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Whole cell transformation of the alfalfa fungal pathogen *Colletotrichum trifolii*

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Summary. A transformation system for *Colletotrichum trifolii*, a fungal pathogen of alfalfa, has been developed using whole cells as recipients. Hygromycin B and benomyl resistant colonies were isolated following treatment of fungal tissue with lithium acetate and separate plasmids containing the respective genes which confer resistance to these antibiotics. The DNA was stably integrated into the fungal chromosome. This approach to transformation has general utility for phytopathogenic fungi and represents an initial step in the molecular analysis of virulence determinants in this race specific fungus.

Key words: Fungal transformation – *Colletotrichum trifolii* – Race-cultivar specificity

Introduction

The fungus *Colletotrichum trifolii* causes anthracnose of alfalfa. This highly evolved host-pathogen interaction exhibits race-cultivar specificity, which is poorly understood, particularly at the molecular level. Major genes for resistance have been incorporated into alfalfa, and although initially these cultivars resisted fungal attack, a physiologically specific race (race 2) has appeared, which is indistinguishable from the avirulent race 1 isolate except for its ability to infect certain alfalfa cultivars. Classical genetic studies show that one or very few genes govern this type of interaction (Day 1974). The alfalfa *Colletotrichum* system possesses many features desirable for studying fungal pathogenicity gene(s). The differential recognition of fungal virulence and/or avirulence by the plant shows a clear distinction between races and cultivars. In addition, fungal races are stable; that is when single spore isolations of either race are cultured and sporulated, essentially all of the conidia derived are of the parental phenotype. Similarly, the alfalfa cultivars are quite predictable in their responses when challenged by the fungal races (O'Neill, personal communication).

Investigation of virulence determinants requires manipulating genes at the DNA level. Central to such a study is reliable DNA transfer. DNA mediated transformation is now well established for yeast (Hinnen et al. 1978), and the filamentous Ascomycetes *Neurospora crassa* (Case et al. 1979) and *Aspergillus nidulans* (Yelton et al. 1984), all of which are non-plant pathogens. There are few examples of successful transformation with phytopathogenic fungi. Transformation of pathogenic *Cochliobolus heterostrophus* (Turgeon et al. 1985), *Magnaporthe grisea* (Parsons et al. 1987), *Glomerella cingulata* (Rodriques and Yoder 1987), *Ustilago maydis* (Leong et al. 1987), and *Fusarium solani* f. sp. *pisi* (Dickman and Kolattukudy 1987), has been reported.

Transformation is most readily demonstrated with dominant, selectable antibiotic resistance genes. In this report, transformation of *C. trifolii* with the gene encoding hygromycin B phosphotransferase [which inactivates the aminoglycoside hygromycin B (Kaster et al. 1984)], and the β-tubulin gene [which confers resistance to the fungicide, benomyl (Orbach et al. 1986)] is described. The transformation protocol uses whole fungal cells, rather than protoplasts. Alkali salts, coupled with polyethylene glycol treatment and brief heat shock, induce competence of fungal tissue for uptake and incorporation of foreign DNA. This procedure, originally developed for yeast (Ito et al. 1983), and later extended to *Neurospora crassa* (Dhawale et al. 1984), is simple, rapid and reliable. This method should prove generally useful for transformation of fungal phytopathogens.

Materials and methods

Strains and culture conditions. *E. coli* strain DH5 (F^-, end A1, hsd R17 (r_k^-, m_k^+), *sup* E44, *thi*-1, *rec*A1, *gyr* A96, *rel* A1, (*arg*F-*lac*zya) U169, φ 80dlacZ M15, $^-$, Hanahan 1983) was the recipient for bacterial transformation and routine propagation of plasmids. *Colletotrichum trifolii* race 1, isolate 2 sp2 was the recipient in fungal transformation experiments, and was kindly provided by Dr. N. O'Neill. The fungus was maintained on either 0.5x oatmeal agar or 10% V-8 juice agar (VJA) at 28 °C. For liquid cultures, the fungus was grown on cutin mineral medium (CMM) using 2% glucose as the carbon source (Dickman et al. 1982).

