Research Paper 研究报告

微生物学报 *Acta Microbiologica Sinica*
52(12):1449 – 1457; 4 December 2012
ISSN 0001 – 6209; CN 11 – 1995/Q
http://journals.im.ac.cn/actamicrocn

Agrobacterium tumefaciens-*mediated* transformation as a tool for insertional mutagenesis in thermophilic fungus *Thermomyces lanuginosus*

Hua Han[#], Xiaoxue Xu[#], Yanjie Peng, Dehui Kong, Duochuan Li[*]
Department of Environmental Biology, Shandong Agricultural University, Tai'an 271018, China

Abstract: [**Objective**] To establish a stable transformation system of the thermophilic fungus *Thermomyces lanuginosus* for its insertional mutagenesis. [**Methods**] *Agrobacterium tumefaciens*-*mediated* transformation (ATMT) was applied to establish transformation system of *T. lanuginosus*. Southern blotting of *hph* gene and cloning of transforming DNA (T-DNA) flanking sequences were used to determine insert number and site of T-DNA in the fungal genome, respectively. [**Results**] A reliable transformation method is established for *T. lanuginosus*. Specifically, pre-germinating spores of *T. lanuginosus* used at co-cultivated period was a prerequisite. *T. lanuginosus* germinating spores co-cultivated with *Agrobacterium tumefaciens* at 28℃ for 48 h achieved the highest transformation efficiency. Addition of Acetosyringone (AS) during pre-culture of *A. tumefaciens* and co-cultivation of *T. lanuginosus* germinating spores with *A. tumefaciens* was essentially required, and the best results were obtained with AS at the concentration of 500 μM. Southern blotting analysis showed that majority of transformants (79.2%) contained a single insertion of T-DNA. Thermal asymmetric interlaced PCR (TAIL-PCR) analysis showed random insertion of T-DNA in the fungal genome. Using the transformation system, some stable phenotypic mutants of T. lanuginosus were obtained. [**Conclusion**] We report, for the first time, a simple and efficient method for transforming *T. lanuginosus* by using ATMT. This approach provides a tool for insertional mutagenesis gene tagging in this thermophilic fungus.

Keywords: *Agrobacterium tumefaciens*-*mediated* transformation (ATMT), insretional mutagenesis, *Thermomyces lanuginosus*, thermophilic fungus
CLC number: Q933 **Document code**: A **Article ID**: 0001-6209 (2012) 12-1449-09

Thermomyces lanuginosus is one of the thermophilic fungi having the ability to thrive at high temperature [1]. Current studies on *T. lanuginosus* are stimulated by the prospect of finding fungus capable of

Supported by the National Natural Science Foundation of China (30870006), by the Chinese Marine Renewable Energy Special Foundation (SDME2011SW01) and by the National Programs for High Technology Research and Development of China (2012AA10180402)

[*] Corresponding author. Tel: +86-538-8249071; Fax: +86-538-8226399; E-mail: lidc20@sdau.edu.cn
[#] These authors contributed equally to this work.

Received: 25 June 2012/Revised: 26 September 2012

secreting high levels of enzymes having high temperature optima and a long "shelf-life"[2]. The optimal use of recombinant DNA techniques in genetic study of *T. lanuginosus* requires the availability of an efficient transformation system, which can provide a way of over-expressing the thermophilic enzymes genes for industry application. In addition, the transformation system also facilitates obtainment of *T. lanuginosus* insertional mutagenesis for functional analysis. In particular, some insertional mutagenesis that loses the thermophilic ability may have significance in exploring thermophilic mechanism of *T. lanuginosus*.

Agrobacterium tumefaciens-mediated transformation (ATMT) as a simple and efficient method for transforming and insertional mutagenesis was used in variety of plants and fungi. An obvious advantage of ATMT does not require protoplasts of biological materials for transformation. It can be used to transform a broad spectrum of starting material. For insertion mutagenesis, ATMT offers a great potential to generate a high percentage of transformants with a single insertion of T-DNA in fungal genome, which will facilitate subsequent isolation of tagged genes[3].

In contrast to mesophilic fungi, transformation of thermophilic fungi developed relatively slowly, and only three methods were reported, such as PEG-mediated transformation[4], lithium acetate transformation[5], and electroporation[6]. ATMT has been developed for many mesophilic filamentous fungi, but application of this technology in any thermophilic fungus has not been achieved. Here we report the first successful ATMT analysis of *T. lanuginosus*.

