Curr Genet (1993) 23:343–350

Current Genetics

Gibberella pulicaris transformants: state of transforming DNA during asexual and sexual growth

Yangkyo P. Salch, Marian N. Beremand*

USDA/ARS, National Center for Agricultural Utilization Research, 1815 N. University St., Peoria, IL 61604, USA

Received: 5 July 1992 / Accepted: 14 September 1992

Abstract. A genetically fertile, trichothecene-producing plant pathogen, *Gibberella pulicaris* (*Fusarium sambucinum*), was transformed with three different vectors: cosHyg1, pUCH1, and pDH25. All three vectors carry *hph* (encoding hygromycin B phosphotransferase) as the selectable marker. Transformation frequency was 0.03 transformants per μmg of DNA for pDH25 and 0.5 for pUCH1 or cosHyg1. The vector DNA sequences integrated at different sites into the fungal genome. Transformants were classified into three types based upon distinctive integration patterns: type A contained a single, intact copy of the vector at one site per genome; type B contained multiple tandem copies or a combination of single and multiple tandem copies at one or more sites per genome; type C contained a partial vector copy at one site per genome. While the transformants with cosHyg1 and pUCH1 were type A or B, type C was unique to pDH25 transformants. Type A and C transformants were both meiotically and mitotically stable. However, type B multiple inserts were unstable in mitosis and meiosis since: (1) multiple tandem copies were deleted; (2) rearrangements occurred during premeiosis; and (3) inserts in one of the type B transformants became methylated during premeiosis. Differential expression of transforming sequences between spore germination and mycelial growth was also observed among type B transformants. The ability to transform *G. pulicaris* with the resulting varied features of integration patterns and the behavior of transforming DNA during mitosis and meiosis provides a means to isolate, manipulate, and study cloned genes in this mycotoxin-producing plant pathogen.

Key words: Transformation – *Gibberella pulicaris* – *Fusarium* – Mitotic/meiotic stability

Mention of companies or products by name does not imply the endorsement by the U.S. Department of Agriculture over others not cited

* *Present address:* Department of Plant Pathology and Microbiology, Texas A and M University, College Station, TX 77843, USA
Correspondence to: Y.P. Salch

Introduction

Gibberella pulicaris is an agriculturally important trichothecene-producing heterothallic ascomycete that infects a wide range of plant species (Booth 1971). The genetic fertility, toxigenicity, and ability of some strains to overcome several host plant natural defense mechanisms by degrading phytoalexins make *G. pulicaris* useful for investigating secondary metabolism and plant-pathogen interactions (Beremand 1989; Desjardins and Gardner 1989; Desjardins et al. 1989;). A transformation system that could facilitate the isolation and analysis of structural and regulatory genes would be an important tool for studying these areas at the molecular level.

A variety of transformation systems have been developed for filamentous fungi (Fincham 1989). While each fungus usually requires unique optimized conditions, most transformation protocols consist of protoplast production followed by the introduction of plasmid DNA in the presence of polyethylene glycol and divalent cations. In filamentous fungi, the introduced DNA generally integrates into the genome, often in a tandem array, and does not replicate autonomonously (Hynes 1986; Goosen et al. 1992; Upshall 1992). The mitotic stability of the transforming DNA is generally so high that the phenotypic loss of an introduced marker gene is in the range of the normal mutation rate (Timberlake and Marshall 1989). During sexual crosses, the integrated transforming DNA may be stably maintained, modified, or deleted. In *Neurospora crassa*, for example, tandemly integrated plasmid DNA is deleted and/or heavily methylated during sexual reproduction by a process designated repeat-induced point mutation (RIP) (Goyon and Faugeron 1989; Selker 1990). Accordingly, the fate of the transforming DNA during asexual and sexual reproduction is an important parameter which defines the unique properties of each transformant and each fungal transformation system.

In this report, we describe a molecular transformation system for *G. pulicaris*. In order to evaluate the fate of the transforming DNA sequences, representative trans-

formants were analyzed with respect to the integration pattern and mitotic/meiotic inheritance of the transforming DNA. A preliminary report of this work has been presented elsewhere (Salch and Beremand 1988).

Materials and methods

Strains, plasmids, and culture conditions. G. pulicaris strains were obtained from P. Nelson, *Fusarium* Research Center, Pensylvania State University. Strain R-6380 was originally isolated from potato tubers in Germany and is a mating-type 1 hermaphrodite. Strain R-5455 was originally isolated from corn plants and is a mating-type 2 hermaphrodite. Plasmid pDH25 (7.0 kb) (obtained from D. J. Henner, Genentech, Inc.) is a derivative of pBR322 carrying the coding region of the *Escherichia coli* hygromycin B phosphotransferase gene (*hph*) flanked by the promoter and transcription terminator of the *Aspergillus flavus trpC* gene (Cullen et al. 1987). Cosmid cosHyg1 and plasmid pUCH1 (5.7 kb), both obtained from B. G. Turgeon and O. C. Yoder, Cornell University, are derivatives of plasmid pUC18. They carry the *hph* coding region fused to promoter 1 from *Cochliobolus heterostrophus* (Turgeon et al. 1987). *G. pulicaris* and *E. coli* strains were maintained as previously described (Desjardins and Beremand 1987; Salch and Shaw 1988). Conidiospores of *G. pulicaris* were obtained from cultures grown on V-8 juice agar medium (Desjardins and Beremand 1987).

