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Genetic transformation of the plant pathogens *Phytophthora capsici* and *Phytophthora parasitica*

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ABSTRACT

***Phytophthora capsici* and *P.parasitica* were transformed to hygromycin B resistance using plasmids pCM54 and pHL1, which contain the bacterial hygromycin B phosphotransferase gene (hph) fused to promoter elements of the *Ustilago maydis* heat shock hsp70 gene. Enzymes Driselase and Novozyme 234 were used to generate protoplasts which were then transformed following exposure to plasmid DNA and polyethylene glycol 6000. Transformation frequencies of over 500 transformants per μg of DNA per 1×10^6 protoplasts were obtained. Plasmid pCM54 appears to be transmitted in *Phytophthora* spp. as an extra-chromosomal element through replication, as shown by Southern blot hybridization and by the loss of plasmid methylation. In addition, transformed strains retained their capacity of infecting Serrano pepper seedlings and Mc. Intosh apple fruits, the host plants for *P.capsici* and *P.parasitica*, respectively.**

INTRODUCTION

The name for the genus *Phytophthora* De Bary derives from the Greek 'phyton' for plant and 'phthora' for destruction. The genus has been very well named considering that all the species are parasites often of economically important crops (1). Many have been isolated from soil where they attack underground parts of plants; other species attack aerial parts causing collar rots, trunk cankers, twig blights, leaf fall, leaf blights, and fruit rots. A wide host range is typical, although a few species are restricted in their host range, e.g. *P.infestans* (2).

Despite their importance, *Phytophthora* sp. have not yet been transformed. The development of an efficient DNA-transfer system in *Phytophthora* is essential to study the genes that govern host specificity and the design of new strategies to generate resistant plants. Transformation systems for a number of other plant pathogenic fungi have been recently developed (3, 4, 5, 6, 7, 8). One of the most advanced systems is that for *Ustilago maydis* (9), a basidiomycete that causes the boil smut on maize and has been widely use as a model system for genetic studies. Recently, a self replicating plasmid that confers resistance to hygromycin B (pCM54) has been developed for *U.Maydis* (10).

In this report, we describe the use of the plasmids pHL1 and pCM54 from *U.Maydis* for the genetic transformation of *P.capsici* and *P.parasitica*, based on acquisition of resistance to hygromycin B. Extraction of intact pCM54, Southern blot analysis, methylation and mitotic instability studies of transformants show that pCM54 is maintained as a self replicating plasmid in the two species of *Phytophthora*.

MATERIALS AND METHODS

Strains and plasmids

Phytophthora capsici P1314 and *P.parasitica* P991 were kindly supplied by Dr. M.D.Coffey (University of California, Riverside). Strains were cultured in 10%-cleared V8-juice agar at 28°C for 2 days in 90 mm petri dishes. Plasmid pCM54 (10) was kindly provided by Dr. W.K.Holloman (Cornell University Medical College, New York).

Chemicals and reagents

Novozyme 234 was purchased from Novo Biolabs (Bagsraerd, Denmark). PEG 6000 was from SERVA (Hilderberg, N.Y.) and Mannitol, hygromycin B and other chemicals were from Sigma.

DNA isolation and manipulation

DNA from *Phytophthora* spp. was isolated by the method of Hynes et al. (11). DNA enriched for plasmids was prepared from protoplasts by the method of Hirt (12). A rapid alkaline extraction procedure was used to prepare plasmid DNA from *E.coli* (13). Restriction enzyme digestions, transfer of DNA from gels to nitrocellulose paper and DNA hybridizations were performed according to standard procedures (14). EcoRI and HindIII digested pUC18 DNA was labeled with random primers according to Feinberg and Vogelstein (15) and used as a probe.

Preparation of protoplasts

The initial experiments of protoplasts isolation were based on the method of Layton and Kuhn (16). Mycelium disks of *P.capsici* and *P.parasitica* were taken from cultures grown in petri dishes containing 15 ml of V8-juice broth for 2 days at 28°C. The V8-broth was poured off and the mycelium from 5 dishes was mixed together with 15 ml of a 1M mannitol and 7mM $MgSO_4$ solution in a petri dish and was incubated at room temperature for 30–45 min. Driselase and Novozyme 234, at 50 μg/ml each one, were filter sterilized after suspension in the mannitol-$MgSO_4$ solution and added to the mycelium. Plates

were shaken at 50 rpm at room temperature for 1–2 h. Protoplasts were filtered through sterile Miracloth to remove mycelial fragments, collected in Corex tubes, centrifuged at 5000 rpm/10 min at 4°C and diluted to 10^7 cells/ml in STC solution (50 mM $CaCl_2$, 10 mM tris-HCl, pH 7.5, 1M sorbitol). Approximately 1×10^7 protoplasts were obtained from 5 petri dishes inoculated with five 5 mm diameter disks of mycelium. Approximately 20% of the protoplasts could be regenerated to form viable colonies.

