Gene transfer system for the phytopathogenic fungus *Ustilago maydis*

(DNA transformation/hygromycin B/corn smut)

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ABSTRACT A selectable marker for transformation was constructed by transcriptional fusion of a *Ustilago maydis* heat shock gene promoter with the hygromycin B phosphotransferase gene of *Escherichia coli*. *U. maydis* was transformed to hygromycin B resistance by polyethylene glycol-induced fusion of spheroplasts following exposure to plasmid DNA that carried the marker gene. Transformation frequencies of 50 and 1000 transformants per μg of DNA per 2×10^7 spheroplasts were obtained for circular and linear vector DNA, respectively. In the majority of transformants, the vector was integrated at a single chromosomal site, in either single copy or tandem duplication, as determined by Southern hybridization analysis of electrophoretically separated chromosomes and of restriction-endonuclease-cleaved DNA. The predominant form (82%) of vector integration was by nonhomologous recombination; the remainder carried the plasmid at the homologous heat shock gene locus. No evidence for gene conversion or gene replacement was obtained in 28 transformants. Hygromycin B phosphotransferase activity and resistance to hygromycin B were roughly correlated with the copy number of the integrated vector at the homologous location. Transforming DNA was stably maintained during mitosis and meiosis. This transformation procedure and associated vector should permit the cloning of genes by direct complementation in *U. maydis*.

Research on the basidiomycete *Ustilago maydis*, a pathogen of corn, has deepened our understanding of the genetics and biochemistry of DNA replication, repair, and recombination (1, 2). This fungus can be cultured with ease on simple, chemically defined medium, and the yeast-like, uninucleate haploid stage of growth facilitates genetic analysis. *U. maydis* infects corn and forms smut galls, plant neoplastic structures. Due to the rapid expansion of knowledge of the molecular biology and genetics of corn, *U. maydis* should prove to be valuable for molecular studies of host–parasite interactions. Studies on this fungus may also provide models for research on other basidiomycete phytopathogens including the cereal smuts and rusts. These fungi are causative agents of many severe plant diseases that can result in significant crop losses (3).

The long-term goal of our work is to elucidate the structure, regulation, and role of genes involved in phytopathogenic and saprophytic growth of *U. maydis*. Molecular-genetic analysis of these genes and others requires the development of a gene-transfer system. Genetic transformation in *U. maydis* based on kanamycin resistance has been reported by Banks (4); however, only low-frequency, unstable transformants were obtained. Here we report the establishment of an efficient and reproducible DNA transformation system for *U. maydis* based on resistance to hygromycin B (HygB), an antibiotic that has been successfully

utilized as a selective agent in a variety of organisms ranging from yeast to mouse cells (5–9).

MATERIALS AND METHODS

Strains and Media. *Escherichia coli* HB101 (F^- *hsdS20* (r_B^- , m_B^-) *recA13 ara-14 proA2 leu-6 lacY gal K2 rpsL20* (Str) *xyl-5 mtl-1 supE44 g^-*) was employed. *U. maydis* strains obtained from R. Holliday (National Institute for Medical Research, Mill Hill, London) were 518(*a2b2*), 521(*alb1*), 227(*adel-1*, *met1-2*, *nar1-6*, *rec2-1*, *a2b2*), and 288(*pan1-1*, *inol-3*, *nar1-1*, *rec1-1*, *alb1*). Other *U. maydis* strains were natural isolates collected in Wisconsin. Minimal, complete, and plate-mating media used in fungal studies were as described (1) except that Casamino acids were used at a concentration of 1% in the plate-mating medium.

Chemicals and Reagents. HygB was a gift of Eli Lilly and was later purchased from Calbiochem. Novozym 234 was obtained from Novo Laboratories (Wilton, CT). Dimethyl sulfoxide was from EM Science (Cherry Hill, NJ). D-Sorbitol, PEG 3350, and other chemicals were from Sigma.