1 MATERIALS AND METHODS

1.1 Strains and culture conditions

Agrobacterium tumefaciens strains LBA4404, EHA105, AGL-1, and GV3101 were used for fungal transformation. Strain *Thermomyces lanuginosus* $HSAUP_{03}$ 80006 used as a recipient strain in transformation was grown on potato dextrose agar (PDA) medium. To produce conidia for transformation, mycelia were inoculated on YPS plates containing per liter: 4 g yeast extract; 1.5 g soluble starch; 1 g KH_2PO_4 and 0.5 g $MgSO_4 \cdot 7H_2O$ (pH 7.0). Transformants were screened on MT medium containing per liter: KH_2PO_4 1.5 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; KCl 0.5 g; L-arginine 2 g; Vishniac 1 ml; Biotin 1 ml; Glucose 20 g; Agar 20 g and Vishniac containing per liter: EDTA 10 g; $ZnSO_4 \cdot 7H_2O$ 4.4 g; $MnCl_2 \cdot 6H_2O$ 1 g; $CoCl_2 \cdot 6H_2O$ 0.32 g; $CuSO_4 \cdot 5H_2O$ 0.32 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.22 g; $CaCl_2 \cdot 2H_2O$ 1.47 g; $FeSO_4 \cdot 7H_2O$ 1 g.

1.2 Plasmid construction

The T-DNA binary vector pROK Ⅱ-hygro was constructed on the backbone of pROK Ⅱ[7]. The hygromycin B resistance gene cassette containing the bacterial hygromycin B phosphotransferase gene under the control of the *Aspergillus nidulans trpC* promoter and *trpC* terminator was isolated by digesting pUCATPH[8] with *Hind* Ⅲ and *Kpn* Ⅰ. The plasmid pROK Ⅱ with the Cauliflower Mosaic Virus 35S (CaMV35S) promoter removed digested with *Hind* Ⅲ and *Kpn* Ⅰ was ligated to the hygromycin B resistance gene cassette to produce pROK Ⅱ-hygro (Fig. 1).

1.3 *Agrobacterium tumefaciens-mediated* transformation

The pROK Ⅱ-hygro vector was transformated into *A. tumefaciens* strain LBA4404 by electroporation, and the *A. tumefaciens* strain LBA4404 containing the pROK Ⅱ-hygro vector was cultivated at 28℃, 200rpm for 48 h in a minimal medium (MM)[9] that was supplemented with kanamycin (50 μg/ml) and rifampicin (200 μg/ml). Cells were harvested by centrifugation, washed two times and finally resuspended in induction medium (IM)[10] and the cell density was adjusted to an optical density at 600 nm to 0.25. The cells were grown for an additional 6 h prior to co-cultivation.

To produce conidia for transformation, *T. lanuginosus* mycelia were inoculated on YPS plates, incubated at 50℃ for 7 – 10 days and spores were harvested by rinsing the culture with sterile water containing 0.9% NaCl and 0.05% Tween-20. Spores of *T. lanuginosus* were inoculated in 100 ml YPS and