Transformation of *Phytophthora* spp.

Protoplasts were mixed with plasmid DNA (1–15 μg DNA/10^6 protoplasts in 100 μl of STC buffer), 15 μg of heparin were added and the mixture was incubated on ice. After 20 min, 0.5 ml of STC buffer containing 15–66% (wt/wt) polyethylene glycol 6000 was added, and the sample was incubated for an additional 10 minutes at room temperature. Five milliliters of regeneration broth (80% 1M mannitol + 20% V8 broth) was added, and the mixture was incubated for 12–16 h. After incubation at 28°C, plates of a two-layer regeneration agar (10 ml each layer of 80% 1M mannitol + 20% V8 broth + 1.5% agar) containing in the lower layer 200, 400, 600, or 800 μg/ml of hygromycin B and in the top layer only regeneration agar were overlaid with 0.5 ml of regenerated protoplasts and incubated at 28°C. After 3–4 days, putative transformed colonies (5 mm or larger radial growth) were counted. The amount of growth in the no DNA control plates was 2–3 mm with no further development. The regeneration frequency of protoplasts after the above treatment was 10% on antibiotic-free medium.

Plant inoculation and fungal reisolation

One month old bare-rooted seedlings of Serrano peppers and McIntosh apple fruits were inoculated with zoospores of *P.capsici* or *P.parasitica* transformants, respectively. Zoospores were obtained by growing *Phytophthora* transformants from 5 mm disks of mycelium in 15 ml cleared V8-juice broth containing 400 μg/ml hygromycin B for 2 days at 28°C. The mycelial mats were rinsed three times with demineralized sterile water, placed in 15 ml sterile soil extract (10 g of soil per liter of water), and incubated 2–3 days at 28°C to induce sporangium formation. To initiate zoospore release, the mats were placed in sterile demineralized water and chilled at 4°C for 20 min. to 1 h. The zoospore suspension was collected by centrifugation at 5000 rpm for 10 min at 4°C. Three pepper seedlings were placed in a 355 ml Styrofoam cup, containing 200 ml of water and 5×10^5 zoospores per transformant. Ten days after inoculation, the roots and stem of each seedling were sliced and plated on PARP, a selective medium containing V8-agar, PCNB (pentachloronitrobenzene), ampicillin, rifampicin, pimaricin and hygromycin B, for the selection of transformants. This medium is used for the isolation of *Phytophthora* from soil and infected plants (17). Zoospore suspensions from *P.parasitica* transformants were inoculated into 5 mm holes made on apple fruits and were covered with a cotton plug. Infected fruits were incubated on a growth chamber at 28°C and was covered with plastic to maintain 100% humidity. Results were recorded after 5 days and sections of rotten apples were plated on PARP and HygB medium for reisolation.

RESULTS

Transformation of *Phytophthora* spp.

To identify a selectable agent for the design of a transformation protocol for *Phytophthora*, the toxicity of kanamacyn, G418 and hygromycin B was tested. It was found that only Hygromycin B (HygB) was useful as a selective agent for *P.capsici* and *P.parasitica*, as it completely inhibits mycelium growth and zoospore germination in both species at 400 μg/ml. At this concentration no spontaneous resistance or background growth was observed. Figure 1 shows the effect of increasing concentrations of hygromycin on the ability of *Phytophthora* to form colonies from protoplasts. In these experiments 1×10^6

