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

Zeqing Feng

Jilin University

Dan He

Jilin University

Song Gao

Jilin University

Shuaishuai Han

Jilin University

Yunyun Wei

Jilin University

Li Wang (✉ wli99@jlu.edu.cn)

Jilin University <https://orcid.org/0000-0002-7386-3232>

Methodology article

Keywords: Fusarium oxysporum, Agrobacterium tumefaciens, T-DNA, insertional mutation, neomycin phosphatase II gene

Posted Date: October 23rd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-94347/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License. [Read Full License](#)

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 10^4 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, monoterpene 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 OD₆₀₀ 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 OD₆₀₀ of 0.8 and an *F. oxysporum* conidia concentration of 1 × 10⁴ CFU/ml were used for co-culturing. The resulting transformation efficiency was 250 mutants per 10⁴ 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
Analysis of sequences flanking the inserted T-DNA in *F. oxysporum* mutants.

<i>F. oxysporum</i>	...	LB	T-DNA	RB	...	<i>F. oxysporum</i>
Mut1	cgagtctgtgtgaagcgct	...	TGGCAGGATATATTGTGGTGTAACAA	— —	GTAACCTAAGAGAAAGAGCGTTTA	... acgtcttattatttcagctt
Mut2	gcaagactcatctaaatgtt	...	TGGCAGGATATATTGTGGTGTAACAA	— —	GTAACCTAAGAGAAAGAGCGTTTA	... ttggaacttgagggtggagg
Mut3	atgtagaagaccctcggttc	...	TGGCAGGATATATTGTGGTGTAACAA	— —	GTAACCTAAGAGAAAGAGCGTTTA	... tgagatcggttgagtgagg
Mut4	gcatttgatgcgatgcgatg	...	TGGCAGGATATATTGTGGTGTAACAA	— —	GTAACCTAAGAGAAAGAGCGTTTA	... gacgagaggtttcagacgct
Mut5	ttgtttgagactaaaaggcc	...	TGGCAGGATATATTGTGGTGTAACAA	— —	GTAACCTAAGAGAAAGAGCGTTTA	... tggacttggtttgggagtg
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 OD₆₀₀ of 0.8, an *F. oxysporum* conidia concentration of 1 × 10⁴ 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 10⁴ 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

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 *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 FNeor-f and FNeor-r primers. The FtrpC-f and FNeor-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- μ l conidia suspensions (1×10^4 CFU/ml) were treated with various G418 concentrations (0, 50, 100, 150, 200, 250, and 300 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 μ M acetosyringone [10]. The optical density at 600 nm (OD₆₀₀) of the medium was adjusted to 0.2–0.3. A pre-culture step was completed at 28 °C and 160 rpm until the OD₆₀₀ 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 CFU/ml. An aseptic filter membrane was placed on the solid induction medium containing 200 μ M acetosyringone. Equal volumes (1 ml) of the *A. tumefaciens* cells and *F. oxysporum* conidia were mixed, after which 200 μ l was applied uniformly to the filter. After incubating at various temperatures (22, 25, and 28 °C) for different times (24, 36, 48, and 60 h), the filter membrane was transferred to the screening medium (PDA medium containing 200 μ g/ml cefotaxime sodium and 100 μ g/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 μ g/ml) and cefotaxime sodium (200 μ M) and then incubated at 25 °C with shaking (120 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'-CAGAAGATGATATTGAAGGAGCATTTT-3'
FtrpC-r	5'-CTTGTTCAATCATATCGATATCGATGCTTCGGTAGAATA-3'
FNeor-f	5'-CATCGATATCGATATGATTGAACAAGATGGATTGCACGCA-3'
FNeor-r	5'-TTTATTCTGTCTTTTATTGCCGTCATA-3'
tRPCNf	5'-CGGGGTACCCAGAAGATGATATTGAAGGAGC-3' Kpn1 restriction site
tRPCNr	5'-CGCGGATCCTTTATTCTGTCTTTTATTGCC-3' Bam H1 restriction site
neoF	5'-ATCTCCTGTCATCTCACCTTGCTC-3'
neoR	5'-GTCTCCTTCCGTGTTTCAGTTAGC-3'
LB1	5'-GGGTTCTATAGGGTTTCGCTCATG-3'
LB2	5'-CATGTGTTGAGCATATAAGAAACCCT-3'
LB3	5'-GAATTAATTCGGCGTTAATTCAGT-3'
RB1	5'-GGCACTGGCCGTCGTTTACAAC-3'
RB2	5'-AACGTCGTGACTGGGAAAACCCT-3'
RB3	5'-CCCTTCCCAACAGTTGCGCA-3'
AD1	5'-TGAGNAGTANCAGAGA-3'
AD2	5'-AGTGNAGAANCAAAGG-3'
AD3	5'-CATCGNCNGANACGAA-3'
AD4	5'-CAAGCAAGCA-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

Acknowledgments

We thank the staff of the Jilin University Mycology Research Center. We also thank Liwen Bianji, Edanz Editing China for editing the English text of a draft of this manuscript.