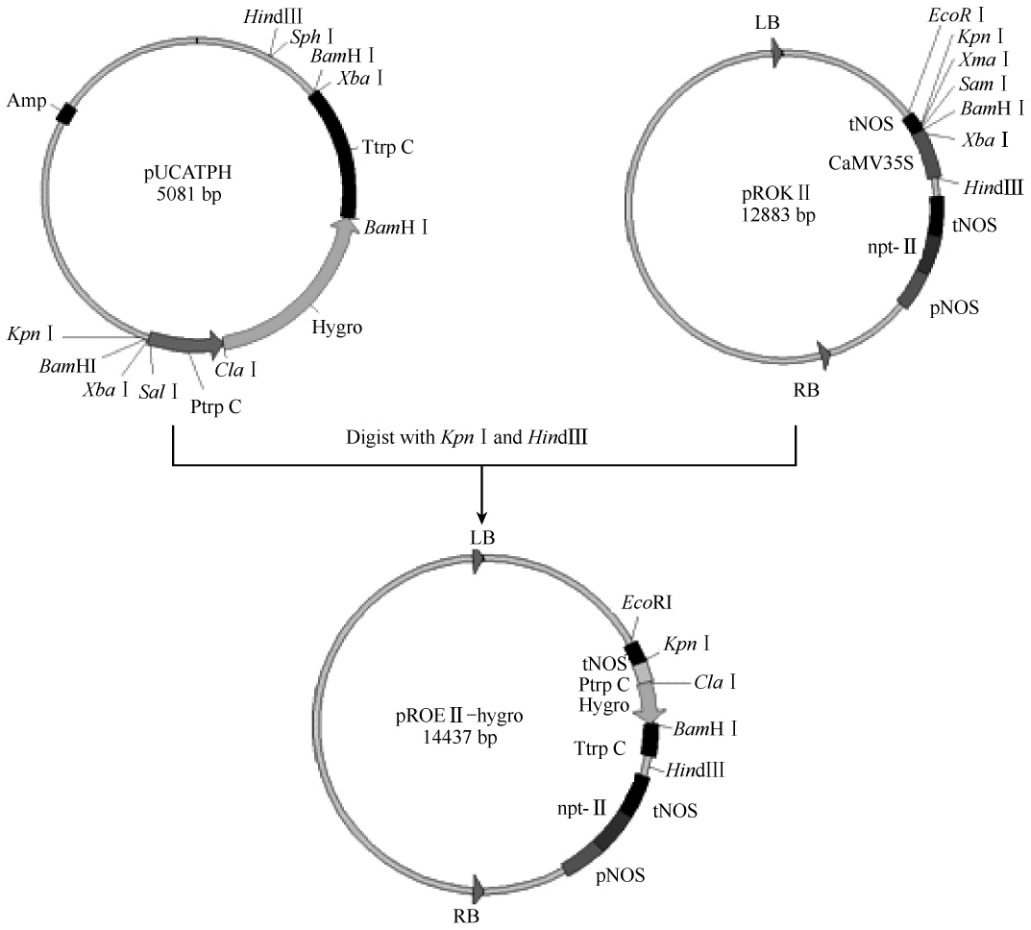

Fig. 1 Construction of the transformation plasmid pROK Ⅱ-hygro. Amp, Ampicillin resistance gene for selection in bacteria; hygro, hygromycin B resistance gene for selection in fungi.

incubated at 50℃, 160rpm for 4 hours. Germinated spores were collected by centrifugation (8000rpm, 5min) and resuspended in sterile water. The germinated spores titre was estimated by a haemocytometer count and adjusted to 10^6/ml. For co-cultivation, 100 μL of the spores suspension and 200 μL of the bacterial culture (IM) were mixed and plated on a cellophane sheet on co-cultivation medium (CM; same as IM, except it contains 5 mM of glucose instead of 10 mM glucose) in the presence of 200 μM AS. Following co-cultivation at 28℃ for 48 h, the filter was transferred to MT plate containing hygromycin B (1000 μg/ml). Transformants usually appeared after 4 – 6 days of incubation at 50℃.

1.4 Analysis of transformants

The putative transformants were carefully excised and transferred to fresh MT plates and one germinating conidium from each transformant was picked and transferred to cerate mono-conidial cultures. Genomic DNA of the wide-type strain and transformants was extracted from mycelia grown on PDA agar medium. Polymerase chain reaction (PCR) detection of *hph* gene was using primers hph-1 (5′-ATGAAAAAG CCTGAACTC-3′) and hph-2 (5′-CTATTCCTTTGCCC TCGG -3′), which defined a 1026bp sequence spanning the *hph* gene.

To assess the mitotic stability of the transformants, the true transformants screened by PCR were transfered to nonselective medium (PDA). Following colonization of each plate, a mycelial plug was taken from the edge of the culture and transferred onto fresh PDA medium. This procedure was repeated six times. Resistances of these cultures to hygromycin B were tested and extracted genomic DNA again to detect the *hph* gene as

described above.

Genomic DNA from the wide type and transformants was digested with the *Hin*d Ⅲ restriction endonucleases, separated by 0.8% agarose gel electrophoresis, and transferred onto nylon membrane (Amersham Biosciences, Piscataway, NJ, USA). A 379 bp DNA fragment was amplified from the *hph* gene as the probe with the oligonucleotide primers hph-s (5′-CGGATTTCGGCTCCAACAA-3′) and hph-a (5′-TCTACACAGCCATCGGTCCA-3′). DNA hybridization probe was labeled and detected using DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Mannheim, Germany). Procedures for probe labeling, hybridization, and detection were carried out according to the manufacturer's recommendations.

1.5 Cloning and characterization of T-DNA insertion junction DNA by TAIL-PCR

DNA fragments spanning the insertion junction of T-DNA were amplified by TAIL-PCR according to Liu and Huang [11]. 7 randomly selected transformants genomic DNA was used as template in successive reactions using left border-specific primers LB-1, LB-2, LB-3 or right border-specific primers RB-1, RB-2, RB-3, and arbitrary degenerate primers AD1, AD2, AD3 and AD4. The sequences of primers were listed in Table 1. The reaction conditions and thermal cycling settings were as described by Liu and Huang [11]. Amplified products from primary, secondary and tertiary PCR reactions were analysed by agarose gel electrophoresis. A single PCR product was generally obtained following the third round of PCR.

