***Agrobacterium*-Mediated Transformation of *Fusarium oxysporum*: An Efficient Tool for Insertional Mutagenesis and Gene Transfer**

E. D. Mullins, X. Chen, P. Romaine, R. Raina, D. M. Geiser, and S. Kang

First, second, third, fifth, and sixth authors: Department of Plant Pathology, The Pennsylvania State University, University Park; and fourth author: Department of Biology, The Pennsylvania State University, University Park 16802.
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ABSTRACT

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Agrobacterium tumefaciens-mediated transformation (ATMT) has long been used to transfer genes to a wide variety of plants and has also served as an efficient tool for insertional mutagenesis. In this paper, we report the construction of four novel binary vectors for fungal transformation and the optimization of an ATMT protocol for insertional mutagenesis, which permits an efficient genetic manipulation of *Fusarium oxysporum* and other phytopathogenic fungi to be achieved. Employing the binary vectors, carrying the bacterial hygromycin B phosphotransferase gene (*hph*) under the control of the *Aspergillus nidulans* *trpC* promoter as a selectable marker, led to the production of 300 to 500 hygromycin B resistant transformants per 1×10^6 conidia of *F. oxysporum*, which is at least an order of magnitude higher than that previously accomplished. Transformation efficiency correlated strongly with the duration of cocultivation of fungal spores with *Agrobacterium tumefaciens* cells and significantly with the number of *Agrobacterium tumefaciens* cells present during the cocultivation period ($r = 0.996$; $n = 3$; $P < 0.01$). All transformants tested remained mitotically stable, maintaining their

hygromycin B resistance. Growing *Agrobacterium tumefaciens* cells in the presence of acetosyringone (AS) prior to cocultivation shortened the time required for the formation of transformants but decreased to 53% the percentage of transformants containing a single T-DNA insert per genome. This increased to over 80% when *Agrobacterium tumefaciens* cells grown in the absence of AS were used. There was no correlation between the average copy number of T-DNA per genome and the colony diameter of the transformants, the period of cocultivation or the quantity of *Agrobacterium tumefaciens* cells present during cocultivation. To isolate the host sequences flanking the inserted T-DNA, we employed a modified thermal asymmetric interlaced PCR (TAIL-PCR) technique. Utilizing just one arbitrary primer resulted in the successful amplification of desired products in 90% of those transformants analyzed. The insertion event appeared to be a random process with truncation of the inserted T-DNA, ranging from 1 to 14 bp in size, occurring on both the right and left border sequences. Considering the size and design of the vectors described here, coupled with the efficiency and flexibility of this ATMT protocol, it is suggested that ATMT should be regarded as a highly efficient alternative to other DNA transfer procedures in characterizing those genes important for the pathogenicity of *F. oxysporum* and potentially those of other fungal pathogens.

In the filamentous fungi transformation with DNA that does not exhibit homology with the fungal genome results in heterologous integration of transforming DNA into the genome, which makes it possible to use the transforming DNA as an insertional mutagen to disrupt genes and ultimately assist in the study of plant disease. A big advantage of this technique over chemical or radiation mutagenesis is that the mutated gene is tagged by the transforming DNA, which can be used to clone the corresponding wild-type gene by plasmid rescue or polymerase chain reaction (PCR). An insertional mutagenesis technique, termed restriction enzyme-mediated integration (REMI), has been successfully used to transform diverse filamentous fungi (1-3,11,16,23,26,30-32,34,37). This technique has allowed for the efficient isolation of genes important for pathogenicity from several fungal pathogens (1-3, 16,23,30,34,37).