Protoplast isolation. Several methods were initially tested to optimize protoplast formation from *G. pulicaris* strain R-6380. Throughout this study, protoplasts were isolated by the following procedure: conidiospores (1×10^8) were incubated in 100 ml of YEPD broth (0.3% yeast extract, 1% Bacto-peptone, and 2% glucose) for 15–18 h at 30°C with vigorous shaking (150 rpm). Mycelia were then collected by centrifugation at 1,000 *g* for 20 min and washed twice in 1.2 M KCl. The cells were digested at 30°C for 4 h with gentle shaking in 50 ml of 1.2 M KCl containing 1 g of Novozym 234 (Novo Biolabs), 1 g of driselase (Sigma), and 4 mg of chitinase (Sigma). The protoplasts were collected by centrifugation at 1,000 *g* for 5 min at 4°C, and then washed once in 1.2 M KCl and twice in STC buffer (1.2 M sorbitol, 10 mM Tris-HCl, 10 mM $CaCl_2$). Protoplasts were either used for transformation immediately or stored at 4°C overnight. This technique converted 90% of the mycelia to protoplasts, when the mycelia were grown in YEPD broth and then incubated with lytic enzymes in 1.2 M KCl.

Transformation. Transformation was performed using the procedure developed for *C. heterostrophus* by Turgeon et al. (1985) except that the protoplasts were regenerated at room temperature overnight and then overlaid with 10 ml of 1% top agar containing 1.5 mg of hygromycin B (HygB) (Sigma) to yield a final concentration of 50 μg/ml. HygB-resistant colonies emerged after 3–14 days incubation at 25°C. These colonies were then subcultured onto fresh YEPD-agar containing HygB (30 μg/ml) and finally transferred to V-8 juice agar slants containing 300 μg/ml HygB for storage at 4°C.

DNA isolation. Vector DNA was isolated by the cesium chloride-ethidium bromide gradient procedure described by Johnston and Gunsalus (1977). *G. pulicaris* genomic DNA was isolated using the procedure developed for *C. heterostrophus* by Garber and Yoder (1983) with modifications. Lawns of sporulating mycelia growing on V-8 juice agar plates were used to inoculate 250 ml of YEPD-broth. Cultures were incubated at 30°C for 1–2 days with shaking (150 rpm). Mycelia were collected by vacuum filtration using a Buchner funnel with a Whatman No. 1 filter. The filtered mycelial mats were freeze-dried and ground to a fine powder in a mortar with a pestle. The powdered mycelium was resuspended in 20 ml of isolation buffer (150 mM Na_2EDTA, 50 mM Tris-HCl, pH 8.0, 1% sarkosyl) with vigorous vortex-mixing for 1 min. Following centrifugation at 1,000 *g* for 25 min, 40 μl of an RNase A solution (10 mg/ml) and 5 μl of an RNase T1 solution (100,000 units/ml) (Boehringer Mannheim Biochemical) were added to the supernatant. This mixture was incubated at 37°C for 30 min and was extracted twice with a phenol-chloroform (1:1) mixture and once with a chloroform-isoamyl alcohol (24:1) mixture. DNA was precipitated with 0.3 M sodium acetate (pH 5.2) and an equal volume of isopropyl alcohol, followed by centrifugation at 1,000 *g* for 30 min at room temperature. The DNA pellet was dissolved in 16 ml of sterile water and mixed with 11 ml of 20% polyethylene glycol 8000 in 2.5 M NaCl. Following the incubation of the mixture on ice for 1 h, the final DNA pellet was recovered by centrifugation as above and then dissolved in 1 ml of TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 8.0). The DNA yield was approximately 1 mg per gram (dry weight) of mycelium.

Molecular analysis of transformants. The integration pattern of the transforming DNA was identified by Southern-hybridization analysis in which the vector DNA was hybridized as a probe to fungal genomic DNA digested with various restriction enzymes. The DNA from transformants recovered from protoplasts treated with either cosHyg1 or pUCHI was digested with *Xho*I, *Hin*dIII, or *Eco*RI. Both vectors have no *Xho*I, one *Hin*dIII, and two *Eco*RI cleavage sites. DNA from pDH25 transformants was digested with *Hin*dIII, *Xba*I, or *Eco*RI. pDH25 has no *Hin*dIII, one *Xba*I, and two *Eco*RI cleavage sites. Restriction enzymes (Promega) were used as specified by the manufacturer. The digested genomic DNA (5 μg) was then separated in an 0.7% agarose gel prepared and run in Tris-acetate buffer (Maniatis et al. 1982). The DNA was transferred to Genescreen plus membranes (New England Nuclear) and the resulting blots were hybridized and washed as recommended by the manufacturer. The probe DNA was labeled with [α-^{32}P]dCTP using a nick-translation kit (BRL) as recommended. The specific activity was approximately 1×10^8 cpm/μg of DNA.

Mitotic stability test. V-8 juice agar plates without hygromycin were each inoculated with a transformant by using agar plugs excised from the slant cultures. The plates were incubated at 25°C for 7–14 days. Conidiospores were harvested and 10^3 spores were spread on V-8 juice agar plates without hygromycin B. After incubating the plates at 25°C for 7–14 days, conidiospores were again harvested and subcultured under non-selective conditions five times. In the sixth cycle, conidiospores (200 per plate) were spread in parallel onto YEPD-agar plates with and without HygB (30 μg/ml) and the germination rates compared. Since some HygB-resistant transformants might only express the HygB resistance gene during mycelial growth, and not during germination of the conidia, 100 colonies from the YEPD plates without HygB were subcultured in parallel onto YEPD medium with and without HygB. The data from the above experiments was used to assess whether the transformants expressed the HygB gene during conidial germination and/or mycelial growth and to determine what percent of the conidia retained the ability to express the HygB resistance gene following extended vegetative growth. The HygB-resistant colonies were further analyzed by Southern hybridization in order to examine the state of the transforming DNA at the molecular level.

Meiotic stability test. Genetic crosses were performed as described by Desjardins and Beremand (1987). Six representative transformants (derived from the strain R-6380) were reciprocally crossed to the opposite mating type strain R-5455. Tetrad analysis was performed with ascospores obtained from six to seven asci derived from a single perithecium. The isolated ascospore progeny were subcultured on YEPD-plates containing 30 μg/ml of HygB to test HygB resistance. The inheritance of the *hph* gene was also determined by Southern hybridization analysis as described above. In order to detect methylation of transforming sequences, *Sau*3AI and *Mbo*I digests of genomic DNA were compared by Southern-hybridization analysis using plasmid DNA as the probe.

