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RESEARCH ARTICLE

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Optimization of *Agrobacterium tumefaciens*-Mediated Transformation of *Xylaria grammica* EL000614, an Endolichenic Fungus Producing Grammicin

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ABSTRACT

An endolichenic fungus *Xylaria grammica* EL000614 produces grammicin, a potent nematicidal pyrone derivative that can serve as a new control option for root-knot nematodes. We optimized an *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocol for *X. grammica* to support genetic studies. Transformants were successfully generated after co-cultivation of homogenized young mycelia of *X. grammica* with *A. tumefaciens* strain AGL-1 carrying a binary vector that contains the bacterial hygromycin B phosphotransferase (*hph*) gene and the *eGFP* gene in T-DNA. The resulting transformants were mitotically stable, and PCR analysis showed the integratin of both genes in the genome of transformants. Expression of eGFP was confirmed via fluorescence microscopy. Southern analysis showed that 131 (78.9%) out of 166 transformants contained a single T-DNA insertion. Crucial factors for producing predominantly single T-DNA transformants include 48 h of co-cultivation, pretreatment of *A. tumefaciens* cells with acetosyringone before co-cultivation, and using freshly prepared mycelia. The established ATMT protocol offers an efficient tool for random insertional mutagenesis and gene transfer in studying the biology and ecology of *X. grammica*.

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ATMT; fungal transformation; gene manipulation; *Xylaria grammica*

1. Introduction

Xylaria, the largest genus of the family Xylariaceae, includes about 500 known species [1–3]. *Xylaria* spp. play an important ecological role and are prolific producers of potentially valuable natural compounds [4]. Identification of *Xylaria* spp. using morphological traits is difficult, and the production of specific metabolites has served as an identification key for *Xylaria*. Strains belonging to *X. grammica* produce a unique mixture of metabolites, one of which is grammicin, an isomer of mycotoxin patulin [5].

Grammicin can be easily obtained from *X. grammica* cultures and displays a potent nematicidal activity against root-knot nematodes [6]. Grammicin, a pyrone derivative, is synthesized from desoxypatulinic acid [7] and was initially identified while characterizing the structure of patulin. Patulin exhibits strong antibacterial activity but weak nematicidal activity [6,7]. In contrast, grammicin displayed weak or moderate antibacterial activity [6]. Efficient tools for genetic manipulation of *X. grammica* were needed to study its genes involved in synthesizing grammicin and regulating its production so that the grammicin biosynthetic capability of *X. grammica* can be effectively harnessed for pathogenic nematode control. Unfortunately, however, no efficient transformation method has been developed for *Xylaria* spp.

In this study, we aimed to establish an efficient genetic manipulation system for *X. grammica* using *Agrobacterium tumefaciens*-mediated transformation (ATMT). During the last two decades, ATMT has been successfully applied to introduce DNA to diverse fungi for genetic manipulation. Its advantages over other methods for DNA delivery, such as electroporation, protoplasting, and cell permeabilization with lithium acetate, are multiple [8–10]. One advantage is that ATMT can introduce DNA into diverse tissues/cells such as conidia, mycelium, gill tissues from mushrooms, and fruiting bodies without requiring the removal of their cell wall [9]. Other potential advantages of ATMT include an efficient performance with diverse fungi, high frequency of homologous recombination, and generation of transformants via mostly single copy DNA integration [11,12].

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ATMT has been used widely as an insertional mutagenesis tool due to high frequency mitotically stable, random, mostly single-copy integration of T-DNA into fungal genomes [13,14]. Genome sequences flanking inserted T-DNAs can be isolated via thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) [15] or inverse PCR [16]. The efficiency of isolating mutants via ATMT-mediated targeted gene replacement was enhanced using a dual selection scheme [17]. ATMT has been applied to manipulate diverse fungi, including *Umbilicaria muehlenbergii*, a lichen-forming fungus difficult to transform [18]. For optimal ATMT of *U. muehlenbergii*, the presence of acetosyringone (AS) when culturing *A. tumefaciens* cells before co-cultivation with fungal cells, the amount of hyphal mass used, and the duration of co-cultivation were important. Here, we report an optimized transformation protocol for *X. grammica* using a strain isolated from the thallus of the lichen *Usnea* sp.

