants from plant tissue. The filters were hybridized with ^{32}P-labelled pBHM-1 DNA. DNA digested with *Hind*III – *Eco* RI was used as a size marker, with *bars* indicating the location of the molecular weights. From top to bottom, the sizes are 21.7, 5.5, 3.4, and 2.0 kb

transformants/μg pBHM-1 and 0.2–1 stable isolates of R-4839 with pSV50 were obtained. Transformation rates are often strain- and vector-dependent (Hynes 1986). M-1068 repeatedly yielded lower frequencies (~50%) than R-4839 independent of the vectors, and pSV50 also yielded lower transformation frequencies than pBHM-1 with both fungi. Transformation rates did not differ significantly between linear and circular donor DNA. A determination of both mitotic stability and vegetative growth rates was made for each transformant. As with other fungal systems, the majority (70%–80%) of the presumptive transformants were abortive, losing their resistant phenotype. Isolates retaining expression of the molecular marker and exhibiting wild-type rates of growth were then further analyzed by Southern blotting and plant inoculations.

Hybridization analysis

Single spores from different mitotically stable hygromycin-resistant M-1068 transformants were isolated and grown on selective media. Spores were harvested and transferred to liquid medium containing 100 μg/ml hygromycin B. Following a 48-h incubation at 25 °C, the fungal tissue was harvested. Total DNA was isolated and analyzed by filter hybridization. Southern blots of undigested as well as restricted DNA are shown in Fig. 1. These blots were probed with nick-translated ^{32}P-labeled pBHM-1. Figure 1 A shows the result of probing undigested genomic DNA from the wild-type M-1068 and selected transformants. The untransformed fungus bears no detectable sequence homology to the vector, whereas the transformants display hybridization signals in the region of high molecular weight DNA. There are no signals in the lower molecular weight areas suggesting the absence of autonomous replicating plasmids. Attempts to transform *E. coli* (DH5) to ampicillin resistance with total genomic transformant DNA were unsuccessful. These data strongly suggest that transformation involved chromosomal integration of the hygromycin gene.

The nature of the integration events can be inferred from Fig. 1 B and C, which show blot analysis of digested fungal DNA. Restriction endonuclease *Sty*-1 (Fig. 1 B) does not cut within pBHM-1, while *Sal*-I (Fig. 1 C) restricts the vector at one site. Figure 1 B indicates that integration most likely occurred at different loci for each transformant and possibly at more than one site for MH1, MH3, and MH4, and MH5. In Fig. 1 C the pattern for MH1, MH3, and MH5 exhibits two unequally intense bands. This could be due to either tandem integration or perhaps insertion at a single site with rearrangement of flanking chromosomal sequences. MH4 has three bands in the *Sal*-I digestion, two of which are equal in intensity but less than the third. This strongly suggests a tandem duplication. MH2 digestion produced two bands of different sizes and indicating a single copy insertion.

DNA analysis of transformants with resistance to MBC are shown in Fig. 2. Undigested fungal DNA from these transformants hybridized only to high molecular weight DNA (not shown) and *E. coli* could not be transformed to carbenicillin resistance with genomic DNA from any of the transformants. As with the hyg^r isolates,

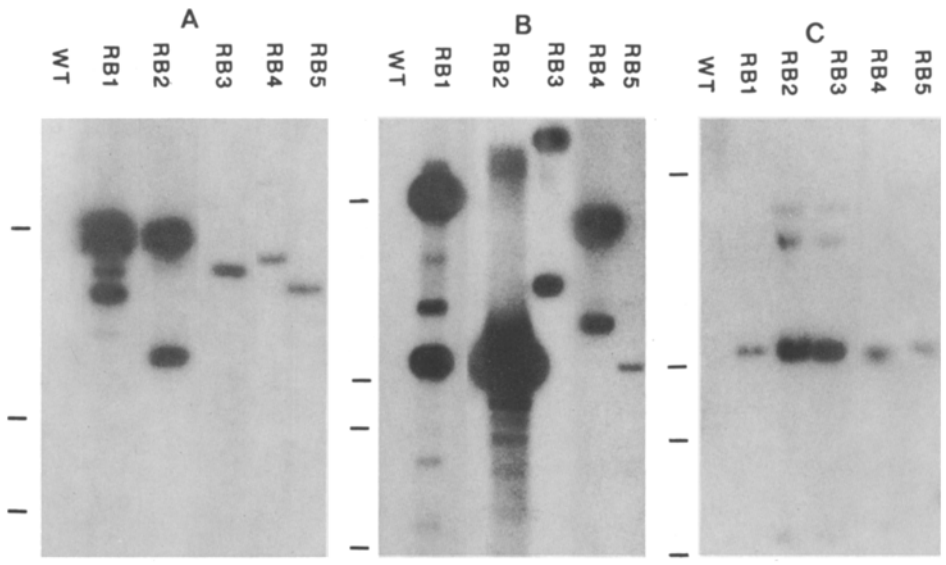

Fig. 2A–C. Southern blot analysis of *Fusarium graminearum* wild type (WT) and selected MBC^r transformants. *Stu*-1 digested genomic DNA is shown in **A** and *Pvu*-1 digestions of total genomic DNA are shown in **B** and **C**. Panel **C** is of reisolated fungal transformants from plant tissue. Blots were probed with ^{32}P-labelled pSV50. All other details are the same as given in Fig. 1