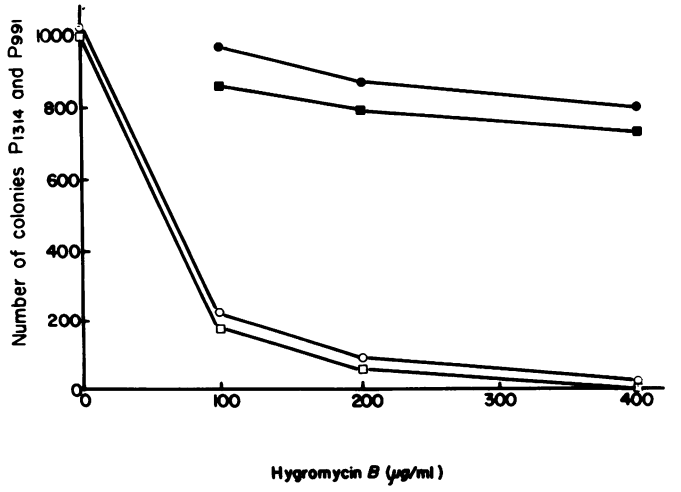

Fig. 1. Transformation of *P.capsici* P1314 and *P.parasitica* P991 to HygB resistance and toxicity curves to HygB. Approximately 1×10^6 regenerable protoplasts from *P.capsici* and *P.parasitica* were treated with 7 μg plasmid DNA, 25% PEG-6000, regenerated and spread onto regeneration medium containing 100, 200 and 400 μg/ml HygB. Colonies were counted after 4 days of incubation at 28°C. Approximately 1×10^6 control protoplasts of *P.capsici* and *P.parasitica* were treated as above without plasmid DNA and their sensitivity recorded.

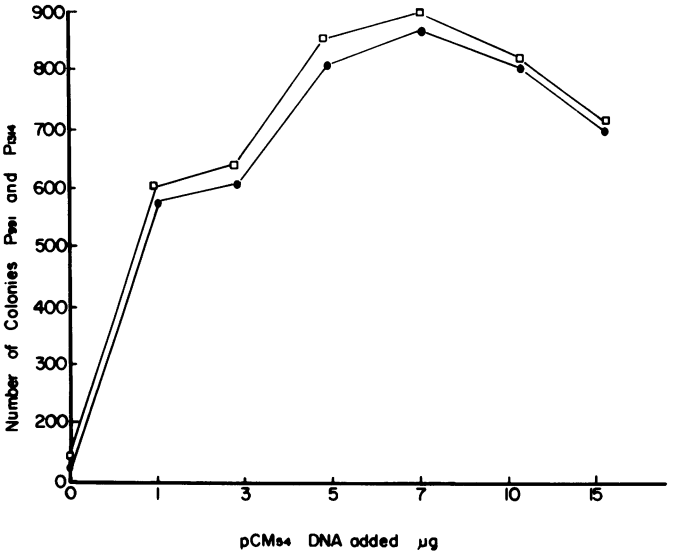

Fig. 2. Transformation of *P.parasitica* P991 and *P.capsisi* P1314 to HygB resistance. Approximately 1×10^6 regenerable protoplasts were treated with various amounts of pCM54 DNA, 25% PEG-6000, regenerated and spread onto regeneration medium containing 200 μg/ml HygB. Colonies were counted after 4 days of incubation at 28°C.

protoplasts of each strain were treated with 25% PEG and plated on 100, 200 and 400 μg/ml HygB.

As donors of HygB resistance genes, we decided to test two plasmids developed for the transformation of *U.maydis*. pHL1 is an integrative vector and pCM54 is an autonomously replicating plasmid derived from pHL1 (9). Both plasmids contain a bacterial HygB phosphotransferase gene under the control of a *U.maydis* hsp 70 gene promoter. pCM54 also harbours a 383 bp fragment containing the autonomous replicating sequence UARS1 from *U.maydis*. Our choice of these plasmids to transform *Phytophthora* spp. was based on the possibility that the hsp 70 gene promoter could be functional in *Phytophthora spp.* since

