Agrobacterium Tumefaciens-mediated T-DNA Insertional Mutagenesis of Fusarium Oxysporum

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Methodology article

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Abstract

Background

*Fusarium* species are important pathogenic organisms, which can cause many diseases in plants and humans. Characterizing the mechanism underlying their pathogenicity and drug resistance is critical. *Agrobacterium tumefaciens*-mediated genetic transformation has been widely used for the molecular analysis of many species.

Results

In this study, we constructed the pXEN recombinant plasmid carrying the neomycin phosphatase II gene (*neo*) and established a simple and efficient procedure for the transformation of resistant *Fusarium oxysporum* mediated by *A. tumefaciens*. The transformation efficiency was as high as 250 mutants per 10^5 conidia. A total of 1,450 stably transformed mutants were generated, resulting in a small-scale library of *F. oxysporum* mutants containing T-DNA tags. Some of the mutants exhibited phenotypic changes in growth, metabolism, and development. Additionally, the sequences flanking the inserted T-DNA were obtained by touchdown-TAIL PCR, the insertion sites and genes associated with the phenotypic changes could be determined.

Conclusions

The developed method may enable to analyze gene functions and study biological characteristics, which will lay the foundation for future analyses of the mechanism underlying *F. oxysporum* pathogenicity and resistance. Furthermore, it may be applicable to investigations of other important pathogenic fungi.

Background

*Fusarium* species are widely distributed diverse fungal organisms. They have a broad host range and can infect humans, animals, and plants [1]. *Fusarium* spp. is the most common pathogens causing fungal keratitis, with a very high blindness rate [2, 3]. Because antibiotic and hormone use as well as organ transplantations are relatively common, the number of patients with AIDS, malignant tumors, diabetes, or hematological diseases has recently been increasing. Consequently, there has been a yearly increase in the number of invasive and disseminated *Fusarium* infections, which are second only to *Aspergillus* infections as the most common infections caused by filamentous fungi [2, 4]. *Fusarium* spp. is also naturally resistant to multiple drugs, including almost all antifungal drugs, such as azoles, echinococcins, and polyene drugs [5]. Thus, effectively treating *Fusarium* infections is extremely difficult, leading to a very high fatality rate [2, 6].

In terms of their effects on agriculture, *Fusarium* spp. can infect important crops and are difficult to control, with the resulting extensive damages potentially leading to annual economic losses totaling billions of dollars [7]. They also produce many highly toxic mycotoxins, including deoxynivalenol, monoterpane toxin, and fumonisin [7, 8]. These toxins can kill plants and accumulate in cereal grains. The consumption of food and feed contaminated with these mycotoxins can cause liver and kidney failure and increase the risk of cancer in humans and livestock [9].

Characterizing the mechanism underlying *Fusarium* spp. pathogenicity and drug resistance is critical. *Agrobacterium tumefaciens*-mediated transformation (ATMT), which is applicable to a wide range of species, is an inexpensive and highly efficient method for the genetically stable random insertion of a DNA segment into the genome, with a high single-copy rate, making it ideal for the functional analysis of genes [10]. T-DNA insertion mutant libraries have been constructed and applied for the functional characterization of genes from various fungi, including *Aspergillus fumigatus* [11], *Sporothrix schenckii* [12], *Magnaporthe oryzae* [13], *Cryptococcus neoformans* [14], and *Aspergillus terreus* [15]. Analyses of these mutant libraries have uncovered mutants related to pathogenicity, drug resistance, and morphology, and the related gene functions have been explored. Therefore, ATMT may be useful for the high-throughput screening of mutants with specific phenotypes as well as for elucidating the functions of the associated genes.

During the development of an ATMT system, screening for resistance is an important process. In this study, hygromycin B, phleomycin, bleomycin, glyphosate, neomycin, and geneticin (G418) were evaluated, with G418 ultimately selected. A new plasmid, pXEN, was constructed and an ATMT system was established and optimized. A *Fusarium oxysporum* T-DNA insertion mutant library based on G418 resistance was preliminarily constructed. The *F. oxysporum* T-DNA insertion mutants were analyzed by PCR and touchdown-TAIL PCR [16] and the T-DNA insertion sites were determined. Moreover, the transformation efficiency was substantially improved by using the plasmid pXEN. By analyzing the phenotypic changes in mutants, the associated genes were identified. The study data provide the basis for future investigations of the mechanisms underlying *F. oxysporum* pathogenesis and drug resistance and may be useful for detecting new drug targets.