2. Materials and methods

2.1. Fungal isolate and culture conditions

An endolichenic fungal isolate *X. grammica* EL000614, available at the Korean Collection for Type Cultures (KCTC) under accession number KCTC13121BP was used in this study. This stain and its transformants were cultured at 25 °C under constant dark on potato dextrose agar (PDA, Difco Laboratories) or in PD broth (PDB, Difco Laboratories).

2.2. Sensitivity of X. grammica to hygromycin B

Hygromycin B sensitivity of EL000614 was evaluated by placing drops of a homogenized hyphal suspension, prepared by macerating a 2 day-old culture in PDB, on PDA amended with different amounts of hygromycin B (0, 25, 50, 75, and 100 µg/mL). After incubating the PDA plates at 25 °C for 7 days, colony growth was checked to determine the optimal concentration of hygromycin B for selecting transformants. This evaluation was performed in three replicates.

2.3. Agrobacterium tumefaciens-mediated transformation of X. grammica

The protocol was mainly based on a previously described procedure [19] with some modifications. AGL-1 strain of *A. tumefaciens* carrying the binary vector pSK1044 [18] was cultured in 5 mL minimal medium (MM) [19] containing kanamycin (50 µg/mL) for 2 days at 28 °C with shaking (150 rpm). Bacterial cells were collected via centrifugation and suspended in 5 mL induction medium (IM) [20] in the presence or absence of 200 µM AS. They were then cultured for an additional 6 h at 28 °C with shaking to induce the virulence of *A. tumefaciens*.

The initial inoculum was prepared by homogenizing agar block of 3-days old culture on PDA in 10 mL sterile distilled water using a homogenizer (IKA Works Asia, Bhd, Malaysia). Subsequently, the homogenized mycelia were transferred into 100 mL PDB and incubated for another 2 days at 25 °C with shaking 150 rpm in the dark. The mycelia were washed twice using 50 mL of sterilized distilled water and then collected using Miracloth (Calbiochem, San Diego, CA, USA). Before the transformation, the mycelia were mixed with 5 mL sterilized distilled water and homogenized again.

For co-cultivation with *X. grammica* mycelial fragments and *A. tumefaciens* cells, 20, 40, and 60 mg of *X. grammica* mycelia were mixed with 100 µL virulence-induced *A. tumefaciens* cells (OD = 0.6) and spread onto sterilized cellulose membrane (cellulose nitrate, 47 mm diameter and 0.45 µm pore, Whatman Ltd, Maidstone, UK) overlaid on co-cultivation medium amended with and without 200 µM AS.

Following co-cultivation at 28 °C for 24, 36, and 48 h, the membranes were transferred onto PDA supplemented with 100 µg/mL hygromycin B to select *X. grammica* and 250 µg/mL cefotaxim to inhibit *A. tumefaciens* cells. After two weeks, hygromycin B-resistant colonies were transferred to 24-well plates (SPL, Suwon, Korea) containing PDA amended with 100 µg/mL hygromycin B and 250 µg/mL cefotaxime. For purification of transformants, their hyphal tips were transferred to PDA containing 100 µg/mL hygromycin B.

2.4. Genomic DNA extraction and PCR analysis to confirm the insertion of T-DNA

Fungal genomic DNA was extracted from mycelia cultured in 5 mL PDB at 25 °C for 7 days. Mycelia from wild-type and transformants were harvested, lyophilized, and ground using Mini-Beadbeater-24 (Biospec Products, Bartlesville, OK, USA) after freezing in liquid nitrogen. Genomic DNA was purified using NucleoSpin Plant kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction.

PCR reactions were carried out using genomic DNAs of 14 randomly selected transformants in an ABI 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). PCR amplification of the *hph* and *eGFP* genes was carried out using 20 ng of genomic DNA and 10 pmol of each primer (Table 1) using i-StarMAX II PCR master mix system (iNtRON Biotechnology Inc., Seongnam,

Table 1. Primers used in this study.

