Agrobacterium-Mediated Co-transformation of Multiple Genes in Metarhizium robertsii

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Abstract Fungi of the Metarhizium genus are a very versatile model for understanding pathogenicity in insects and their symbiotic relationship with plants. To establish a co-transformation system for the transformation of multiple M. robertsii genes using Agrobacterium tumefaciens, we evaluated whether the antibiotic nourseothricin has the same marker selection efficiency as phosphinothricin using separate vectors. Subsequently, in the two vectors containing the nourseothricin and phosphinothricin resistance cassettes were inserted eGFP and mCherry expression cassettes, respectively. These new vectors were then introduced independently into A. tumefaciens and used to transform M. robertsii either in independent events or in one single co-transformation event using an equimolar mixture of A. tumefaciens cultures. The number of transformants obtained by co-transformation was similar to that obtained by the individual transformation events. This method provides an additional strategy for the simultaneous insertion of multiple genes into M. robertsii.

Keywords Agrobacterium, Co-transformation, Metarhizium, Nourseothricin

Fungi of the Metarhizium genus are a versatile study model based on their biotechnological applications, which have been implemented for over 100 years [1]. Aside from insects, these fungi have been demonstrated to be safely compatible with other organisms (plants, animals, and humans) due to their entomopathogenic status. This specificity has allowed organisms from the Metarhizium genus to be approved by different environmental agencies, including the United States Environmental Protection Agency [2] and the Canada Pest Management Regulatory Agency [3], for marketing and extensive field use.

Metarhizium fungi are the most widely studied entomopathogens at both the molecular and biochemical level [1]. These organisms are globally distributed, and they colonize a wide range of environments, including forests, savannas, marshes, coastal areas and deserts [1]. M. robertsii has become an excellent model organism for exploring several aspects of ecology and evolution, such as host specificity, changes, and specialization mechanisms [1, 4, 5]. In addition, Metarhizium-focused research is currently at the forefront of efforts to develop alternatives to chemical insecticides, such as biological control agents, for use in commercial agricultural products and disease vector control programs, and many of these agents are now on the market or in development [1, 6, 7].

Recently, the ecological impact of members of the Metarhizium genus and their potential as biological control agents has been reinforced by reports that Metarhizium fungi perform more environmental functions than previously believed. For example, Metarhizium fungi can interact with plants by colonizing their roots in a mycorrhizal and endophytic manner, establishing a symbiotic relationship with the plants, which stimulates their root growth [8], activates their defense system and increases their resistance to abiotic factors, such as salinity [9]. Metarhizium fungi also transfer nutrients, such as nitrogen, that are obtained from insect fungal parasites from the soil to the plant roots [10]. In turn, plants transfer carbon compounds to the fungi [11], indicating that Metarhizium fungi and plants have a mutualistic symbiotic relationship [11].

The numerous, diverse interactions between Metarhizium fungi and the environment has supported genomic sequencing of the species M. robertsii, M. acridum, M. rileyi,
**MATERIALS AND METHODS**

**Fungal and bacterial strains.** The *M. robertsi* strain ARSEF 2575 was obtained from the United States Department of Agriculture Collection of Entomopathogenic Fungal Cultures (Ithaca, NY, USA). Cultures were grown on potato dextrose agar at 27°C for 10 days for conidia harvest. *Escherichia coli* XL-Blue competent cells were used for vector construction using standard molecular techniques [23]. *A. tumefaciens* AGL1 was used as the carrier for the individually constructed vectors for *M. robertsi* by simultaneously inserting multiple genes in separate vectors in a single event.