Table 1 The primers for TAIL-PCR

Primer name	Nucleotide sequence (5′→3′)
AD1	NTCGASTWTSGWGTT
AD2	NGTCGASWGANAWGAA
AD3	WGTGNAGWANCANAGA
AD4	AGWGNAGWANCAWAGG
LB-1	GCCATCGCCCTGATAGACGGTT
LB-2	AACACTCAACCCTATCTCGGGCTATT
LB-3	TCGGAACCACCATCAAACAGG
RB-1	TAGTGACCTTAGGCGACTTTTGAACG
RB-2	AGTGGCTCCTTCAACGTTGCGGTT
RB-3	ATCAGATTGTCGTTTCCCGCCTTC

2 RESULTS AND DISCUSSION

2.1 The minimum inhibitory concentration of hygromycin B for *T. lanuginosus*

To determine the minimum inhibitory concentration of hygromycin B for the wild-type strain of *T. lanuginosus*, 10^6 spores were inoculated onto MT plates supplemented with hygromycin B at different concentrations (0, 400, 600, 800, 1000, 1200 μg/ml). The results showed that conidia germination and mycelial growth of *T. lanuginosus* was inhibited on MT mediu containing ≥ 1000 μg/ml hygromycin B. Therefore, 1000 μg/ml was considered the minimum hygromycin B inhibitory concentration for the selection of *T. lanuginosus* transformants in the ATMT experiments (data not shown).

2.2 Optimization of the ATMT system of *T. lanuginosus*

In our initial transformation assay, *A. tumefaciens* stain LBA4404 carrying plasmid pROK Ⅱ-hygro was co-cultivated with the germinated conidia of *T. lanuginosus* at 28℃ for 48 h on CM containing 200 μM AS, and approximately 4 colonies resistant to hygromycin B were obtained per 10^6 spores (per plate). The transformation efficiency was too low, and the transformation conditions of the initial transformation assay need to be optimized.

The chromosomal background of *A. tumefaciens* plays a key role in the efficiency of the ATMT system. In addition to the function of the *vir* region, the recognition and binding of *A. tumefaciens* to the host surface also depend on the genes encoded on the bacterial genome [12]. In this study, independent transformation experiments with *A. tumefaciens* strains LBA4404, EHA105, GV3101 and AGL-1 containing the plasmid pROK Ⅱ-hygro were performed. This study data show that EHA105 resulted in the highest transformation efficiency, LBA4404 and AGL-1 produced lower transformation efficiency than EHA105, and no transformant was obtained using GV3101 strain (Fig. 2-A). It was also reported that two similar transformations of *Monascus purpureus* and *penicillium*

marneffei [13-14] showed the usage of *A. tumefaciens* strains derived from the super-virulent A281 strain EHA105 (high level of *vir* gene expression) resulted in a higher frequency. Therefore, various parameters were then assessed to optimize the transformation process with *A. tumefaciens* strain EHA105.

At higher temperatures (> 28℃) the T-DNA transfer machinery of *A. tumefaciens* does not function properly [15]. However, *T. lanuginosus* hardly have growth at 28℃. In addition, the higher temperatures (> 28℃) are also unfit for the infection of *A. tumefaciens* to *T. lanuginosus*. To solve this problem, spores of *T. lanuginosus* were pre-germinated at 50℃ and co-cultivation proceeded at 28℃ to ensure the infection of *A. tumefaciens* to *T. lanuginosus*. Transformation rates were influenced by germinated and non-germinated conidia. No transformant was obtained using non-germinated conidia. Germination of the spores of 4 hours gave the highest transformation rates, up to 88 transformants per 10 plates (Fig. 2-B).