Target	Name	Sequence (5′–3′)
hph	HygB_F	TCAGCTTCGATGTAGGAGGG
	HygB_R	TTCTACACAGCCATCGGTCC
eGFP	eGFP_F	ATGGTGAGCAAGGGCG
	eGFP_R	TTACTTGTACAGCTCGTC
TAIL-PCR	RB1	GGCACTGGCCGTCGTTTTACAAC
	RB2-1	CTGGCGTAATAGCGAAGAGG
	RB3	CCCTTCCCAACAGTTGCGCA
	AD1	NGTCGASWGANAWGAA
	AD2	TGWGNAGSANCASAGA
	AD3	AGWGNAGWANCAWAGG
	AD4	WAGTGNAGWANCANGAA
	AD6	WGTGNAGWANCANAGA

Korea). The amplification conditions were as follows: (a) initial denaturation at 94 °C for 3 min; (b) 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 56 °C, and 1 min elongation at 72 °C, and (c) 10 min elongation at 72 °C. PCR products were resolved via electrophoresis using a 1% agarose gel, stained with Ecodye™ DNA staining solution (Solgent Co. Daejeon, Korea), and visualized under UV light.

2.5. Southern blot analysis

Southern blot analysis was carried out to determine the copy number of inserted T-DNA in the genome of *X. grammica*. 1 µg of genomic DNA from the wild-type strain and individual transformants was digested with *Hin*dIII and was resolved via gel electrophoresis using 0.8% agarose gel at 50 V for 4 h in 0.5% Tris-Acetate-EDTA buffer. Separated DNA fragments were transferred to Hybond N^+ membrane (Amersham International, Little Chalfont, England) using 10 × SSC (1.5 M NaCl and 0.15 M Sodium citrate) and UV crosslinked.

A 600 bp probe corresponding to the *hph* gene was obtained by PCR amplification using pBHt2 [21] as a template and primers HygB_F and HygB_R (Table 1), and the resulting fragment was labeled using ^{32}P-dCTP via random priming (Rediprime II DNA labeling system, GE Healthcare Life Sciences). Hybridization was carried out overnight at 65 °C in 6 × SSPE (1 × SSPE: 0.18 M NaCl, 1 mM EDTA, and 10 mM sodium phosphate at pH 7.4) containing 1% sodium dodecyl sulfate (SDS) and 100 µg of denatured salmon sperm DNA per mL. Hybridized blots were washed twice in 2 × SSPE and 0.1% SDS for 5 min at 65 °C. Signals were detected using autoradiography films and BAS-MS imaging plate (Fuji Film).

2.6. Observation of eGFP expression in transformants

Individual transformants were observed using a Zeiss Axio Imager A1 fluorescence microscope (Carl Zeiss, Oberkochen, Germany) with the following filter settings: 470/40 nm excitation and 525/50 nm emission.

2.7. Identification of T-DNA insertion sites by characterizing genomic sequences flanking inserted T-DNA

To recover genomic sequences flanking each inserted T-DNA, TAIL-PCR was used as previously described [22]. The secondary or tertiary PCR products were purified using ExoSAP-IT® (USB, Cleveland, OH, USA) according to the manufacturer's instruction. Purified PCR amplicons were sequenced using primer RB3 (Table 1) to determine *X. grammica* genome sequences flanking the right border of inserted T-DNA.

2.8. Mitotic stability of transformants

To test the mitotic stability of hygromycin B resistance, 100 randomly selected transformants were tested by growing them on PDA without hygromycin B for three generations at 25 °C. Subsequently, mycelia at the growing edge of the colony after the third subculture were inoculated on PDA containing 100 µg/mL hygromycin B to test whether they are still hygromycin B resistant.

3. Results and discussion

3.1. Establishment and optimization of ATMT for X. grammica

Because the *hph* gene has been shown to be expressed in many filamentous fungi [23], the gene has been widely used as a marker for selecting transformants. Growth of *X. grammica* isolate EL000614 on PDA supplemented with 50, 75, and 100 µg/mL of hygromycin B (Figure 1(A)) showed that 100 µg/mL hygromycin B completely blocked its growth. This concentration was chosen for selecting transformants.

