Transformation of the Oomycete Pathogen, *Phytophthora infestans*

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A stable transformation procedure has been developed for *Phytophthora infestans*, an oomycete fungus that causes the late blight diseases of potato and tomato. This is the first description of reliable methods for transformation in an oomycete pathogen. Drug-resistant transformants were obtained by using vectors that contained bacterial genes for resistance to hygromycin B or G418 fused to promoters and terminators from the *Hsp70* and *Ham34* genes of the oomycete, *Bremia lactucae*. Using polyethylene glycol and $CaCl_2$, vector DNA was introduced into protoplasts as a complex with cationic liposomes or with carrier DNA only. Transformants were obtained at similar frequencies with each combination of promoter and selectable marker and were confirmed by DNA and RNA hybridization and phosphotransferase assays. Transformation occurred through the integration of single or tandemly repeated copies of the plasmids into genomic DNA, conferring mitotically stable drug-resistant phenotypes. The sizes of the marker gene mRNAs in each transformant and the results of transcript mapping studies were consistent with the function of the *B. lactucae* regulatory sequences in *P. infestans*. A hygromycin-resistant transformant was tested and found to maintain pathogenicity, indicating that the gene transfer procedure will be useful for the molecular analysis of genes relevant to disease.

Phytophthora infestans (Mont.) de Bary causes the late blight diseases of potato and tomato (Clarke 1983). It is one of the many oomycete fungi that cause a wide range of destructive diseases on plants. Race/cultivar specificity in potato and tomato late blight is thought to be determined by "gene-for-gene" interactions (Flor 1956) involving matching pairs of pathogen avirulence genes and host resistance genes. Eleven dominant genes for resistance have been identified in potato and one in tomato (Black *et al.* 1953; Malcomson and Black 1966; Gallegly and Marvel 1955). Several of the corresponding genes in *P. infestans* have been characterized genetically (Spielman *et al.* 1989, 1990).

Procedures for gene transfer will be essential for the characterization of genes controlling specificity and pathogenicity. Methods developed for the transformation of non-pathogenic fungi such as *Saccharomyces cerevisiae* Meyen ex Hansen, *Neurospora crassa* Shear et Dodge, and *Aspergillus nidulans* (Eidam) G. Wint. have been successfully applied to several pathogenic ascomycetes and basidiomycetes (rev. by Fincham 1989). However, the oomycetes have proven recalcitrant to transformation when vectors and protocols developed for these fungi are used. In part, this may be due to the weak taxonomic affinity between the oomycetes and the higher fungi; comparisons of the sequences of rRNA genes place the oomycetes closer to the algae than to the higher fungi (Forster *et al.* 1990). Therefore, specific vectors may be required for the oomycetes.

We had previously developed methods for detecting the transient expression of genes introduced into protoplasts of *P. infestans*, using the reporter gene β-glucuronidase

(GUS) as a first step toward the development of transformation procedures (Judelson and Michelmore 1991). These assays were used to evaluate methods for introducing DNA into protoplasts and to identify promoters and terminators that functioned in *P. infestans*. These studies indicated that promoters from the heat shock gene *Hsp70* (Judelson and Michelmore 1989) and the putative structural protein gene *Ham34* (Judelson and Michelmore 1990) of the related oomycete, *Bremia lactucae* Regel (lettuce downy mildew), functioned well in *P. infestans* (Judelson and Michelmore 1991). In contrast, promoters from four ascomycete genes, one basidiomycete gene, and three plant genes performed poorly in *P. infestans* (unpublished observations). In this paper, we report the development of procedures for the stable integrative transformation of *P. infestans*, using plasmids containing fusions between transcriptional regulatory sequences from the *Ham34* and *Hsp70* genes of *B. lactucae* and two bacterial genes conferring resistance to hygromycin or G418. This is the first description of reliable procedures for stable transformation of an oomycete plant pathogen.