**Binary vector construction.** The pPK2-BAR vector was constructed by inserting a PtrpC-BAR *Xbal*/EcoRI fragment from pBARKS1-Spel previously cut and self-ligating at the SpeI sites from pBARKS1 [25] into the *Xbal*/EcoRI sites in binary pPK2 vector [26]. The pPK2-BAR-mCherry vector was constructed by amplifying the TrrpC fragment with the primers TrrpC-*Xbal*-Up (5'-TCTCTAGAGGATCCGAGGATCCGAGAGTTAATAAACA-3') and TrrpC-Spel-BamHI-Down (5'-GAGGATCCAGGCAGACTAGTCTAAGGTACTTACTAATC-3') from the pBARGEM7-2 vector [25] and then inserting the amplified fragment into the SpeI/BamHI sites in pPK2-BAR. The PgpD-A-mCherry fragment was subsequently released from the pGG464 vector [27] and cloned into the EcoRI/BamHI sites. The pPK2-NTC vector was constructed by amplifying the T-DNA vector with the primers PNT-NAT-*Xbal* (5'-TCTCTAGAGCGAGGAGCGGGA-3') and 5NAT-BglII (5'-ATAGATCTTCTAGCCGCGCGCGAT-3') using the 12N-NTC-OSCAR vector as a template [28] and then inserting the amplified fragment into the pPK2-BAR vector using the *Xbal*/BglII sites. The pPK2-NAT-eGFP vector was constructed by amplifying the pPK2 vector with the primers 5GST-EF1α (5'-ATGAAGGATTTGAGGCTGCGGCTGCGGCTGGT-3') and 3GST-EF1α (5'-ATAGATCTTCTAGCCGCGCGCGAT-3') from the pBENGFL vector [24] and then inserting the amplified fragments into the EcoRI/BglII sites in the pPK2-NTC plasmid. The T-DNA vector sequences of pPK2-BAR, pPK2-NTC, pPK2-NTC-eGFP, and pPK2-BAR-mCherry were deposited into the NCBI GenBank database under the accession numbers MF169981, MF169982, MF169983, and MF169984, respectively.

**Fungal transformation.** The four vectors generated were introduced individually into *A. tumefaciens* strain AGL1 cells. For the *Metarhizium* agro-transformation, we followed the protocol previously described by Fang and collaborators [24] with some modifications. Briefly, 1 × 10⁷ conidia suspended in 0.01% Triton X-100 were placed in a 1.5-mL Eppendorf tube and centrifuged for 5 min at 4,000 × g. The Triton X-100 solution was removed to preserve the conidia buttons, and the buttons were then mixed with 1 mL of *A. tumefaciens* cells, induced (OD₆₆₀ = 0.7–0.8) and vortexed for 30 sec. Next, 200 µL of the mixture was spread on black filter paper to continue the protocol described by Fang et al. [24]. Nourseothricin (Gold Biotechnology, St. Louis, MO, USA) and phosphinothricin (herbicide Finale; Bayer Crop Science AG, Leverkusen, Germany) were used at concentrations of 350 µg/mL and 250 µg/mL, respectively, for individual selection on M-100 agar medium [24].

For co-transformation of *M. robertsi* mediated by *A. tumefaciens*, separate *A. tumefaciens* cultures containing the pPK2-NTC-eGFP and pPK2-BAR-mCherry vectors were induced with acetosyringone as described [24]. After the cells were induced (OD₆₆₀ = 0.7–0.8), they were mixed equimolarly in a 1:1 ratio with *M. robertsi* conidia to continue the protocol described above. For double selection of the transformants, 350 µg/mL nourseothricin and 250 µg/mL phosphinothricin were simultaneously added on M-100 agar medium.

**Transformant verification.** The obtained transformants were verified by three passes on their respective plates containing the antibiotics. DNA extraction was then performed by grinding frozen mycelia and adding phenol-chloroform [23]. Next, 100 ng of the extracted DNA was used as a template to verify the transformants using PCR to detect the presence of the corresponding resistance and fluorescence cassettes. The nourseothricin-resistance cassette was amplified using the oligonucleotides PNAT-*Xbal* and SNAT-BglII, the eGFP cassette was amplified using the oligonucleotides 5CS-EGFP and 3CS-EGFP, the phosphinothricin-resistance cassette was amplified using the oligonucleotides PttrpC-*Xbal* (5'-ATCTTGAATTCTAGGGACCCGCTGATAG-3') and Bar-down (5'-TCAGATCTTAGGGGCTTCTGACG-3') and the mCherry cassette was amplified using the oligonucleotides Ppgd-ds (5'-GGGAGGAGACCCGAGGAC-3') and cherry-down (5'-TTACTTTTGTACAGCAGCTGCCCAGC-3'). Protein expression...
of the fluorescent reporter protein was confirmed by visual inspection using a Nikon OPTIPHOT-2 microscope (Nikon, Tokyo, Japan) equipped with epifluorescent illumination.

