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# Transformation of a Filamentous Fungus *Cryphonectria parasitica* Using *Agrobacterium tumefaciens*

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**Abstract** As *Agrobacterium tumefaciens*, which has long been used to transform plants, is known to transfer T-DNA to budding yeast, *Saccharomyces cerevisiae*, a variety of fungi were subjected to the *A. tumefaciens*-mediated transformation to improve their transformation frequency and feasibility. The *A. tumefaciens*-mediated transformation of chestnut blight fungus, *Cryphonectria parasitica*, is performed in this study as the first example of transformation of a hardwood fungal pathogen. The transfer of the binary vector pBIN9-Hg, containing the bacterial hygromycin B phosphotransferase gene under the control of the *Aspergillus nidulans trpC* promoter and terminator, as a selectable marker, led to the selection of more than 1,000 stable, hygromycin B-resistant transformants per $1 \times 10^6$ conidia of *C. parasitica*. The putative transformants appeared to be mitotically stable. The transformation efficiency appears to depend on the bacterial strain, age of the bacteria cell culture and ratio of fungal spores to bacterial cells. PCR and Southern blot analysis indicated that the marker gene was inserted at different chromosomal sites. Moreover, three transformants out of ten showed more than two hybridizing bands, suggesting more than two copies of the inserted marker gene are not uncommon.

*Keywords*: *Agrobacterium tumefaciens*-mediated transformation, *Cryphonectria parasitica*, fungal transformation

Filamentous fungi play a vital role in nature as primary decomposers. They have a large impact on food production as detrimental plant pathogens and on direct food sources as edible mushrooms. In addition, filamentous fungi are used in a variety of industrial processes, including the production of fermented foods, primary metabolites such as organic acids and vitamins, and secondary metabolites [1]. Moreover, filamentous fungi can produce homologous and heterologous proteins by the secretion of a variety of extracellular enzymes.

Since the first transformation of filamentous fungi [2, 3], several transformation techniques have become available. The vast majority of transformation protocols require preparing protoplasts, followed by the permeabilization of the cell membranes with electroporation or with a combination of $CaCl_2$ and polyethylene glycol (PEG). However, these transformations are relatively inefficient due to the isolation of protoplasts or osmotically-sensitive cells, which are troublesome to prepare and can even be a limiting factor in the case of obligate parasites [4]. Intact fungal cells have also been used [5], but usually with lower transformation frequencies. A fundamentally different transformation technique was recently developed for filamentous fungi using *Agrobacterium tumefaciens* [6,7-9]. *A. tumefaciens* is a gram-negative soil bacterium, which has the ability to transfer sections of plasmid borne DNA to plants. *Agrobacterium* has been used as a major genetic tool for plant molecular breeding, and the molecular mechanism by which it genetically transforms the host cells has been studied intensively. One of the principal advantages of *Agrobacterium tumefaciens*-mediated transformation (ATMT), over that of conventional transformation for filamentous fungi, is the versatility in choosing the starting biosample, including hyphae, spores or blocks of mushroom fruiting body tissue i.e., there is no need to isolate the protoplast. The direct transformation of fungal conidia makes ATMT a powerful and easy tool for genetic manipulation of filamentous fungi. ATMT is also useful for species that are recalcitrant to transformation by other means [10,11].

The ascomycetous filamentous fungus *Cryphonectria parasitica* (Murr.) Barr, the causal agent of chestnut blight, was responsible for the virtual disappearance of chestnut forests in North America in the beginning of the early 20th century. However, strains containing the double-stranded (ds) RNA Cryphonectria hypovirus (CHV) showed characteristic symptoms of reduced virulence, hypovirulence [12,13] and displayed hypovirulence-associated changes, such as reduced pigmentation, sporulation, laccase production and oxalate accumulation [14-16]. Moreover, symptoms of hypoviral infection appear to be the result of aberrant expressions of specific sets of

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fungal genes in the hypovirulent strain. Therefore, the chestnut blight fungus, *C. parasitica*, and its hypovirus, is a useful system for studying the mechanisms of fungal gene regulation by mycoviruses. A number of hypoviral-regulated *C. parasitica* genes have been identified, based on their expression patterns or homologies to the known genes in other organisms [17-20]. Nonetheless, there are still more genes to be identified to explain the phenotypic changes in the fungal host. ATMT generates a high percentage of transformants with single-copy integrations of the marker gene at different chromosomal sites, rendering this system suitable for genome mutagenesis studies, such as gene tagging, promoter entrapment and gene activation. An insertional mutagenesis has been used extensively in filamentous fungi [21,22] as a tool for massive and high through-put screening of genes set(s) involved in important phenotypic changes. Thus, without the necessity of preparing the protoplasts, ATMT can be implemented as an easy method for the transformation of *C. parasitica* and the concomitant construction of the tagged-mutant library of *C. parasitica*. This paper is the first to report the *A. tumefaciens*-mediated transformation of hardwood-pathogenic fungus, *C. parasitica*, and provides an easy means for its genetic manipulation.