Results

Transformation

G. pulicaris protoplasts were transformed with pUCH1, pDH25, or cosHyg1, all of which confer resistance to hygromycin B. Upon incubation of the protoplasts in the presence of HygB, both large and small colonies arose. The small colonies failed to grow after being transferred to fresh YEPD medium containing 30 μg/ml of HygB. They are most likely abortive transformants similar to those previously reported for other fungi (Hynes 1986). Therefore, only the large and stable HygB-resistant colonies were considered as true transformants and they arose at a frequency of 0.2–0.5 transformants per μg of pUCH1 or cosHyg1 DNA. Transformation by pDH25 occurred at a frequency of less than 0.03 transformants per μg of DNA. Thus, pUCH1 and cosHyg1 appear to be more suitable vectors for the transformation of *G. pulicaris*. Mock transformations conducted without vector DNA did not give rise to HygB-resistant colonies.

Molecular analysis of transformants

Southern-hybridization analysis of DNA derived from 24 HygB-resistant isolates confirmed that they all contained vector DNA sequences and therefore were true transformants. When the undigested DNA from these transformants (12 cosHyg1, nine pUCH1, and three pDH25) was hybridized with the corresponding vector DNA, hybridization occurred only with high molecular weight DNA. These results suggest the absence of autonomously replicating vector DNA sequences and indicate that these transformants arose by integration of the vector DNA into the fungal genome. No hybridization was observed with genomic DNA from the untransformed strain R-6380.

Digestion of genomic DNA with restriction enzymes that recognize one site (*Hin*dIII for cosHyg1 and pUCH1; *Xba*I for pDH25), two sites (*Eco*RI for all three vectors), or no sites (*Xho*I for cosHyg1 and pUCH1; *Hin*dIII for pDH25) on the original transforming vector revealed several types of integration. Data for the cosHyg1 transformants C6 (Fig. 1, lanes 2), C3 and C9 (summarized in Table 1) are consistent with the insertion of a single copy of the vector at a single site (type A integration). *Xho*I and *Hin*dIII digests yielded one and two hybridizing bands respectively, and digestion with *Eco*RI released the expected *Eco*RI vector fragment. The presence of only two hybridizing bands in the *Eco*RI digest of C6 suggests that integration occurred near one of the *Eco*RI sites on the vector. Alternatively, a small portion of the vector containing one of the *Eco*RI sites could have been deleted.

Data for transformants C5, C7, H2, and H5 (Fig. 1, lanes 1, 3, 4, 6) and for transformants C1, C2, C4, C8, C10, C11, C12, and C14 (summarized in Table 1) indicate that they contained multiple copies of the transforming vector DNA sequences inserted in a tandem array (type B integration). DNA digests produced by enzymes which have two recognition sites on the transforming vector included two hybridizing bands equivalent in size to the vector fragments expected for tandem multi-copy inserts. The presence of more than three hybridizing fragments in the one-recognition-site enzyme digests, and multiple hybridizing bands in the zero-recognition-site enzyme digests, suggested that insertions occurred at more than one site in transformants C5, H2 and H5. However, the data in Fig. 1 do not reveal if the additional insertion site(s) involved single or multi-copy inserts.

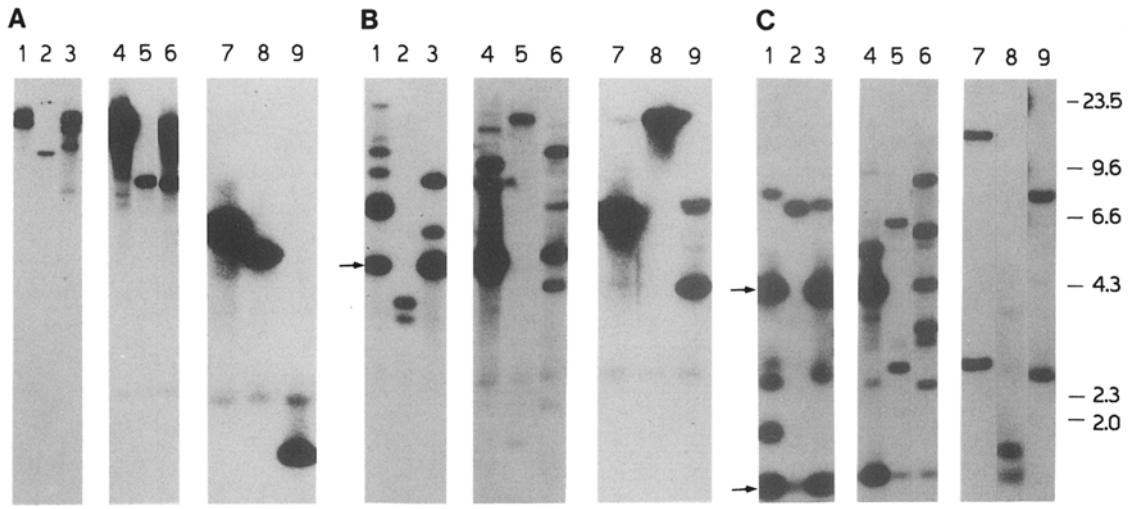

Fig. 1 A–C. Southern hybridization analysis of restriction digests of DNA from *G. pulicaris* transformants. DNA from various transformants was isolated and digested with restriction endonucleases that **A** recognize no cleavage site (*Xho*I for cosHyg1 and pUCH1; *Hin*dIII for pDH25), **B** one cleavage site (*Hin*dIII for cosHyg1 and pUCH1; *Xba*I for pDH25), and **C** two cleavage sites (*Eco*RI for all). The DNA digests were separated by electrophoresis in 0.7–0.8% agarose gels and blots were prepared. The probes were cosHyg1, pUCH1, or pDH25. *Lanes 1*, C5; *2*, C6; *3*, C7; *4*, H2; *5*, H3; *6*, H5; *7*, D1; *8*, D2; *9*, D3. *C* stands for cosHyg1 transformants, *H* stands for pUCH1 transformants and *D* stands for pDH25 transformants. *Arrows* indicate internal vector fragments. Molecular markers based on the lambda *Hin*dIII digest are indicated on the right