**RESULTS AND DISCUSSION**

Co-transformation is defined as the simultaneous introduction of multiple genes in a cell, and the genes can be present on the same plasmid or on separate plasmids [29]. The main advantage of co-transformation for the transfer of multiple genes is that a single transformation event can result in the integration of multiple transgenes as opposed to sequential transformation, which requires multiple time-consuming transformation events [29]. Co-transformation can be adapted to a variety of transformation methods, such as electroporation, biolistic, protoplasts and *Agrobacterium*-mediated transformation [29-31]. Several different strategies have been employed in *Agrobacterium*-mediated co-transformation for the independent delivery of T-DNA in plants [31], but *Agrobacterium*-mediated co-transformation in fungi has not yet been reported. T-DNA can be held in mixed bacterial strains in which two T-DNA molecules are contained in separate binary vectors in different *Agrobacterium* cells or in a single bacterial strain, whereby

![Diagram of T-DNA vector construction](image)

**Fig. 1.** T-DNA vector construction. A, T-DNA in the pPK2-NTC vector for nourseothricin selection; B, T-DNA in the pPK2-BAR vector for phosphinothricin selection; C, T-DNA in the pPK2-NTC-eGFP vector for nourseothricin resistance using green fluorescence for visual selection; D, T-DNA in the pPK2-BAR-mCherry vector with phosphinothricin resistance using cherry fluorescence for visual selection. PpgpA, promoter of glyceraldehyde-3-phosphate dehydrogenase gene from *Aspergillus nidulans*; NTC, nourseothricin resistance *nat1* gene from *Filobasidiella neoformans*; Ptef, promoter of translation elongation factor gene from *Aureobasidium pullulans*; eGFP, enhanced green fluorescent protein gene; T, termination region of the glucoamylase gene from *Aspergillus awamori*; PtpC, promoter of tryptophan C gene from *Aspergillus nidulans*; BAR, phosphinothricin resistance gene from *Streptomyces hygroscopicus*; TtrpC, terminator of tryptophan C gene from *Aspergillus nidulans*; mCherry, cherry fluorescent protein gene; RB and LB are the right and left border of T-DNA, respectively.

![Graph of transformation and co-transformation efficiency](image)

**Fig. 2.** The transformation and co-transformation efficiency in *Metarhizium robertsii* using the vectors generated in this work. Each transformation was performed three times, and the transformants were counted. The bars represent the standard error.
two T-DNA molecules could either be held in distinct regions on the same binary plasmid or on separate binary plasmids [31].

In this study, we used a mixed approach involving Agrobacterium containing two different T-DNA molecules on separate binary vectors to evaluate co-transformation mediated by Agrobacterium in M. robertsii. To identify a selection marker with the same efficiency as phosphinothricin for use in co-transformation, we evaluated the efficiency of the antibiotic nourseothricin, which has been used to transform M. robertsii, but its efficiency in this fungus has not been analyzed [21]. This antibiotic inhibits protein synthesis by inducing miscoding [32], and resistance is conferred by the nourseothricin acetyltransferase 1 (nat1) gene from Streptomyces noursei [32]. We determined that the concentration of nourseothricin required to inhibit M. robertsii growth on agar medium M-100 at 28°C is 350 µg/mL. The plates were incubated for 20 days without observing spontaneous mutant resistance. Once the efficiency of nourseothricin in M. robertsii on M-100 agar medium was established, the binary vectors pPK2-NTC (Fig. 1A), pPK2-NTC-eGFP (Fig. 1B), pPK2-BAR (Fig. 1C), and pPK2-BAR-mCherry (Fig. 1D) were constructed as described in the materials and methods section.

There was no difference in the number of transformants obtained using either nourseothricin or phosphinothricin as the selection agent in the Agrobacterium-mediated transformation experiments (Fig. 2), demonstrating that nourseothricin as well as phosphinothricin can be used as a dominant selection marker in Metarhizium.

These results lead us to perform an Agrobacterium-mediated co-transformation using both nourseothricin and phosphinothricin simultaneously to avoid pleiotropic effects. We co-transformed Metarhizium using the vectors pPK2-NTC-eGFP and pPK2-BAR-mCherry, and the number of resulting transformants was similar to that resulting from the individual transformation experiments in which the individual vectors were used. The high transformation efficiency using Agrobacterium-mediated co-transformation
in *M. robertsii* suggests a synergistic effect. This transformation efficiency is consistent with co-transformation in plants, as a larger number of genes involved in the co-transformation reportedly increases the efficiency of the transformants obtained when *Agrobacterium* is used [31]. Transgene integration was confirmed by PCR (Fig. 3) and fluorescence microscopy (Fig. 4).

In this study, for the first time in fungi, we reported the use of *Agrobacterium*-mediated co-transformation to integrate multiple genes into the *M. robertsii* genome using mixed *Agrobacterium* cells containing two different T-DNA molecules on separate binary vectors, thus improving the genetic strategies for studying this fungus. The advantages of this co-transformation method can facilitate the study of *Metarhizium* by reducing valuable time spent on constructing complex or long T-DNA molecules in binary vectors and sequential transformations.

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