Single colonies of *A. tumefaciens* strains, containing the pBIN19-Hg vector, were inoculated in 5 mL of minimal medium (MM) [23] containing kanamycin (50 μg/mL), and were grown at 28°C for 48 h, vigorously agitating at 250 rpm. pBIN19-Hg was constructed by the insertion of a 2.5 kb *Sma*I/*Hin*dIII fragment, containing the hygromycin B phosphotransferase (*hph*) gene from pSHG25, into the *Sma*I/*Hin*dIII sites of the pBIN19 binary vector [24]. The pSHG25 was constructed by cloning the 2.5 kb of the *Sal*I fragment, containing the *hgh* gene from pDH25 [25], into the *Sal*I site of pUC19. The *E. coli* plasmid was introduced into the *A. tumefaciens* by triparental mating, using pRK2013 as a mobilization helper [26]. Recombinant plasmids were recovered from *A. tumefaciens* by the boiling minipreparation method [27]; then restriction analysis was carried out to confirm the presence of the introduced plasmid.

The bacterial cells harvested from a 2 mL aliquot of MM at different growth phases, with the ranges of 24 to 54 h after inoculation, were washed with an induction medium (IM) [28] and subsequently resuspended in 5 mL of IM containing 200 μM acetosyringone (AS). The cells were grown for an additional 6 h. The amount of Agrobacterium cell was again accurately determined by plating out dilutions. Subsequently, 100 μL of the Agrobacterium cells were adjusted with 100 μL of fungal conidia to the ratio of 100 *A. tumefaciens* cells to 1 *Cryphonectria parasitica* EP155/2 (ATCC 38755) conidium. The concentrations of conidia of *C. parasitica* for the initial transformation were $10^6$ and $10^7$ conidia/mL. 200 μL of the mix was plated on IM, containing 5 mM of glucose instead of 10 mM, in the presence, or absence, of 200 μM AS. The plates were incubated at 30°C for 2 days, and then overlaid with 10 mL of top agar, containing 150 μg/mL of hygromycin B (Roche Diagnostics Co., Mannheim, Germany), to select the hygromycin B resistant transformant, and 200 mM of cefotaxime to inhibit the growth of the *Agrobacterium*. The overlaid plates were incubated for 5 to 7 days at 30°C. The putative transformants growing on the top agar transferred to the new medium containing hygromycin.

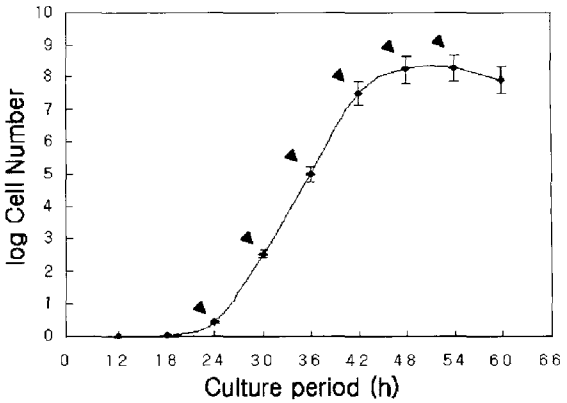


**Fig. 1.** The growth curve of *A. tumefaciens* AGL1. Arrowheads indicate the specific time of bacterial cell sampling to determine the transforming efficiency depending on growth phases. Vertical bars represent standard deviation. All measurements were performed in triplicates, with similar results obtained for each replicate.

*C. parasitica* conidia were obtained by growing the strain on a potato dextrose agar, supplemented with methionine and biotine (PDAmb), for 7 days, and harvesting with 10 mL of sterile water per plate. The number of conidia was determined using a hemacytometer. The conditions and methods for preparing the primary inoculum for liquid cultures have previously been described [25].

To measure the transforming efficiency depending on the growth phase of the bacterial cultures, the growth curve for *A. tumefaciens* AGL1 was measured every 6 h, using the optical density at 560 nm (O.D. 560 nm), and corresponding number of cells in plated dilution cultures (on MM containing rifampicin). Fig. 1 represents a typical growth curve for a bacterial culture with four typical phases of growth: the lag, log, stationary, and death phases. *A. tumefaciens* started their exponential growth phase 24 h after inoculation, reached the stationary phase at 48 h and entered the death phase since then. As shown in Table 1, bacterial cells, which were harvested at different growth phases within 24 to 54 h after inoculation, were used to transform *C. parasitica*. It appeared that the transformation efficiency increased as the culture proceeded to the early stationary phase and decreased since then. More than 1,000 putative transformants were obtained using bacterial cells prepared 48 h after inoculation at a concentration of $10^7$ conidia/mL. However, the transformation efficiency decreased gradually as the growth phase of bacterial culture entered the late stationary phase. Ten putative transformants were selected for further studies from the top agar containing hygromycin B, single-spored, and serially transferred three times onto non-selective PDAmb with no hygromycin B, followed by the transfer onto selective PDAmb with hygromycin. All