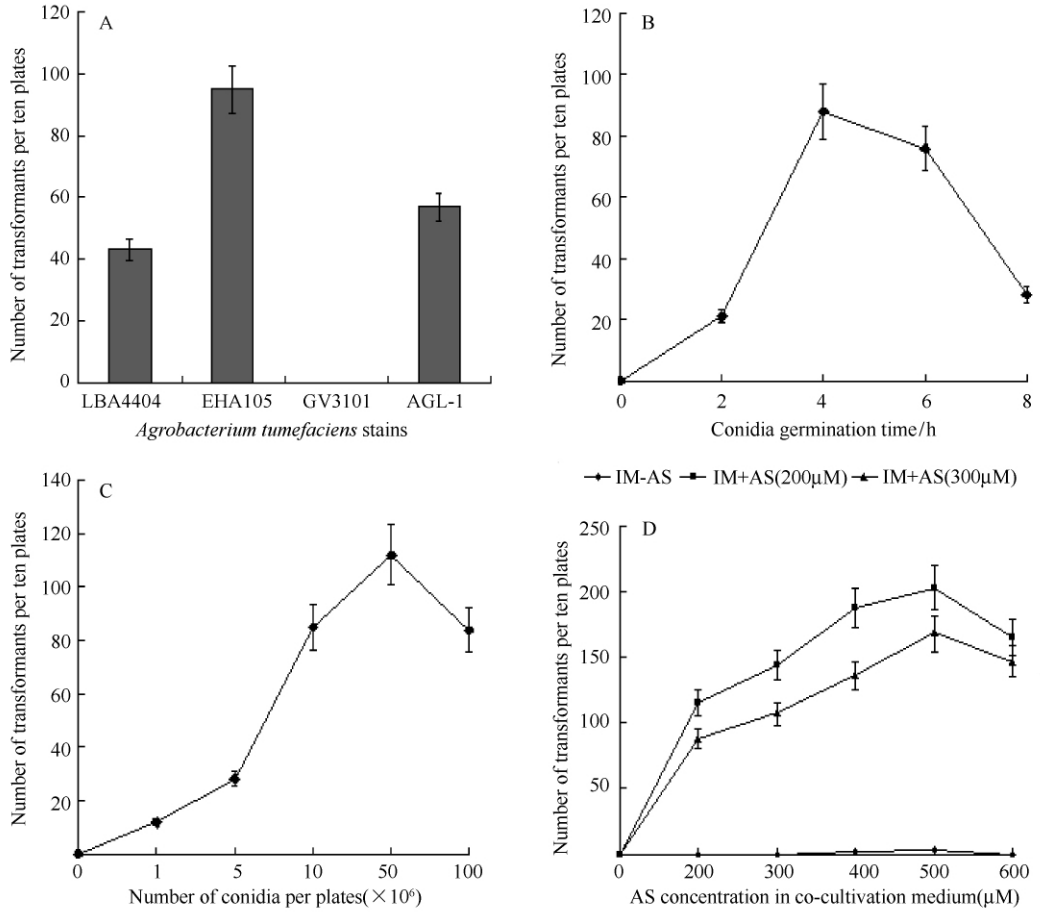

Fig. 2 Optimal parameters of *Agrobacterium tumefaciens*-*mediated Thermomyces lanuginosus* transformation. A: Effect of different *A. tumefaciens* stains. *A. tumefaciens* stains LBA4404, EHA105, GV3101 and AGL-1 harboring plasmid pROK Ⅱ-hygro were grown for 6 h in IM in the presence of AS (200 μM) prior to co-cultivation. Conidia of *T. lanuginosus* germinating for 4 h at 50℃ were adjusted to 10^6 per plate. 200 μl of *A. tumefaciens* and conidia suspension were pipetted onto a cellophane sheet and incubated for 48 h at 28℃ Transformants were selected on selection medium for 5 d at 50℃. B: Effect of conidia germinating time. *A. tumefaciens* stain EHA105 harboring pROK Ⅱ-hygro was used and the conidia germinated for 0, 2, 4, 6 and 8 h. Other parameters used were same to A. C: Effect of the input number of *T. lanuginosus* conidia. Different number of *T. lanuginosus* conidia germinated for 4 h were inputted and the transformation procedure was described as B. D: Effect of AS concentrations in IM and CM. *A. tumefaciens* stain EHA105 and 5×10^6 conidia per plate were used. Different concentrations of AS in IM and CM were tested and the other conditions were same to C.

To test the effect of the number of *T. lanuginosus* conidia to transformation, different densities of spore germinated for 4 h were used for transformation. The results show that transformation efficiency increased as a function of the initial number of germinated spores up to 5×10^6. Further increase of the spore number did not improve the transformation efficiency of *T. lanuginosus* (Fig. 2-C).

The insertion of T-DNA into the host genome requires activation and high expression of the *vir* region genes on the Ti plasmid [16]. Transcription of the *vir* region genes was found to be silent when *A. tumefaciens* was grown in LB medium. The addition of AS or other phenolic compounds has been shown to induce the activation and high expression of *vir* region genes [17]. Therefore, the addition of AS during the *A. tumefaciens* co-cultivation period was necessary for transformation, but the addition of AS during the pre-culture of *A. tumefaciens* did not seem to be an absolute requirement [18]. In the study, no transformants were observed on MT when AS was absent from the pre-culture, and the appropriate concentration of AS in IM and CM for transformation was detected to be 200 μM and 500 μM, respectively (Fig. 2-D). These results indicated that the addition of AS to the *A. tumefaciens* pre-culture was essential for the transformation of *T. lanuginosus* and the absence of AS in the pre-culture can serve as a negative control. Using optimal transformation conditions, the transformation efficiency improved form 40 to 217 transformants per 10 plates.

2.3 Analysis of transformants

To investigate the state of the T-DNA in these fungal transformants, we isolated total DNA from 78 random transformants and confirmed integration of the *hph* gene by PCR analysis. The expected 1026bp PCR product was detected in 73 transformants, indicating that T-DNA integrated into the fungal genome (data not shown).

With other filamentous fungi, transformants have been reported to be relatively stable during growth under non-hygromycin B conditions [18-19]. This is an important feature of an effective mutagenesis system. In this study, transformants cultivated for 5 generations on nonselective medium (PDA) still appeared the resistances to hygromycin B and the *hph* gene also could be detected by PCR.