Results

**Minimum inhibitory concentration of G418 against *F. oxysporum***

*F. oxysporum* growth was completely inhibited on PDA medium containing G418 (50 μg/ml). Therefore, this concentration was used as the minimum inhibitory concentration for screening the mutants.

**Optimization of the transformation conditions**

Increases in the co-culture time resulted in gradual increases in the transformation efficiency (Fig. 1a). However, overlapping colonies were observed at 60 h, making it difficult to isolate single mutants. Additionally, at each analyzed co-culture time-point, the transformation efficiency was highest at 25 °C (Fig. 1a).
Therefore, a 48-h culture at 25 °C was considered optimal for the transformation of *F. oxysporum*.

The transformation efficiency was highest when the OD_{600} of the *A. tumefaciens* culture was 0.8 (Fig. 1b). Increases in the *F. oxysporum* conidia concentration gradually increased the transformation efficiency (Fig. 1b). However, excessively high transformation efficiencies were not conducive to the subsequent selection of individual mutants. Therefore, an *A. tumefaciens* culture with an OD_{600} of 0.8 and an *F. oxysporum* conidia concentration of 1 × 10^{4} CFU/ml were used for co-culturing. The resulting transformation efficiency was 250 mutants per 10^{4} conidia.

## Screening mutants with phenotypic changes

The mutants were cultured and compared with the wild-type strain to detect changes to colony morphology (Fig. 2). The Mut1, Mut2, Mut3 and Mut4 mutants grew more slowly than the wild-type, whereas Mut5 exhibited increased hyphal growth. Additionally, Mut4 produced a dark-blue pigment, Mut3 and Mut5 produced less pigment. These changes may be related to the pathogenicity of the mutants. Accordingly, the mutants obtained in this study may be valuable materials for future research on the pathogenicity of *Fusarium* spp.

### Analysis of T-DNA insertion sites

To confirm the T-DNA fragment was inserted in the genomes of the putative mutants, we randomly selected 15 mutants for a PCR amplification of the neomycin phosphatase II gene (*neo*) fragment. The amplified 700-bp fragment was 99% similar to the *neo* fragment. After five rounds of inoculations of PDA lacking G418, the mutants were still able to grow on PDA medium containing G418 (50 µg/ml) and tested positive for the *neo* fragment in one PCR amplification (Fig. 3a). These observations implied the T-DNA was stably inserted into the *F. oxysporum* genome.

The T-DNA flanking sequences were amplified by touchdown-TAIL PCR (Fig. 3b). The T-DNA insertion site was located via an alignment with the pXEN sequence and the *F. oxysporum* genome sequence (Table 1). The mutated genes and the resulting phenotypic changes may be useful for the functional characterization of *F. oxysporum* genes.

### Table 1

| *F. oxysporum* | ... | LB | T- DNA | RB | ... | *F. oxysporum*
|---------------|-----|----|--------|----|-----|---------------|
| Mut1          | cgagtctgtgtgaagcgct | ... | TGGCAGGATATATTGTGGTGAACCA | -- | -- | GAAAACCTAAGAAAGACGTTTA | ... | acgtctattatttcagctt |
| Mut2          | gcagaactctaaatggtt | ... | TGGCAGGATATATTGTGGTGAACCA | -- | -- | GAAAACCTAAGAAAGACGTTTA | ... | tggacttggagtggaggg |
| Mut3          | atgtagagacccctgttgc | ... | TGGCAGGATATATTGTGGTGAACCA | -- | -- | GAAAACCTAAGAAAGACGTTTA | ... | tgaagactttcgagttccg |
| Mut4          | gcattgtagctgtgactg | ... | TGGCAGGATATATTGTGGTGAACCA | -- | -- | GAAAACCTAAGAAAGACGTTTA | ... | gcagagagttcagcctt |
| Mut5          | tgtgtgagactaaagccccgggcaagtcctgtgtgaagcgct | ... | TGGCAGGATATATTGTGGTGAACCA | -- | -- | GAAAACCTAAGAAAGACGTTTA | ... | tggacttggctttggaggtt |