Transformation vectors. pSV50 was obtained from Dr. C. Yanosky. This 7.9 kb cosmid vector harbors a 2.6 kb fragment containing a *N. crassa* β-tubulin gene which confers resistance to benomyl (Vollmer and Yanofsky 1986). For transformation of hygromycin B resistance (hyg^r), a plasmid was constructed in the phagemid Bluescript + (Stratagene). pITH, generously provided by Dr. O. Yoder, contains a truncated hygromycin phosphototransferase gene translationally fused to promoter elements of *Cochliobolus hetereostrophus* (Turgeon et al. 1987). To isolate these segments, pITH was restricted with *Sal*I and *Hind*III and the 2.4 kb fragment was gel purified and ligated to a similarly digested Bluescript vector. The resulting 5.4 kb plasmid has been designated pBHM-1.

Transformation procedure. *C. trifolii* tissue was made competent for DNA uptake with the alkali salt lithium acetate. A 5 mm agar plug of the fungus was placed in 100 ml of CMM. Cultures were grown for 48 h and harvested by vacuum filtration. The fungal mat was washed extensively with TE pH 7.6 and then resuspended in 0.1 M lithium acetate (5 ml/gm). The suspension was incubated at 30 °C with shaking and then centrifuged (10,000 rpm, 4 °C). The pellet was resuspended in 0.5–1 ml of 0.1 M lithium acetate creating a thick slurry. Vector DNA, 10–20 μg in 10–50 μl TE pH 7.6, containing 4 mM spermidine (Sigma) and 1 mM spermine (Sigma) was added and the mixture was incubated with shaking for 0.5 h at 30 °C. At this time, 10 volumes of 40% PEG 4,000 (Sigma) in 0.1 M lithium acetate was added and incubation was continued for an additional hour. The suspension was then heated for 5 min at 37 °C and the tissue was collected by centrifugation and washed with sterile water. The centrifugation was repeated and the pellet was resuspended in sterile water. The tissue was densely spread on VJA plates. Following a 20 h incubation at room temperature, plates were overlaid with 5 ml of 1% agar containing 3 mg of hygromycin B (Sigma) or 30 μg of carbendazim (MBC). MBC is the active ingredient in benomyl and is manufactured by Dupont and was kindly provided by Dr. C. P. Woloshuk. 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 and MBC^r (carbendazim resistant) isolates on rich nonselective media (VJA). Following 7 days growth, agar plugs of colony edges were transferred back 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 two day intervals for 10 days.

Isolation of DNA. *C. trifolii* genomic DNA was isolated by a miniprep procedure. Spore suspensions were inoculated into 100 ml CMM and grown with shaking (100 rpm) at 28 °C. 24 h later either hygromycin or MBC (final concentration 100 μg/ml, and 1 μg/ml, respectively) were added. Cultures were then harvested and washed 48 h following inoculation. At this point the tissue was frozen in liquid nitrogen and either lyophilized or ground into a fine powder with a mortar and pestle, after which lysis buffer (TE pH 8, 5% SDS, 25% sucrose) was added. Following vortexing and a 65 °C incubation for 30 min, cell wall and other debris was centrifuged. The supernant was transferred, 1 g/ml CsCl added and spun for 10 min. The solution was precipitated with ethanol, the pellet dried and resuspended in 50 μl of TE pH 8, containing 150 mM NaCl. RNase A was added and the solution was incubated for 15 min at 37 °C. The solution was phenol extracted and precipitated. DNA prepared by this procedure was of high molecular weight (~40 kb) and suitable for restriction analysis and Southern blotting.

DNA manipulations. DNA was digested and ligated according to manufacturer's recommendations. Large scale plasmid preparations and minipreps were done by the alkaline lysis procedure (Birnboim and Doly 1979). Bacterial transformation was performed using $CaCl_2$ (Mandel and Higa 1970) with selection on LB or 2 YT plates containing 50 μg/ml carbenicillin. For hybridization analysis, DNA probes were isolated and labeled by nick translation (Rigby et al. 1977) to a specific activity of 5×10^7–10^8 cpm/μg. DNA was transferred to nitrocellulose (Schleicher and Schuell) by the method of Southern (1975) as described by Maniatis et al. (1982). Autoradiography employed Kodak X-OMAT film with Dupont Cronex intensifying screens.

Results

Colletotrichum trifolii race 1, isolate 2sp2 served as the recipient in the transformation experiments. The minimum concentration of antibiotic which completely inhibited fungal growth was determined by incorporating serial dilutions of either hygromycin B or MBC into VJA, transferring an agar plug of the fungus to the edge of the plate and comparing growth rates. At 100 μg/ml of hygromycin B or 1 μg/ml of MBC fungal growth could not be detected and these antibiotic concentrations were used for selection. Using the transformation protocol of the preceding section, resistant colonies appeared within five days for hygromycin and seven days for MBC. Resistant colonies were recovered for up to two weeks. No transformation was seen with plasmid DNA alone. No spontaneous revertants to resistance were observed with either antibiotic even after repeated platings.