DNA Isolation and Manipulation. DNA was isolated from the fungus by the method of Specht *et al.* (10). For analysis of large numbers of transformants, a protocol for rapid DNA isolation from spheroplasts (11) was adopted. DNA hybridization was carried out as described by Amasino (12) except that PEG and NaCl were omitted from the hybridization buffer. Orthogonal-field-alternation gel electrophoresis (OFAGE) was performed essentially according to the method of Carle and Olson (13).

Preparation of Spheroplasts. Haploid cells of *U. maydis* were incubated at 28°C in a 250-ml Erlenmeyer flask containing 40 ml of Holliday's complete medium until the density had reached 2×10^7 cells per ml (OD_{600} 1.0–1.2). These early logarithmic-phase cells, consisting mostly of actively budding cells, were harvested by centrifugation at $1100 \times g$ for 8 min in a table-top centrifuge and resuspended in 20 ml of a freshly prepared solution of 5 mM EDTA, pH 8.0/25 mM 2-mercaptoethanol. After gentle shaking at room temperature for 20 min, cells were collected by centrifugation and resuspended by brief Vortex mixing in 10 ml of buffer I (1 M sorbitol/50 mM sodium citrate, pH 5.8). A filter-sterilized stock solution of Novozym 234 (lot PPM86, 100 mg/ml in buffer I) was added to the suspension to give a final enzyme concentration of 0.25%. Spheroplast formation was monitored with a phase-contrast microscope. Within 2–5 min at room temperature, 20–30% of the cells were converted to spheroplasts. At this time spheroplasts were collected by centrifugation at $140 \times g$ for 8 min in a table-top centrifuge. Spheroplasts continued to form during centrifugation. The pellet, containing mostly spheroplasts, was

washed twice with 10 ml of buffer II (25 mM CaCl_2 /25 mM Tris-HCl, pH 7.5/1 M sorbitol). Spheroplasts were diluted to 10^8 cells per ml in buffer II. Between 5×10^8 and 7×10^8 spheroplasts were routinely obtained from a 40-ml culture with this protocol, corresponding to a yield of >60%.

DNA Transformation. Spheroplasts (2×10^7 spheroplasts in 200 μl of buffer II) were incubated with 0.5–1 μg of plasmid DNA at room temperature (20°C–26°C) for 20 min in a mixture containing 2 μl of 2-mercaptoethanol (final concentration 110 mM) and 50 μl of 66% (wt/vol) PEG 3350 in 25 mM CaCl_2 /25 mM Tris-HCl, pH 7.5. Then, a total of 2.5 ml of 66% (wt/vol) PEG 3350 in 25 mM CaCl_2 /25 mM Tris-HCl, pH 7.5, was added in a stepwise manner as follows. First one drop was added, then 0.5 ml was added, and finally the remainder of the solution was added and gently shaken to mix, with an intermission of 1 min between additions. After an additional 15 min of incubation at room temperature, the spheroplast-DNA suspension was diluted to a volume of 35 ml with buffer II by the sequential addition of 1 ml, of 5 ml, and followed by the remainder of the buffer. The spheroplasts were collected by centrifugation as before and resuspended in 0.2 ml of $2\times$ liquid complete medium containing 1 M sorbitol and shaken gently (100 rpm) for 3 hr at room temperature. No evidence for cell division was obtained during this period. The spheroplasts were spread with a glass rod onto the surface of a two-layer selective medium plate containing a 12-ml lower layer (complete medium in 1 M sorbitol and HygB at 700 $\mu\text{g}/\text{ml}$) and a 12-ml top layer (complete medium in 1 M sorbitol). The top layer was poured immediately before the transformed spheroplasts were spread. Any residual liquid on the surface of the plate was removed with a sterile cotton swab before spreading the protoplasts. The plate was incubated at 28°C, and resistant colonies appeared within 3–4 days. The regeneration frequency of spheroplasts after the above treatment was 20–30% on antibiotic-free medium. Less than 0.01% of the spheroplast population was observed to regenerate if the spheroplast suspension was pretreated with water.