Note: Mut1–Mut5 represent five selected mutants; lowercase letters represent the sequences flanking the T-DNA in mutants; uppercase letters represent the left arm (LB) and right arm (RB) sequences at the T-DNA boundaries; hyphens represent the sequence between the LB and RB, ellipses represent the pXEN sequences.

### Discussion

The efficient and simple detection of mutants is a critical part of fungal transformation systems. The wild-type *F. oxysporum* analyzed in the current study is resistant to multiple drugs and chemicals used for screening mutants. Regarding the commonly used G418 [17], we determined that G418 (50 µg/ml) can completely inhibit *F. oxysporum* growth. However, there is currently no plasmid carrying a G418-resistance gene that can be used to transform *F. oxysporum*. In this study, we constructed the pXEN recombinant plasmid containing the G418-resistance *neo* gene. This plasmid can be used for the ATMT of *F. oxysporum*. Because it has two multiple cloning sites between the LB and RB and resistance genes, it is also useful for gene knockout experiments. Furthermore, it may be applicable for functional analyses of genes in other strains sensitive to geneticin.

The transformation efficiency of ATMT is restricted by many factors. The optimal ATMT conditions for *F. oxysporum* were determined based on analyses of the co-culture time and temperature as well as the ratio of *A. tumefaciens* to *F. oxysporum*. We determined that an *A. tumefaciens* culture with an OD_{600} of 0.8, an *F. oxysporum* conidia concentration of 1 × 10^{4} CFU/ml, and a 48-h co-culture at 25 °C are the optimal conditions for transforming *F. oxysporum*. The resulting transformation efficiency was 250 mutants per 10^{4} conidia, which was approximately 30-times higher than that reported for other transformation systems [18]. The higher transformation efficiency of the current study may be due to the use of G418 resistance to detect mutants. To the best of our knowledge, this is the first study to identify the mutants based on G418 resistance. Thus, our method may be a better option for the ATMT of *F. oxysporum* than previously described procedures. Additionally, changing the method used to screen mutants may enhance the transformation of some strains with low transformation efficiencies or special resistance, which may enable the study of gene functions in these strains.

### Conclusions

The T-DNA anking sequences were amplified by touchdown-TAIL PCR (Fig. 3b). The T-DNA insertion site was located via an alignment with the pXEN sequence and the *F. oxysporum* genome sequence (Table 1). The mutated genes and the resulting phenotypic changes may be useful for the functional characterization of *F. oxysporum* genes.
In this study, we constructed the pXEN recombinant plasmid carrying the neomycin phosphotransferase II gene (neo) and established a simple and efficient procedure for the transformation of resistant \( F. \) oxysporum mediated by \( A. \) tumefaciens. Some of the mutants exhibited phenotypic changes in growth, metabolism, and development. By analyzing of the flanking sequence, the insertion sites and genes associated with the phenotypic changes could be determined. The data presented herein reveal that the ATMT system developed in this study can efficiently insert T-DNA into the \( F. \) oxysporum genome. Because the insertion sites can be determined, our system may enable researchers to analyze gene functions and study biological characteristics. Furthermore, our method may be applicable to investigations of other important pathogenic fungi.

Methods

Strains and plasmids

Wild-type \( F. \) oxysporum JLCC31768, which was isolated from patients with clinical infections in Jilin province, China, was used as the parent strain for transformations. Agrobacterium tumefaciens AGL-1 carrying the pBHT1 plasmid, \( A. \) tumefaciens Agr0, as well as the pEGFP-N3 and pXEH plasmids was used in this study. All strains are preserved in the Fungi Research Center of Jilin University.