Another type of integration was observed in the pDH25 transformants, D1, D2, and D3 (Fig. 1, lanes 7, 8, and 9). The presence of a single, less than vector size (<7.0 kb) hybridizing band following digestion of the genomic DNA with *Hin*dIII (which has no recognition sites on pDH25) suggested that these transformants contained a single-site insertion with a partial deletion of the vector DNA (type C integration). This was verified by a further analysis of transformants D1 and D3 which showed that they each lacked plasmid pBR322 sequences and a portion of the *A. nidulans trp*C promoter (data not shown). Transformant H3 did not appear to fall within the above three general categories. The presence of only one hybridization band following digestion of the genomic DNA with an enzyme with no recognition sites in pUCH1 (Fig. 1, lane 5) indicated that the vector sequences integrated at a single site; however, digestions with enzymes which have one and two recognition sites on pUCH1 were not consistent with those expected for either a partial-copy, single-copy or multi-copy insert (the digestion patterns were consistent with those expected for an insert that involves a partial duplication).

Finally, the diversity in the digestion patterns for the above group of transformants and the occurrence of integrations at multiple sites within the same transformant suggest that these vector DNAs can integrate at different locations in the *G. pulicaris* genome.

Mitotic stability of HygB resistance

The mitotic stability of the marker phenotype in the transformants was investigated using the following approaches (details in Materials and methods). First, spores obtained after five cycles of subculturing under non-selective conditions were plated in parallel on selective and non-selective media and scored for growth. Then, colonies which arose on the non-selective medium were transferred to test their ability to grow in the presence of HygB. These two tests were conducted in order to provide a measure of the mitotic stability of the transforming DNA during prolonged periods of vegetative growth under non-selective conditions and to reveal any differences in the expression of HygB resistance between germinating spores and growing mycelia.

The stability and expression of the transformed phenotypes varied depending upon the transformant (Table 1). Stable inheritance means that 100% of the spores retained the HygB resistance trait. All type A or C transformants showed a stable inheritance and sufficient expression of the HygB gene in both germinating spores and mycelia to confer resistance to HygB. On the other hand, only three of the ten type B transformants (C1, C7, and C14) showed stable inheritance of HygB resistance by at least one of the above tests. Furthermore, the entire group of type B transformants displayed differences in the ability of germinating spores and growing mycelia to express HygB resistance. In two cases, transformants C1 and C4, there were more HygB-resistant colonies as measured by germinating spores than as measured by the transfer of growing mycelia. In the remaining eight transformants, there were a greater number of HygB-resistant colonies as measured by the transfer of growing mycelia than as measured by germinating conidia. The most notable was transformant C14. In the presence of HygB, C14 showed less than 1% survival for germinating conidia and, conversely, total survival for growing mycelia. This sharp contrast in the ability of these different cell types to grow in the presence of HygB suggests that the integrated *hph* genes in transformant C14 may be stably inherited but differentially expressed during spore germination and mycelial growth. Finally, in these expreriments and others (data not shown), there was no correlation between the number of vector copies and the level of HygB resistance expressed.

Table 1. Mitotic stability of transforming DNA

Transformant[a]	Integration type[b]	% HygB^R colonies		Rearrangement of DNA[e]
		I[c]	II[d]	
C3	A	100	100	–
C6	A	100	100	–
C9	A	100	100	–
D1	C	100	100	–
D2	C	100	100	–
D3	C	100	99	–
C1	B	100	81	–
C4	B	96	82	–
C7	B	92	100	–
C2	B	78	97	+
C8	B	55	82	+
C5	B	43	93	+
C10	B	24	85	–
C12	B	14	78	–
C11	B	6	50	+
C14	B	<1	100	–

[a] C, cosHyg1 transformants
D, pDH25 transformants
[b] A, type A transformants with a single-copy insertion at a single site
B, type B transformants with a combination of a single- and multiple-tandem copy integrations or multiple-tandem copy insertions at a single or multiple sites
C, type C transformants with a partial-vector copy insertion at a single site
[c] % of conidia that germinate and grow in the presence of HygB
[d] % of mycelia that grow in the presence of HygB upon transfer from HygB-free media
[e] (–) for no rearrangement and (+) for rearrangement of the transforming DNA sequences based upon Southern-hybridization analysis

Mitotic stability of integrated DNA

Stability of the transforming DNA during mitosis was further examined by Southern-hybridization analysis. The data are summarized in Table 1. As observed with the HygB-resistant phenotype, stability of the inserted vector DNA sequences varied depending upon the transformant. All of the type A or C transformants examined, and six of the type B transformants, underwent no obvious DNA rearrangements. Four of the type B transfor-

mants showed some rearrangement of the transforming DNA (Table 1).