Following transformation, a "lag" period prior to the addition of antibiotic was essential to initiate expression of drug resistance. Short periods (2–12 h) were insufficient, since fungal growth was not evident, but 20 and 24 h proved sufficient. Periods in excess of 24 h resulted in extensive background.

Using 10–20 μg of vector DNA, frequencies ranging from 5–10 stable transformants/μg pBHM-1 and 0.4–1

Table 1. The effect of heparin, spermine and spermidine on transformation of *C. trifolii*

Treatment[a]	No. of Transformants	
	Experiment 1	Experiment 2
Heparin – 10 μg	20	28
Heparin – 100 μg	15	22
Spermine – 1 mM	150	170
Spermidine – 4 mM	47	92
Heparin (10 μg), spermine (1 mM), spermidine (4 mM)	201	185
Spermine (1 mM), spermidine (1 mM)	191	254
Control	22	18

[a] *C. trifolii* race 1 was transformed as indicated in the text using 10 μg of circular pBHM-1. 0.5 g of fungal tissue was used in each case

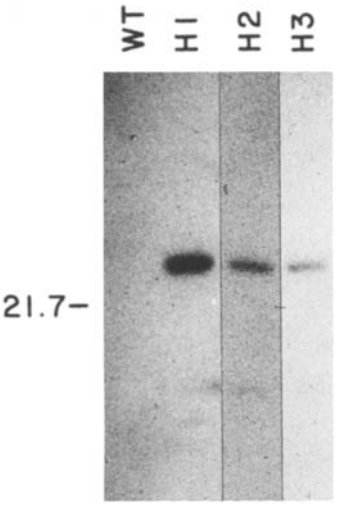


Fig. 1. Southern blot hybridization of undigested genomic DNA from *C. trifolii* wild type and selected hygromycin resistant transformants. The blot was hybridized with ^{32}P-labeled hygromycin B phosphotransferase DNA. *Lane* 1, untransformed wild-type; *lane* 2–4, transformants H1, H2, H3. Molecular weight standards of DNA digested with *Hind*III and *Eco*RI are indicated *on the left*. Electrophoresis was on 0.7% agarose

stable transformants/μg of pSV50 were obtained. As in other fungal systems, many putative transformed isolates were unstable (Hynes 1986), possibly due to nonintegration of the plasmid vector. With *C. trifolii*, about 75% of the original resistant colonies lost resistance on transfer.

Mitotic stability

The stability of the transformants was evaluated by repeated vegetative transfer from selective antibiotic containing media to non-selective VJA. An isolate was considered stable if following transfer growth for 10 days, and re-transfer to the selective media, its growth rate was undiminished. Such isolates were considered as stable transformants and screened by Southern for vector DNA sequences.

Factors affecting C. trifolii transformation

In attempts to enhance transformation efficiency experimental parameters were varied. The age of the fungal tissue significantly affected transformation efficiency. Young, actively growing tissue took up significantly more DNA than old, slower growing cells. In other words, the log phase cells are more competent than stationary phase cells. Heparin facilitates transformation of yeast (Schweizer et al. 1981) presumably by inhibiting endogenous nucleases. However, incubating heparin (10 or 100 μg) with vector DNA (20 min on ice) prior to mixing with lithium acetate treated fungal tissue (Table 1) did not improve transformation. Polyamines, spermine and spermidine, however, each increased transformation and together increased transformation about 10 fold (Table 1). Spermine and spermidine are now routinely mixed with vector DNA before addition to fungal tissue. Linear and circular plasmid DNAs transformed with similar efficiencies (data not shown).

Hybridization analysis

Selected hygromycin resistant, mitotically stable *C. trifolii* transformants were grown in liquid medium containing 100 μg/ml hyg B, and total DNA was isolated and analyzed by hybridization. Gel blots of undigested, *Sty*I-digested and *Sal*I-digested DNA were probed with the radiolabeled hygromycin gene. The hygromycin probe was made from nick translated (Rigby et al. 1977) pBHM-1 or a 1.7 kb *Bam*HI-*Hind*III fragment containing the Hyg^r gene. Undigested DNA from transformants (Fig. 1) yielded hybridization signals only in the chromosomal DNA region, whereas signals were absent in the wild type *C. trifolii* lane. Attempts to transform *E. coli* to ampicillin resistance with total DNA from the transformants were unsuccessful. These data strongly suggest that transformation involved chromosomal integration of the vector.