HygB Phosphotransferase Assay. Fungal cells were first made into spheroplasts, as described above, and then disrupted by sonication in ice-cold 10 mM Tris-HCl/10 mM MgCl_2 /25 mM NH_4Cl /0.6 mM 2-mercaptoethanol, pH 7.8. The S30 supernatant, obtained after centrifuging the sonicated cells at $30,000 \times g$ for 45 min, was assayed for enzyme activity by the method of Haas and Dowding (14) with HygB as substrate. Enzyme activity was linear over 10 min with up to 0.5 μg of protein per ml under these assay conditions.

Genetic and Cytological Procedures. *U. maydis* diploid strains were constructed as described (1). Pathogenicity tests and genetic crosses were carried out on the maize cultivar Golden Bantam according to standard methods (1, 15). To analyze the random segregants of meiosis, the following procedure was adopted: diploid teliospores from maize smut galls were rinsed briefly with ether to kill vegetative cells and then with water before suspending in a solution of 0.4% tetracycline. Teliospores were spread on complete medium plates and incubated at 28°C for 24–30 hr. The resulting haploid basidiospores were collected with a moistened cotton swab and suspended in water. This solution was then spread on complete medium plates. After 2–3 days of growth, colonies were replica-plated onto various test media plates.

Mithromycin staining (16) was used to detect fungal nuclei.

RESULTS

Characteristics of Vector pHL1. A derivative of pUC12, pHL1 contains a 3.3-kilobase (kb) *Hind*III insert that carries the bacterial HygB phosphotransferase gene (5) in transcriptional fusion with a *U. maydis* *hsp70* gene promoter (Fig. 1).

FIG. 1. Construction of transformation vector pHL1. pSL7 consists of a 4.6-kb *Hind*III fragment of *U. maydis* genomic DNA inserted into the *Hind*III site of pUC12. This insert contains a member of the *hsp70* gene family of this fungus (D.W.H. and S.A.L., unpublished data). The two internal *Bgl* II fragments of pSL7 were deleted to give rise to pSL11. pDHW10, a deletion derivative of pSL11, was obtained by linearization at the unique *Bgl* II site, treatment with BAL-31 nuclease, and ligation to *Bgl* II linkers. The endpoint of this deletion is located ≈ 25 base pairs downstream of the transcription initiation site of this *hsp70* gene (D.W.H. and S.A.L., unpublished data). pHL1 was constructed by insertion of the 1.05-kb *Bam*HI fragment, carrying the HygB phosphotransferase gene coding sequence of pLG90 (5), into the *Bgl* II site of pDHW10. Among the unique restriction sites in pHL1 are *Sst* I and *Bam*HI (in the polylinker) and *Xho* I (in the *U. maydis* insert). A detailed restriction map of pSL7 will be presented elsewhere (D.W.H. and S.A.L., unpublished data). *U. maydis* DNA sequences on the 5' and 3' side of the HygB phosphotransferase gene coding sequence are 1.25 kb and 1.0 kb long, respectively. H, *Hind*III; RI, *Eco*RI; X, *Xho* I; Bg, *Bgl* II; and PL, polylinker. pUC12 is indicated by the thinnest line; the HygB phosphotransferase gene coding sequence is represented by a medium-thick line; and *U. maydis* DNA is represented by the thickest line.

This plasmid confers ampicillin but not HygB resistance in *E. coli*, and HygB resistance in *U. maydis*. HygB is particularly useful as a selection for *Ustilago* as it completely inhibits growth of this fungus at 150 $\mu\text{g}/\text{ml}$, spontaneous resistance is low, and background growth is marginal (unpublished observations). In contrast, *U. maydis* is highly resistant to the related drugs G418, neomycin, and kanamycin.