**Table 1.** Effects on the transformation efficiency of the growth phases of *A. tumefaceins*

| Number of conidia (conidia/mL) | Culture time (hour after the inoculation) | | | | | |
|---|---|---|---|---|---|---|
| | 24 | 30 | 36 | 42 | 48 | 54 |
| $1 \times 10^6$ | 235 ± 11[a] | 490 ± 25 | 500 ± 33 | 552 ± 41 | 600 ± 43 | 510 ± 40 |
| $1 \times 10^7$ | 440 ± 23 | 700 ± 59 | 751 ± 54 | 904 ± 78 | 1134 ± 73 | 700 ± 48 |

[a] Values are means ± standard deviation.

**Table 2.** Effect of different ratios of *A. tumefaciens* cells to *C. parasitica* conidia

| Number of spores (conidia/mL) | Number of bacteria cells (cells/mL) | Number of transformants |
|---|---|---|
| $1 \times 0^4$ | $1 \times 10^5$ | 0 |
| | $1 \times 10^6$ | 0 |
| | $1 \times 10^7$ | 0 |
| $1 \times 10^5$ | $1 \times 10^6$ | 0 |
| | $1 \times 10^7$ | 0 |
| | $1 \times 10^8$ | 0 |
| $1 \times 10^6$ | $1 \times 10^7$ | 120 ± 21[a] |
| | $1 \times 10^8$ | 600 ± 43 |
| | $1 \times 10^9$ | 590 ± 41 |
| $1 \times 10^7$ | $1 \times 10^7$ | 180 ± 19 |
| | $1 \times 10^8$ | 850 ± 67 |
| | $1 \times 10^9$ | 1134 ± 73 |

[a] Values are means ± standard deviation.

transformants were hygromycin B-resistant and mitotically stable with no difference in the radial growth rate or culture morphology even after a successive transfer onto the non-selective medium.

Most other studies used bacterial cells that were grown overnight, which corresponded to the lag or early log phases of this study. However, a three-fold increase in transformation efficiency was observed using bacterial cell from the early stationary phase. Further studies are required to establish the reason for better yields of transformation in stationary phase cultures; it is unlikely to be the strain specificity since a different strain of *A. tumefaciens*, PC2760, also gave a better yield when the early stationary phase, rather than an overnight (≈ 14 h)-grown culture (data not shown), was used.

However, during co-cultivation in the absence of acetosyringone (AS) (an essential phenolic compound that induces the expression of various virulence genes in *A. timefaciens*), no transformant was obtained in any trial, suggesting that the transfer of T-DNA depends on the induction of bacterial virulent genes, a similar transformation mechanism operating in both plant and fungi.

The transformations were examined with various ratios of bacteria to conidia. Ratios of 10,100 and 1,000 bacterial cells to 1 conidium were applied to the conidial concentrations of $1 \times 10^4$, $10^5$ and $10^6$, and ratios of 1, 10 and 100 were applied to conidial concentrations of $1 \times 10^7$ (Table 2). The ratio of 1,000 to 1 at the conidial concentration of $1 \times 10^7$ was not possible since bacterial cells covered the whole plate prior to selection. It was possible to obtain hygromycin B-resistant transformants using more than $1 \times 10^6$ conidia and, at ratios of 10 and 100, more transformants were obtained as more conidia used. More than 1,000 transformants were obtained through the co-cultivation of $1 \times 10^7$ bacteria cells with $1 \times 10^7$ conidia. At the same conidia concentration, a 100 to 1 ratio of bacteria to conidia yielded the most transformants.

Also, the transformation efficiency was compared using five different strains of *A. tumefaciens* (Table 3). The *A. tumefaciens* strains, AGL1, A281, PC2760, LBA4404, and GV3101, were used for the transformation of *C. parasitica*. A ratio of 100 to 1 was applied to two different concentrations of conidia, $1 \times 10^6$ and $1 \times 10^7$ conidia/mL. There were enormous differences in transformation efficiencies among the strains. LBA4404, one of the most common strains for plant transformation, was not good for fungal transformation and GV3101 was not able to transform *C. parasitica*. Among AGL1, A281 and PC2760 strains, which yielded more than 1,000 trans-