To determine whether the pretreatment of *A. tumefaciens* cells with AS, which induces the expression of virulence genes in *A. tumefaciens*, is essential for ATMT of *X. grammica*, the transformation was conducted with and without AS treatment. Transformants were formed only when *A. tumefaciens* cells were grown in the presence of 200 µM AS before co-cultivation with fungal mycelia (Figure 1(B)). This result is consistent with the finding by de Groot et al. [20] that AS is required for successful ATMT of fungi.

To determine the amount of mycelia for maximal transformation efficiency, 20, 40, and 60 mg of mycelia (fresh weight)/mL were used. The

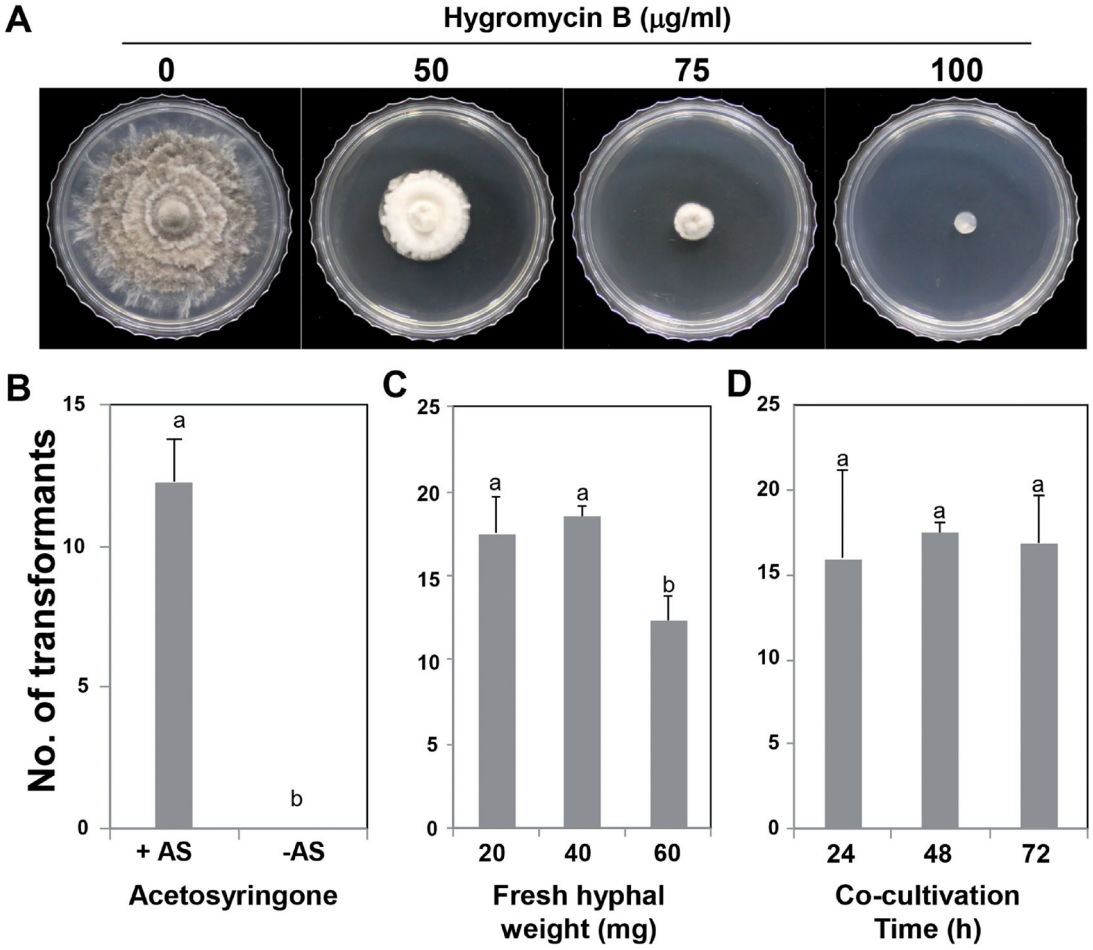


Figure 1. Hygromycin B sensitivity and factors affecting the efficiency of *Agrobacterium tumefaciens*-mediated transformation of *Xylaria grammica*. (A) Sensitivity of *X. grammica* to hygromycin B after incubation for 7 days at 25 °C on PDA; (B) effect of pre-induction of *A. tumefaciens* cells with acetosyringone (AS); (C) Effect of the amount of mycelia used; (D) effect of co-cultivation time. Data presented are the mean of three independent experiments. Error bars indicate standard deviation. *t*-Test (B) or ANOVA (C and D) was performed to test statistical differences at $p \leq 0.05$.

transformation efficiencies when using 20 and 40 mg/mL mycelia were significantly higher than when using 60 mg/mL of mycelia (Figure 2(C)).