MATERIALS AND METHODS

Transformation vectors. Vectors were constructed from pUC19-based expression plasmids containing promoters and terminators from the *Ham34* and *Hsp70* genes of *B. lactucae*, separated by *Sma*I linkers (Judelson and Michelmore 1991). A 988-nucleotide (nt) *Pvu*I fragment containing the neomycin phosphotransferase (*nptII*) gene (blunt-ended with Klenow polymerase), or a 1,253-nt *Sma*I fragment containing hygromycin phosphotransferase (*hpt*), were excised from pMON200 or pMON408, respectively (Rogers *et al.* 1987), and inserted into the *Sma*I site of the expression vectors (Fig. 1). DNA for transformation experiments was prepared in *E. coli* strain DH5α and purified by alkaline lysis followed by centrifugation on CsCl-ethidium bromide gradients.

Growth of *P. infestans* and generation of protoplasts.
P. infestans cultures (kindly provided by Richard Bostock, University of California, Davis, and Michael Coffey, University of California, Riverside) were maintained by serial culture on V8 agar at 19° C. A potato race 0 isolate (i.e., virulent only on hosts lacking resistance genes) was used as the recipient strain for transformation in most experiments. Protoplasts were isolated from young mycelia as described (Judelson and Michelmore 1991). Briefly, sporangia were washed from V8 agar cultures and placed in ALBA medium (Bruck et al. 1980) at 19° C. After 48 hr, the mycelia were washed once in KC osmoticum (0.64 M KCl–0.2 M CaCl$_2$) and incubated at room temperature with 5 mg/ml of Novozym 234 (Novo Laboratories, Wilton, CT) and 2.5 mg/ml of cellulase (Sigma, from *Trichoderma reesi*) in KC at 5 ml per milliliter of packed mycelia. Digestion was generally complete after 1 hr, at which time the protoplasts were filtered through 35-μm mesh, pelleted by centrifugation at $600 \times g$, washed twice in KC, and washed once in MTC-10 or MTC-50 (1 M mannitol, 10 mM Tris 7.5, plus 10 or 50 mM CaCl$_2$, respectively), depending on the transformation procedure that was to be used.

Transformation procedure. Protoplasts were treated as described previously, using methods shown to result in maximum DNA uptake (Judelson and Michelmore 1991), except that mannitol was used instead of sorbitol as an osmoticum. For transformations that used cationic liposomes, vector DNA (30 μg) was incubated for 15 min with 60 μg of Lipofectin reagent (Bethesda Research Laboratories, Gaithersburg, MD) and then gently mixed with 10^8 protoplasts in 1 ml of MTC-10. For transformations without liposomes, 30 μg of vector DNA was mixed with 200 μg of salmon DNA as carrier, and then added to 10^8 protoplasts in MTC-50. After 10 (liposome method) or 1 min (carrier DNA method), 1 ml of 50% PEG 3350 (Sigma)

containing 20 mM CaCl$_2$ and 10 mM Tris, pH 7.5, was slowly added. After 10 min, protoplasts were diluted into a 10-fold volume of clarified V8 medium containing 1 M mannitol. After 24 hr at 19° C, either 30 μg/ml of hygromycin B (Calbiochem Corp., La Jolla, CA) or 4 μg/ml of G418 (Sigma) was added, and after an additional 24 hr the young mycelia were recovered by centrifugation and spread on V8 agar containing hygromycin (70 μg/ml) or G418 (10 μg/ml). Colonies appeared within 7–12 days and were subsequently propagated on plates that generally contained either 140 μg/ml of hygromycin or 20 μg/ml of G418. The frequency of protoplast regeneration was determined by microscopic examination of the liquid cultures, 24 hr after DNA treatment. The regeneration rates ranged from 2 to 15%, with an average of 5%.

Biochemical and molecular analysis of transformants. DNA and RNA blot hybridizations were performed by standard techniques using DNA or RNA isolated from mycelia grown in liquid V8 medium (Raeder and Broda 1985; Frederick and Kinsey 1990). Primer extension analysis was performed as described (Judelson and Michelmore 1989), using a 30-nt oligonucleotide complementary to bases 16–45 of the *hpt* open reading frame. Phosphotransferase assays were performed as described (Mohr 1989), omitting the nitrocellulose binding step. Assays were performed in duplicate, using hygromycin or G418 as substrates.