Transformation of *U. maydis*. Unless specified otherwise, the transformation experiments were carried out with *U. maydis* 518, a prototrophic, wild-type strain. All of the fungal strains we have examined have shown similar sensitivity to HygB. The frequency of spontaneous resistance to the antibiotic at a concentration of 250 $\mu\text{g}/\text{ml}$, a level lower than the one used in the selective medium for transformation experiments, was $<10^{-9}$ for whole cells and spheroplasts. *U. maydis* isolates originating from a variety of sources have been transformed successfully with pHL1. Utilizing the standard transformation protocol, we have routinely obtained >500 stable transformants per μg of linearized pHL1 DNA per 2×10^7 spheroplasts. The numbers of transformants obtained were proportional to the amount of plasmid used when 0.1–1 μg of DNA was added.

To assess the role played by the *hsp70* gene promoter in the transformation vehicle, two additional vectors, pHL2 and pHL1-B, were constructed. pHL2 bears the same fea-

tures as pHL1 except that the orientation of the *Bam*HI insert, which contains the HygB resistance gene, was inverted relative to the *hsp70* gene promoter. pHL2 failed to transform *U. maydis* to HygB resistance. Inversion of the *Hind*III fragment of pHL1 gave rise to plasmid pHL1-B, with the *E. coli lac* promoter of the vector directed away from the HygB phosphotransferase gene. pHL1-B was functionally equivalent to pHL1 in transformation experiments.

The protocol we employed for *U. maydis* DNA transformation shares some basic similarities with other fungal gene transfer systems. Three factors contributed greatly to the efficiency of transformation. First was the use of early logarithmic-phase cells to prepare spheroplasts. Since younger or older cells could be converted into spheroplasts and regenerated at the same frequency as early logarithmic-phase cells, this difference in transformation frequency must be due to intrinsic physiological properties of early logarithmic-phase cells. Second, linearization of pHL1, either at the *Xho*I site (on the 5' side of the heat shock promoter) or in the pUC12 polylinker sequence, increased transformation frequency by up to 20-fold. This is in marked contrast to results obtained with other filamentous fungi, where linearization of the plasmid usually has little or no effect on transformation frequency (17). In *Saccharomyces cerevisiae* improved transformation has also been achieved with linear DNA and has been attributed to the recombinogenic nature of free ends of DNA (18). Third, the genetic background of the recipient strains also influenced the frequency of transformation. Compared to strain 518, strain 227 gave a transformation frequency that was lower by a factor of 50–100, whereas strain 288 consistently gave a 2- to 3-fold increase of HygB-resistant colonies, perhaps because the *rec1* mutation exhibits increased spontaneous mitotic recombination (19).

Fate of Transforming DNA. Twenty-eight HygB-resistant colonies arising on selective medium after transformation with linear pHL1 were purified by three rounds of single-colony isolation under continuous drug selection. All of the colonies contained uninucleate cells as revealed by mithromycin staining. No heterokaryons were detected. Total cellular DNA was isolated from each of the 28 colonies, and Southern transfers of undigested, *Hind*III- or *Eco*RI-digested DNA were hybridized with two probes: (i) the *Bam*HI fragment of pLG90 containing the HygB phosphotransferase gene coding sequence was used to detect the presence of the antibiotic resistance determinant, and (ii)

intact pHL1 was employed to determine the location as well as copy number of the integrated plasmid.

The 28 transformants displayed several common features. (i) They all hybridized strongly with the *Bam*HI fragment; DNA from untransformed recipient strains failed to hybridize. (ii) Southern blots of undigested, sheared DNA failed to show hybridization to bands other than sheared DNA, even after an extended period of exposure (data not shown). This argues against the presence of free monomeric plasmid in the transformants. The integration pattern of selected *U. maydis* transformants is illustrated in Figs. 2 and 3. Two types of integration events were found: integration at the resident chromosomal site of the *hsp70* gene by way of a single homologous recombination or integration elsewhere in the genome, corresponding respectively to type I and type II integration in *Aspergillus nidulans* as described by Yelton *et al.* (20). No gene replacement or type III integration events (20) were detected.