pSV50 appears to have integrated into the chromosome. Prolonged exposure of the autoradiograms and reduced stringency revealed hybridization signals with wild-type R-4839 DNA to the *N. crassa* β-tubulin gene. This is not surprising since the β-tubulin gene is widely conserved among diverse species (Neff et al. 1983), although we cannot rule out the remote possibility of weak plasmid homology to regions of the fungal genome.

The organization of the insertions is shown in Fig. 2A and B. RB1 and RB2 contain high molecular weight bands as a result of *Stu*-1 digestion (*Stu*-1 does not cut pSV50). These isolates have complex patterns, indicative of multiple insertions, some of which may be in tandem. The others appear to result from single integration events.

Plant inoculations and analysis

Classical stalk rot disease symptoms of tissue decomposition occur in mature plants well after the infection and penetration processes are complete. Since a primary aim of these studies was to study the behavior of these transformants following plant inoculation, with respect to initial in planta movement, DNA organization, and marker stability, both sets of transformants were inoculated into corn plants as described in 'Materials and Methods'. Under these conditions, the ramification of introduced organisms may be grossly observed by discoloration of vascular tissue. By the 2nd day after inoculation, vascular discoloration in inoculated plants was observed past the second nodal plate above the inoculation site. At 2-, 7-, and 21-day intervals, isolations were made. Selection for antibiotic resistance permitted reliable identification of the transformed fungi. Both hyg^r and MBC^r isolates were obtained at each time point. Southern analysis of *Stu*-1 digested DNA for hyg^r transformants or *Pvu*-1 restriction for MBC^r fungi was performed from isolations made 21 days following inoculation (Figs. 1D and 2C). Comparison of Figs. 1C and D, and 2B and C illustrates the hybridization patterns. Considerable deletions and/or rearrangements are apparent. A significant feataure of these results is the uniformity of both sets of transformants.

Southern blotting of transformants following passage through the host plant indicates that rearrangement may result in a minimal function unit. Since normal vascular discoloration symptoms occurred in the plant, these changes had no obvious effect on virulence or transcription and translation of the drug-resistance gene.

To investigate whether the pronounced alteration in banding pattern arose from plant interaction or simply time, we analyzed the original transformants maintained on petri dishes containing the appropriate antibiotic over a 21-day period. The hybridization pattern for the ten transformants was the same as shown in Figs. 1C and 2B with one exception. In this case (RB1), fewer bands were present (not shown). The marker gene was shown to be stable in culture over a 3-month period.

Discussion

This report demonstrates the development of a gene transfer system for two economically important species of *Fusarium, F. moniforme,* and *F. graminearum*. In addition we have shown the feasibility of using recombinant DNA technology to study a multiple pathogen disease complex. Via DNA transformation, different molecular markers have been stably integrated into separate fungal genomes with specific molecular tags. The presence and

expression of antibiotic resistance genes are easily observable on selective medium and are sufficiently stable to be recovered from the plant 3 months after the original transformation.

Following passage through host plant tissue, extensive rearrangement of the fungal transformant DNA occurred. The homogeneous pattern from both sets of the transformants was somewhat surprising. It will be of interest to determine whether any alteration in banding patterns occur following reinoculation of these isolates. Possibly, the foreign DNA has now rearranged to become very stable. Since we could only detect viable transformed isolates, there is no data as to what extent other possibly lethal alterations occurred.

While it has been determined that culture for 3 months in the laboratory or 21 days in the plant did not adversely affect the ability to reisolate the transformants, there is still a need to verify the stability of transformants over longer periods as well as in competition experiments in greenhouse or field situations. In addition, position effects and copy number with respect to stability and gene expression need to be examined. The success of these experiments, however, permits the investigation of many different parameters of stalk rot. The temporal and spatial rates of spread, the effect of stress on virulence and plant defense, and competitiveness between the two species within the plant can now be experimentally addressed. In addition, the route and effect of both kernel and seed infection as well as the effect(s) of soil inoculum can now be studied. The strategy of using molecular tags should be of general use with complex multiple pathogen diseases.

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