RESULTS AND DISCUSSION

Characteristics of transformation vectors. The design of vectors was based on data obtained earlier from transient expression assays using the GUS reporter gene (Judelson and Michelmore 1991). These experiments indicated that promoters from the *B. lactucae Hsp70* and *Ham34* genes functioned well in *P. infestans*, and that vectors containing 3' sequences from the *Ham34* gene conferred higher levels of marker gene expression than did vectors with other terminators. Consequently, vectors were constructed for the expression of the marker genes hygromycin phosphotransferase (*hpt*) and neomycin phosphotransferase (*nptII*) using the *Ham34* terminator and either the *Hsp70* or *Ham34* promoters (Fig. 1).

Isolation of transformants. Stable, drug-resistant transformants were obtained with each combination of promoter and marker gene, and two different methods for introducing DNA into protoplasts. The methods for DNA treatment had previously been developed for *P. infestans* with the aid of transient assays (Judelson and Michelmore 1991). In typical experiments, 10^8 protoplasts derived from young mycelia were either mixed with 30 μg of vector DNA complexed with cationic liposomes (Felgner et al. 1987), or with vector plus carrier DNA only. After treatment with polyethylene glycol (PEG) and CaCl$_2$, the protoplasts were allowed to regenerate overnight in liquid medium without drug prior to the application of drug selection. This regime resulted in maximum rates of regeneration of the protoplasts, which averaged 5%. Using these protocols, in 12 separate experiments involving a total of 28 vector treatments, we obtained 64 colonies stably resistant to G418 or hygromycin. Subsequent DNA and RNA hybridization
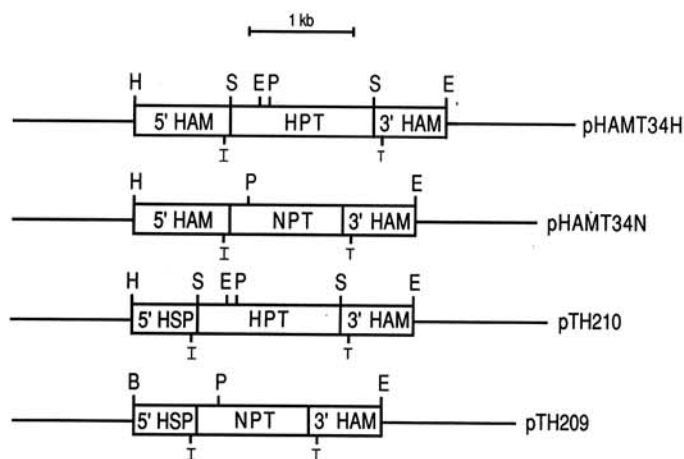