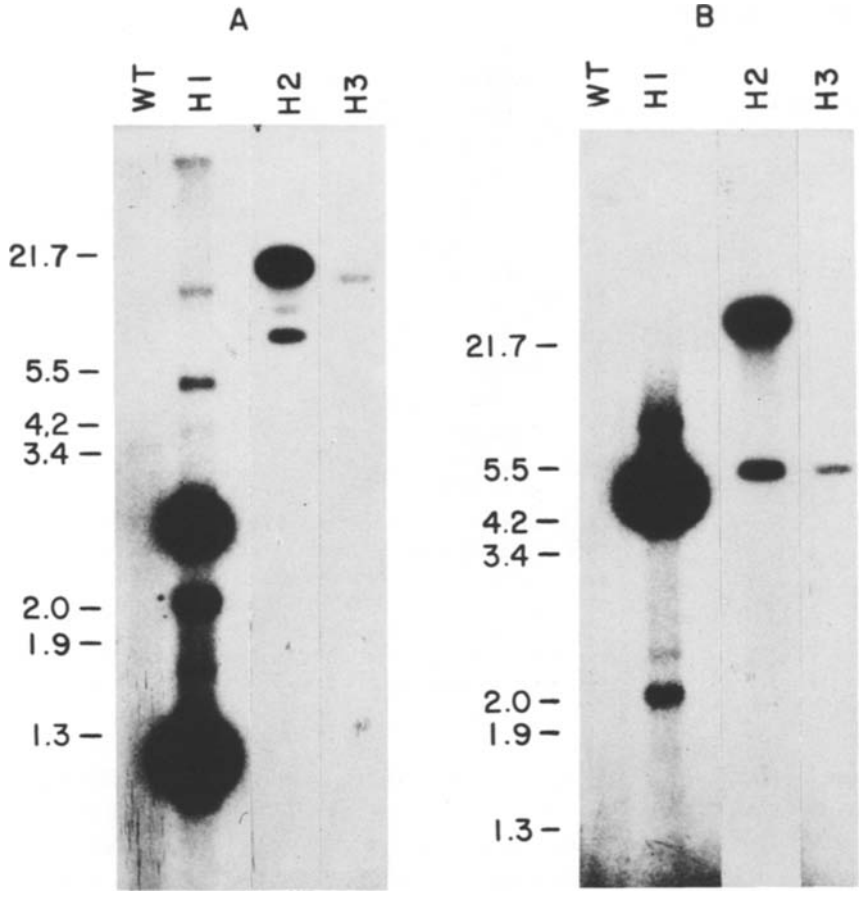
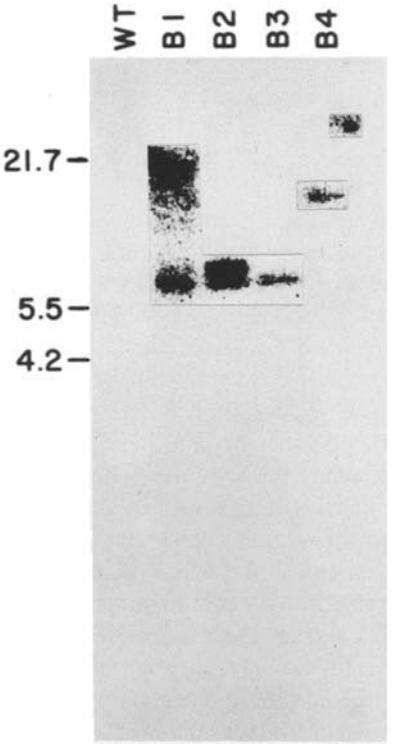


Fig. 2A, B. Southern analysis of genomic DNA from hygromycin resistant transformants digested with either *Sty*I (**A**) or *Sal*I (**B**). Lane 1 in both cases represents wild-type (WT) DNA. Electrophoresis was on 0.7% agarose and ^{32}P labelled pBHM-1 was used as the probe. Molecular weight standards are as described for Fig. 1