Construction of the G418 resistance plasmid

The \( trpC \) promoter region in pBHT1 was amplified with the FtrpC-f and FtrpC-r primers. The \( neo \) fragment in pEGFP-N3 was amplified with the FNeo-f and FNeo-r primers. The FtrpC-f and FNeo-r primers were then used to generate the complete \( trpC \) promoter–\( neo \) expression cassette in a staggered extension process. The \( trpC \) promoter–\( neo \) sequence was amplified with the trpcNf and trpcNr primers, after which the amplicon was digested and linked to pXEH. The resulting pXEN recombinant plasmid was sequenced (Table 2) and then inserted into \( A. \) tumefaciens Agr0 cells to produce the AgrN strain.

ATMT of \( F. \) oxysporum

To determine the minimum inhibitory concentration of G418 against wild-type \( F. \) oxysporum, 100-µl conidia suspensions \((1 \times 10^4 \text{ CFU/ml})\) were treated with various G418 concentrations \((0, 50, 100, 150, 200, 250, \text{ and } 300 \text{ mg/ml})\) on PDA medium at 25 °C for 7 days. The minimum inhibitory concentration was determined based on whether the conidia germinated.

\( F. \) oxysporum was transformed according to an optimized version of a previously described method [10, 12]. \( A. \) tumefaciens AgrN cells carrying the pXEN plasmid were activated, collected, and resuspended in liquid induction medium containing 200 µM acetosyringone [10]. The optical density at 600 nm \((\text{OD}_{600})\) of the medium was adjusted to 0.2–0.3. A pre-culture step was completed at 28 °C and 160 rpm until the \( \text{OD}_{600} \) reached 0.4, 0.6, 0.8, and 1.0. Additionally, PDA medium was inoculated with wild-type \( F. \) oxysporum and incubated at 25 °C for 5 days. Conidia were suspended in a saline solution for final concentrations of \( 10^3, 10^4, \) and \( 10^5 \text{ CFU/ml} \). An aseptic filter membrane was placed on the solid induction medium containing 200 µM acetosyringone. Equal volumes \((1 \text{ ml})\) of the \( A. \) tumefaciens cells and \( F. \) oxysporum conidia were mixed, after which 200 µl was applied uniformly to the filter. After incubating at various temperatures \((22, 25, \text{ and } 28 \text{ °C})\) for different times \((24, 36, 48, \text{ and } 60 \text{ h})\), the filter membrane was transferred to the screening medium (PDA medium containing 200 µg/ml cefotaxime sodium and 100 µg/ml G418) to screen for mutants, while simultaneously inhibiting \( A. \) tumefaciens growth. The screening medium was incubated at 25 °C for 3–4 days until mutant colonies appeared.

Screening of phenotypic mutants

The center of Petri plates containing PDA medium was inoculated with the mutants. After incubating at 25 °C for 7 days, the colony morphology was analyzed.

Analysis of the genetic stability of the T-DNA insertion

The stability of the T-DNA insertion was assessed based on the G418 resistance of the mutants. Specifically, 15 randomly selected mutants were examined for the presence of the \( neo \) fragment via the amplification with the neoF and neoR primers (Table 2). The mutants were then cultured on PDA lacking G418 for 3 days. An inoculation needle was used to transfer mycelia from the colony edge to fresh PDA lacking G418 for another 3-day culture. After five rounds of inoculation, the mycelia were transferred to screening medium and the presence of the \( neo \) fragment was confirmed by PCR.

Analysis of the T-DNA flanking sequences

The putative mutants were added to PDB medium containing G418 (50 µg/ml) and cefotaxime sodium (200 µM) and then incubated at 25 °C with shaking \((120 \text{ rpm})\) for 72 h. Genomic DNA was extracted from the cultured mutants [19], and used as the template for the amplification of the \( neo \) fragment to determine whether the T-DNA was inserted into the genome of these putative mutants.