Fig. 1. Transformation vectors. Neomycin phosphotransferase (*nptII*) or hygromycin phosphotransferase (*hpt*) genes were inserted into expression vectors that contained promoters and terminators from the *Ham34* and *Hsp70* genes of *B. lactucae*, forming transcriptional fusions. Indicated are the 920-nt promoter fragment from *Ham34* (5' HAM), the 590-nt promoter element from *Hsp70* (5' HSP), the 550-nt *Ham34* terminator (3' HAM), the native *B. lactucae* transcriptional start (I) or termination (T) points, and pUC19 sequences (solid line). B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sma*I.
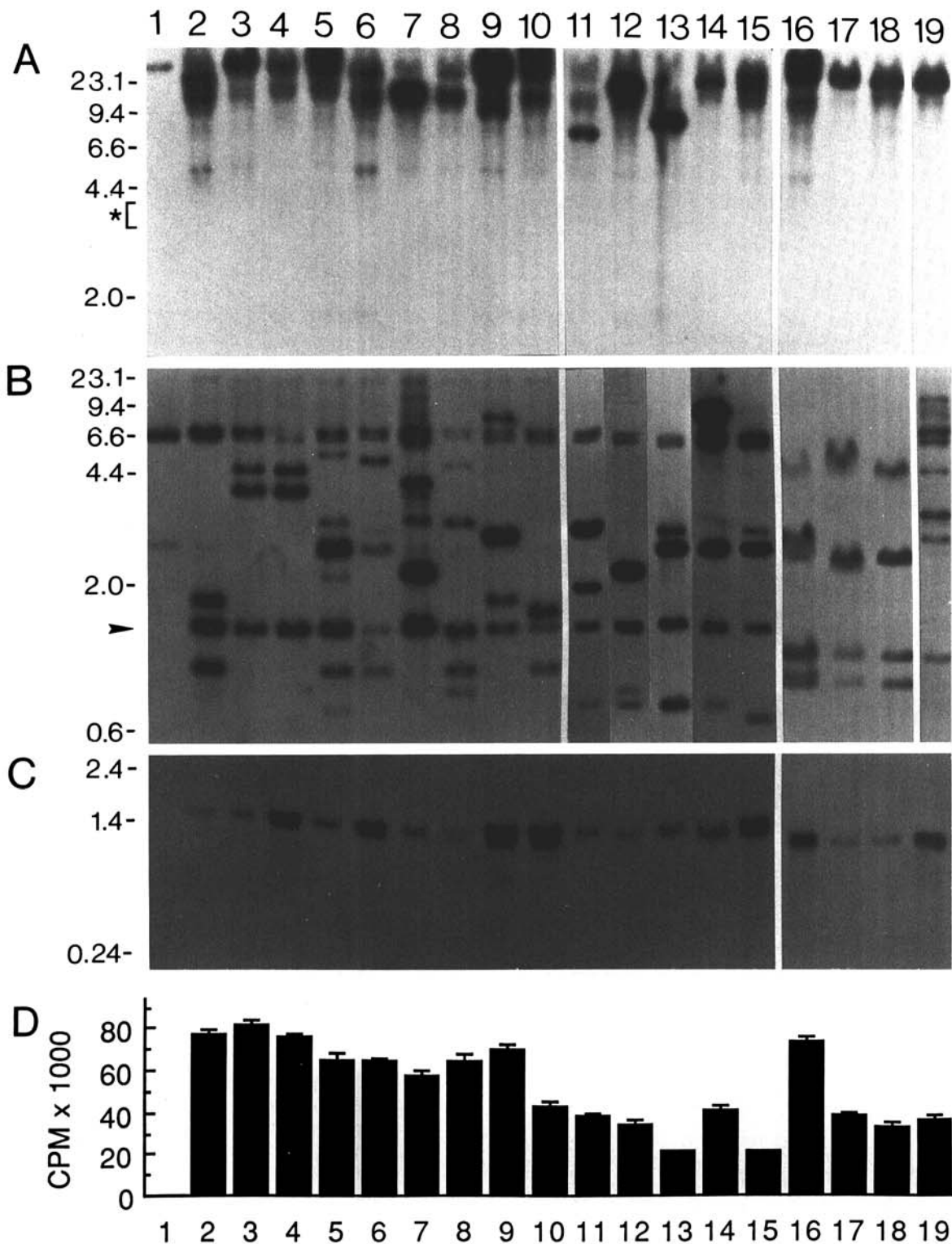
Fig. 2. Characterization of transformants by **A, B,** DNA hybridization; **C,** RNA blotting; and **D,** phosphotransferase assays. Lanes correspond to the untransformed recipient strain (1), hygromycin-resistant transformants obtained with pHAMT34H (2–10), or pTH210 (11–15), and G418-resistant transformants generated using pHAMT34N (16–18) or pTH209 (19). Transformants were obtained using the cationic liposome method (lanes 2–4, 8–17, 19) or with carrier DNA only (5–7, 18). In A and B, genomic DNA (3 μg) was digested with *Bcl*I (A, 1–19), *Eco*RI plus *Hin*dIII (B, 1–10), or *Eco*RI, *Pst*I, and *Hin*dIII (B, 11–19) and hybridized to radiolabeled probes from the corresponding transformation vectors (pHAMT34H for wild type). Size standards (in kb) are indicated in the left margin. In A, the asterisk in the left margin indicates where undigested transformation plasmids appeared on gels run in parallel. Arrows in the left and right margins of B denote bands that contain the *Ham34* terminator and most of the *hpt* gene (lanes 2–15), or the terminator and most of the *nptII* gene (lanes 16–19), respectively. In C, total RNA from wild type or each transformant (4 μg) was separated by electrophoresis on formaldehyde gels and hybridized to probes for *hpt* (1–15) or *nptII* (16–19). Size standards (in kb) are indicated in the margin. D indicates the activity of hygromycin phosphotransferase (lanes 1–15) and neomycin phosphotransferase (determined using G418 as substrate, lanes 16–19), expressed as cpm $\times$ 10^3/10 μg of protein per hour. Average of two determinations; error bars indicate range of values.

studies and phosphotransferase assays confirmed that these colonies were authentic transformants (below). Occasionally, additional colonies grew on the selection plates but they failed to grow after being transferred to fresh plates.

