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Agrobacterium-mediated transformation (AMT) of *Trichoderma reesei* as an efficient tool for random insertional mutagenesis

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Abstract Filamentous fungus *Trichoderma reesei* QM9414 was successfully transformed with *Agrobacterium tumefaciens* AGL-1 for random integration of transforming DNA (T-DNA). Co-cultivation of *T. reesei* conidia or protoplasts with *A. tumefaciens* in the presence of acetosyringone resulted in the formation of hygromycin B-resistant fungal colonies with high transformation frequency. Nine randomly selected resistant clones were proved to be stable through mitotic cell division. The integration of the *hph* gene into *T. reesei* genome was determined by PCR and dot blot analysis. Transgenic *T. reesei* strains were analyzed using TAIL-PCR for their T-DNA contents. The results showed that T-DNA inserts occurred evidently by fusing DNA at T-DNA borders via random recombination, which suggests that *Agrobacterium*-mediated transformation is a potentially powerful tool towards tagged mutagenesis and gene transfer technology for *T. reesei*.

Keywords Agrobacterium-mediated transformation · Trichoderma reesei · T-DNA · Insertional mutagenesis · hygromycin B resistance · TAIL-PCR

Introduction

Trichoderma reesei is a biotechnically important filamentous fungus known as an efficient producer of enzymes and proteins. Because of its capacity to secrete enzymes in high yields, *T. reesei* has been exploited as an industrial host for homologous and heterologous protein production (Penttilä 1998; Punt et al. 2002). For example, several mutant strains can produce cellulase yields in the range of 40 g l^{-1} of culture medium and the major cellulase, CBH I, forms about 50% of all protein secreted (Durand et al. 1988). Though large amounts of homologous proteins can be obtained from this organism, the production of heterologous proteins is usually rather low (Conesa et al. 2001; Punt et al. 2002). In view of the importance of fungal protein secretory systems, the low productivity of heterologous proteins has accelerated research into the molecular mechanisms of protein secretion in filamentous fungi (Iwashita 2002). However, our knowledge on the fungal secretion pathway is still at an early stage, which was contributed only by the models developed in yeast and higher eukaryotes (Conesa et al. 2001). Therefore, isolation of the genes involved in protein secretion and characterization of their role and pathways are crucial for fungal molecular biology, which may be approached by genetic mutagenesis analysis.

Though *T. reesei* complete genome has been sequenced recently (Hsiang and Baillie 2004), as a contrast, the functions of large amounts of genes are little known and need to be elucidated. Therefore, developing high-throughput methods to work out the biological function of these genes is of great significance. Genetic approaches, producing a large mutant population by disruption or replacement of genes and then studying the related fungal phenotypes, are powerful tools for deciphering gene function, of which a potential tool is *Agrobacterium*-mediated transformation (AMT) that can perform large-scale random and targeted mutagenesis.

Agrobacterium tumefaciens T-DNA transfer has succeeded not only in natural plant hosts, but also in yeast (Bundock et al. 1995), filamentous fungi (de Groot et al. 1998) and even human cells (Kunik et al. 2001). AMT has

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proven to be a simple and reproducible filamentous fungal transformation method in many instances (Michielse et al. 2005). The decisive advantage of T-DNA insertional mutagenesis over chemical or radiation mutagenesis in fungi is that the mutated genes are tagged by T-DNA, which can then be used to identify the disrupted genes or the flanking sequences (Bundock and Hooykaas 1996; Mullins et al. 2001). Therefore, AMT has been used for insertional mutagenesis as the T-DNA is integrated at random chromosomal sites in the fungi host genome (Combier et al. 2003; Mullins et al. 2001; Sugui et al. 2005). de Groot and colleagues once transformed *T. reesei* using AMT system, but it was a tentative work for *T. reesei* without further analysis (de Groot et al. 1998). Despite that the method is more convenient than the conventional $CaCl_2$/PEG-based scheme, there are no further reports and applications for this in *T. reesei* since then.

In this study, *T. reesei* QM9414 was successfully transformed by applying the AMT method, and a procedure for higher efficient AMT was established. Furthermore, the T-DNA junction fragments were recovered after thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) and the characterization indicated that AMT is a suitable tool for insertional mutagenesis in *T. reesei*.