Although an efficient tool for tagging and cloning pathogenicity genes from fungal pathogens, a significant limitation of REMI is that a substantial portion (20 to 100%) of generated mutants appear to be untagged by the transforming DNA (2,11,23,31,34). It has been proposed that cutting of the chromosome by a restriction enzyme followed by imperfect DNA repair during transformation is responsible for those untagged mutations (34). In *Gibberella*

fujikuroi, the transformation process itself caused deletions and chromosome translocations unlinked to the inserted plasmid in both the presence and absence of restriction enzymes (23). Genetic crosses have been used to identify untagged mutants in which the mutant phenotype fails to cosegregate with the selectable marker on the inserted plasmid. However, for fungi lacking a sexual stage, the cloning of tagged genes and subsequent functional complementation by transformation are necessary to verify if a given mutant has been tagged. Especially, when REMI generates a high percentage of mutants with multiple copies of inserted plasmid, such a verification process can be quite time consuming.

Agrobacterium tumefaciens-mediated transformation (ATMT) has long been used to transfer genes to a wide variety of plants and has also been used extensively as a tool for insertional mutagenesis in *Arabidopsis thaliana* (9,12,20). More recently, several fungi have been transformed with *Agrobacterium tumefaciens* (4,8,15). For insertional mutagenesis, this technique offers huge potential as an alternative tool to REMI (10). One of the principal advantages of ATMT over conventional transformation techniques is the versatility it provides in choosing which starting material to transform. Applicable to several fungi, *Agrobacterium tumefaciens* can transform protoplasts, hyphae, spores (8), and blocks of mushroom mycelial tissue (P. Romaine, unpublished data).

Binary vector pUR5750 for transforming fungi to hygromycin B resistance, which was reported by de Groot et al. (8), is approximately 14 kb long. Although its large size is unlikely to become a hindrance during the generation of hygromycin B-resistant

Corresponding author: S. Kang; E-mail address: sxk55@psu.edu

transformants, the cloning of additional genes on the vector for gene transfer to fungi is likely to be difficult. In addition, pUR5750 contains only four unique restriction sites for gene cloning, which also limits its utility as a vehicle for delivering genes into fungi. One of the main objectives of this work was to construct new binary vectors that are more versatile than pUR5750 for gene transfer in various fungi.

Although *Fusarium* vascular wilt disease, caused by *Fusarium oxysporum*, is both widespread in nature and very destructive, our knowledge on its mechanisms of pathogenesis is very limited. Application of ATMT as a tool for insertional mutagenesis is likely to provide such knowledge. Given the asexual nature of *F. oxysporum*, it is important to optimize conditions of ATMT for maximizing the number of transformants carrying a single copy of T-DNA. Additional objectives of this work were to (i) find such conditions and (ii) develop an efficient procedure that would allow for the rescue of inserted T-DNA along with the adjacent host sequences.

MATERIALS AND METHODS

Fungal material. *F. oxysporum* strain O-685 was obtained from the Fusarium Research Center collection (The Pennsylvania State University, University Park) and was stored as a monoconidial culture at -80°C . Material was revitalized on 1% water agar supplemented with irradiated carnation leaf pieces (CLA) (13). The incubation period required to induce conidial formation was typi-

cally 12 days at 22°C under continuous fluorescent light. Conidia were collected by adding sterile water to the plates and rubbing the surface of the CLA with the end of a sterile micropipette tip. Conidial suspension was filtered through Miracloth (Calbiochem-Behring, La Jolla, CA) to remove large particles.