**Table 3.** Effect of various *A. tumefaciens* strains on the transformation efficiency

| Strains | Genotype and /or Bacterial Phenotype | Number of conidia (spores/mL) | Number of transformants |
|---|---|---|---|
| AGL1 | AGL0 *recA::bla* pTiBo542ΔT-region Mop$^+$ Cb$^R$ [31] | $1 \times 10^6$ | 600 + 43 [a] |
| | | $1 \times 10^7$ | 1134 + 73 |
| A281 | C58 pTiBo542 [31] | $1 \times 10^6$ | 503 + 32 |
| | | $1 \times 10^7$ | 1,013 + 71 |
| PC2760 | T-DNA deletion derivative of pTiA6NC [32] | $1 \times 10^6$ | 620 + 46 |
| | | $1 \times 10^7$ | 1,264 + 103 |
| LBA4404 | A *vir* helper; harbors disarmed Ti plasmid pAL4404, a T-DNA deletion derivative of pTiAch5 [33] | $1 \times 10^6$ | 170 + 23 |
| | | $1 \times 10^7$ | 120 + 21 |
| GV3101 | C58, pMP90RK, a T-DNA deletion derivative of pTiC58 [34] | $1 \times 10^6$ | 0 |
| | | $1 \times 10^7$ | 0 |

[a] Values are means ± standard deviation.

formants, PC2760 was found to be the most effective, followed by AGL1 and then A281. PC2760 resulted in more than 1,200 transformants per $1 \times 10^6$ recipients. Although this transformation efficiency of *C. parasitica* was not as high as that of the protoplasts described previously [25], it was still comparable to the transformation efficiency of other filamentous fungi [9,21,29].

No differences in growth phases were observed among the different strains, suggesting that the difference in the transformation efficiency was due to the genetic background of each strain and not to the differences in the growth phases. It will be interesting to compare the genetic discrepancy in strain GV3101 from that of the other strains. Unraveling the molecular basis for these differences might help expand the host range of *Agrobacterium* as a genetic tool. Moreover, it might help identify the bacterial, as well as host, factors participating in infection, which will, in turn, contribute a better understanding of the basic biological processes, such as cell-to-cell communication and responses.

Using DNA from successively transferred transformants and internal primers for the coding region of *hph*, PCR amplification was carried out to confirm the presence of the introduced gene. Genomic DNA from *C. parasitica* was extracted using the method described by Kim *et al.* [25]. All the transformants showed the PCR amplicons of *hph* with the expected size of around 1.0 kb, but no amplification was observed in wild type EP155/2 (data not shown).

To determine the T-DNA copy number per genome, genomic Southern blot analysis was performed. The genomic DNA (5 μg) was digested with *Eco*RI which cut the inserted DNA only once due to the presence of a restriction site at the right border of the T-DNA, separated on 0.8% agarose gel, and then transferred onto a Hybond N$^+$ membrane (Amersham Pharmacia Biotech, UK). The blot was hybridized with the [α-$^{32}$P]-dCTP-labeled 0.8 kb *Eco*RI-*Bam*HI fragment, containing the coding region of *hph* [30]. Southern blot analysis, using *Eco*RI and a probe corresponding to the *hph* coding region, showed hybridizing bands of different sizes, indicating that the T-DNA was integrated at different chromosomal locations (Fig. 2). However, three transformants (lanes 4, 6, and 10) out of ten showed more than two hybridizing bands, suggesting that more than two copies of the inserted DNA are not uncommon.

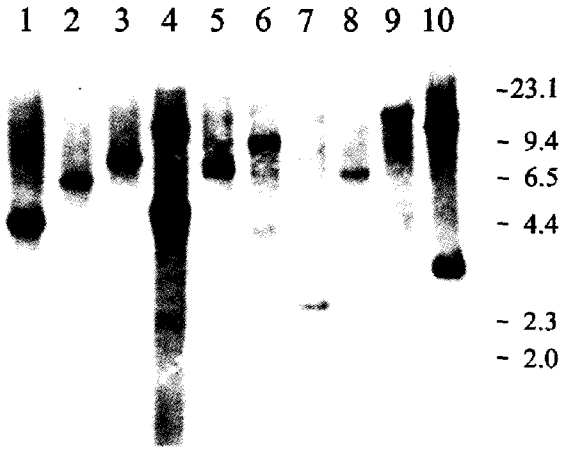


**Fig. 2.** The genomic Southern blot analysis. Genomic DNA from EP155/2, and randomly selected transformants, was digested with *Eco*RI, which has a restriction site at the right border of the T-DNA. The digested DNA underwent electrophoresis on a 0.8% agarose gel, and was transferred to a nylon membrane. The blots were hybridized with the [α-$^{32}$P]dCTP-labeled 0.8 kb *Eco*RI-*Bam*HI fragment of pDH25 with the *hph* gene. Lanes 1-10: DNA from the transformants. Numbers on the right indicate DNA sizes in kb.

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