Two type B transformants, C2 and C14, were chosen for further analysis. Thirty single spores were isolated from each transformant and tested for HygB resistance. Inheritance of the transforming DNA was examined by Southern-hybridization analysis in ten of these isolates. All the single-spore isolates from transformant C14 were resistant to HygB in the mycelial stage and the integrated DNA retained the same hybridization pattern as the original C14 transformant (data not shown). In contrast, C2 was unstable. Only 83% of the tested C2 single-spore isolates showed resistance to HygB and none of the ten examined single-spore isolates retained the exact *Xho*I (zero recognition sites in cosHyg1) hybridization pattern of the parent C2 (Fig. 2). The primary C2 transformants displayed five *Xho*I-generated hybridizing bands (27, 23, 19, 14, and 8.6 kb) and all of the single-spore isolates displayed a reduction or complete loss of the higher molecular weight bands and/or an increase in the lower molecular weight bands. In fact, two single-spore isolates, SS10 and SS11, lacked all of the C2 parental hybridizing bands and were sensitive to HygB. Other isolates displayed non-parental *Xho*I fragments, such as the 10-kb band present in SS2, SS3 and SS6. *Eco*RI digests demonstrated that isolates SS1 through SS9 had the same border fragments (11 kb and 2.5 kb) as the C2 parent (Fig. 3). The above data indicate that C2 has tandem copies of the cosHyg1 vector sequences inserted at a single site in the genome and that the repeated copies were excised during mitotic growth, presumably by recombination between the duplicate vector sequences. The loss of multiple tandem vector sequences in C2 also occurred under selective conditions (data not shown). Finally, an instability of multiple tandem-copy insertions was observed in two other type B transformants which have integrations at different sites (data not shown).

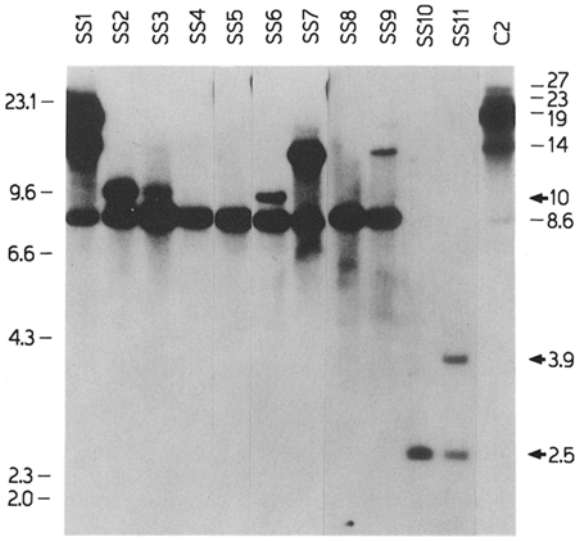

Fig. 2. Southern hybridization analysis of *Xho*I digests of DNA from the first round of single-spore isolates of transformant C2. *Xho*I does not cleave cosHyg1 DNA. The DNA digests were analyzed by agarose-gel electrophoresis (0.7%) followed by Southern hybridization using cosHyg1 as a probe. *SS* stands for single spore. Standard molecular markers are indicated on the left and the size of the inserted DNA is indicated on the right. *Arrows* indicates non-parental type of fragments (10, 3.9, and 2.5 kb)

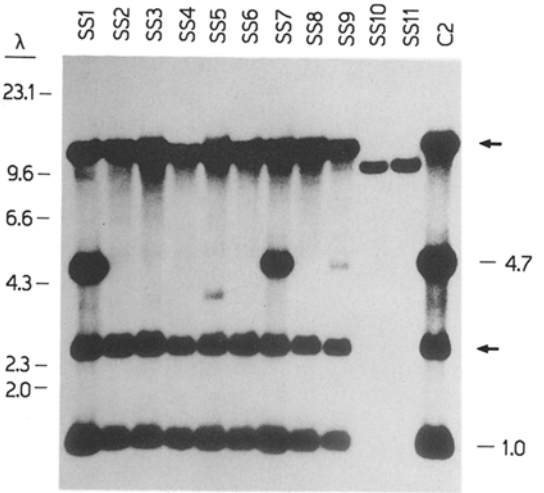

Fig. 3. Southern hybridization analysis of *Eco*RI digests of DNA from the first round of single-spore isolates of transformant C2. *Eco*RI cleaves cosHyg1 twice. The DNA digests were resolved on an 0.7% agarose gel and analyzed by Southern hybridization using cosHyg1 as a probe. Standard molecular markers and the size of internal vector fragments are indicated on the left and on the right, respectively. The border sequences are indicated with *arrows*

Meiotic stability of HygB resistance

Six different transformants, representing each of the three integration types, were reciprocally crossed to the opposite mating-type strain which was not resistant to HygB (Table 2). Ascospore progeny from tetrads from each cross were tested for viability and the inheritance of HygB resistance. As shown in Table 2, all transformants readily crossed as male parents, but only two transformants (D2 and C6) produced viable ascospores when used as the female parent. The reason for the low viability of ascospores in the 1756A and 1758A crosses is unknown.

The segregation ratio of the $HygB^R$: $HygB^S$ progeny reflects the fate of the transforming DNA sequences during meiosis (Selker 1990). A 1:1 ratio (due to a 4:4 ratio of resistant to sensitive progeny in each ascus) indicates complete retention of the HygB-resistant phenotype. Crosses made with the type A and type C transformants (C6, D1, D2 and D3) yielded a 1:1 $HygB^R$: $HygB^S$ segregation ratio (Table 2) and no 0:8 tetrads were observed. In contrast, the absence of any HygB-resistant progeny is indicative of a loss of the HygB resistance trait prior to fertilization. The cross with the type B transformant, C4, yielded a 0:50 $HygB^R$: $HygB^S$ segregation ratio with most of the asci containing seven or eight viable spores. Finally, an intermediate ratio with less resistant progeny than sensitive progeny (due to a mixture of individual asci with 4:4 and 0:8 segregation ratios) is indicative of a loss of the HygB-resistant trait after fertilization, but prior to karyogamy. The cross with the type B transformant, C2, yielded a 11:17 ratio of $HygB^R$: $HygB^S$ progeny and one

Table 2. Meiotic stability of transforming DNA sequences of *G. pulicaris* transformants