The effect of the co-cultivation period on transformation efficiency was assessed by culturing *A. tumefaciens* cells in IM-AS for 24, 28, and 72 h. The duration of co-cultivation did not appear to influence transformation efficiency significantly (Figure 2(D)).

3.2. Confirmation of the integration of the hph and eGFP genes in the genome of X. grammica and transformant stability

PCR analysis was performed to confirm the presence of both the *hph* and *eGFP* genes in the genome of *X. grammica* transformants. 14 randomly selected transformants were analyzed by PCR using sets of primers targeting the *hph* and *eGFP* genes. Both genes (1 kb for the *hph* gene and 0.7 kb for the *eGFP* gene were amplified, indicating that both genes were integrated into the fungal genome after ATMT with pSK1044 (Figure 2(A)) [18].

Transformants were also analyzed for the expression of eGFP using fluorescence microscopy. The *eGFP* gene used was placed under the control of the *Cochliobolus heterostrophus GAPD* gene promoter. Mycelia of three randomly chosen transformants displayed strong green fluorescence (Figure 2(B)).

The mitotic stability of transformants was checked by sub-culturing hygromycin B-resistant transformants for three rounds on medium in the absence of hygromycin B and then transferring them to medium containing hygromycin (100 μg/mL). 100% of the transformants tested maintained hygromycin B resistance, suggesting that inserted T-DNA is mitotically stable.

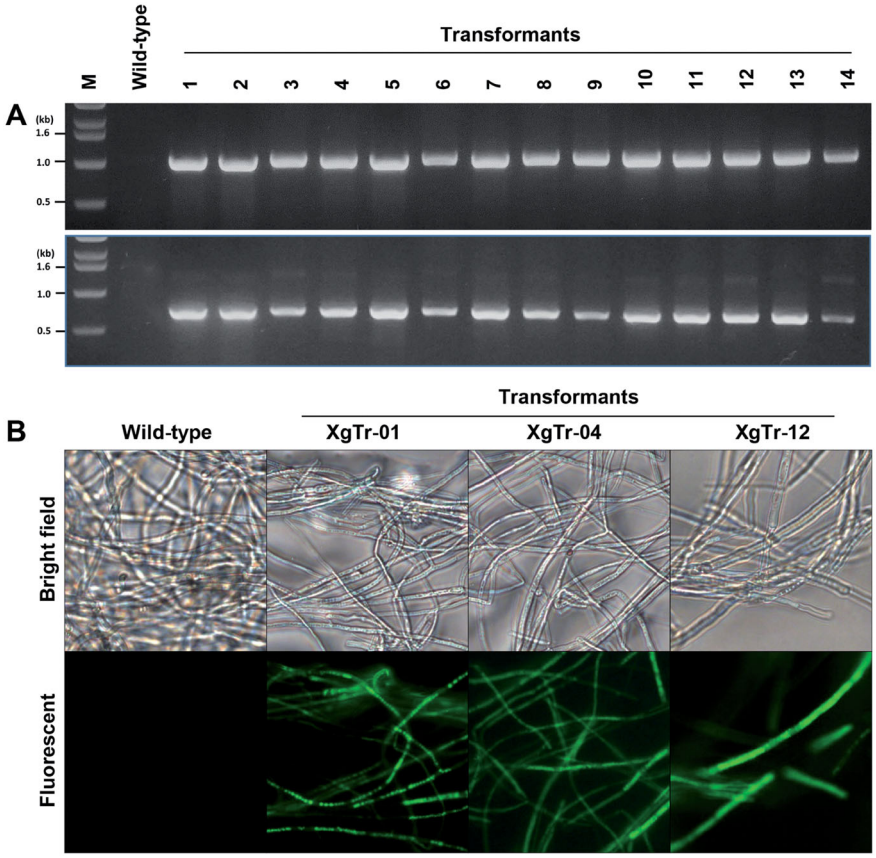
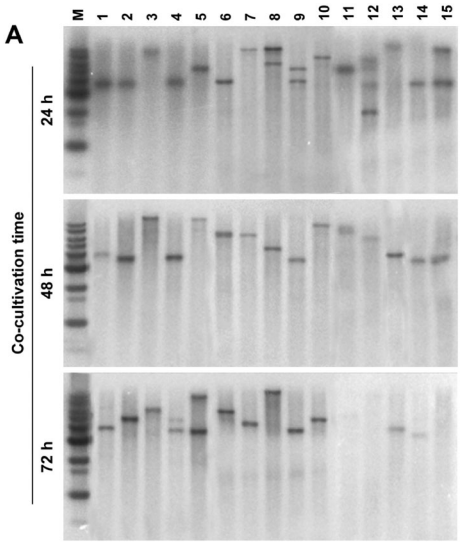