Materials and methods

Strains and growth conditions

Escherichia coli strain DH5α was used as a host for the propagation of plasmid DNA. *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) was used as a T-DNA donor for maintenance of constructs and for fungal transformation. These bacterial strains were maintained on LB media (Sambrook et al. 1989) at 37°C and YEP media (Walkerpeach and Velten 1994) at 28°C, respectively. *Trichoderma reesei* QM 9414 (ATCC 26921) was used as a recipient, which was sporulated and maintained on potato dextrose agar (PDA) at 28°C. For liquid cultivations, QM 9414 was grown in liquid minimal medium (Ilmen et al. 1997) on a rotary shaker (28°C, 180 rpm).

Construction of the T-DNA binary vector

The T-DNA binary vector pBI-hph was constructed on the backbone of pBIN121 (Chen et al. 2003). First, a 4.1-kb fragment of pAN7-1(Punt et al. 1987), containing the hygromycin B (Hyg) phosphotransferase (*hph*) gene under control of *Aspergillus nidulans gpdA* promoter (P*gpdA*) and *trpC* terminator (T*trpC*), was isolated by digesting pAN7-1 with *Hin*dIII and *Eco*RI. Subsequently, the fragment was introduced to a *Hin*dIII/*Eco*RI site of pBI121. The resultant plasmid carrying the *hph* gene cassette between the right and left borders was named pBI-hph (Fig. 1).

Agrobacterium-mediated fungal transformation

The transformation protocol was a modification of the method developed by de Groot et al. (1998). Protoplasts of *T. reesei* were prepared as described by Penttilä et al. (1987). *T. reesei* conidia were obtained by growing the strain on PDA plate for 5 days and washing the plate gently with a sterile physiologic salt solution. *A. tumefaciens* strain AGL-1, containing the binary vector pBI-hph, was grown at 28°C for 2 days in YEP media supplemented with kanamycin (50 μg/ml) and rifampicin (50 μg/ml). The *A. tumefaciens* cells were diluted to (optical density) OD_{660}=0.15 in induction medium (IM) with the presence or absence of 200 μM acetosyringone (AS). The cells were grown for an additional 6 h before mixing them with an equal volume of a conidial or protoplast suspension from QM9414 (10^5, 10^6, and 10^7 conidia or protoplasts per milliliter). This mix (200 μl per plate) was plated on a 90-mm diameter cellophane sheet and placed on cocultivation medium (same as induction medium except that it contains 20 g agar per litre) in the presence or absence of 200 μM AS.

After incubation at 25°C for 48 h, the cellophane sheet was transferred to select medium (same as minimal medium

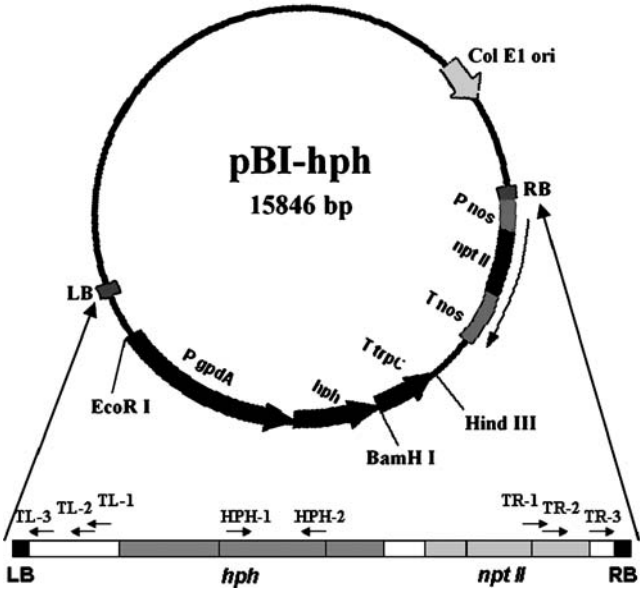

Fig. 1 Organization of binary vector pBI-hph. *hph* and *npt II* correspond to the hygromycin B resistance gene and the neomycin phosphotransferase II gene conferring resistance to kanamycin, respectively. *LB* left border, *RB* right border. *TL-1*, *TL-2*, *TL-3* (at left border) and *TR-1*, *TR-2*, *TR-3* (at right border) are the specific primers employed for TAIL-PCR. HPH-1 and HPH-2 are the primers used for detection of the *hph* gene. *Arrows* above the lower diagram show the position and orientation of the PCR primers. The sequences of the primers are listed in Table 1

except that it contains 1 mol sorbitol and 20 g agar per litre) containing Hyg (150 μg/ml) as a selection agent for transformants and cefotaxime (200 μM) to kill the *A. tumefaciens* cells. Individual transformants were transferred into test tubes containing 3 ml PDA slant media until conidiogenesis. Conidia of individual transformants were suspended with sterile physiologic salt solution. To create monoconidial cultures, one germinating conidium from each transformant was picked and transferred to another PDA slant media. Spores from these monoconidial cultures and individual transformants were stored in 20% glycerol at −70°C until further analysis.