Cross no.	Parents[a] F × M	Integration type[b]	% of ascospore viability	No. of asci analyzed[c]	$HygB^R$ vs $HygB^S$ spores	Rearrangement of DNA[d]
1755A	R-5455 × D1	C	95	7	26:26	–
1755B*	D1 × R-5455	C	–	(+)[e]	–	NA
1756A	R-5455 × D2	C	50	7	14:11	–
1756B	D2 × R-5455	C	98	7	25:25	–
1757A	R-5455 × D3	C	90	6	18:17	–
1757B*	D3 × R-5455	C	–	–	–	NA
1758A	R-5455 × C2	B	45	7	11:17	+
1758B*	C2 × R-5455	B	–	–	–	NA
1759A	R-5455 × C4	B	93	7	0:50	+
1759B*	C4 × R-5455	B	–	–	–	NA
1760A	R-5455 × C6	A	93	7	25:26	–
1760B	C6 × R-5455	A	96	6	25:21	–

* Crosses were infertile and no ascospores were obtained
[a] F and M indicate female and male, respectively
[b] Refer to Table 1 footnote b
[c] All of the asci originated from a single perithecium in each cross; those asci which produced five to eight viable ascospores were chosen for the meiotic stability test
[d] (–), no rearrangement and (+), rearrangement. NA, not applicable
[e] One ascus was observed but no viable ascospores were obtained

tetrad displayed a 0:5 segregation pattern for resistant to sensitive progeny. However, the low viability (< 50%) of the ascospore progeny must be considered when interpreting the segregation data for this cross.

Meiotic stability of integrated DNA

Two sets of tetrads were selected from each cross shown in Table 2 and subjected to Southern-hybridization analysis using vector DNA as a probe. Except in crosses 1758A (transformant C2) and 1759A (transformant C4), no alterations were observed in the hybridization patterns obtained for any of the progeny, as compared to those for the original transformants (Table 2). This indicated that the introduced vector sequences in the type A and C transformants were stably inherited in a Mendelian fashion but that rearrangements occurred in the transforming DNA sequences present in the progeny of the two type B transformants.

As shown in the previous mitotic stability section, C2 had a multiple tandem-copy insert, at a single site, which yielded multiple hybridizing bands when the genomic DNA was digested with an enzyme that had no recognition sites on the original transforming vector (Fig. 4, lane m). In the present experiment, the vector-containing progeny from both ascus 4 and 6 had only the smallest band (8.6 kb; Fig. 4). These data are consistent with a deletion of vector sequences prior to fertilization. However, further studies with additional restriction enzymes, that have one or two recognition sites on the vector DNA, revealed some differences in the digestion patterns for these progeny which indicated that the multiple tandem copies of the vector DNA sequences were lost after fertilization (data not shown).

Another type of rearrangement of integrated DNA sequences was observed in cross 1759A with the C4 transformant. Analysis of DNAs digested with *Xho*I (no recognition sites in the vector DNA) demonstrated that C4 had two large hybridizing bands (Fig. 4, lane m; the two bands are shown as one due to overexposure of the blot), while the C4 progeny had only the smaller hybridizing band. Despite the presence of vector DNA sequences in the chromosomes of only half of the progeny, all of them were sensitive to HygB (Table 2). This suggests that the smaller hybridizing band does not have a functional *hph* gene.

To determine if the rearrangements in the vector DNA sequences involved a RIP effect (Foss et al. 1991), genomic DNA from each parent transformant and the corresponding progeny was digested with a pair of methylation-specific isoschizomers, *Sau*3A and *Mbo*I. These enzymes recognize the DNA sequence GATC, but *Sau*3A cannot cleave if the C-residue is methylated. None of the

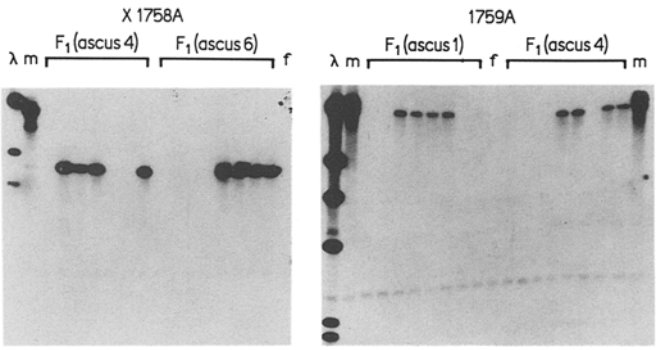

Fig. 4. Southern hybridization analysis of ascospore progeny from crosses 1758A and 1759A. Total DNA from tetrads was digested with *Xho*I, fractionated in an 0.7% agarose gel, and transferred to a Genescreen Plus membrane. The blot was hybridized with cosHyg1 as a probe. The *left lanes* of each blot contain lambda *Hin*dIII fragments. The *m lanes* contain genomic DNA from the parental transformant C2 in the cross 1758A and C4 in the 1759A. The *f lanes* contain genomic DNA from untransformed female parent strain R-5455. (The smallest fragments across the blot in 1759A is from non-specific hybridization onto the membrane)