Although transformants were reproducibly obtained, the efficiency of transformation was lower than that observed for many filamentous fungi. At these frequencies it was difficult to make accurate comparisons of vector efficacy. However, transformants were obtained at similar rates regardless of the selectable marker used (63 hygromycin-resistant colonies in 20 attempts, 46 G418-resistant derivatives in 13 trials), or the promoter employed (54 transformants with *Ham34* in 17 attempts, and 55 transformants in 16 attempts using *Hsp70* sequences). In side-by-side comparisons, the frequency of transformation using the liposome method was higher than that observed when the vectors were mixed with carrier DNA only (sum of four comparisons, 12 vs. two). This was consistent with the results of earlier transient assays, where cationic liposome treatments resulted in optimal levels of marker gene expression (Judelson and Michelmore 1991). We have attempted to raise the frequency of transformation by adding homologous sequences of *P. infestans* DNA to the vectors (12 vectors tested, each containing 1–5 kb inserts) and by linearizing the vectors, but these efforts have so far been unsuccessful. In part, the low transformation frequencies may be a consequence of the low regeneration rates of the protoplasts (5%). This regeneration rate is less than that often obtained for many filamentous ascomycetes and basidiomycetes; however, lower rates would be expected for *P. infestans* since *Phytophthora* mycelia are coenocytic and therefore many protoplasts derived from mycelia will be anucleate and inviable.

DNA hybridization analysis. Fourteen colonies resistant to hygromycin (nine using the *Ham34* promoter, four using the *Hsp70* promoter) and four resistant to G418 (three with the *Ham34* promoter, one with the *Hsp70* promoter) were selected for further study to confirm transformation and characterize the fate of vector DNA. Southern analysis indicated that transformation had occurred by the chromosomal integration of vector DNA. When vector DNA was hybridized to genomic DNA digested with *Bcl*I, which does not cut within the vectors, hybridization was observed to bands of higher molecular weight than would be expected if the vectors were present as free monomeric plasmids (Fig. 2A).

Data from other digests were also consistent with the presence of vector DNA in the transformants. For example, a 1.6-kb band was observed in *Eco*RI-*Hin*dIII digests of DNA from each hygromycin-resistant transformant (Fig. 2B), which corresponded in size to a vector fragment containing most of *hpt* and the *Ham34* terminator. Similarly, in digests of DNA from the G418-resistant transformants, a 1.3-kb *Eco*RI-*Pst*I band was detected that was consistent with a terminator-*nptII* fragment present in the vectors.

Southern analysis also indicated that transformation had usually occurred through the integration of single copies of the vectors. In the digests shown in Figure 2B, simple integration should result in two bands representing vector DNA plus two species corresponding to border fragments

(plus, in the *Hin*dIII and *Eco*RI digests, a 6.6-kb doublet hybridizing in both the untransformed recipient and the transformants). Digestion patterns consistent with a simple integration event were observed in 12 cases. However, in three instances (lanes 3, 4, and 10) fewer bands were detected, which suggested partial deletion or rearrangement of vector DNA. In three other transformants (lanes 13, 16, and 18), the structure of the integrated DNA was consistent with the insertion of tandemly repeated vector molecules, which is also frequently observed in the transformation of ascomycete (Yelton *et al.* 1984) and basidiomycete fungi (Wang *et al.* 1988). In these instances all bands expected for the vector alone, plus putative border fragments, were observed in these digests and other enzyme combinations (not shown). For example, in the *Hin*dIII-*Eco*RI digest shown in lane 13 (transformant obtained using pTH210), the 0.8-, 1.6-, and 2.6-kb bands expected for the vector alone were observed plus more weakly hybridizing 2.1- and 2.9-kb species. In the transformants containing nontandem integration events, borders with genomic DNA occurred within pUC19 sequences nine times, within the promoter fragment in three cases, and within both promoter and pUC19 sequences three times, but curiously never within the 3′ terminator region. In all transformants, the number of total bands, including border fragments, was consistent with integration at a single genomic site.