It is evident from Fig. 3 that integration of the pHL1 vector into the *hsp70* locus by homologous recombination disrupted a large (13.2-kb) *Eco*RI fragment and gave rise to two new *Eco*RI fragments of 7.2 kb and 7.4 kb. This type of integration is represented by transformant 2-4 (Fig. 2). Upon probing with pHL1 (Fig. 2D, lane 3), the only other *Eco*RI fragment that hybridized was 4.6 kb long. This corresponds to the larger *Eco*RI fragment of the vector. When subjected to *Hind*III digestion (Fig. 2B, lane 2), three fragments of 3.3 kb, 2.7 kb, and 4.5 kb corresponding to vector fragments and the recombinant *Hind*III genomic fragment carrying the *hsp70* gene were revealed as expected. These data as well as the intensity of hybridization signals suggests that a single copy of pHL1 had integrated at the *hsp70* locus in transformant 2-4. Transformant 4-1 (Fig. 2D, lane 4) also lacked the large 13.2-kb *Eco*RI fragment and possessed the same new *Eco*RI fragments characteristic of homologous recombination. In addition, a third *Eco*RI fragment of 1.4 kb, corresponding to the smaller *Eco*RI fragment of pHL1, was observed. These data can be best explained by a tandem integration of two copies of the plasmid. The same hybridization pattern of *Hind*III fragments displayed by transformants 2-4 and 4-1 when probed with the *Bam*HI fragment or pHL1 (Fig. 2A, lanes 2 and 3, and B, lanes 2 and 3) also supported this interpretation.

Homologous integration was also suggested by hybridization of the *Bam*HI fragment probe to intact chromosomes

FIG. 2. Southern hybridization analysis of selected HygB-resistant transformants. Approximately 5 μ g of total genomic DNA or 2.5 ng of pHL1 was digested with *Hind*III (A and B) or *Eco*RI (C and D) and electrophoresed in 0.5% agarose gels by using phage λ DNA digested with *Hind*III as a size marker. Southern transfers of gels were hybridized with a radiolabeled 1.05-kb *Bam*HI fragment encoding the HygB phosphotransferase gene from pLG90 (A and C) or pHL1 (B and D) and autoradiographed. In A and B, the lanes contain DNA as follows: 1, strain 518; 2, transformant 2-4; 3, transformant 4-1; 4, transformant 4-2; 5, transformant 4-5; and 6, pHL1. In C and D, the lanes contain DNA as follows: 1, pHL1; 2, strain 518; 3, transformant 2-4; 4, transformant 4-1; 5, transformant 4-2; and 6, transformant 4-5.

FIG. 3. Integration of pHL1 by homologous recombination into the *U. maydis* genome. The predicted structure of the *hsp70* locus after integration of the vector by a single crossover at the *Xho* I site in pHL1. Abbreviations are as in Fig. 1. The 4.6-kb *Hind*III fragment that encodes the *hsp70* gene is located in the 13.2-kb *Eco*RI fragment of *U. maydis* genomic DNA and is indicated by the hatched box; the corresponding homologous fungal DNA in pHL1 is indicated by the thickest line; the HygB phosphotransferase gene coding sequence indicated by a medium-thick line; other fungal DNA is indicated by a thin line; and pUC12 is indicated by the thinnest line.

separated by OFAGE (Fig. 4). The probe hybridized to chromosome cluster XII–XIII, where the *hsp70* gene used to construct the vector is known to be located (T. Kinscherf and S.A.L., unpublished data).