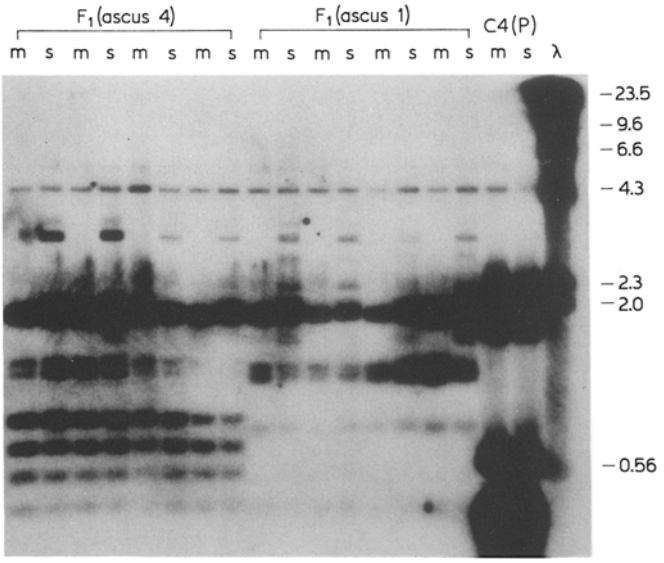

Fig. 5. Detection of methylated DNA sequences. Total DNA from ascospore progeny from the cross 1759A was isolated, digested with *Sau*3AI (*s lanes*) or *Mbo*I (*m lanes*) followed by fractionation in 1.0% agarose gel, and then blotted. The blot was hybridized with cosHyg1. *C(4)P* stands for parent transformant C4. Standard molecular markers are on the right. (The largest bands across the blot are due to non-specific hybridization onto the membrane)

progeny from all the crosses with type A or C transformants showed methylation. Only the progeny from cross 1759A exhibited a *Sau*3A hybridization pattern different from that of the parental transformant C4 (Fig. 5, S lanes). The presence of larger fragments in the *Sau*3A digests in comparison to *Mbo*I digests (Fig. 5, M lanes) is evidence for the *Sau*3A partial cleavage due to the methylated cytosine residues. This and the low intensity of partial *Sau*3A fragments indicates a low degree of methylation. In addition, different hybridization patterns in *Mbo*I digests among progeny from different asci of the same perithecium indicate that these modifications occurred after fertilization. Similar results have been shown in *N. crassa* (Selker et al. 1987) and *Ascobolus immersus* (Goyon and Faugeron 1989). The progeny of cross 1758A showed no methylation and only the deletion of tandem repeats. High frequency deletion of tandem repeats without methylation has also been shown in *Sordaria macrosporaca* (Le Chevanton et al. 1989) and in *Podospora anserina* (Coppin-Raynal et al. 1989).

Discussion

We have developed a transformation system for *G. pulicaris* and studied genetic inheritance of the vector DNAs during asexual and sexual reproduction. The frequency of tranformation with cosHyg1 and pUCH1 was comparable to that of other plant pathogenic filamentous fungi such as *C. heterostrophus* (Turgeon et al. 1985), *M. grisea* (Parsons et al. 1987), and *F. oxysporum* (Kistler and Benny 1988). pDH25 repeatedly produced a very low transformation frequency for *G. pulicaris* in the present study. However, pDH25 yielded 10^5 transformants per μg DNA in *Cryphonectria parasitica* (Churchill et al. 1990). As seen with other fungal systems (Hynes 1986), transformation frequency is dependent both on the vector and on recipient combination.

Presumably, many factors could influence the lower transformation frequency of *G. pulicaris* with pDH25. First, the *A. nidulans trp*C promoter sequence in pDH25 may not be as stable in *G. pulicaris*as is the *C. heterostrophus* promoter 1 in cosHyg1 and pUCH1. We have observed that transformants D1 and D3 lacked the *A. nidulans trp*C promoter (data not shown). This observation suggests that the integrated *hph* gene may be expressed from *G. pulicaris*-specific promoter sequences which could have become located at the 5'-end of the gene during integration. Second, the pBR322 sequences present in pDH25 may be more unstable in *G. pulicaris* than are the pUC18 sequences present in pUCH1 and cosHyg1. The instability of pBR322 sequences has been reported in some eukaryotic transformation systems. In *A. nidulans* (Johnstone et al. 1985), the pUC3-derived vectors gave a 25-fold higher transformation frequency than pBR322-derived vectors carrying the same marker genes. Lusky and Botchan (1981) reported that the presence of a *cis*-acting sequence near the *ori* site in pBR322 destabilized the transfection of chimeric SV40-pBR322 vectors in animal cell cultures. In-vivo alteration of pDH25 and other sequences has also been observed in the transformants of *F. oxysporum* (Kistler and Benny 1988) and *A. nidulans* (Razanamparany and Bequeret 1988).

The transformation of *G. pulicaris* occurred by integration of vector DNA into the genome as partial- (type C), single- (type A), or multiple-tandem (type B) copies at one or more sites per transformant. There was no correlation between the number of vector copies and the level of the HygB resistance expressed (data not shown). This has also been observed in other fungal systems (Kelly and Hynes 1985; Turgeon et al. 1985; Wernars et al. 1985; Hynes 1986; Oliver et al. 1987; Rodriquez and Yoder 1987; Farman and Oliver 1988).

Among the multicopy transformants, the HygB resistance phenotype was differentially expressed during spore germination and mycelial growth. These differences probably reflect position effects created by integration of the vectors at different sites with respect to both vector and host chromosome DNA sequences. The most notable response was demonstrated by transformant C14: when tested on HygB-medium, C14 showed 100% survival during vegetative growth but less than 1% survival in germinating conidia. One possible explanation is that expression of the *hph* gene(s) in C14 is constitutive, but insufficient to confer resistance during spore germination. Alternatively, the *hph* gene(s) may not be expressed at all during spore germination. If the latter possibility were true, then either only one of the multiple copies of the gene is functional or else all copies must be coordinately regulated.