Molecular analysis of transformants

Total fungal genomic DNA was prepared as described by Penttilä et al. (1987). The sequences of the primers used in this study are listed in Table 1 and their positions are shown in Fig 1. PCR detection of the *hph* gene was done using primers HPH-1 and HPH-2, which defined a 937-bp sequence spanning the *hph* gene. The PCR reaction was performed using an automated thermocycler (Whatman Biometra, Gottingen, Germany). PCR conditions included an initial denaturing step of 5 min at 94°C, followed by 35 cycles consisting each of 1 min at 94°C (denaturation), 1 min at 52°C (annealing) and 1 min at 72°C (elongation). A final elongation step consisting of 10 min at 72°C was included. For further Dot blot analysis of transformants, the PCR-amplified 937-bp-long fragment of the *hph* gene was used to detect the hygromycin B phosphotransferase resistance gene. Probes were labeled with the non-radioactive DIG (digoxigenin) method and hybridization procedure was carried out according to kit instructions (Roche Diagnostics, No. 11 585 614 910).

Table 1 Primers used in this study

Primer name	Nucleotide sequence (5′ to 3′)	Melting temperature (°C)
HPH-1	AAGTTCGACAGCGTCTCC	55
HPH-2	TTCCACTATCGGCGAGTA	53
TR-1	TGCGGTTCTGTCAGTTCCAAAC	63
TR-2	AAACGTAAAACGGCTTGTCCC	61
TR-3	ATTGTCGTTTCCCGCCTTCAGT	65
TL-1	CCCGTCTCACTGGTGAAAAGAA	62
TL-2	TGAAAAGAAAAAACCACCCCAG	61
TL-3	GTTATTAAGTTGTCTAAGCGTCA	62
AD-1	GTNCGASWCANAWGTT	Ave. 46
AD-2	NGTCGASWGANAWGAA	Ave. 46
AD-3	WGTGNAGWANCANAGA	Ave. 46

Cloning and sequencing of genomic DNA flanking the T-DNA insert

The thermal asymmetrical interlaced-polymerase chain reaction (TAIL-PCR) protocol was used to clone the genomic DNA fragment flanking inserted T-DNA from *T. reesei* transformants. The specific right border (RB) primers (TR-1, TR-2, and TR-3) and left border (LB) primers (TL-1, TL-2, and TL-3) and three arbitrary degenerate primers (AD-1, AD-2, and AD-3) used (Table 1) were designed as described previously (Liu et al. 1995). The reaction conditions and thermal cycling settings were carried out according to Liu et al. (1995). The tertiary TAIL-PCR product of each transformant showing the highest intensity was purified using Qiagen Gel Extraction kit (Qiagen) and sequenced at Shenggong (Shanghai, China). The innermost specific primer, either RB-3 or LB-3, was used as the sequencing primer. Sequence comparison was performed using the BLAST programs available on the NCBI web site (http://www.ncbi.nlm.nih.gov).

Mitotic stability of transformants

To assess the mitotic stability of Hyg resistance, the transformants was tested by culturing on PDA for five generations in the absence of Hyg. Subsequently, monoconidial cultures that were derived from each transformant after the fifth generation were tested for resistance to Hyg (150 μg/ml).

Results

Hygromycin B sensitivity of *T. reesei*

Growth inhibition of *T. reesei* QM9414 was tested by plating 10^5conidia on minimal medium plate supplemented with Hyg in different concentrations, i.e. 0, 50, 100, 150, 200, and 300 μg/ml. Growth was totally inhibited on medium containing 150 μg Hyg/ml, so this was considered suitable for the selection of resistant colonies in transformation experiments.