Plasmid construction. All the vectors described in this study (Fig. 1) were constructed on the backbone of pCambia1300 (Cambia, Canberra, Australia). A 1.4-kb fragment of pCSN44 (33), containing the hygromycin B resistance (*hph*) gene under the *Aspergillus nidulans trpC* promoter, was isolated by digesting pCSN44 with *Bam*HI and *Hind*III, and subsequently the ends of the fragment were blunted with DNA polymerase I large fragment in the presence of dNTPs. The *hph* gene under the *Cauliflower mosaic virus* 35S (CaMV35S) promoter was removed from pCambia1300 by digesting the plasmid with *Xho*I and *Bst*XI, and its restricted ends were blunted as described above. This fragment was ligated to the 1.4-kb fragment of pCSN44 to produce pBHt1 (8.4 kb). Plasmid pDHt (7.1 kb) was constructed by self-ligating the digested pCambia1300 without an insert. To construct pBHt2 (8.4 kb), the 1.4-kb *Hpa*I fragment of pCB1004 (6) carrying the modified *hph* gene under the *Aspergillus nidulans trpC* promoter, was ligated to the same fragment of pCambia1300 that was used to construct pBHt1. In both pBHt1 and pBHt2 the 3' end of the *hph* gene was ligated to the CaMV35S poly (A) signal. Plasmid pKHt (10.6 kb) contains a 2.2-kb fragment containing the ColE1 replication of origin and the gene con-

Fig. 1. Map of novel binary vectors, pBHt1, pBHt2, and pKHt, completed with a pCambia1300 backbone. The multiple cloning site composed of restriction sites for *Eco*RI, *Sac*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Sal*I, *Pst*I, *Sph*I, and *Hind*III. *hph*, *trpC*, and *cam* correspond to the hygromycin B resistance gene, *Aspergillus nidulans trpC* promoter and chloramphenicol resistance gene, respectively. Arrows denote direction of their transcription.

ferring chloramphenicol resistance between the *EcoRI* and *PstI* sites of pBHt2. This 2.2-kb fragment was amplified by PCR from pBCSK (Stratagene, La Jolla, CA) by the following two primers: ECO-1 (5'-GGGCGAATTCTCTTCCGCTTCCTCGCT-3') and PST-1 (5'-AAGGAAGTGCAGTATGATATTCAACATT-3'). The underlined sequences in these primers indicate the *EcoRI* and *PstI* sites, respectively, that were introduced to facilitate the subsequent cloning of this fragment in pBHt2. The amplified DNA was separated on a 0.7% agarose gel. A DNA fragment of approximately 2.2 kb was cut from the gel, purified with a gel ex-

traction kit (QIAquick; Qiagen, Valencia, CA), and digested with *EcoRI* and *PstI*. The resulting fragment was cloned between the *EcoRI* and *PstI* sites of pBHt2 to produce pKHt.

Transformation. *Agrobacterium tumefaciens* strain AGL-1 (provided by M. Guiltinan at Penn. State), containing an appropriate binary vector, was grown at 28°C for 2 days in minimal medium (MM) (17) supplemented with kanamycin (50 µg/ml). The *Agrobacterium tumefaciens* cells were diluted to (optical density) OD₆₀₀ = 0.15 in induction medium (IM) (4), both in the presence (IM+AS) and absence (IM-AS) of 200 µM acetosyringone (AS). The cells were grown for an additional 6 h before mixing them with an equal volume of a conidial suspension from strain O-685 (1 × 10⁶ conidia per ml). This mix (200 µl per plate) was plated on a 0.45-µm pore, 45-mm diameter nitrocellulose filter (Whatman, Hillsboro, OR) and placed on cocultivation medium (same as IM except that it contains 5 mM glucose instead of 10 mM glucose) in the presence and absence of 200 µM AS. Following incubation at 25°C for 2 days, the filter was transferred to MM containing hygromycin B (75 µg/ml) as a selection agent for transformants and cefotaxime (200 µM) and moxalactam (100 µg/ml) to kill the *Agrobacterium tumefaciens* cells. Individual transformants were transferred into 24-well plates (Costar Scientific Corp., Cambridge, MA) containing 1.5 ml of CLA amended with hygromycin B (75 µg/ml) and incubated until conidiogenesis. Conidia of individual transformants were suspended with sterile water and plated on potato dextrose agar (PDA; Difco Laboratories, Detroit) supplemented with hygromycin B (75 µg/ml). To create monoconidial cultures, one germinating conidium from each transformant was picked and transferred to CLA containing hygromycin B (75 µg/ml) in either a small petri plate (60 × 15 mm) or a 15-ml plastic tube. Spores from these monoconidial cultures were stored in 20% glycerol at -80°C until further analysis.