The majority of the transformants possessed pHL1 at locations elsewhere in the genome as represented by transformants 4-2 and 4-5 (Fig. 2). Unlike 2-4 or 4-1, transformants 4-2 and 4-5 retained the large 13.2-kb *Eco*RI fragment. In the case of 4-2 (Fig. 2 A, lane 4, B, lane 4, C, lane 5, and D, lane 5), the 3.3-kb *Hind*III fragment and 1.4-kb *Eco*RI fragment have been replaced by two new fragments suggesting the insertion of a single copy of the vector. For transformant 4-5 (Fig. 2B, lane 5), however, all three *Hind*III frag-

ments (2.7 kb, 3.3 kb, and 4.5 kb) were retained in addition to four new *Hind*III fragments, which implies vector insertion at two locations. Data for *Eco*RI digestion of transformant 4-5 (Fig. 2 C, lane 6, and D, lane 6) DNA also support this interpretation.

In summary, 5 of 28 transformants examined by Southern analysis resulted from homologous integration (type I integration). Of the 5 transformants, one involved a single-copy insertion, whereas 4 were the result of tandem insertion of the vector. The remaining 23 were generated by illegitimate recombination of pHL1 into the host genome (type II integration). Included in this group were single-copy insertions (3 transformants) and tandem integration of the vector with or without additional rearrangements or deletions (10 transformants in each case). No evidence for type III integration (double-homologous recombination or gene conversion) was obtained.

HygB Phosphotransferase Activity. HygB phosphotransferase activity was readily detected in transformed fungal cells, and the enzyme levels were roughly correlated with both the levels of resistance to the drug and vector copy number for type I transformants (Table 1). No enzyme activity was found in the recipient strain.

Mitotic and Meiotic Stability of Transformants. Of the 28 transformants subjected to Southern analysis, 3 with type I integration and 3 with type II integration were examined further for stability of the transforming DNA. Purified single colonies of each transformant were grown in liquid complete

FIG. 4. Southern hybridization analysis of the electrophoretic karyotype of transformants. OFAGE was carried out essentially as described by Carle and Olson (13) but with 2×10^9 protoplasts embedded in 5 ml of 2.5% low-melting agarose in 1 M sorbitol/0.5 M EDTA, pH 7.4. Chromosomal DNA was liberated from protoplasts by incubation of the agarose sheet in proteinase K at 1 mg/ml and 1% *N*-laurylsarcosine, sodium salt, in 0.5 M EDTA (pH 7.4) at 50°C for 12 hr. Southern transfer was probed with the 1.05-kb *Bam*HI fragment containing the coding region of the HygB phosphotransferase gene of pLG90 and then autoradiographed. The lanes contain the chromosome complement as follows: 1, strain 518; 2, transformant 2-4; 3, transformant 4-1; and 4, transformant 4-2. (Left) Ethidium bromide-stained gel. (Right) Autoradiogram of Southern transfer.

Table 1. HygB phosphotransferase activity of *U. maydis* transformants

Extract source	Vector, no. of copies	Specific activity, pmol/min per μ g of protein
Strain 518	0	<0.01 + 0.00
Transformant 2-4	1	0.73 + 0.04
Transformant 4-1	2	1.60 + 0.05
Transformant 5-10	Multiple	2.50 + 0.08

All transformants carried the pHL1 vector at the homologous *hsp70* locus. Enzyme activities were determined in triplicate and are expressed as means + SEM.

medium without HygB for ≈ 30 generations before spreading on complete medium plates free of antibiotic. The resulting colonies were transferred onto selective and nonselective medium to test for sensitivity to HygB. For each sample, 200 colonies were scored. All of these retained the HygB-resistant phenotype.

The meiotic stability of transformed *U. maydis* cells was investigated by crossing four transformants of strains 518, 2-1, 2-4, 4-1, and 4-2 with strain 521, a wild-type *U. maydis* isolate. Two hundred basidiospore segregants from each cross were examined, and half of these were found to be resistant to HygB (data not shown). From each cross six HygB-resistant segregants as well as six HygB-sensitive meiotic segregants were subjected to Southern analysis. No hybridization of the *Bam*HI fragment probe was found in any of the antibiotic sensitive segregants (data not shown). Moreover, the plasmid integration patterns of the HygB-resistant segregants remained unchanged when compared to original parental transformants (data not shown).