In applying ATMT to generate mutant libraries, single copy T-DNA and random site integration throughout the genome are preferred. T-DNA gene tagging has been proven to be a very valuable mutagenic tool in fungi. The majority of fungal transformants receive a single copy of the T-DNA [3], so the analysis of most T-DNA tagged mutagenesis would be straightforward. In this study, southern analysis of transformants demonstrated that each possessed the inserted DNA fragment containing the *hph* gene, 79.2% (19 of 24) of which displayed single hybridization bands indicating a single insert of T-DNA in each of these transformants (Fig. 3).

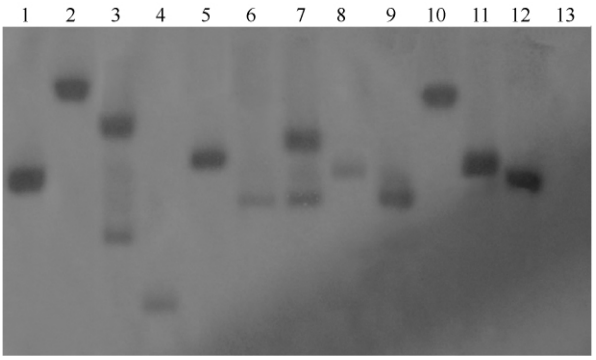

Fig. 3 Southern blot analysis of the transformants. Genomic DNA of 12 randomly selected transformants was digested with *Hind* Ⅲ which cuts once in T-DNA, gel size-fragmented, transferred to a nylon membrane and probed with the partial sequence of *hph* gene. Lane 1 – 12: independent transformants; Lane 13: untransformed wild-type strain.

2.4 Cloning and characterization of T-DNA and its flanking sequences

We amplified the left border and right border fragments in 7 transformants containing only a single insert of T-DNA tested by southern blot. The sizes of the tertiary PCR reaction products ranged from 0.5 to 1.2 kb. As a result of the nested arrangement of the LB and RB primers, the size of the desired PCR products resulting from the tertiary PCR reaction were 56bp and 105bp respectively shorter than that obtained following the secondary PCR reaction. Sequence analysis of the

TAIL-PCR products showed that we successfully cloned 6 left and 5 right border sequences corresponding to the left or right border of the T-DNA. The LB and RB parts of the inserted T-DNA were variable length (Fig. 4). Sequence analysis suggested that the insertion of T-DNA within the host genome appeared to be a random event. Genome-wide randomness of T-DNA integration has been also observed in other fungi [3]. Along with the southern blot results, we suggested ATMT was a system that provided a powerful mean for insertional mutagenesis in *T. lanuginosus*. Furthermore, other methods of insertional mutagenesis, such as restriction enzyme-scale insertions (REMI), can lead to high rates of genetic deletions, rearrangements, untagged mutations, and multiple insertions in other fungi [20], making identification of causative mutant genes difficult. Unquestionably, ATMT has more advantages than other methods in insertional mutagenesis system.

Fig. 4 Sequence of T-DNA with flanking *Thermomyces lanuginosus* DNA. NO. 0: the expected left and right border sequences of the T-DNA from plasmid pROK Ⅱ-hygro. NO. 1 – 6: partial nucleotide sequences of TAIL-PCR products obtained from randomly selected transformants. The right border of NO. 5 was not obtained and is indicated by ×, while dots represent truncations of the T-DNA ends.

2.5 Screening for phenotypic mutants

Mutant is a valuable tool that could be used for both forward and reverse genetics. In our *T. lanuginosus* transformation experiments, some phenotypic mutants of *T. lanuginosus* were screened and cultured on PDA medium. As shown in Fig. 5, the mutant strain Mut1 and Mut2 grew slower than the wild type strain, and the mutant strain Mut3 grew faster than the wild type strain but produced few spores. The mutant strain Mut1 lost the capacity to sporulate, Mut4 and Mut5 nearly did not produce spores and their aerial hyphae grew vigorously.