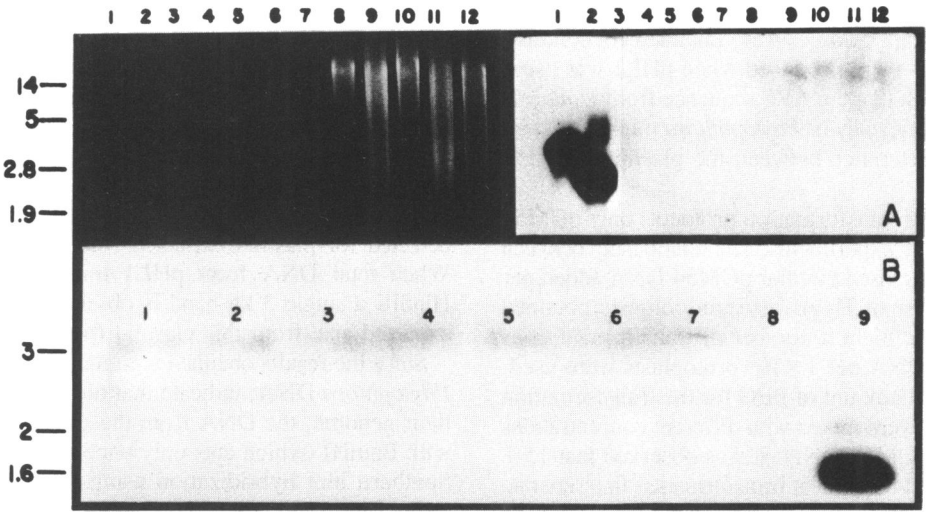

Fig. 3. Southern blot hybridization analysis of pHL1 *Phytophthora* transformants. (A) Total DNA (11) was isolated from *Phytophthora* transformants grown in V8-juice broth containing 400μg/ml HygB. Approximately equal amounts of DNA were loaded on each lane of an agarose gel. After electrophoresis the gel was blotted and hybridized against ^{32}P-labeled pUC18. Lane 1 is EcoRI digested pHL1; lane 2 HindIII digested pHL1; lane 3, λ PstI molecular weight markers; lanes 5 and 6, untrasformed DNA from *P.capsici* and *P.parasitica*; lanes 8−10, DNA from *P.capsici* transformants; lanes 11−12, DNA from *P.parasitica* transformants. (Left) ethidium bromide-staine gel. (Right) authoradiogram of Southern blot hybridization. *Phythophthora* total DNA was digested with HindIII and hybridized against ^{32}P- labelled pUC18. Lane 1, pHL1 digested with Hind III; lanes 2−4, DNA from *P.capsici* transformants; lanes 5−7 DNA from *P.parasitica* transformants; lane 8, DNA from untransformed *P.capsici*; lane 9 molecular wieght markers.