Expression of marker genes. To confirm that the *hpt* and *nptII* marker genes were being expressed, we assayed the 18 transformants described above for the appropriate phosphotransferase activity (Fig. 2D). Each transformant expressed the expected activity, although some variation in specific activity was detected among the transformants that might reflect position effects or cultural variation. With some exceptions, higher levels of activity were typically detected in transformants containing the *Ham34* promoter. This is in contrast to the results previously obtained in transient assays, where genes fused to the *Hsp70* promoter were expressed at higher levels than genes fused to 5′ *Ham34* sequences (Judelson and Michelmore 1991). These differences may be explained by activation of the *Hsp70* promoter by stress in the protoplasts used in the those assays. Transformants containing tandemly repeated copies of the vectors generally did not have higher levels of phosphotransferase activity.

Northern analysis also confirmed the expression of the two marker genes (Fig. 2C). Regardless of the nature of the transformation event, the size of the RNAs that hybridized to probes for *hpt* or *nptII* in each transformant was consistent with the initiation and termination of transcription at the native *B. lactucae* sites, rather than in flanking regions of *P. infestans* DNA, assuming a 100-nt poly(A) tail. Transcriptional start points were mapped by primer extension analysis of RNA isolated from a hygromycin-resistant transformant that contained the entire 920-nt *Ham34* promoter, and RNA samples from two hygromycin-resistant transformants in which approximately 450 nt of the *Ham34* promoter fragment was missing; in these latter transformants contiguous DNA from *P. infestans* could possibly have been providing promoter activity (transformants shown in lanes 2–4, Fig. 2). In all three cases, the extension products indicated that transcription was

initiated only at the sites observed for the native *Ham34* gene in *B. lactucae* (Fig. 3).

Potential utility of transformation. Additional experiments indicated that the gene transfer procedure will be useful for studying *P. infestans* and interactions in late blight. Transformants were obtained at similar frequencies using three different strains of *P. infestans*, suggesting that the technology will be applicable to most strains. Also, one randomly selected hygromycin-resistant transformant was tested for pathogenicity and found to be vigorously pathogenic on a tomato host, despite having experienced extended *in vitro* culture (>10 subcultures) and transformation procedures involving regeneration from protoplasts.

Further experiments indicated that transformed phenotypes were mitotically stable. Fifteen single-zoospore derivatives (which are usually mononuclear) of a hygromycin-

Fig. 3. Primer extension analysis of *hpt* mRNA in transformants containing the *Ham34* promoter. A primer complementary to bases 16–45 of the *hpt* open reading frame was annealed to RNA from the untransformed recipient strain (1) or three hygromycin-resistant transformants (lanes 2–4, corresponding to lanes 2–4 of Fig. 2), and extended with reverse transcriptase. The size of the extension products were determined by comparison to a sequence ladder of pHAMT34H, obtained using the same primer; shown in the left- and right-most lanes are the T and A reactions, respectively (other reactions not shown). The positions of the 5′ mRNA termini (represented by dots next to the sequence indicated in the left margin) are coincident with those described for *Ham34* transcripts in *Bremia lactucae*, 69 and 70 nt upstream of the native *Ham34* initiation codon.

resistant transformant, isolated after 3 wk of growth of the transformant without drug selection, all retained the drug-resistant phenotype. These results also indicated that the transformant was not a heterokaryon. In addition, two hygromycin-resistant and two G418-resistant transformants cultured without drug for 2 mo also maintained their resistant phenotypes. Phosphotransferase assays performed on two of these transformants (one with the *Ham34* promoter and one with *Hsp70*) indicated that the marker genes were expressed in the absence of drug selection at levels similar to those in parallel cultures maintained on drug, and that gene inactivation had not occurred. It follows that these two promoters could be used to express genes other than selectable markers.

In the future, sequential rounds of transformation may be required to introduce genes of interest. In addition, since the oomycetes have no stable haploid form, strategies for gene disruption may require serial transformation events. We have demonstrated that this will be possible in *P. infestans* by retransforming a hygromycin-resistant strain to G418 resistance, using the different selectable markers in successive experiments (not shown).