Funding

This study was partially supported by grants from the National Natural Science Foundation of China (81772162 and U1704283).

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

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.

References

1. Al-Hatmi AMS, Hagen F, Menken SB, Meis JF, Sybren de Hoog G. Global molecular epidemiology and genetic diversity of *Fusarium*, a significant emerging group of human opportunists from 1958 to 2015. *Emerg Microbes Infect.* 2016;5(12):e124.
2. Al-Hatmi AMS, Bonifaz A, Ranque S, Sybren de Hoog G, Verweij PE, Meis JF. Current antifungal treatment of fusariosis. *Int J Antimicrob Agents.* 2018;51(3):326–32.
3. Oliveira Dos Santos C, Kolwijck E, van Rooij J, Stoutenbeek R, Visser N, Cheng YY, et al. Epidemiology and clinical management of *Fusarium* keratitis in the Netherlands, 2005–2016. *Front Cell Infect Microbiol.* 2020;10:133.
4. Lockhart SR, Guarner J. Emerging and reemerging fungal infections. *Semin Diagn Pathol.* 2019;36(3):177–81.
5. Al-Hatmi AM, Meis JF, Sybren de Hoog G. *Fusarium*: Molecular diversity and intrinsic drug resistance. *PLoS Pathog.* 2016; 12(4): e1005464.
6. Garcia RR, Min Z, Narasimhan S, Bhanot N. *Fusarium* brain abscess: Case report and literature review. *Mycoses.* 2015;58(1):22–6.
7. Haile JK, N'Diaye A, Walkowiak S, Nilsen KT, Clarke JM, Kutcher HR, et al. *Fusarium* head blight in durum wheat: Recent status, breeding directions, and future research prospects. *Phytopathology.* 2019;109(10):1664–75.
8. Munkvold GP. *Fusarium* species and their associated mycotoxins. *Methods Mol Biol.* 2017;1542:51–106.
9. Sewram V, Mshicileli N, Shephard GS, Vismer HF, Rheeder JP, Lee YW, et al. Production of fumonisin B and C analogues by several *Fusarium* species. *J Agric Food Chem.* 2005;53(12):4861–6.
10. Michiels CB, Hooykaas PJ, van den Hondel CA, Ram AF. *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. *Curr genet.* 2005;48(1):1–17.
11. Sugui JA, Chang YC, Kwon-Chung KJ. *Agrobacterium tumefaciens*-mediated transformation of *Aspergillus fumigatus*: an efficient tool for insertional mutagenesis and targeted gene disruption. *Appl Environ Microbiol.* 2005;71(4):1798–802.
12. Zhang Y, Li G, He D, Yu B, Yokoyama K, Wang L. Efficient insertional mutagenesis system for the dimorphic pathogenic fungus *Sporothrix schenckii*, using *Agrobacterium tumefaciens*. *J Microbiol Methods.* 2011;84(3):418–22.
13. Chen XL, Yang J, Peng YL. Large-scale insertional mutagenesis in *Magnaporthe oryzae* by *Agrobacterium tumefaciens*-mediated transformation. *Methods Mol Biol.* 2011;722:213–24.
14. Li Z, Pan J, Zhu X. *Agrobacterium tumefaciens*-mediated transformation (ATMT) for the screening for genes involved in laccase glucose repression in the pathogenic yeast *Cryptococcus neoformans*. *Wei Sheng Wu Xue Bao.* 2012;52(1):44–51.
15. Wang D, He D, Li G, Song Gao S, Lv H, Shan Q, et al. An efficient tool for random insertional mutagenesis: *Agrobacterium tumefaciens*-mediated transformation of the filamentous fungus *Aspergillus terreus*. *J Microbiol Methods.* 2014;98(1):114–8.
16. Gao S, He D, Li G, Zhang Y, Lv H, Wang L. A method for amplification of unknown flanking sequences based on touchdown PCR and suppression-PCR. *Anal Biochem.* 2016;509:79–81.
17. Chinswangwatanakul W, Lewis JL, Manning M, Roberts IA, Gordon MY. Use of G418 resistance to select cells retrovirally transduced with the Neo(R) gene-response. *Exp Hematol.* 1998;26(3):185–7.
18. Mullins ED, Chen X, Romaine P, Raina R, Geiser DM, Kang S. *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: An efficient tool for insertional mutagenesis and gene transfer. *Phytopathology.* 2001;91(2):173–80.
19. Wang L, Yokoyama K, Miyaji M, Nishimura K. The identification and phylogenetic relationship of pathogenic species of *Aspergillus* based on the mitochondrial cytochrome *b* gene. *Med Mycol.* 1998;36(3):153–64.

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 1×10^3 , 1×10^4 , and 1×10^5 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.