Agrobacterium-mediated fungal transformation

Cocultivation of *T. reesei* conidia or protoplasts with *A. tumefaciens* in the presence of AS led to the formation of Hyg-resistant fungal colonies approximately 4 days after transfer to selective medium (Fig. 2). The numbers of Hyg-resistant transformants in different experiments are shown in Table 2. In the absence of AS during cocultivation, no transformants were formed. The transformation frequency was in the range of 200 to 500 transformants per 10^7

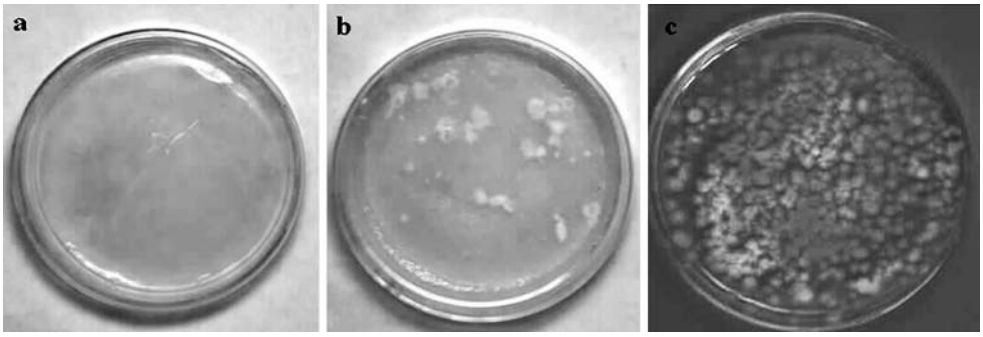

Fig. 2 Selection of putative hygromycin-resistant transformants of *T. reesei*. Some 1×10^5 protoplasts (**b**) and 2×10^6 protoplasts (**c**) were cocultivated with *A. tumefaciens* strain AGL-1 carrying the vector pBI-hph. Protoplasts were also cultivated without that bacterium as the control (**a**). Shown is the appearance of the cultures after 4 days on selective medium with hygromycin at 150 μg/ml

conidia. When protoplasts were used, the transformation frequency varied from approximately 2,000 to 9,000 transformants per 10^7 protoplasts.

An assessment of the mitotic stability of nine randomly selected transformants originating from different transformation experiments showed that they all maintained their Hyg resistance after being sub-cultured for five generations in the absence of Hyg (data not shown).

Confirmation of *hph* gene integration into the fungal genomic DNA

Nine transformants, which had been proved to retain their mitotic stability, were selected and designated HK1-HK9. Genomic DNA from the nine transformants was tested for the presence of the *hph* gene by PCR using specific primers HPH-1 and HPH-2 (Fig. 3a). Furthermore, Dot blot analysis using the DIG-labeled *hph* gene as a probe was carried out for the confirmation of *hph* gene integration into *T. reesei* genome (Fig. 3b). The expected 937-bp PCR products, confirmed by sequencing, and hybridization signals were all detected from the nine transformants, indicating that *hph* gene cassette between the left and right borders of T-DNA integrated into the fungal genome. In contrast, *hph* gene was never detected with untransformed *T. reesei* genomic DNA (Fig. 3).

TAIL-PCR analysis of the T-DNA insertion junctions

To determine the sequences of the junctions between the T-DNA and flanking fungal DNA, TAIL-PCR was performed for the nine transformed fungal genomic DNA. Using T-DNA RB-specific primers along with the degenerate primer AD3, we successfully amplified junction fragments in five out of the nine transformants: HK1, HK2, HK5, HK7, and HK8. The primary PCR reactions yielded a complex amplification pattern, and the number of PCR products was reduced after secondary PCR reactions (data not shown). When the tertiary PCR reactions were performed, a single junction product ranging in size from 0.6 to 0.8 kb was generated (Fig. 4). As for the other four transformations, none of the AD primers used in combination with the three RB-specific primers could amplify RB border junctions. Using the AD1 primer with the LB-specific primers, the sequences flanking the integrated T-DNA junction were successfully cloned from four transformants, which contained HK1, HK2, HK4, and HK5. For the tertiary PCR reactions, a single junction product ranging in size from 1.0 to 1.5 kb was obtained (Fig. 4). Neither AD2 nor AD3 allowed the amplification of the LB junction in all the transformants. Thus, the nine TAIL-PCR products in six out of nine transformants were subsequently sequenced.