Transformation of Diploid *U. maydis*. Utilizing the same transformation procedure, we have also introduced pHL1 into a diploid *U. maydis* strain D-2, which was constructed by fusing haploid strain 227 with 288. A low frequency of 10 transformants per μ g of plasmid DNA was obtained. The transformants were uninucleate as demonstrated by mithromycin straining, maintained the ability to grow on minimal medium, and were solopathogenic—that is, they were able to elicit corn galls after inoculation of host plants. The teliospores recovered from these galls were germinated on complete medium and resulting basidiospore segregants were examined for HygB resistance. Two hundred segregants were checked for each of 4 transformants, and a 1:1 numerical ratio of HygB-resistant/HygB-sensitive cells was found (data not shown).

DISCUSSION

We have established an efficient and reliable DNA transformation procedure for the phytopathogenic fungus *U. maydis* that uses the drug HygB as a selective agent. The vector pHL1 contains a transcriptional fusion of a *U. maydis hsp70* gene promoter and a structural gene encoding the HygB-resistance determinant. Several factors contributed to the high transformation frequencies that we obtained. Among these were the use of early logarithmic-phase cells in the preparation of spheroplasts, the inclusion of 2-mercaptoethanol in the transformation mixture, and linearization of the plasmid vector DNA.

hsp70 gene promoters from other eukaryotes have been used in construction of selectable markers (6, 21, 22). Yeast and *Drosophila hsp70* promoters have also been fused with the *E. coli* β -galactosidase gene (23, 24). In both cases, a substantial basal-level expression of the gene was observed under normal growth conditions. Results from S1-protection analysis show that the *U. maydis hsp70* gene employed in the development of the selection marker is expressed at normal growth temperatures (D.W.H. and S.A.L., unpublished data). Since pHL2 failed to transform the fungus to HygB resistance and pHL1B was as effective as pHL1, we conclude that it is the *hsp70* promoter fused to the HygB phosphotransferase gene that drives the expression of the antibiotic resistance determinant in transformants.

In all of the transformants exhibiting type I integration, the crossover event always occurred within the *U. maydis* DNA sequence on the 5' side of the HygB phosphotransferase gene in the vector. Evidently, the linearization of pHL1 (at an *Xho* I site) promoted recombination at this region, or the insertion of a bacterial DNA sequence somehow interfered with homologous exchange between the DNA sequence on the 3' side of the insert and its counterpart on the fungal

chromosome. This would also explain our failure to detect a single case of type III integration among the transformants analyzed, since this type of integration requires a double crossover or gene conversion at the *hsp70* locus. Another explanation for the failure to see replacement of the wild-type *hsp70* gene by the disrupted *hsp70* gene (carrying HygB phosphotransferase gene within it) is that this *hsp70* gene is essential for growth. Disruption of a cognate *hsp70* gene of *S. cerevisiae* is a lethal event (25). Transformation of pHL1 into diploid *U. maydis* cells would make it possible to investigate such recessive lethal mutations.

The integration pattern observed here in *U. maydis*, in which pHL1 was integrated predominantly at chromosomal sites other than the *hsp70* locus, resembles that found in *Neurospora crassa* and *A. nidulans* but differs from *S. cerevisiae* where all transformants are the result of homologous recombination (17). This phenomenon may be marker dependent as has been shown in *A. nidulans* (26).

U. maydis transformants were stable mitotically and meiotically, whether they carried single or multiple copies of the vector. The data obtained for meiotic passage of vector suggest that it segregates as a single Mendelian trait when integrated at one location. No evidence for autonomous replication of the transforming plasmid was obtained. Considering the ease with which large numbers of *U. maydis* spheroplasts can be generated, it should now be feasible to clone genes directly by complementation in this fungus with the methods described here.

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