The nature of integration can be inferred from Fig. 2 which shows DNA blots of digested fungal DNA probed with pBHM-1 (Fig. 2A). *Sty*I does not cut pBHM-1 while *Sal*I restricts the vector at one site (Fig. 2B). Transformant H1 shows a complex pattern, suggesting multiple sites of integration. Since the *Sty*I digestion also yields bands greater than 21 kb, tandem duplications have likely occurred. The intensity of the signals may also reflect copy number as seen for this isolate. Some of the insertion events apparently have been modified by rearrangements, deletions, or insertions. The banding pattern of transformant H2 and H3 also indicates tandem integration of the vector. Transformant H3 appears to have inserted at two loci, one of which also contains tandem duplications. In all cases, including several not shown, different patterns

Fig. 3. Southern analysis of genomic DNA from MBC resistant transformants digested with *Ava*I (4B). Lane 1, represents wild-type (WT) DNA. All other experimental conditions were as described in Fig. 1 except that radiolabelled pSV50 was used as the probe

of hybridization suggest varied integration sites resulting from random insertions.

The carbendazim resistant transformants similarly displayed no evidence of extrachromosomal plasmids since hybridization signals coincided with chromosomal DNA (not shown) and since total DNA from these isolates failed to transform *E. coli*.

DNA of wild type fungus hybridized faintly upon prolonged exposure of X-ray film (not shown), suggesting partial homology to the *N. crassa* β-tubulin gene. Southern blot analysis of digested DNA from these transformants is seen in Fig. 3. *Hpa*I does not cut pSV50. When *Hpa*I digests of these isolates were probed with nick translated pSV50 only bands of chromosomal size were observed (not shown). This strongly suggests tandem repeats in each case. When MBC^r transformants were restricted with *Ava*I, which cuts at a single site in the vector, hybridizing signals appear at 7.9 kb, the size of the pSV50 (Fig. 3). Transformant B3 has a single hybridizing band at 7.9 kb. In addition, the other three isolates contain a second band in different locations. These three transformants also yielded an additional small, faint band not apparent in Fig. 3. These results support the interpretation that the integration event resulted in tandem repeats. It is unclear if homologous recombination influenced the integration events. The possibility of *N. crassa* β-tubulin gene or weak plasmid homology to *C. trifolii* DNA cannot be ruled out in the explanation of the data.

Discussion

An essential requirement for investigating the molecular basis of virulence and host specificity in the *C. trifolii*-alfalfa race specific interaction is DNA transformation. Transformation of whole cells with the alkali salt lithium acetate is such a system. The general utility of antibiotic resistance genes as dominant selectable markers is now extended to *C. trifolii*, and drug resistance appears to be a useful molecular tag to monitor selection with fungal phytopathogens. Virtually any fungus sensitive to either of these antibiotics is a potential recipient for transformation, obviating the need for mutant strains or sexual crosses.

In all reported cases, transformation with phytopathogenic fungi has been integrative. Chromosomal integration may be the only means by which these fungi stably maintain foreign DNA. Fungal transformation commonly involves tandem duplication. Stable extrachromosomal (plasmid) transformation has not been seen in fungal phytopathogens.

The plasmid pBHM-1 presumably can integrate only by nonhomologous recombination and at one or more apparently random sites. Plasmid integration by nonhomologous recombination has been observed in *Aspergillus neurospora* and *Colletotrichum lindemuthianum*, as well as plant and animal systems. From preliminary evidence presented in these and other studies (Dickman and Partridge, submitted) nonhomologous recombination involves random insertion. In mammalian transformation, random nonhomologous integration greatly exceeds (by 10^3) homologous or targeted gene insertion (Roth and Wilson 1986). Although the mechanism is as yet unknown, transformation by a nonhomologous recombination is potentially useful. Providing transformation is efficient and selectable, genes can potentially be tagged by insertional inactivation. The vector containing a selectable marker can then be recovered (i.e. marker rescue) with adjacent sequences of interest. More examples are necessary to confirm the generality of random nonhomologous insertion before employing it as a molecular tag.

An important adjunct of this work is the generality of fungal transformation in the presence of alkali salts. This procedure is rapid, simple and consistent. It is also applicable to many fungal genera (Dickman, unpublished). Where few transformants are needed, but numerous separate transformations must be performed (i.e. promoter deletion analysis) this protocol is especially favorable. The primary drawback of this technique is its relative inefficiency. When comparing protoplasts and whole cells, using equal amounts of starting tissue, protoplasts yield more transformants, at least with *C. trifolii* and pBHM-1. Where gene complementation by transformation is necessary, protoplasts may be required.

Libraries of race 2 are being constructed in pBHM-1 for en masse transformation into race 1. Gene(s) responsible for virulence will be sought. This paper provides a framework for isolation, characterization and regulation of *C. trifolii* race specific virulence genes.

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