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Agrobacterium tumefaciens-Mediated Transformation of *Aspergillus fumigatus*: an Efficient Tool for Insertional Mutagenesis and Targeted Gene Disruption

Janyce A. Sugui, Yun C. Chang, and K. J. Kwon-Chung*

Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland

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Agrobacterium tumefaciens **was used to transform** ***Aspergillus fumigatus*** **by either random or site-directed integration of transforming DNA (T-DNA). Random mutagenesis via** ***Agrobacterium tumefaciens*****-mediated transformation (ATMT) was accomplished with T-DNA containing a hygromycin resistance cassette. Cocultivation of** ***A. fumigatus*** **conidia and** ***Agrobacterium*** **(1:10 ratio) for 48 h at 24°C resulted in high frequencies of transformation (>100 transformants/10^7 conidia). The majority of transformants harbored a randomly integrated single copy of T-DNA and were mitotically stable. We chose** ***alb1*****, a polyketide synthase gene, as the target gene for homologous integration because of the clear phenotype difference between the white colonies of Δ*alb1* mutant strains and the bluish-green colonies of wild-type strains. ATMT with a T-DNA-containing** ***alb1*** **disruption construct resulted in 66% albino transformants. Southern analysis revealed that 19 of the 20 randomly chosen albino transformants (95%) were disrupted by homologous recombination. These results suggest that ATMT is an efficient tool for transformation, random insertional mutagenesis, and gene disruption in** ***A. fumigatus*****.**

Aspergillus fumigatus, an airborne fungal pathogen, is the major cause of allergic bronchopulmonary aspergillosis, aspergilloma, and invasive aspergillosis. Of particular importance is the invasive aspergillosis that starts with inhalation of conidia and progresses to life-threatening infection in immunocompromised patients (11). Due to the rising prevalence of cancer, organ transplantation, and other causes of immunosuppression, the number of patients at risk of invasive aspergillosis is on the rise. Despite aggressive antifungal therapy, the overall mortality of invasive aspergillosis remains high, and new strategies to prevent and treat this disease are urgently needed (7). Several factors such as polyketide synthase, which is involved in melanin synthesis (12, 17), and a Ras-related protein, RhbA, presumably involved in nutrient sensing (15), have been reported to be associated with *A. fumigatus* virulence. Further identification of the genes necessary for virulence will enable us to address the key pathobiological questions for this fungus and provide a foundation for better strategies to manage aspergillosis.

As genomic sequencing of *A. fumigatus* is in its final stages, identification of potential virulence genes can be more rapidly assessed. Investigation of the functions of such genes can be accomplished via mutational analysis either by reverse or forward genetic approaches. Transformation of fungi with DNA that does not possess homology with the fungal genome results in random integration into the fungal genome and can cause gene disruption as an insertional mutagen. A decisive advantage of insertional mutagenesis over chemical or radiation mutagenesis is that the mutated genes are tagged by the transforming DNA (T-DNA), which can be used to identify the disrupted genes. Although recombinational analysis by classical genetics is not possible in *A. fumigatus*, the corresponding wild-type genes can be disrupted by homologous integration of a knockout construct to confirm its function.

To disrupt a gene by homologous integration in *A. fumigatus*, transformation via the spheroplast method has been most widely used; this is a laborious and time-consuming method, and the homologous recombination frequency can be relatively low (3). Electroporation and biolistic methods were also used to transform *A. fumigatus*; these methods, however, resulted in a high frequency of multiple integrations and a low frequency of homologous recombination (3). As an alternative method, *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been used to transform several *Aspergillus* species such as *Aspergillus niger*, *Aspergillus awamori*, and *Aspergillus giganteus* (5, 10, 13). de Groot and colleagues used *A. tumefaciens* to deliver T-DNA containing a hygromycin resistance gene into conidia as well as spheroplasts of *A. awamori* (5). The transformation frequency via ATMT was significantly higher in comparison to frequency with the traditional method, regardless of whether conidia or spheroplasts were used for transformation. Most of the transformants obtained by ATMT contained a randomly integrated single T-DNA copy. Homologous integration via ATMT was also accomplished in *A. awamori*. The frequency of homologous recombination with T-DNA containing the *pyrG* gene of *A. awamori* was higher compared to results with conventional transformation methods (10). A comparison of ATMT with biolistic, electroporation, and spheroplast methods showed that ATMT is superior to the other methods for the transformation of *A. giganteus*. The transformation frequency was enhanced, and all the transformants contained a randomly integrated single copy of T-DNA (13). In contrast, ATMT was described as being less efficient