Application to other oomycetes. The expression of promoters from *B. lactucae* (order Peronosporales, family Peronosporaceae) in *P. infestans* (family Pythiaeae) suggests that our vectors may be useful for molecular studies of other oomycetes such as *Pythium* spp., other *Phytophthora* spp., etc. Transient assays using the GUS reporter gene have demonstrated that the *Ham34* and *Hsp70* promoters functioned in the soybean pathogen, *Phytophthora megasperma* Drechsler f. sp. *glycinea* (also order Peronosporales), and in the more distantly related saprophytic water mold, *Achlya ambisexualis* J. R. Raper (order Saprolegniales; unpublished results). In the only other report of gene transfer in the oomycetes, the production of drug-resistant derivatives of *A. ambisexualis* were reported using a fusion between *nptII* and the SV40 early promoter (Manavathu *et al.* 1988). However, the sizes of *nptII* transcripts in putative transformants were variable and did not reflect the expected function of the SV40 sequences, suggesting that *nptII* expression depended on cryptic vector sequences or integration near endogenous promoters. In transient assays, we have shown that this SV40 promoter, as well as promoters from higher fungi and plants, do not function in oomycetes (unpublished results). In contrast, the results presented in this paper indicated that authentic *B. lactucae* sequences and not flanking *P. infestans* promoters were functioning in our transformants. Thus, our vectors may enable the reliable manipulation of most oomycete fungi.

NOTE ADDED IN PROOF

Bailey *et al.* (1991) have reported the transformation of *P. capsici* Leonian and *P. parasitica* Dastur using pCM54, a vector that contains a *hsp70* promoter and autonomously replicating sequence (ARS) from *U. maydis* (DC) Cda. In side-by-side comparisons, we have failed to obtain transformants of *P. infestans* using several preparations of pCM54. Inclusion of the ARS in our vector, pHAMT34H, provided no increase in the frequency of transformation,
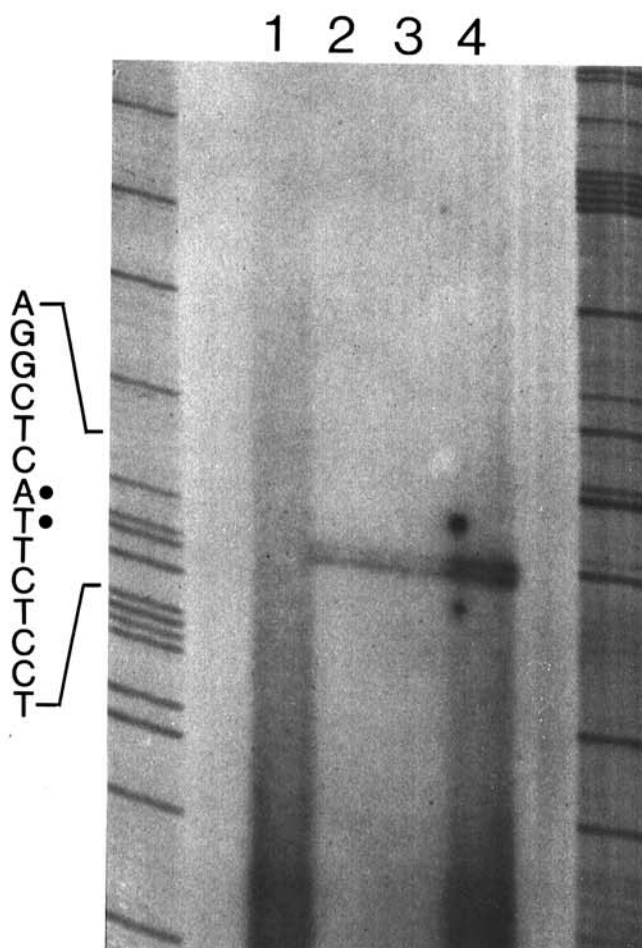
and transient assays using the GUS reporter gene indicated that the *U. maydis hsp70* promoter was not functional in *P. infestans*. These observations may indicate that significant heterogeneity exists within the *Phytophthora* genus in the sequences involved in transcription and DNA replication.

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