Figure 2. Confirmation of transformants. (A) PCR amplification of the hph (Upper) and eGFP (Lower) genes in 14 randomly selected transformants. M indicates DNA size marker; (B) expression of eGFP in transformants. Bright field and fluorescence images of the wild-type and three randomly selected transformants were shown.

3.3. Analysis of T-DNA insertion patterns among the transformants

One of the advantages of ATMT over other transformation methods is that most of the resulting transformants carry a single copy T-DNA [10–12]. While longer co-cultivation times tend to increase transformation efficiency, the number of T-DNA insertions per transformant also increased [21,24].

To determine the effect of co-cultivation time on a single copy T-DNA integration in *X. grammica*, we performed a Southern blot analysis of the transformants derived from 24, 48, and 72 h of co-cultivation (Figure 3). The co-cultivation for 48 h showed higher single-copy T-DNA integration than 24 and 72 h. Three independent biological replicates showed that all transformants after 48 h of co-cultivation harbored a single T-DNA insert. We further analyzed the number of T-DNA insertions in 166 randomly selected transformants by Southern blot. About 78.9% of transformants (131 in number) appeared to have a single copy of T-DNA. Small fractions of transformants showed two (32 transformants, 19.3%), three (2 transformants, 1.8%), and four or more (1 transformant, 0.3%) copies of T-DNA inserted in their genome (Table 2).



B

No. of T-DNA insertion	No. of transformants (%)		
	24 h	48 h	72 h
1	11 (73.3)	15 (100)	12 (85.7)
2	3 (20.0)	0	2 (14.3)
≥3	1 (6.7)	0	0

Figure 3. Effects of co-cultivation time on the copy number of inserted T-DNA. (A) Southern blot analysis of transformants. Genomic DNAs of 15 randomly selected transformants from each co-cultivation time setting were probed with a labeled *hph* gene after digestion with *Hin*dIII, a restriction enzyme that does not cut the *hph* cassette. M indicates 1 kb DNA ladder; (B) proportion of transformants with different T-DNA copy numbers.

For an efficient forward genetics analysis via ATMT-mediated insertional mutagenesis, easy identification of the genes/sites tagged by T-DNA is crucial [21]. The tagged genes and the orientation of T-DNA insertion can be easily identified by amplifying genomic sequences flanking inserted T-DNA using primers based on the RB and LB sequences [21,25]. We analyzed genome sequences flanking inserted T-DNA using TAIL-PCR [15]. Primary (RB1), secondary (RB2-1), and tertiary (RB3) PCR reactions were carried out. TAIL-PCR of 16 randomly selected transformants resulted in PCR amplicons ranging from 0.2 to 2 kb in size (Figure 4). These PCR amplicons were sequenced to identify the integration sites (Table 3). All these sequences were unique, suggesting random integration, supporting that ATMT is suitable for genome-wide random insertional mutagenesis of *X. grammica*.