DNA extraction and analysis. Transformants were grown for 5 days at room temperature in 50 ml of liquid potato dextrose broth (Difco Laboratories) amended with hygromycin B (75 µg/ml).

TABLE 1. Conditions used for thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) protocol^a

Primary PCR reaction	Secondary PCR reaction	Tertiary PCR reaction
92°C: 2 min	94°C: 10 s***	94°C: 10 s
95°C: 1 min	66°C: 1 min***	94°C: 15 s****
94°C: 30 s*	72°C: 2 min***	42°C: 15 s****
66°C: 1 min*	94°C: 10 s***	72°C: 2 min****
72°C: 2 min*	66°C: 1 min***	72°C: 5 min
94°C: 30 s	72°C: 2 min***	—
30°C: 3 min	94°C: 10 s***	—
Ramp to 72°C over 3 min	44°C: 1 min***	—
72°C: 2 min	72°C: 2 min***	—
94°C: 5 s**	72°C: 5 min	—
44°C: 1 min**	—	—
72°C: 2 min**	—	—
94°C: 5 s***	—	—
66°C: 1 min***	—	—
72°C: 2 min***	—	—
94°C: 5 s***	—	—
66°C: 1 min***	—	—
72°C: 2 min***	—	—
94°C: 5 s***	—	—
44°C: 1 min***	—	—
72°C: 2 min***	—	—
72°C: 5 min	—	—

^a *, **, ***, and **** represents ×5, ×10, and ×15, and ×30 cycles, respectively.

Fig. 2. A, Right border (RB) and B, left border (LB) ends of the T-DNA sequence showing the position and melting temperatures (T_m 's) of the primers employed for thermal asymmetric interlaced polymerase chain reaction. Bold uppercase letters indicate the 24-bp imperfect repeat that was cleaved at the indicated positions prior to insertion. C, The sequence and T_m of the arbitrary degenerate primer (AD-1); W = A and T; and N = A, C, G, and T.

Mycelial mats were collected by vacuum-infiltration on Whatman No. 1 filter paper and washed several times with sterile water before drying between paper towels. Mats were ground to a fine powder in liquid nitrogen, and DNA was extracted as previously described (29). A miniprep procedure developed for *Magnaporthe grisea* (35) was also employed for isolating DNA from some transformants.

To determine the copy number of T-DNA (number of T-DNA insertions throughout the host genome), extracted DNA was digested with *Eco*RI and the products separated by electrophoresis on a 0.7% agarose gel in 1× TAE (40 mM Tris-acetate, pH 8.5 and 2 mM EDTA) buffer containing ethidium bromide. The gel was treated with 0.25 M HCl before blotting onto a nylon membrane (Hybond-XL; Amersham Pharmacia, Buckinghamshire, U.K.) with 0.4 M NaOH as transfer buffer. Prehybridization, hybridization, and high stringency washes of the membrane were completed at 65°C as described by the manufacturer. The 1.4-kb *Hpa*I fragment of pCB1004 described above was labeled with ³²P-labeled dCTP and used as a probe to determine the T-DNA copy number.

Cloning and sequencing of genomic DNA flanking T-DNA. Employing novel primers, a thermal asymmetric interlaced PCR (TAIL-PCR) protocol (Table 1; 24,25) was used for cloning genomic DNA flanking inserted T-DNA from 10 randomly selected transformants. The right border primers (RB-1, -2, and -3) and the left border primers (LB-1, -2, and -3) and the arbitrary degenerate (AD-1) primer used (Fig. 2) were designed such that their melting temperatures (T_m 's) would ensure maximum thermal asymmetric priming. T_m 's were calculated as previously described (27). A thermal cycler (PTC-100; MJ Research Inc., Watertown, MA) was used for the TAIL-PCR. The tertiary TAIL-PCR product of each transformant showing the highest intensity was purified using QIAquick columns (Qiagen) and sequenced on an automated DNA sequencer (ABI Prism 377) by a terminator cycle sequencing kit (ABI Prism BigDye; Applied Biosystems, Foster City, CA). The innermost specific primer, either RB-3 or LB-3, was used as the sequencing primer.