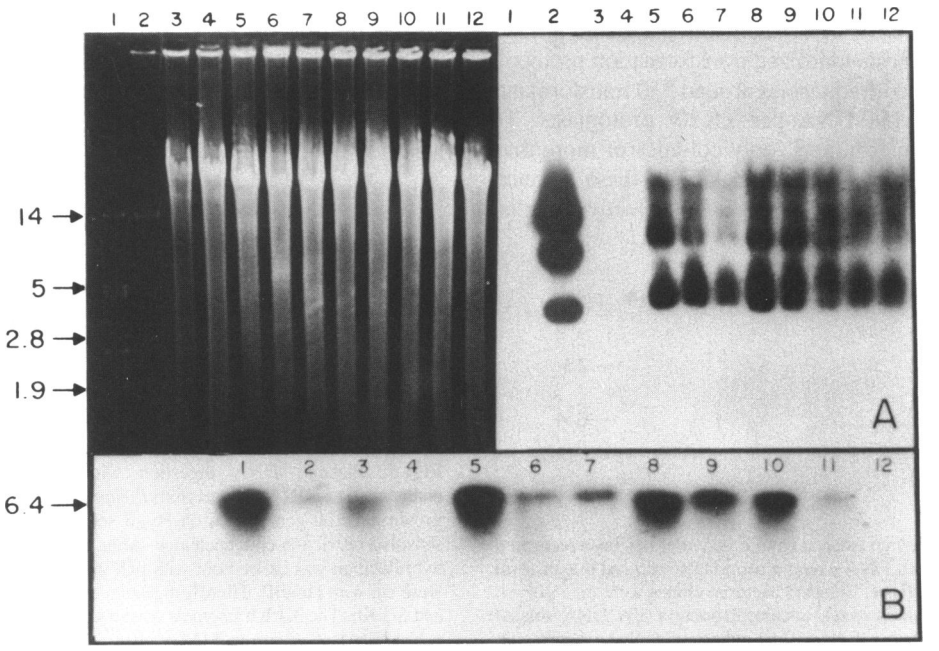

Fig. 4. Southern blot Hybridization analysis of pCM54 *Phytophthora* transformants. (A). Total DNA (11) was isolated from *Phytophthora* transformants grown in V8-juice broth with 400 μg/ml hygromycin B. Approximately equal amounts of DNA were loaded on each lane of a 0.8% agarose gel. After electrophoresis and blot transfer, Southern hybridization was carried out with ^{32}P-labeled pUC18 DNA as the probe. Lane 1 is PstI marker, numbers in the left margin are in Kb; lane 2 uncut pCM54; lanes 3 and 4 untransformed DNA controls of *P.capsici* and *P.parasitica*; lanes 5−8 *P.capsici* transformants; lanes 9−12 *P.parasitica* transformants. (Left) ethidium bromide-stained gel. (Rigth) autoradiogram of Southern transfer. (B) BamHI digested DNA was separated by electrophoresis, transfered and hybridized using ^{32}P-labeled pUC18 as a probe. Lane 1 correspond to BamHI digested pCM54 control plasmid. Lanes 2−6 DNA digested with BamHI from *P.capsici* transformants. Lanes 7−11 show BamHI digested *P.parasitica* transformants. Lane 12 corresponds to BamHI digested DNA from *P.capsici* untransformed. The number in the left margin indicate the position of pCM54 (in Kb) produced by BamHI digestion after comparison with a standard lambda HindIII marker.

it is known that mechanisms that regulate the expression of heat shock genes are conserved in many species. These plasmids confer ampicillin resistance in *E.coli* and HygB in *U.maydis*.

To test whether these plasmids were functional in *Phytophthora*, approximately 1×10^6 regenerable protoplasts were mixed with different amounts of pHL1 and pCM54 and treated with 25% PEG 6000 as described in material and methods. It was observed that in all concentrations of pCM54 tested, several hundreds of Hyg B resistant colonies were obtained. In contrast, only a few transformants were produced when pHL1 was used. These experiments suggest that the ARS sequence from *U.maydis* somehow enhances the efficiency of *Phytophthora* transformation, since that is the only difference between the plasmids pCM54 and pHL1.

To further optimize the transformation protocol, only pCM54 was used in transformation experiments. The relationship between the amount of covalently closed circular pCM54 DNA added per treatment and the number of HygB resistant colonies obtained is shown in figure 2. The highest number of transformants was obtained when 7 μg of DNA per 1×10^6 protoplasts were used. To determine the optimal amount of PEG for the transformation experiments, protoplasts were mixed with different concentrations of PEG 6000 and 7 μg pCM54 DNA. It was observed that 15% PEG produced the highest number of transformants. In a separate experiment, we used PEG 4000, but the transformation frequencies and the regeneration ability of the treated protoplasts were very low (data not shown). To assess the number of transformants and their sensitivity to HygB, we treated protoplasts with 25% PEG 6000, 7 μg pCM54 DNA and selected them on 100, 200 and 400 μg HygB/ml of regeneration agar. The number of transformants remained very constant with the different concentrations used, mainly between 200 and 400 μg/ml of HygB (Fig. 1).

All the experiments above mentioned were repeated at least three times. Utilizing this standardized transformation protocol, we have routinely obtained frequencies around 550 transformants per μg of circular pCM54 DNA per 1×10^6 protoplasts. To score the number of transformants, only colonies of more than 5 mm in diameter were consider. Over 95% of these colonies continue growing after several passages in selective media. Colonies that grew more than the control but did not reach 5 mm in diameter were not counted, since they may represent abortive transformation events.

Fate of plasmid DNA in *Phytophthora* spp. transformants

To demonstrate that the hygromycin resistant colonies were indeed transformed, total DNA was isolated from a number of resistant colonies, electrophoresed and analyzed by Southern blot hybridization using pUC18 as a probe. When undigested DNA from several independent transformants was used , it was observed that for pHL1 transformants the hibridization signal comigrates with the bulk of genomic DNA, as can be expected for transformation events in which the plasmid has integrated in the genome of *Phytophthora* (panel A figure 3). In the case of pCM54 three bands are observed that have similar mobility to the bands detected for plasmid extracted from *E.coli* (panel A figure 4). When total DNA from pHL1 transformants is digested with HindIII a single 3 kb band is observed, that corresponds to the expected size from this plasmid (panel B figure 3).