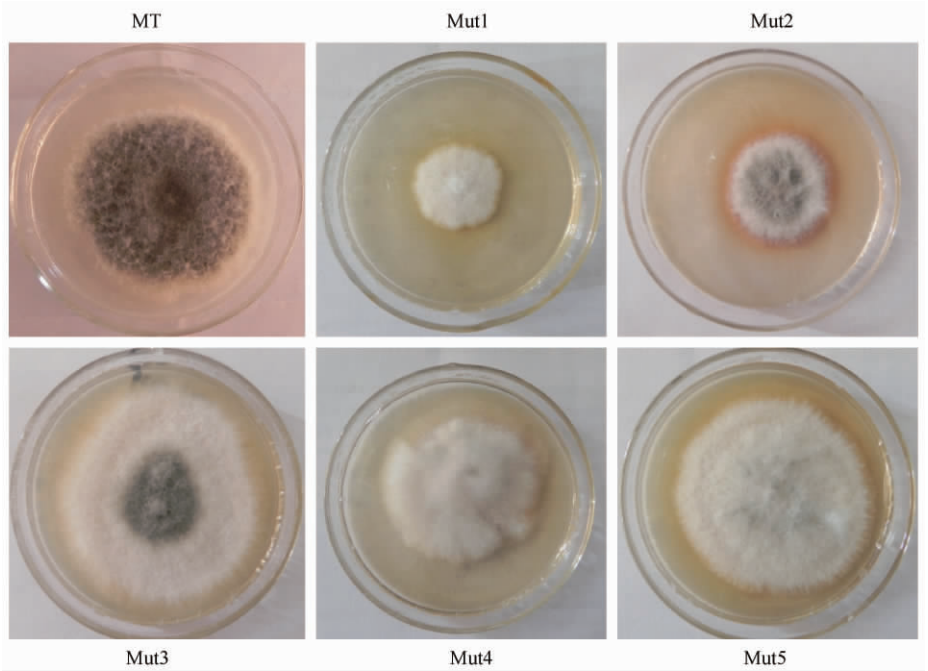

Fig. 5 The colonial morphology of the wild type and T-DNA insertional mutants of *Thermomyces lanuginosus*. The wild type host strain (WT) and mutant strains (Mut1 to Mut5) were inoculated onto PDA medium and incubated at 50℃ for a week.

In conclusion, we report, for the first time, a simple and efficient method for transforming thermophilic fungus *T. lanuginosus* by using *A. tumefaciens*-*mediated* DNA transfer, but also the first time for all of the thermophilic fungi. This approach provides a tool for insertional mutagenesis gene tagging in this thermophilic fungus. In addition, we prospect this relatively simple and useful methodology can promote the development of the transformation systems of other thermophilic fungi. In this study, we overcome a difficult question that *T. lanuginosus* and *A. tumefaciens* have no common growth temperature in co-cultivated stage, which does not exist in transformation of non-thermophilic fungi. This method may promote the success of other thermophilic fungi transformation system. Compared with non-thermophilic fungi, the transformation efficiency of *T. lanuginosus* is low; two possible explanations are that (i) the non-homologous promoter to control the expression of the selection *hph* gene, (ii) the difference of growth temperature of *T. lanuginosus* and *A. tumefacien* in co-cultivated stage. One approach to solve the low transformation efficiency is to isolate a homologous promoter from *T. lanuginosus* for *hph* gene expression.

REFERENCES

[1] Maheshwari, R, Bharadwaj, G. Bhat, MK. Thermophilic fungi: Their physiology and enzymes. *Microbiology and Molecular Biology Reviews*, 2000, 64 (3) :461-488.

[2] Singh S, Madlala AM, Prior BA. Thermomyces lanuginosus: properties of strains and their hemicellulases. *FEMS Microbiology Reviews*, 2003, 27 (1) :3-16.

[3] Michielse CB, Hooykaas PJ, van den Hondel CA, Ram AF. *Agrobacterium*-*mediated* transformation as a tool for functional genomics in fungi. *Current Genetics*, 2005, 48 (1) :1-17.

[4] Jain S, Durand H, Tiraby G. Development of a transformation system for the thermophilic fungus *Talaromyces sp. CL*240 based on the use of phleomycin resistance as a dominant selectable marker. *Molecular and General Genetics*, 1992, 234 (3) :489-493.

[5] Allison DS, Rey MW, Berka RM, Armstrong G, Dunn-Coleman NS. Transformation of the thermophilic fungus *Humicola grisea var. thermoidea* and overproduction of *Humicola* glucoamylase. *Current Genetics*, 1992, 21 (3) :225-229.

[6] Gangavaram LP, Mchunu N, Ramakrishnan P, Singh S, Permaul K. Improved electroporation-mediated non-integrative transformation of *Thermomyces lanuginosus*. *Journal of Microbiological Methods*, 2009, 77 (2) :159-164.

[7] Nair GR, Liu Z, Binns AN. Reexamining the role of the accessory plasmid pAtC58 in the virulence of *Agrobacterium tumefaciens* strain C58. *Plant Physiology*, 2003, 133 (3) :989-999.

[8] Lu S, Lyngholm L, Yang G, Bronson C, Yoder O, Turgeon BG. Tagged mutations at the Tox1 locus of Cochliobolus heterostrophus by restriction enzyme-mediated integration. *Proceedings of the National Academy of Sciences of the United States of America*, 1994, 91 (26) :12649-12653.

[9] Hooykaas P, Roobol C, Schilperoort R. Regulation of the transfer of Ti plasmids of *Agrobacterium tumefaciens*. *Journal of General and Applied Microbiology*, 1979, 110 (1) :99-109.

[10] Bundock P, Dulk-Ras AD, Beijersbergen A, Hooykaas P. Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. *EMBO Journal*, 1995, 14 (13) :3206-3214.

[11] Liu YG, Huang N. Efficient amplification of insert end sequences from bacterial artificial chromosome clones by thermal asymmetric interlaced PCR. *Plant Molecular Biology Reporter*, 1998, 16 (2) :175-181.