Table 2 Hyg-resistant fungal transformants after *Agrobacterium*-mediated transformation of *T. reesei* in three different experiments

Experiment	Medium	No. of protoplasts or conidia	No. of Hyg-resistant transformants	No. of Hyg-resistant transformants per 10^7 recipients
1	−AS	1×10^7 conidia	0	0
	+AS	1×10^7 conidia	220	220
2	−AS	2×10^6 conidia	0	0
	+AS	2×10^6 conidia	103	515
3	−AS	2×10^6 conidia	0	0
	+AS	2×10^6 conidia	89	445
1	−AS	2×10^6 protoplasts	0	0
	+AS	2×10^6 protoplasts	250	2,500
2	−AS	1×10^5 protoplasts	0	0
	+AS	1×10^5 protoplasts	22	2,200
3	−AS	1×10^5 protoplasts	0	0
	+AS	1×10^5 protoplasts	85	8,500

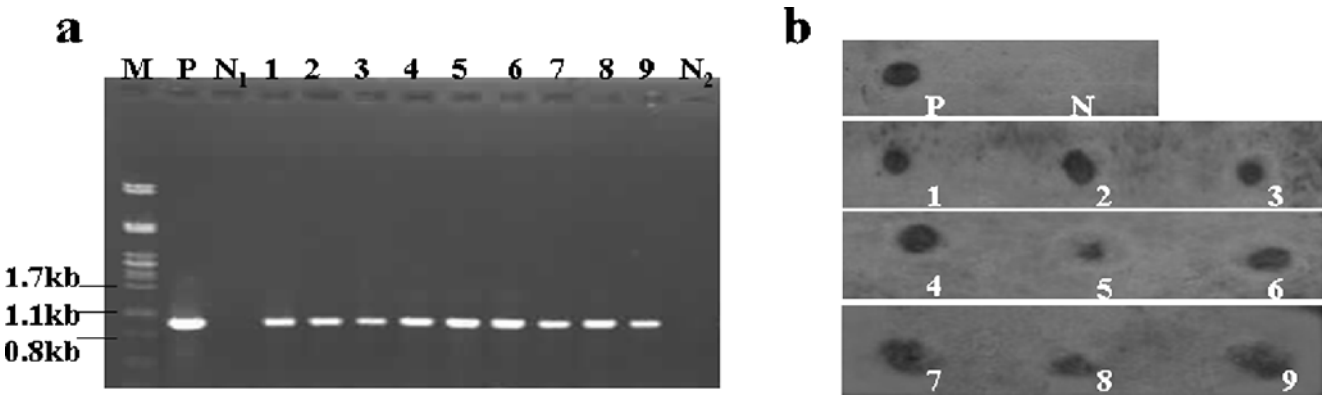

Fig. 3 Molecular analysis of integration of the T-DNA into randomly chosen Hyg-resistant transformants fungal genomic DNA. **a** Polymerase chain reaction assay with primers specific for the amplification of an internal 937-bp fragment of the *hph* gene. Lanes *1–9* genomic DNA isolated from putative transformants HK1-HK9, *P* positive control with vector pBI-hph, N_1 negative control without template, N_2: wild-type *T.reesei* QM9414 genomic DNA, *M* DNA molecular weight markers with kilobases indicated on the left. **b** Dot blot analysis with a DIG-labeled *hph* gene probe. *P* positive control with vector pBI-hph, *N* wild-type *T.reesei* QM9414 genomic DNA, *1–9* genomic DNA isolated from putative transformants HK1-HK9

Characterization of the T-DNA insertion junctions

Sequence analysis of the nine recombination junctions from TAIL-PCR revealed that they contained sequences corresponding to either RB or LB of the T-DNA (Fig. 5). Truncation of the inserted T-DNA was observed at one or both borders, but more frequently at the left than the right, as has been reported in plant, yeast, and other filamentous fungi (Tinland 1996; Bundock and Hooykaas 1996; de Groot et al. 1998). In the two RB junctions (HK1 and HK2) sequenced, the intact border nucleotides were retained. Furthermore, the vector sequences adjacent to RB were found in HK7, which exceeded more than 400 bp in size. This event also occurred in a prior report (Tsuji et al. 2003).

The fungal sequences flanking the inserted T-DNA from six transformants were compared and no homology was found among them. This suggests that T-DNA integration occurs at random positions in *T. reesei* genome by a process of non-homologous recombination. Thus, we may presume that this kind of integration is sequence-independent in a mechanism of illegitimate recombination (IR) mechanism, just as described previously in yeast (Bundock and Hooykaas 1996).Therefore, AMT can be used as a potential tool for random insertional mutagenesis in *T. reesei*.