Since the results obtained with undigested pCM54 transformed *Phytophtora* DNA, indicate that the plasmid is not integrated in their genome, the DNA from these transformants was digested with BamHI (which cuts only once in pCM54) and analyzed by Southern blot hybridization using pUC18 as a probe. All the transformants thus far analysed showed a unique 6.4 Kb band that comigrates with pCM54 and is not present in DNA extracted from non-transformed colonies (panel B figure 4). The presence of a unique band in all the colonies analyzed suggests that either pCM54 is maintained as an independent replicon or that multiple copies are integrated in tandem into the genome of *Phytophthora*. In addition, DNA was extracted from pCM54 *Phytophthora* transformants by the Hirt method (13), that enriches for small molecules and used to transform *E.coli*. It was found that indeed this DNA is able to transform *E.coli* and that the transformants contain the original pCM54 (Fig. 5). Similar experiments carried out with DNA from pHL1 transformants, failed to produce *E.coli* strains carring pHL1. All these evidence strongly suggest that

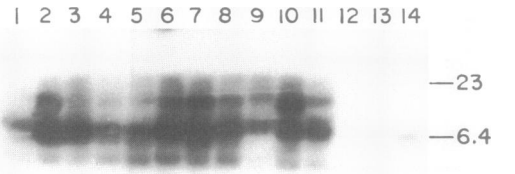

Fig. 5. Analysis of pCM54 plasmid isolated from *E.coli* after had been recovered from *P.capsici* and *P.parasitica*. DNA from transformants was used to transform *E.coli* DH5 to ampicillin resistance. Selected bacterial clones were then grown, and plasmid DNA was prepared by a rapid alkaline extraction (13). DNA isolated from cells was partially digested with BamHI as indicated by electrophoresis in 0.8% agarose gel containing 40 mM Tris-acetate (ph 8.0) − 2 mM EDTA. After electrophoresis and transfer, Southern hybridization was carried out with ^{32}P-labeled pUC18 as the probe. Lanes 1 − 5 correspond to the partial restriction pattern of pCM54 digested with BamHI from DH5 clones transformed with DNA of 5 *P.capsici* transformants. Lanes 6 − 10 correspond to clones obtained from 5 *P.parasitica* transformants. Lane 11 is a pCM54 control partially digested. Lanes 12 and 13 are untransformed *P.capsici* and *P.parasitica* DNA. Lane 14 is the pCM54 control DNA digested with BamHI. The numbers in the right margin indicate the position of plasmid bands in Kb.

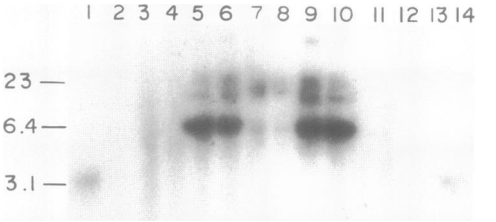

Fig. 6. Plasmid DNA digestion with methylation sensitive restriction endonucleases. DNA isolated from *P.capsici* and *P.parasitica* transformants (12) grown after 20 generations on HygB selection was digested with methylation sensitive restriction endonucleases. After electrophoresis and transfer, Southern hybridization was carried out with pUC18 as the probe. Lanes 1, 2, 13 and 14 were cut with HindIII. HindIII digestion of pCM54 yields two fragments of 3.1 and 3.3 Kb. The 3.1 Kb fragment contains pUC12 and hybridizes with the pUC18 probe. Lanes 1 − 2 are pCM54 control DNA isolated from dam+ *E.coli* DH5 and double digested with HindIII and MboI and with HindIII and DpnI, respectively. Lanes 3 − 4 and 11 − 12 are DNA from *P.capsici* and *P.parasitica* transformants digested with MboI. Lanes 5 to 7 and 8 to 10 are DNA from *P.capsici* and *P.parasitica* treated with DpnI. Lanes 13 − 14 are DNA from *P.capsici* transformant double digested with HindIII and MboI and with HindIII and DpnI, respectively. The largest fragment expected from digestion of pUC18 with MboI or DpnI is approximately 300 bp. Thus, in this gel only plasmids resistant to DpnI digestion appear; fragments smaller than 500 bp ran off the gel. The numbers on the left margin indicate the position of plasmid bands in Kb.

pCM54 is replicating autonomously in *Phytophthora*.

Further evidence for the autonomous replication of pCM54 in *Phytophthora spp.* transformants was obtained by monitoring the loss of methylation of DNA using the restriction endonucleases DpnI and MboI (Fig.6). pCM54 prepared from dam+*E.coli* DH5 which methylates adenine was sensitive to DpnI (lane 2) but not to the isoschizomer MboI (lane 1). In contrast, DNA extracted from *Phytophthora* transformants grown for 20 generations, was found to be resistant to DpnI (lanes 5−10 and 14) but sensitive to MboI (lanes 3−4 and 11−13), indicating a loss of methylation. If we assume that changes in methylation can only occur during replication (23, 24) this result agrees with a mode of transmission of plasmids that replicate autonomously.