* Corresponding author. Mailing address: LCID, NIAID, Bldg. 10, Room 11C304, National Institutes of Health, Bethesda, MD 20892. Phone: (301) 496-1602. Fax: (301) 480-3240. E-mail: june_kwon-chung@nih.gov.

than conventional transformation methods for *A. niger* (5). Here we report transformation of *A. fumigatus* mediated by *Agrobacterium tumefaciens* as a tool for insertional mutagenesis as well as for gene disruption.

MATERIALS AND METHODS

Strains and media. A clinical strain of *A. fumigatus*, B-5233, and transformants obtained by ATMT were maintained on *Aspergillus* minimal medium (17). *Agrobacterium tumefaciens* strain EHA105 (a gift from Seogchan Kang, Pennsylvania State University, University Park, Pa.) was grown either on Luria-Bertani broth supplemented with 50 μg of kanamycin per ml or induction medium (4) supplemented with 0.2 mM acetosyringone (IMAS). Transformants were selected on *Aspergillus* minimal medium (17) supplemented with 200 μg of hygromycin per ml and 200 μg of cefotaxime (SM) per ml.

Plasmid construction. The plasmid used for ectopic mutagenesis, pDHt/*hph*, was constructed by insertion of a 2.7-kb HindIII/SacI fragment from pAN7 containing the hygromycin resistance gene (*hph*) (16) into the HindIII/SacI-restricted pDHt/SK plasmid. pDHt/SK is a gift from Seogchan Kang (Pennsylvania State University) and is derived from the plasmid pDHt (14) by inserting a 0.8-kb HpaI/StuI fragment from pGreenII between the two PvuII sites in pDHt. Gene disruption by homologous recombination was performed with the plasmid pDHt/*alb1*::*hph*. This plasmid was constructed by inserting a 7.2-kb SacI/ApaI fragment from pRGD12 (17), which contained the *hph* gene flanked by *alb1* sequence, into the SacI/ApaI-restricted plasmid pDHt/SK (Fig. 2A). *Agrobacterium tumefaciens* strain EHA105 was transformed by electroporation (6) with pDHt/*hph* or pDHt/*alb1*::*hph* plasmids. Strains of *Agrobacterium* harboring pDHt/*hph* and pDHt/*alb1*::*hph* were named EHA105A and EHA105B, respectively.

ATMT transformation. Strain EHA105A or EHA105B was grown on Luria-Bertani broth supplemented with 50 μg of kanamycin per ml on a rotatory shaker (200 rpm) at 28°C for 12 h. One milliliter of the *Agrobacterium* culture was inoculated into 9.0 ml of IMAS and incubated on a shaker (200 rpm) at 28°C for 6 h or until an optical density at 660 nm of 0.8 was reached. Conidia of B-5233 and *Agrobacterium* were cocultivated at a ratio of 1:10 (conidia to bacteria), unless otherwise specified. A total of 100 μl of the *Agrobacterium* culture (10^8 CFU) was mixed with 100 μl of B-5233 conidia (10^7 conidia) and spread onto a filter placed on an IMAS agar plate. The filters used were nitrocellulose (Protran; Schleicher and Schuell, Keene, N.H.), cellulose (Whatman no. 1; Maidstone, United Kingdom) and nylon (Hybond-N; Amersham Biosciences, Little Chalfont, United Kingdom). Plates containing the filters were incubated at 24°C in the dark for 16, 26, 40, or 48 h. Initial incubations for 16 and 26 h were followed by further incubation at 28°C for 24 h. To select ATMT transformants, filters containing the transformants were transferred to SM agar plates and incubated at 37°C for 3 days.