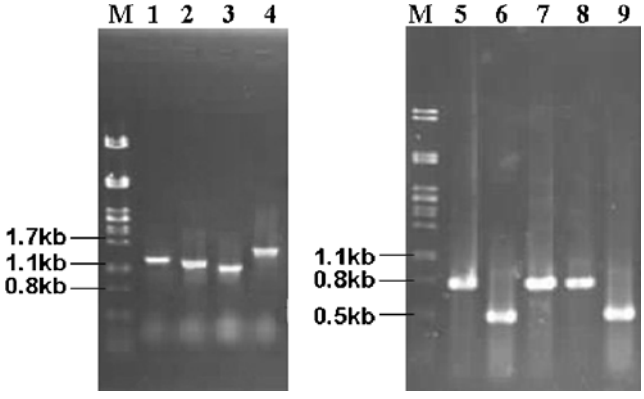

Fig. 4 Agarose gel analysis of TAIL-PCR products generated from the tertiary reactions of the putative transformants. Lanes *1–4* represent the TAIL-PCR products from LB junctions of HK1, HK2, HK4, and HK5, respectively. Lanes *5–9* correspond to those from RB junctions of HK1, HK2, HK5, HK7, and HK8, respectively. *M* DNA molecular weight markers with kilobases indicated on the left

Using the BLASTX algorithm (NCBI, Bethesda, MD, USA) to analyze the possible *T. reesei* genomic sequences flanking T-DNA, the search for homology gave no positive result except HK1. That is, no significant sequence identities were found between the sequences from our eight transformants and the sequences in the GenBank database. Sequences from the transformant HK1 contained a reading frame with significant homology to the *Escherichia coli cbpA* gene, encoding a curved DNA-binding protein, which was an analog of DnaJ functioning as a typical molecular chaperone in a variety of cellular processes (Ueguchi et al. 1994).

Our analysis on T-DNA insertion joint junctions indicates the existence of new unknown sequences at the sites, which excludes the original vector structure and sequence. This strongly suggests that the T-DNA transfer and insertion are successful and the means described in this paper can be a potentially powerful tool for future work on *T. reesei.* Though, unfortunately, the genome database of *T. reesei* is still not available to us, we wish further analysis can be done in the near future or taken by other strategies.

Discussion

In this study, we described the construction of a novel binary vector pBI-hph for insertional mutagenesis, the

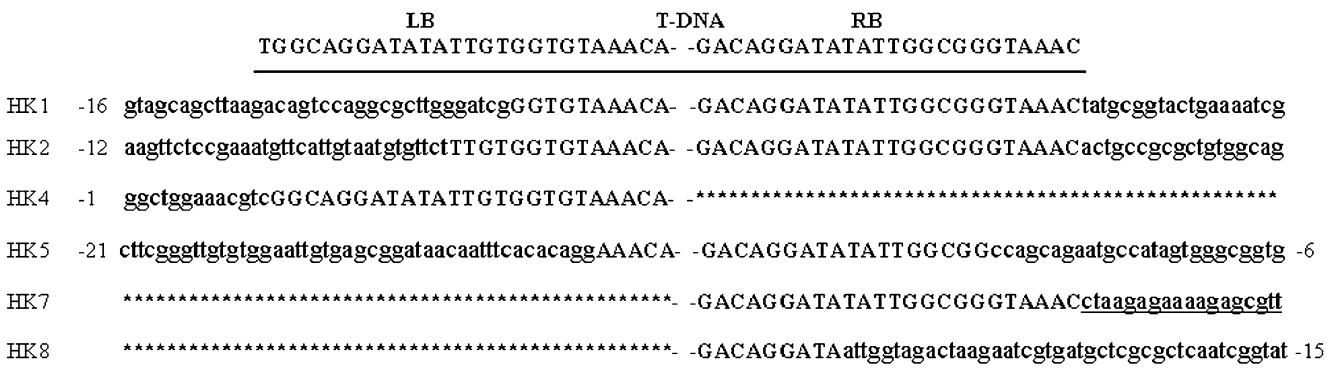

Fig. 5 Sequence analysis of the T-DNA insertion junctions from the AMT transformants. The *top line* shows LB and RB sequences of the T-DNA from binary vector pBI-hph. *Bold uppercase letters* denote nucleotides of the T-DNA borders, while *hyphens* represent the sequences between LB and RB. Flanking genomic sequences are indicated by *lowercase letters*. Sequences from pBI-hph out of the T-DNA zone are *underlined*. Numbers on the *left* and *right* indicate the number of nucleotides truncated from the *left and right borders*, respectively. Sequence data for HK7, HK8 (*left border*) and HK4 (*right border*) were not obtained, and are indicated by *asterisks*. *LB* left border, *RB* right border

appropriate AMT protocol for transformation of *T. reesei* and an efficient TAIL-PCR approach for cloning T-DNA inserts and the flanking genomic DNA sequences.