Mitotic stability of transformants. To determine the stability of transformants, five randomly selected transformants were cultured on PDA without hygromycin B. Following colonization of

each plate, a mycelial plug was taken from the edge of the culture and transferred onto fresh PDA. This procedure was repeated an additional five times after which 13 germinating monoonidia from each transformant line were isolated and cultured. Resistance of these monoonidial cultures to hygromycin B was tested by growing them on PDA containing hygromycin B (75 µg/ml).

RESULTS

***Agrobacterium tumefaciens* mediated transformation of *F. oxysporum*.** We replaced the *hph* gene under the CaMV35S promoter in pCambia1300 with the *hph* gene under the *Asperigillus nidulans trpC* promoter to produce pBHt1. Cocultivation of *Agrobacterium tumefaciens* cells carrying pBHt1 with conidia of *F. oxysporum* strain O-685 in the presence of AS led to the appearance of hygromycin B-resistant colonies approximately 4 to 5 days after transfer to the selection medium. In the absence of AS during cocultivation, no transformants were formed. Interestingly, both pBHt1 and pCambia1300 successfully transformed this strain, suggesting that the CaMV35S promoter can function in *F. oxysporum*. Although the average colony size of transformants by pCambia1300 was consistently smaller than that by pBHt1, no significant difference existed in the number of transformants between these constructs. Subsequently, all the transformation experiments described below were performed with pBHt1 unless indicated otherwise. Transformation efficiency was in the range of 300 to 500 transformants per 1×10^6 conidia when *Agrobacterium tumefaciens* cells were grown in the presence of AS prior to 48 h of cocultivation. An assessment of the mitotic stability of five transformants showed that all of them maintained their hygromycin B resistance after being cultured for six generations in the absence of hygromycin B (data not shown).

Optimization of transformation conditions. We have determined the influence of the following two factors on the transformation efficiency and the rate at which T-DNA is inserted into the host genome: (i) the effect of pretreating the bacterial cells with AS prior to cocultivation, and (ii) the length of the cocultivation period. We grew *Agrobacterium tumefaciens* cells in IM with and without AS for 6 h at 28°C, prior to cocultivation with O-685

Fig. 3. Effect on transformation efficiency of increasing the cocultivation time with which *Agrobacterium tumefaciens* cells and O-685 conidia were incubated. *Agrobacterium tumefaciens* cells were incubated in induction medium (IM) in the presence or absence of acetosyringone (AS). Data presented as the average of four plates per treatment. Error bars indicate standard error.

Fig. 4. T-DNA copy number analysis completed in the presence or absence of acetosyringone (AS) on O-685 transformants (19 for induction medium [IM]+AS; 26 for IM-AS). DNA was digested with *Eco*RI, fragmented on an agarose gel, transferred to a nylon membrane, and probed with a radiolabeled 1.4-kb *Hpa*I fragment of pCB1004.

conidia. Following every 12 h of cocultivation, the nitrocellulose filters overlaid with the mix of *Agrobacterium tumefaciens* cells and fungal conidia were transferred to Aspergillus MM (18) containing hygromycin B to permit selection. Although the presence of AS during cocultivation was imperative for transformation, the inclusion of AS during the 6 h growth of *Agrobacterium tumefaciens* cells prior to cocultivation was not essential (Fig. 3). However, with *Agrobacterium tumefaciens* cells grown in the absence of AS (IM-AS), the appearance of transformants was substantially delayed relative to that recorded with *Agrobacterium tumefaciens* cells grown in the presence of AS (IM+AS). Transformation efficiency increased as the cocultivation period increased under both conditions but was consistently lower under the IM+AS condition when the same cocultivation period was used (Fig. 3). The difference in transformation efficiency between the two conditions was less pronounced after 48 h of cocultivation.