The sequences flanking the inserted T-DNA in the mutant genomes were amplified by touchdown-TAIL PCR [16] (Table 2). The left and right arms of the T-DNA were amplified by two rounds of semi-nested PCR, sequenced, and aligned with the pXEN plasmid. The flanking sequence was compared with the \( F. \) oxysporum f. sp. lycopersici 4287 genome (GCF_000149955.1) to determine the insertion site.
Table 2
Details regarding the primers used in this study.

| Primer name | Nucleotide sequence (5’ to 3’) |
|-------------|--------------------------------|
| FtrpC-f     | 5’-CAGAAGATGATATTGAAGGAGCATTTTT-3’ |
| FtrpC-r     | 5’-CTTGTTCAATCATGATGATGCTGTTGAGAATA-3’ |
| FNeor-f     | 5’-CATCGATATGATGATGATGAAAGATGAGATTGACGAC-3’ |
| FNeor-r     | 5’-TTTATTTCTGTCTTTTTATTGGCCGTACA-3’ |
| tRPCNf      | 5’-CGGGGTACCAGAAGATGATATTGAGGAGC-3’<br>Kpn1 restriction site |
| tRPCNr      | 5’-CGCGATATCGATATGATTGAACAAGATGGATTGCACGCA-3’<br>Bam H1 restriction site |
| neoF        | 5’-GTCTCCCTCGGTCTTCTCCGTAGGCGAC-3’ |
| neoR        | 5’-CTGCAGGCTGTTGTTTATTTGCGTACA-3’ |
| LB1         | 5’-GGGTTCCTATAGGGTTTCGCTCATG-3’ |
| LB2         | 5’-CATGTGTTGAGGACATATAAGAAACCCT-3’ |
| LB3         | 5’-GAATTAATTGGCTGTTAATCCAGT-3’ |
| RB1         | 5’-GGCAGTGGCGTTGTTTACAAAC-3’ |
| RB2         | 5’-AACGTCGTAGCTGGGAAACCT-3’ |
| RB3         | 5’-CCCCAAACAGATTGCGGAC-3’ |
| AD1         | 5’-TGGAGNAGTACAAGA-3’ |
| AD2         | 5’-AGTNAGAANCAAGG-3’ |
| AD3         | 5’-CATCGNCNGAAGCAGA-3’ |
| AD4         | 5’-CAAGCAGAAGCA-3’ |

Abbreviations

*F. oxysporum*: Fusarium oxysporum; *A. tumefaciens*: Agrobacterium tumefaciens; neo: the neomycin phosphatase II gene; ATMT: Agrobacterium tumefaciens-mediated transformation; AIDS: Acquired Immune Deficiency Syndrome; G418: geneticin; PCR: Polymerase Chain Reaction

Declarations

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Abbreviations

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Authors’ contributions

LW received the research grants, and provided guidance to conduct the experiments. ZF and DH constructed the recombinant plasmid and established T-DNA insertion mutant library. SG, SH and YW screened of phenotypic mutants and analyzed the inserted gene. ZF wrote the original draft. DH participated in the design of the study and redrafted the manuscript. LW reviewed, and edited the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.
Consent for publication

Not applicable.

Availability of data and materials

All data generated during this study are included in the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures
Figure 1

Effects of cultural conditions on transformation efficiency. (a) Co-culture time and temperature. Increasing co-culture times gradually increased the transformation efficiency. At each analyzed time-point, the transformation efficiency was highest at 25 °C. (b) Conidia concentrations of A. tumefaciens and F. oxysporum. A–C: F. oxysporum conidia concentrations of 1 × 10³, 1 × 10⁴, and 1 × 10⁵ CFU/ml. Increases in the conidia concentration increased the transformation efficiency. For each conidia concentration, the transformation efficiency was highest when the OD600 of the A. tumefaciens culture was 0.8.

Figure 2

Colony morphology of F. oxysporum wild-type and T-DNA insertion mutants. WT: wild-type; Mut1–Mut5: T-DNA insertion mutants (cultured on PDA medium at 25 °C for 6 days).

Figure 3

Electrophoretic analysis of mutants. (a) PCR-amplified the neo fragment. M: DL2000 DNA marker; 1–15: F. oxysporum mutant strains, with the neo fragment amplified; 16: wild-type, with no neo fragment amplified. (b) Touchdown-TAIL PCR products. M: DL2000 DNA marker; 1–5: the left arm amplified sequence of the mutant; 6–10: the right arm amplified sequence of the mutant.