A *hph* gene expression cassette directly obtained from pAN7-1 was used to select *T. reesei* transformants. This gene cassette has already been proved efficient to be transcribed and translated in *T. reesei* (Penttilä et al. 1987) and other filamentous fungi (Punt et al. 2002). Thus, the binary vector pBI-hph constructed in this study is potentially adapted to a broad large group of filamentous fungi for enhancing the effectiveness of AMT.

Different filters such as nitrocellulose, Hybond, and filter paper have been used in the cocultivation step of various AMT protocols. However, some of the filters containing Hybond C and nitrocellulose led to lower transformation efficiency (Michielse et al. 2005; Vijn and Govers 2003). Though Whatman 3MM filter paper and Hybond N^+ resulted in equally high transformation rates, the cost was high for performing large-scale mutagenesis. In this study, we adopted cellophane sheets as cocultivation filter, which are cheaper than other filters. Our results showed that cellophane sheets could form high transformation rate (Table 2). Furthermore, it is easy to distinguish and screen the resistant colonies from the select media with transparent cellophane sheet (Fig. 2).

In prior reports, it has been shown that protoplasts and conidia can be transformed with similar efficiency by *A. tumefaciens* (de Groot et al. 1998; Zwiers and De Waard 2001). However, this is not always the case. For example, the zygomycetes *Rhizopus oryzae* transformants were only obtained by using protoplasts as recipient (Michielse et al. 2004). In this study, both conidia and protoplasts were successfully used as the starting material, but there is significant difference in transformation frequency between conidia and protoplasts (Table 2). Obtaining protoplasts was time consuming to a certain degree, but their transformation efficiency was more than ten times higher than using conidia. In addition, we found that the selection using conidia as starting material was not strong enough to completely inhibit the growth of untransformed colonies, and it may need subsequent rounds of selection to pass away the pseudo-positive colonies. However, when protoplasts were used for transformation, the colonies grew on the initial selection media could all retain their resistance in the next selection.

Our results showed that *hph* gene had integrated into the *T. reesei* genome by PCR and Dot blot analysis. Moreover, TAIL-PCR successfully rescued the T-DNA borders and the flanking genome sequences from the putative transformants. As for the copy of T-DNA inserts in the fungal genome, it remains to be a further analysis. It was shown that several parameters have an influence on the T-DNA copy number in fungal genome (Michielse et al. 2005). For instance, germinated spores used for AMT led to mostly multi-copy transformants, whereas mycelium formed predominantly single-copy transformants (Mikosch et al. 2001). Acetosyringone induction before cocultivation has been shown to result in either a decrease or an increase in single-copy T-DNA integration (Combier et al. 2003; Mullins et al. 2001). It may be possible to change fungal starting material or the cocultivation conditions to form the single-copy T-DNA events in fungal genome. Therefore, we plan to perform various experiments to obtain single integration in *T. reesei* genome for further study.

Compared with REMI (restriction-enzyme-mediated integration) used as an important insertional mutagenesis method for filamentous fungi, AMT has no main drawbacks of REMI, such as untagged mutation, unpredictable deletion of an inserted marker DNA, or rearrangement of a chromosomal DNA, which often restricts recovery of the

tagged genes from the mutants (Kahmann and Basse 1999). Therefore, T-DNA insertion mutagenesis has become a promising tool for isolation of new genes and the study of their function in filamentous fungi. Moreover, after the protocol optimized for *T. reesei*, it is possible to create a complete collection of insertional mutants, which can be analyzed both phenotypically and genotypically. Thus, our report of a reproducible AMT protocol would promote the development of T-DNA tagging as an efficient tool for transformation, random insertional mutagenesis, and gene disruption in this valuable industrial fungus.