We determined the effect of AS on the percentage of transformants containing a single insert of T-DNA per genome by Southern analysis with the *hph* gene as a probe (Fig. 4). The IM-AS condition increased the number of such transformants relative to the IM+AS treatment considerably (Fig. 4). Only 53% of those transformants generated by *Agrobacterium tumefaciens* cells grown in the IM+AS condition had a single T-DNA, whereas those derived from *Agrobacterium tumefaciens* cells grown in the IM-AS condition increased to over 80%. Among those transformants derived from *Agrobacterium tumefaciens* cells grown in the IM+AS condition, the period of co-incubation did not appear to influence such a frequency. Four of nine transformants (44%) derived from the 36-h cocultivation (Fig. 3) possessed a single insert of T-DNA, whereas four of eight transformants (50%) derived from the 48-h cocultivation treatment had a single T-DNA insert. We also determined the relationship between the size of transformant colonies and the copy number of T-DNA by grouping transformants into three classes (<3-mm diameter, 3- to 6-mm diameter, and >6-mm diameter) after 4 days of growth on the selective medium. Similar to cocultivation time, average colony diameter did not correlate with the copy number of T-DNA in either the IM-AS or IM+AS condition (data not shown).

The effect of *Agrobacterium tumefaciens* cell number on the percentage of transformants containing a single T-DNA insert was also determined. Different volumes of *Agrobacterium tumefaciens* cells grown in the IM+AS condition (ranging from 5 to 100 μ l) were adjusted to 100 μ l (with IM+AS) and cocultivated with 100 μ l of conidia (1×10^6 conidia per ml) for 48 h. Not surprisingly, as the *Agrobacterium tumefaciens* cell number increased, so did the efficiency of transformation ($r = 0.996$; $n = 3$; $P < 0.01$; Table 2). A Southern analysis of six transformants from each condition showed that no significant correlation existed between the average insert number per genome and the quantity of *Agrobacterium tumefaciens* cells present during the cocultivation period. On average, 56% of the 30 transformants analyzed possessed a single insert of T-DNA, which was consistent with the result shown in Figure 4.

TABLE 2. Effect of decreasing the volume of *Agrobacterium tumefaciens* cells available in the cocultivation mix on the efficiency of transformation and the subsequent average copy number of T-DNA^a

Bacterial cell volume (μ l)	Transformation efficiency	Copy number
100	42 $\pm$ 4.9	1.3 $\pm$ 0.21
50	21 $\pm$ 1.1	1.2 $\pm$ 0.16
25	14 $\pm$ 1.99	1.8 $\pm$ 0.31
10	8.5 $\pm$ 0.49	1.5 $\pm$ 0.22
5	4 $\pm$ 0.99	1.3 $\pm$ 0.21

^a Copy number was determined by Southern hybridization using the ³²P-labeled 1.4-kb *Hpa*I fragment of pCB1004 as a probe. Average number of transformants between three plates $\pm$ standard error. Average copy number of six transformants per treatment $\pm$ standard error.

Cloning and characterization of T-DNA and its flanking sequences. TAIL-PCR was used to isolate the genomic DNA of *F. oxysporum* flanking the site of T-DNA insertion. The potential of TAIL-PCR as a tool for such a purpose has already been established (24). We designed a 16-nucleotide (nt) long AD primer, labeled AD-1, with a calculated melting temperature (T_m) of 47°C (Fig. 2). T_m 's of primers specific for each border sequence of T-DNA (LB-1, -2, and -3 for the left border and RB-1, -2, and -3 for the right border), ranging from 20 to 26 nt in size, were designed to be at least 58°C or higher, as previously recommended (25). The 5' end of LB-3 and RB-3 was 74 and 63 bp apart from the 5' end of LB-2 and RB-2, respectively, to facilitate the identification of PCR products corresponding to T-DNA insert junctions by size comparison between the secondary and tertiary reaction products.