DNA analysis. Conidia were inoculated into yeast nitrogen base, (Difco, Sparks, Md.), supplemented with 0.5% glucose, and grown on a shaker (200 rpm) at 37°C for 24 h. DNA extractions from hyphae and Southern hybridizations were carried out as previously described (17). SpeI-restricted DNA from transformants with random insertions was probed with the ^{32}P-labeled pAN7 plasmid. In the case of *alb1*-targeted disruption, AvrII-restricted DNA from albino transformants was probed with an *alb1* fragment amplified from the template pks33 (14) by using the primers ALB9 (GATCTGGAATCGTCCGTGTT) and ALB10 (CCCTGGAGAAGAATCGAGGT). For reverse transcription PCR (RT-PCR), total RNA was isolated from albino conidia as described (17). RT-PCR with a One-Step RT-PCR kit (QIAGEN, Valencia, Calif.) was performed according to the manufacturer's protocol. Specific primers used were INT3.1 (ATGGAGTG GCTCTTACGCCAT) and INT4.1 (ATCTTTCGAGCCAGCGCTTG). Since the amplicon contains two introns in the genomic DNA, the wild-type DNA template (positive control) produced a fragment 120 bp larger than the one observed with the total RNA. Tubulin primers were added to the reaction mixture as a control for the amount and quality of RNA. Tubulin primer sequences were as follows: for β-tubulin2F, AAAGGTCTCATCTGCGTGCT; for β-tubulin2R, GGCGGAGAGCTGTGACTATC.

RESULTS AND DISCUSSION

Agrobacterium tumefaciens, a plant-pathogenic bacteria, has the ability to transfer a fragment of DNA (T-DNA) from its tumor-inducing plasmid to a recipient's genome. The tumor-inducing plasmid also contains a set of genes termed *vir* that

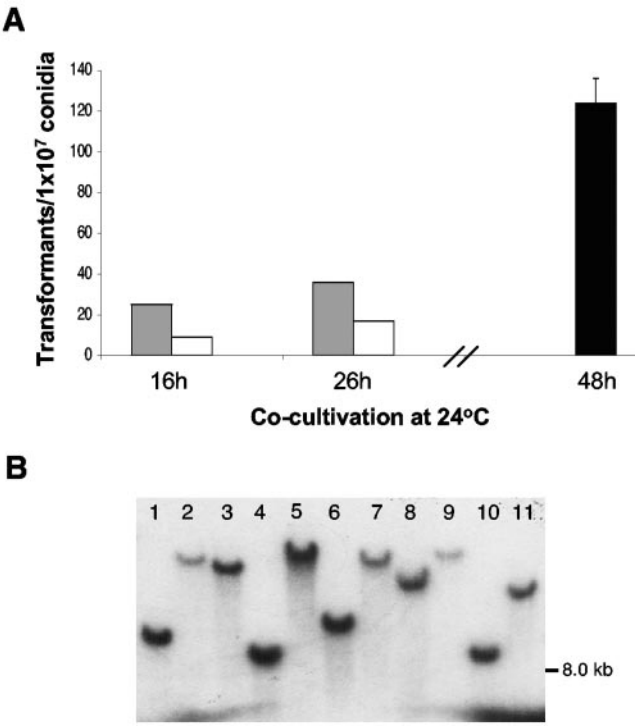

FIG. 1. *A. fumigatus* transformation frequency by ATMT method. (A) *Agrobacterium* and conidia were cocultivated on either a nylon (gray bars) or cellulose (white bars) filter at 24°C for the indicated time period, followed by further incubation at 28°C for 24 h. Cocultivation on nylon (black bar) was at 24°C for 48 h without subsequent incubation at 28°C. (B) Southern analysis. DNAs of *A. fumigatus* transformants were digested with SpeI and hybridized with pAN7 plasmid DNA as a probe containing the *hph* gene.

are essential for the T-DNA transfer. Induction of the *vir* genes by certain chemical signals, such as acetosyringone, initiates the process of transfer and integration of the T-DNA into the recipient's genome (9). Based on previous reports of *Agrobacterium*-mediated transformation in certain species of *Aspergillus* (5, 10, 13), we developed an ATMT system in *A. fumigatus* by using hygromycin resistance (*hph* gene) as the selectable marker. Initially, we inserted the *hph* gene into T-DNA and aimed for its random integration into the *A. fumigatus* genome.