[12] Koukolikova-Nicola Z, Hohn B. How does the T-DNA of *Agrobacterium tumefaciens* find its way into the plant cell nucleus. *Biochimie*, 1993, 75 (8) :635-638.

[13] Campoy S, Perez F, Martin JF, Gutierrez S, Liras P. Stable transformants of the azaphilone pigment-producing *Monascus purpureus* obtained by protoplast transformation and *Agrobacterium*-*mediated* DNA transfer. *Current Genetics*, 2003, 43 (6) :447-452.

[14] Zhang P, Xu B, Wang Y, Li Y, Qian Z, Tang S, Huan S, Ren S. *Agrobacterium tumefaciens*-*mediated* transformation as a tool for insertional mutagenesis in the fungus *Penicillium marneffei*. *Mycological Research*, 2008, 112 (8) :943-949.

[15] Fullner KJ, Nester EW. Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens*. *Journal of Bacteriology*, 1996, 178(6):1498-1504.

[16] Stachel S, Timmerman B, Zambryski P. Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: requirement for 5′virD gene products. *EMBO Journal*, 1987, 6(4):857-863.

[17] Lai EM, Shih HW, Wen SR, Cheng MW, Hwang HH, Chiu SH. Proteomic analysis of *Agrobacterium tumefaciens* response to the *vir* gene inducer acetosyringone. *Proteomics*, 2006, 6(14):4130-4136.

[18] Leclerque A, Wan H, Abschutz A, Chen S, Mitina GV, Zimmermann G, Schairer HU. *Agrobacterium-mediated* insertional mutagenesis (AIM) of the entomopathogenic fungus *Beauveria bassiana*. *Current Genetics*, 2004, 45(2):111-119.

[19] Zhong YH, Wang XL, Wang TH, Jiang Q. *Agrobacterium-mediated* transformation (AMT) of *Trichoderma reesei* as an efficient tool for random insertional mutagenesis. *Applied Microbiology and Biotechnology*, 2007, 73(6):1348-1354.

[20] Bolker M, Bohnert HU, Braun KH, Gorl J, Kahmann R. Tagging pathogenicity genes in *Ustilago maydis* by restriction enzyme-mediated integration (REMI). *Molecular and General Genetics*, 1995, 248(5):547-552.

农杆菌介导的获取疏绵状嗜热丝孢菌插入突变体体系的建立

韩华#,徐晓雪#,彭延杰,孔德慧,李多川*

山东农业大学环境生物系,泰安 271018

摘要:【目的】建立疏绵状嗜热丝孢菌的稳定遗传转化体系并获得插入突变体。【方法】利用农杆菌介导的方法建立疏绵状嗜热丝孢菌的遗传转化体系;分别通过 Southern 杂交、克隆转移 DNA(T-DNA)侧翼序列来确定 T-DNA 在疏绵状嗜热丝孢菌基因组中的拷贝数和插入位点。【结果】成功建立了可靠的疏绵状嗜热丝孢菌的遗传转化体系。共培养过程中使用萌发孢子是成功建立疏绵状嗜热丝孢菌遗传转化体系的必要条件。疏绵状嗜热丝孢菌萌发的孢子与农杆菌在28℃共培养48h时,转化效率最高。乙酰丁香酮(AS)在农杆菌预培养及疏绵状嗜热丝孢菌萌发的孢子与农杆菌的共培养阶段都是必需的,且在共培养阶段当 AS 浓度为500 μM时转化效率最高。Southern 杂交验证表明,79.2% 的转化子为 T-DNA 单拷贝插入,且通过热不对称 PCR(TAIL-PCR)分析得出 T-DNA 在该菌基因组中的插入位点是随机的。通过该转化系统筛选到部分表型突变体。【结论】我们首次报道了利用 ATMT 技术成功转化嗜热真菌-疏绵状嗜热丝孢菌,证明了该方法是一种简单有效的获得插入突变体的方法,并为该嗜热真菌进行基因定位提供了工具。

关键词: 农杆菌介导转化, 插入突变体, 疏绵状嗜热丝孢菌, 嗜热真菌

中图分类号: Q933 **文献标识码**: A **文章编号**:0001-6209 (2012) 12-1457-09

(本文责编:张晓丽)

基金项目: 国家自然科学基金(30870006);国家海洋可再生能源专项基金(SDME2011SW01);国家"863 计划"(2012AA10180402)

* 通信作者。Tel: +86-538-8249071; Fax: +86-538-8226399; E-mail: lidc20@sdau.edu.cn

作者简介: #并列第一作者。韩华(1988-),男,山东济南人,硕士,研究方向为分子真菌学。E-mail: jnhanhua@163.com

收稿日期:2012-06-25; **修回日期**:2012-09-26