Table 2. Southern blot analysis of 166 transformants to determine the number of T-DNA inserted.

No. of T-DNA insertion	No. of transformants (%)
1	131 (78.9)
2	32 (19.3)
3	2 (1.2)
4	1 (0.6)
Total	166

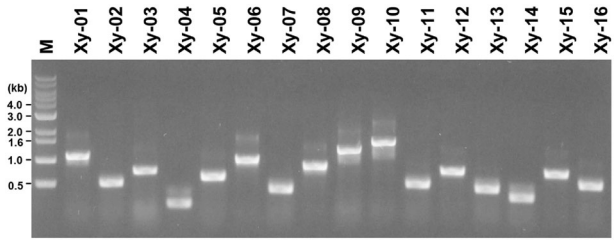


Figure 4. Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) using 16 randomly selected transformants. A right border-specific primer and arbitrary degenerate primers were used. M indicates 1 kb DNA ladder.

4. Conclusion

This is the first report of the genetically transforming *X. grammica*. ATMT has been successfully applied to genetically manipulating a wide variety of fungal species [9]. The main objective of this work was to establish an efficient ATMT protocol for *X. grammica* to support future molecular genetic studies. The highest number of transformants with a single T-DNA insertion was obtained after 48 h co-cultivation of freshly prepared mycelia of *X. grammica* with *A. tumefaciens* cells pre-induced with 200 µM AS. Successful expression of eGFP in *X. grammica* will also help visualize its interaction with other organisms.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Table 3. Genomic sequences flanking the right border of inserted T-DNA in 16 *Xylaria grammica* transformants.

Transformants	T-DNA copy number	Sequences flanking the RB of inserted T-DNA (5′–3′)[a]
XgTr-01	1	GTTTAAACTATCAGTGTTTGA[b]cttacctacttctccaccaggctgaatt
XgTr-02	1	GTTTAAACTATCAGTGTTTGAagtccttgagattagcttgttgcgtatt
XgTr-03	1	GTTTAAACTATCAGTGTTTGAgagataatacatacacatatccctcgatt
XgTr-05	1	GTTTAAACTATCAGTGTTTGAgagcacttgatggttggaatcttaatat
XgTr-06	1	GTTTAAACTATCAGTGTTTGAcccaacaggatgggctgaaatgagaggt
XgTr-08	1	GTTTAAACTATCAGTGTTTGActgcgagattcagttctgcgaggtagac
XgTr-09	1	GTTTAAACTATCAGTGTTTGAgtccaacgcgcactaacacttcgatttt
XgTr-10	1	GTTTAAACTATCAGTGTTTGAaacagcgtcttggcacgcactaaatcta
XgTr-11	1	GTTTAAACTATCAGTGTTTGAggaaacctgggggtaaaaaaacgtgaac
XgTr-13	1	GTTTAAACTATCAGTGTTTGAcagggaagggccgcagatatagagagat
XgTr-14	1	GTTTAAACTATCAGTGTTTGAtaaccaacccctacctacctaggtccatt
XgTr-17	1	GTTTAAACTATCAGTGTTTGActtatattatcatcgtcatcatcatcgc
XgTr-19	1	GTTTAAACTATCAGTGTTTGAcaggtgaccccggtactaaccttgggac
XgTr-20	1	GTTTAAACTATCAGTGTTTGAtcgattttacgcacatatgcgcattctt
XgTr-23	2	GTTTAAACTATCAGTGTTTGAttgttttaactgttgagttaactagagg
XgTr-24	1	GTTTAAACTATCAGTGTTTGAcccgcagccttcggataactcttcataa

[a]Partial genomic sequences flanking the RB of inserted T-DNA in the analyzed transformants are shown. [b]The bold letters correspond to the right border sequences of T-DNA.

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