Contact between recipient and *Agrobacterium* is an essential prerequisite for the T-DNA transfer. A solid substrate allows the recipient and *Agrobacterium* to grow in close proximity so that the T-DNA can be transferred. Three different filters (nitrocellulose, cellulose, and nylon) were tested as substrates for cocultivation of *A. fumigatus* and *Agrobacterium tumefaciens*. The best results were obtained with nylon and cellulose filters (Fig. 1A). Although nitrocellulose filters are most commonly used as the substrates for cocultivation of *Agrobacterium* and filamentous fungi for ATMT (5), our results with *A. fumigatus* indicate that nitrocellulose is not as efficient as nylon or cellulose filters (data not shown). The reason for this difference is unclear. It is possible that the chemical properties of these membranes may affect the distribution of the *Agrobacterium* cells and conidia or inhibit their interaction, thereby resulting in differences in transformation efficiency. Initially, conidia and *Agrobacterium* were cocultivated at 28°C for 72 h.

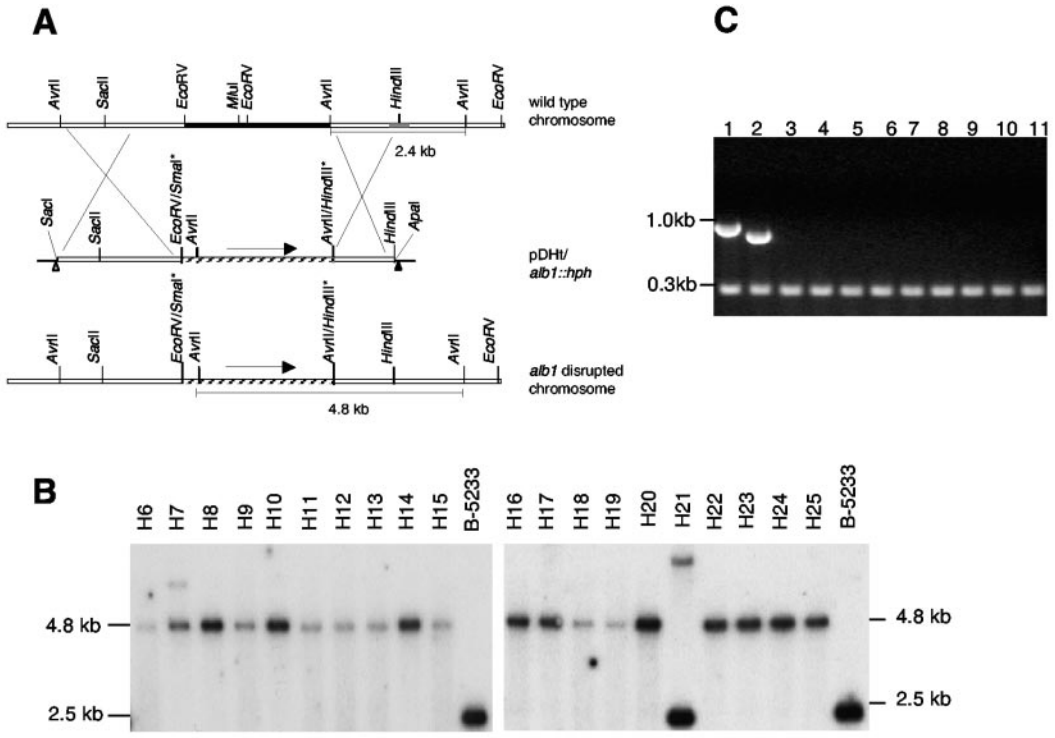

FIG. 2. The *alb1* gene disruption via ATMT. (A) Schematic representation of the *alb1* native gene, the disruption construct, and the disrupted locus. The deleted fragment of *alb1* (filled black line), the fragment used as the probe for Southern analysis (gray line), and the hygromycin resistance cassette (hatched line) are indicated. An asterisk indicates the destroyed restriction sites. Triangles represent left- and right-border repeats in the T-DNA. (B) Southern hybridization of 20 randomly chosen albino transformants. AvrII-restricted DNA was probed with the *alb1* wild-type gene. (C) RT-PCR of *alb1* transcript in albino mutants. Lane 1, B-5233 genomic DNA; lane 2, RNA derived from B-5233; lanes 3 to 11, RNA derived from transformants H6 through H14. The 232-bp fragment is the PCR product of β-tubulin.