Stability of the integrated DNA during vegetative and asexual growth varied depending on the transformant. All type A and C transformants were phenotypically and genotypically stable during both mitosis and meiosis. In contrast, nearly half of the type B transformants were unstable during mitosis. The mitotic instability of integrated DNA in filamentous fungi has also been described

in *C. heterostrophus* (Turgeon et al. 1985; Keller et al. 1991), *Glomerella cingulata* f. sp *phaseoli* (Rodriquez and Yoder 1987), *Trichoderma reesei* (Penttila et al. 1987), and *F. moniliforme* and *F. graminearum* (Dickman and Partridge 1989). One type of mitotic instability observed in the present study was a progressive loss of the multiple copies of the insert that had integrated at a single locus. The propensity for such deletions during mitosis could prove useful for reducing multi-copy inserts to a single copy to yield a stable transformant for subsequent experiment. The analysis of the fate of the multiple tandem-copy DNA sequences during meiosis was complicated by the instability of these sequences during mitosis. While the segregation and hybridization data indicated that deletions in the inserted vector sequences clearly occurred prior to fertilization, there was some evidence that changes, particluarly methylation, also occurred after fertilization both prior to and after karyogamy. In fact, there were no indications of methylation of the integrated vector DNA sequences during vegetative growth. The methylation data suggest that RIP or RIP-like events, analogous to that in *N. crassa* (Selker et al 1987; Selker and Garrett 1988; Selker 1990; Foss et al. 1991) or perhaps to that in *Ascobolus immersus* (Goyon and Faugeron 1989), may occur in *G. pulicaris* during sexual growth. This possibility should be further investigated in future studies.

In conclusion, we have shown that *G. pulicaris* protoplasts can be transformed with non-homologous vectors. This transformation system is the first to be developed for a heterothallic, sexually fertile, trichothecene-producing plant pathogen. Even though the frequency is low, it is currently being utilized to study the molecular genetics of trichothecene production and host-pathogen interactions (Hohn and Desjardins 1992). Attempts are also being made to improve the transformation frequency by the isolation of *G. pulicaris* promoters.

Acknowledgements. We thank D. J. Henner for providing the vector pDH25, and B. G. Turgeon and O. C. Yoder for providing the vectors pUCH1 and cosHyg1. We also thank A. E. Desjardins for many fruitful discussions throughout this study.

References

Beremand MN (1989) Mycopathologia 107:67–74
Booth C (1971) The genus *Fusarium*. Commonwealth Mycological Institute, Kew, England, pp 168–171
Coppin-Raynal E, Picard M, Arnaise S (1989) Mol Gen Genet 219:270–276
Churchill ACL, Ciuffetti LM, Hansen DR, Van Etten HD, Van Alfen NK (1990) Curr Genet 17:25–31
Cullen D, Leong SA, Wilson LJ, Henner DJ (1987) Gene 57:21–26
Desjardins AE, Beremand MN (1987) Phytopathology 77:678–683
Desjardins AE, Gardner HW (1989) Mol Plant-Microbe Inter 2:26–34
Desjardins AE, Gardner HW, Plattner RD (1989) Phytochemistry 28:431–437
Dickman MB, Partridge JE (1989) Theor Appl Genet 77:535–539
Farman ML, Oliver RP (1988) Curr Genet 13:327–330
Fincham JRS (1989) Microbiol Rev 53:148–170Foss ES, Garrett PW, Kinsey JA, Selker EU (1991) Genetics 127:711–717
Garber RC, Yoder OC (1983) Anal Biochem 135:416–422
Goosen TC, Bos J, van dan Broek H (1992) Transformation and gene manipulation in filamentous fungi: an overview. In: Arora DK, Elander RP, Mukerji KG, (eds) Handbook of applied mycology, vol 4. Marcel Dekker, New York, pp 151–195
Goyon C and Faugeron G (1989) Mol Cell Biol 9:2818–2827
Hohn TM, Desjardins AE (1992) Mol Plant-Microbe Inter 5:249–256
Hynes MJ (1986) Exp Mycol 10:1–8
Johnston JB, Gunsalus IC (1977) Biochem Biophys Res Commun 75:13–17
Johnstone IL, Hughes SG, Clutterbuck AJ (1985) EMBO J 4:1307–1311
Keller NP, Bergstrom GC, Yoder OC (1991) Curr Genet 19:227–233
Kelly MK, Hynes MJ (1985) EMBO J 4:475–479
Kistler HC, Benny UK (1988) Curr Genet 13:145–149
LeChevanton L, Leblon G, Lebilcot S (1989) Mol Gen Genet 218:390–396
Lusky M, Botchan M (1981) Nature 293:79–81
Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
Oliver RP, Roberts IN, Harling R, Kenyon L, Punt PJ, Dingemanse MA, van den Hondel CAMJJ (1987) Curr Genet 12:231–233
Parsons KA, Chumley FG, Valent B (1987) Proc Natl Acad Sci USA 84:4161–4165
Penttila M, Nevalainen H, Ratto M, Salminen E, Knowles J (1987) Gene 61:155–164
Pouwels PH (1985) Curr Genet 9:361–368
Razanamparany V, Bequeret J (1988) Gene 74:399–409
Rodriguez RJ, Yoder OC (1987) Gene 54:83–87
Salch YP, Beremand MN (1988) J Cell Biochem suppl 12C:290
Salch YP, Shaw PD (1988) J Bacteriol 170:2584–2591
Selker EU (1990) Annu Rev Genet 24:579–613
Selker EU, Garrett PW (1988) Proc Natl Acad Sci USA 85:6870–6874
Selker EU, Cambareri EB, Jensen BC, Haack KA (1987) Cell 51:741–752
Timberlake WE and Marshall (1989) Science 244:1313–1317
Turgeon BG, Garber RC, Yoder OC (1985) Mol Gen Genet 201:450–453
Turgeon BG, Garber RC, Yoder OC (1987) Mol Cell Biol 7:3297–3305
Upshall A (1992) The application of molecular genetic methods to filamentous fungi. In: Arora DK, Elander RP, Mukerji KG, (eds) Handbook of applied mycology, vol 4. Marcel Dekker, New York, pp 81–99
Wernars K, Goosen T, Wennekes LMJ, Visser J, Bos CJ, van den Broek HWJ, van Gorcom RFM, van den Hondel CAMJJ, Pouwels PH (1985) Curr Genet 9:361–368

Communicated by O.C.Yoder