Employing the AD-1 primer with the RB-specific primers, successful amplification of junction fragments was attained in 7 of the 10 transformants analyzed (Fig. 5). Using AD-1 with the set of LB-specific primers (LB-1, -2, and -3), we successfully amplified junction fragments from eight transformants, two of which corresponded to those that failed to produce a desired product with the RB primers. The sizes of PCR products ranged from 0.3 to 1.6 kb. As a result of the nested arrangement of the RB and LB primers within one another, the size profile of the desired product was staggered, decreasing with each TAIL-PCR reaction (Fig. 5). The primary reaction typically produced several products. As found previously (24,25), the number of PCR products was reduced with each successive reaction. In some transformants, more than one junction product was amplified presumably due to the presence of multiple annealing sites of AD-1 in the vicinity of the inserted T-DNA (Fig. 5).

A sequence comparison of 15 junction fragments (7 RB and 8 LB) suggested that the insertion of T-DNA within the host genome appears to be a random event (Fig. 6). The *F. oxysporum* sequences flanking the insertion sites revealed no significant similarity among each other or with the T-DNA border sequences. Truncation of the inserted T-DNA, ranging from 1 to 25 bp in size, occurred on both the right and left border sequences, but more frequently with the latter (RB 28% versus LB 38%). Using the BLASTX algorithm (NCBI, Bethesda, MD) no significant similarity was found between the genomic DNA sequences flanking inserted T-DNA and those present in the GenBank database.

Construction of additional *Agrobacterium tumefaciens* binary vectors for fungal transformation. Additional *Agrobacte*

Fig. 5. Agarose gel analysis of thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) products generated from four representative transformants. Amplification of transformants Tr. 4 and Tr. 6 was performed with the right border (RB) nest of primers (RB-1, -2, and -3) with arbitrary degenerate primer (AD)-1. Tr. 7 and Tr. 9 were amplified with AD-1 and the left border (LB) set of specific primers (LB-1, -2, and -3). The first, second, and third lanes of each gel (from left to right) represent the primary, secondary, and tertiary reactions, respectively, of the protocol. Arrows label TAIL-PCR products corresponding to junction fragments containing segment of inserted T-DNA and associated flanking fungal DNA.

transformation efficiency was significantly enhanced by prolonging the bacterial-fungal cell cocultivation time and by increasing the number of *Agrobacterium tumefaciens* cells, the percentage of transformants carrying a single T-DNA insert per fungal genome was independent of these variables (Fig. 4 and Table 2). Furthermore, the colony size of hygromycin B resistant transformants was not a reliable indicator for the rate of T-DNA insertion, which rejects the hypothesis that the larger the size of the transformant, the more copies of T-DNA has been inserted therein. The presence of AS is essential for successful ATMT (4,8) and as shown here, when included in the culturing media of the *Agrobacterium tumefaciens* cells, was the only factor that influenced the rate of T-DNA insertion into the host genome (Fig. 4). In its absence from the 6-h bacterial cell pretreatment, over 80% of the tested transformants had experienced single insertions, whereas its inclusion resulted in almost 50% of the generated transformants possessing multiple T-DNA inserts.