The number of transformants was low under these conditions, averaging 10 transformants per 10^7 conidia. The cocultivation temperature was then lowered to 24°C for the first 16 or 26 h, followed by incubation at 28°C for 24 h. The results showed that extending the incubation time at 24°C led to higher numbers of transformants (Fig. 1A). When the cocultivation time at 24°C was extended to 48 h without further incubation at 28°C, over 100 transformants per 10^7 conidia were obtained. This was approximately a fourfold increase compared to the number of transformants from shorter incubations at 24°C (Fig. 1A). Although 28°C is optimal for growth of *A. tumefaciens*, this temperature was not appropriate for T-DNA transfer. It has been proposed that the T-DNA transfer machinery is greatly affected by temperature. For example, the expression of some *vir* genes of *Agrobacterium* that are necessary for interaction between the bacteria and the recipient is impaired at temperatures above 26°C, thus affecting the transformation efficiency (2, 8).

To analyze the number of T-DNA copies integrated in the genome, 15 transformants were randomly selected. Southern blot analysis revealed that most of the transformants harbored a single copy of T-DNA integrated randomly in the genome (Fig. 1B). Similar results have been reported in *A. giganteus* (13) and *A. awamori* (10), where a majority of transformants contained a single copy of T-DNA in the genome. The efficiency of generating mutants by ATMT in *A. fumigatus* was demonstrated in further experiments where we obtained six albino conidial mutants in about 9,000 transformants (data not shown). According to our data, the best conditions for random insertion of T-DNA via ATMT in *A. fumigatus* involve the cocultivation of conidia and *Agrobacterium* on nylon filters at 24°C for 48 h, followed by transfer of the filter to SM agar for further incubation at 37°C for 3 days.

Our second aim was to assess whether a T-DNA-containing *A. fumigatus* gene can be integrated at a specific site via homologous recombination. The gene chosen as the target for homologous integration was *alb1*, a polyketide synthase gene. This gene was chosen because the colonies of Δ*alb1* mutants are readily distinguishable from the wild-type colonies (17). When *alb1* is disrupted, the mutant fails to synthesize the bluish-green conidial pigment and produces albino colonies. Disruption of the *alb1* gene was carried out by using a plasmid construct containing a hygromycin resistance cassette flanked by *alb1* sequence (17) (Fig. 2A). Initially *A. tumefaciens* strain EHA105B was cocultivated with conidia of the wild-type strain B-5233 at 24°C for 48 h. However, the number of transformants was too numerous, and the filters were completely covered with transformants (data not shown). Since previous experiments had shown that cocultivation time influences transformation efficiency, we reduced the time to 40 h in order to obtain a manageable number of transformants. With a 40-h cocultivation time, the number of transformants was significantly lower, averaging 60 colonies per 10^7 conidia (Fig. 3). Among the transformants, 66% produced albino conidia, while