It has been observed that self replicating plasmids which lack centromeric sequences are unstable through mitotic divisions (24). As an additional test to determine whether pCM54 is present in *Phytophthora* as an extrachromosomal self-replicating element, the mitotic stability of pCM54 was determined. It was observed that after 20 transfers in nonselective conditions, only 20% of the zoospores derived from a transformed strain remained resistant to HygB when replated on hygromycin medium. This demonstrates the extrachromosomal nature of pCM54 sequences in *Phytophthora spp.*, since in stable transformants the plasmid should not be lost at high frequency.

Pathogenicity of transformants

Zoospore suspensions of five *P.capsici* and *P.parasitica* transformants selected under hygromycin medium were used to inoculate Serrano peppers seedlings and Mc Intosh apple fruits. All of the transformants tested produced symptoms indistinguishable from untransformed *P.capsici* and *P.parasitica* (data not shown). In addition, HygB resistant *P.capsici* and *P.parasitica* were isolated from the plants and fruits that had been inoculated with transformants.

DISCUSSION

Phytophthora spp. fungi are coenocytic and diploid throughout the vegetative phase. They are predominantly heterotallic, existing as two sexual compatibility types: A1 (homozygous recessive) and A2 (heterozygous) each mating type is bisexual but self sterile (18,19,20). Somatic recombination might be expected where the mycelium is diploid, leading to recombination in heterozygous diploids without the intervention of the A1 homozygous mating type (21). In our study, the somatic diploid phase of *P.capsici* and *P.parasitica* was used for protoplast production and transformation. The frequency of transformation for *P.capsici* and *P.parasitica* obtained was similar and around 550 transformants per μg DNA per 10^6 protoplasts, when the ARS1 sequence of *U.maydis* was part of the transforming plasmid. Only a low number of transformants was obtained when the integrative plasmid pHL1 was used.

The number of transformants produced by pCM54 in *Phytophthora* is less than one-fourth of that obtained for *U.maydis* with the same plasmid (10), which may reflect a less efficient functionality of the ARS1 sequence in *Phytophthora* as compared to that in its normal environment. However, we have demonstrated that *Phytophthora spp.* protoplasts can be readily transformed with a plasmid vector from *U.maydis* carrying the gene encoding the HygB-resistance, the hsp70 gene promoter and the UARS1 autonomous replicating sequence. This plasmid can be detected in transformants grown under selective conditions with the mobility of circular DNA and without any obvious structural alteration, as demonstrated by Southern analysis and transformation of *E.coli* with isolated DNA from *Phytophthora spp.* transformants. Therefore the transforming DNA does not seem to be integrated in the chromosomal DNA of *Phytophthora* spp. It was also proven that pCM54 replicates in *Phytophthora* spp. because (i) the plasmid becomes demethylated after 20 generations of zoospore production from transformants under selective conditions, and (ii) by the mitotic instability observed when the transformants were grown under non-selective conditions. The mitotic instability of pCM54 in *Phytophthora* spp. is similar to that observed for this plasmid in *U.maydis* (10) and yeast plasmids (22,23) where high segregation frequency and strong maternal segregation bias is observed by pedigree analysis in the case of ARS containing plasmids (24).

Zoospores selected on hygromycin produced symptoms indistinguishable from untransformed *P.capsici* and *P.parasitica* without any delay or alteration that could suggest a loss of fitness of the transformants in the pathogenicity process. In addition, HygB resistant *Phytophthora spp.* were isolated from plants and fruits that had been inoculated with zoospores of transformants, suggesting that the presence of foreign DNA had no obvious effect on pathogenicity as previously reported for *Colletotrichum graminicola* (4) and *Glomerella cingulata* f. sp. *phaseoli* (7) transformed with homologous and heterologous selectable genes.

The development of a transformation system as reported in this paper will make possible the cloning of genes from *Phytophthora spp.* by complementation of mutants affected in pathogenicity or virulence with gene libraries built in pCM54. We have begun experiments to improve the stable transformation system and the production of non-pathogenic mutants.

As far as we know this is the first report of a transformation system for *Phytophthora spp.*, and the functionality of an autonomous replicating plasmid from a basidiomycete fungus in an oomycete fungus.

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