Having designed novel primer sets and demonstrated their effectiveness, we have established TAIL-PCR as a rapid, effective strategy for isolating the host DNA sequence flanking a site of T-DNA insertion in *F. oxysporum*. Using only one arbitrary primer (AD-1) in combination with either the LB or the RB primers, we were able to isolate PCR fragments spanning one or both of the insertion junctions of T-DNA from 90% of the transformants tested. It is likely that 100% efficiency could be attained with the use of additional AD primers. One concern with TAIL-PCR is that if the border sequences become truncated beyond the annealing site for the amplifying borders, amplification of the desired product would not be possible. In spite of the fact that such an event appears unlikely in *F. oxysporum* (Fig. 5), where the extent of truncation (2 to 25 bp) was substantially lower than that (1 to 82 bp) seen in *Aspergillus awamori* (8), we constructed the pKHt vector. Containing the ColE1 origin of replication and the chloramphenicol resistance gene within the multiple cloning sites, this vector will permit both TAIL-PCR and plasmid rescue for cloning fungal genes tagged by T-DNA.

The use of *Agrobacterium tumefaciens* as an insertional mutagen in *Arabidopsis* has resulted in the establishment of large T-DNA transformed populations that are now accessible to the scientific community (21). One of the objectives of this study was to test the feasibility of ATMT as a tool for the insertional mutagenesis of *F. oxysporum*. The systematic analysis of fungal genes important for pathogenicity has been greatly assisted by REMI (1–3,16,23,30,34,37) whereby the mutated gene, as a result of being tagged by the foreign DNA, can be cloned easily. We believe that ATMT could offer an alternative approach for the efficient insertional mutagenesis of fungi (10), which may circumvent some of the shortcomings of REMI. A previous study showed that <20% *Arabidopsis* mutants generated by ATMT were untagged by T-DNA (12). However, another study reported a much higher frequency (64%) of untagged mutants in *Arabidopsis* (7). It remains to be determined whether ATMT also generates a significant percentage of untagged mutants in fungi as has also been observed in some fungal mutants generated by REMI (2,11,23,31,34). If the frequency of untagged mutants by ATMT is low in fungi, it will be especially suitable for creating a mutant library of *F. oxysporum* as well as other fungi that lack the sexual stage.

Although we have used *F. oxysporum* as the sole host for ATMT, the binary vectors and protocols developed in this study are applicable to transform other fungi. In a preliminary experiment with pBHt1 (S. Kang, unpublished data), the transformation efficiency of three different strains of *Verticillium dahliae* ranged from less than 50 per 1×10^6 conidia (with an isolate from bell pepper) to 500 per 1×10^6 conidia (with an isolate from cabbage). The efficiency with *M. grisea*, using pBHt2, was approximately 200 per 1×10^6 conidia after 36 h of cocultivation (Y.-H. Lee at Seoul National University, personal communication), which was similar to what we observed with *F. oxysporum*. However, both

pBHt1 and pCAMBIA1300 failed to transform *Agaricus bisporus* (button mushroom), suggesting that neither the *Aspergillus nidulans trpC* promoter nor the CaMV35S promoter function properly in *Agaricus bisporus*.

We have demonstrated in this paper that the vectors we have constructed and the protocol modifications we have made significantly enhance the effectiveness of ATMT to the point that its use can now be broadly adopted to study gene function in a large group of fungal pathogens. Furthermore, our vectors are significantly smaller in size (pBHt1, 8.4 kb and pBHt2, 8.4 kb) than the plasmid (pUR5750, 14 kb) previously used (8). Although the size of a binary vector is unlikely to become a critical factor with respect to transformation efficiency, the disparity between the vectors will be crucial when additional genes need to be cloned into the vectors to carry out gene transfer. The utility of pBHt2 as a vehicle for gene delivery is further enhanced by the fact that it contains nine unique restriction sites, in contrast to the four located on pUR5750.

In spite of the earlier publications describing the potential of this procedure, ATMT has not featured prominently in literature. We believe that because of the successful transformation of a large number of fungi by *Agrobacterium tumefaciens* and the other advantages it possesses over protoplast-based transformation techniques (8), coupled with the availability of our vectors to the community, the full potential of ATMT as a means for facilitating the genetic manipulation of diverse plant pathogenic fungi will be realized.

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