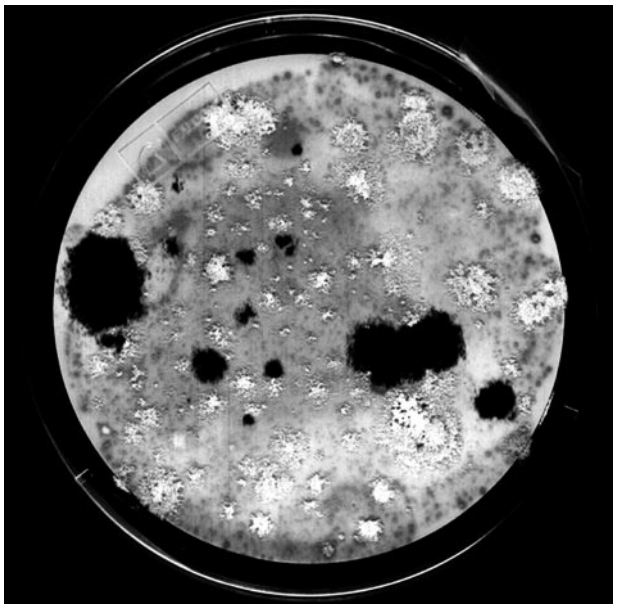

FIG. 3. Gene disruption of *alb1* via ATMT. The cocultivation plate shows transformants generated by targeted mutagenesis. Wild-type colonies (black) presumably contain ectopically integrated T-DNA. Albino colonies suggest the disruption of the *alb1* gene. Adobe Photoshop version 3.0 was used for the image.

the other 34% produced bluish-green conidia. Further experiments with a 40-h incubation time with a conidia-to-bacteria ratio of either 1:100 or 1:1,000 resulted in a greater reduction in transformation frequency. The average numbers of transformants obtained with the 1:100 and 1:1,000 ratios were 15 and 4, respectively. A reduction in the transformation frequency resulting from an increase in the number of *Agrobacterium* cells relative to conidia has also been described in *A. giganteus* ATMT; a fivefold increase of input *Agrobacterium* above the optimum levels resulted in a 16-fold reduction in transformation efficiency (13).

Twenty randomly selected albino mutants were analyzed by Southern hybridization. Upon hybridization with a 560-bp wild-type *alb1* fragment, the strain B-5233 yielded a 2.4-kb band corresponding to the wild-type *alb1* gene (Fig. 2A), while the albino transformants resulting from a double crossover at the homologous site yielded a 4.8-kb band (Fig. 2A). Homologous recombination between the disruption construct and the *alb1* gene occurred in 19 transformants (95%) (Fig. 2B). One of the transformants, H7, contained an extra band in addition to the expected deletion pattern, suggesting that H7 has an additional copy of the disruption construct, one in the *alb1* locus and the other at an ectopic site. Transformant H21 revealed two bands, the wild-type fragment and a fragment of approximately 7 kb (Fig. 2B). The presence of a wild-type-sized band suggested that the albino phenotype was not the result of a double crossover. Further analysis indicated that the albino phenotype in this transformant was due to integration of a T-DNA copy at the *alb1* locus by single crossover (data not shown). Gene disruption was confirmed by a loss of the *alb1* transcript. RT-PCR from nine randomly selected transformants revealed that all strains lacked the *alb1* transcript (Fig. 2C), confirming the gene disruption. High frequencies of gene disruption via ATMT have been reported in various other fungi. For example, ATMT carried out with a disruption construct of the β-1,6-glucanase gene (*VFGlu1*) in *Verticillium fungicola* resulted in a targeted gene deletion in 75% of the transformants (1). In *Trichoderma atroviride*, approximately 60% of ATMT transformants showed targeted disruptions in the *tmk1* as well as the *tga3* genes (18).

The gene *alb1* has been previously disrupted in the wild-type B-5233 by the spheroplast transformation method (17). The disruption construct contained a cassette similar to the one used in the present study, with the same *alb1* flanking regions as well as the selectable marker. Spheroplast transformation yielded 30% of the transformants with albino phenotype (17). Among the albino transformants, only 25% contained a single copy of the disruption construct integrated via double crossover; for the rest, either multiple copies of the disruption construct were present, or the disruption resulted from a single crossover event (unpublished data). Because the precise number of viable spores as well as the input of transforming DNA cannot be standardized, direct comparisons of homologous integration frequencies between spheroplast transformation and ATMT are not possible. As a general outcome, however, the yield of albino transformants by the ATMT method was twofold higher relative to wild-type transformants when compared with results from the spheroplast transformation method. Furthermore, the frequency of homologous recombination via a double crossover without additional ectopic integrations of the disruption construct is higher among albino mutants obtained by the ATMT than by the spheroplast method. In general, the frequency of homologous integration in *A. fumigatus* (B-5233) by spheroplast transformation is between 10 to 30%, depending on the genes targeted, and multiple integrations can range between 40 to 60% (unpublished observations). In conclusion, the ATMT method not only simplifies the transformation procedure but also offers an efficient tool for random as well as targeted integration in *A. fumigatus*.

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