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References

Bundock P, den Dulk-Ras A, Beijersbergen A, Hooykaas PJJ (1995) Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. EMBO J 14:3206–3214

Bundock P, Hooykaas PJJ (1996) Integration of *Agrobacterium tumefaciens* T-DNA in the *Saccharomyces cerevisiae* genome by illegitimate recombination. Proc Natl Acad Sci USA 93:15272–152751

Chen PY, Wang CK, Soong SC, To KY (2003) Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. Mol Breed 11:287–293

Combier JP, Melayah D, Raffier C, Gay G, Marmeisse R (2003) *Agrobacterium tumefaciens*-mediated transformation as a tool for insertional mutagenesis in the symbiotic ectomycorrhizal fungus *Hebeloma cylindrosporum*. FEMS Microbiol Lett 220:141–148

Conesa A, Punt PJ, van Luijk N, van den Hondel CA (2001) The secretion pathway of filamentous fungi: a biotechnological view. Fungal Genet Biol 33:155–171

de Groot MJA, Bundock P, Hooykaas PJJ, Beijersbergen AGM (1998) *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi. Nat Biotechnol 16:839–842

Durand H, Clanet M, Tiraby G (1988) Genetic improvement of *Trichoderma reesei* for large scale cellulase production. Enzyme Microb Technol 10:341–345

Hsiang T, Baillie DL (2004) Recent progress, developments, and issues in comparative fungal genomics. Can J Plant Pathol 26:19–30

Ilmen P, Saloheimo A, Onnela M, Pnettilä M (1997) Regulation of cellulase expression in the filamentous fungus *Trichoderma reesei.* Appl Environ Microbiol 63:1298–1306

Iwashita K (2002) Recent studied of protein secretion by filamentous fungi. J Biosci Bioeng 94:530–535

Kahmann R, Basse C (1999) REMI (restriction enzyme mediated integration) and its impact on the isolation of pathogenicity genes in fungi attacking plants. Eur J Plant Pathol 105:221–229

Kunik T, Tzfira T, Kapulnik Y, Gafni Y, Dingwall C, Citovsky V (2001) Genetic transformation of HeLa cells by *Agrobacterium*. Proc Natl Acad Sci USA 98:1871–1876

Lazo GR, Stein PA, Ludwig RA (1991) A DNA transformation competent *Arabidopsis* genomic library in *Agrobacterium*. Bio/Technology 9:963–967

Liu YG, Mitsukawa LN, Oosumi T, Whittier RF (1995) Efficient isolation and mapping of *Arabidopis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J 8:457–463

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

Michielse CB, Salim K, Ragas P, Ram AF, Kudla B, Jarry B, Punt PJ, van den Hondel CA (2004) Development of a system for integrative and stable transformation of the zygomycete *Rhizopus oryzae* by *Agrobacterium*-mediated DNA transfer. Mol Genet Genomics 271:499–510

Mikosch TS, Lavrijssen B, Sonnenberg AS, van Griensven LJ (2001) Transformation of the cultivated mushroom *Agaricus bisporus* (Lange) using T-DNA from *Agrobacterium tumefaciens*. Curr Genet 39:35–39

Mullins ED, Chen X, Romaine P, Raina R, Geiser DM, Kang S (2001) *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: an efficient tool for insertional mutagenesis and gene transfer. Phytopathology 91:173–180

Penttilä M (1998) Heterologous protein production in *Trichoderma*. In: Kubicek CP, Harman GE (eds) *Trichoderma* and *Gliocladium*, vol 2. Taylor & Francis, London, United Kingdom, pp 365–382

Penttilä M, Nevalainenb H, Rättöa M, Salminenb E, Knowles J (1987) A versatile transformation system for the filamentous fungus *Trichoderma reesei.* Gene 61:155–164

Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, van den Honde CA (1987) Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 56:117–124

Punt PJ, van Biezen N, Conesa A, Albers A, Mangnus J, van den Hondel C (2002) Filamentous fungi as cell factories for heterologous protein production. Trends Biotechnol 20:200–206

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Sugui JA, Chang YC, Kwon-Chung KJ (2005) *Agrobacterium tumefaciens*-mediated transformation of *Aspergillus fumigatus*: an efficient tool for insertional mutagenesis and targeted gene disruption. Appl Environ Microbiol 71:1798–1802

Tinland B (1996) The integration of T-DNA into plant genomes. Trends Plant Sci 1:178–184

Tsuji G, Fujii S, Fujihara N, Hirose C, Tsuge S, Shiraishi T, Kubo Y (2003) *Agrobacterium tumefaciens*-mediated transformation for random insertional mutagenesis in *Colletotrichum lagenarium*. J Gen Plant Pathol 69:230–239

Ueguchi C, Kakeda M, Yamada H, Mizuno T (1994) An analogue of the DnaJ molecular chaperone in *Escherichia coli*. Proc Natl Acad Sci USA 91:1054–1058

Vijn I, Govers F (2003) *Agrobacterium tumefaciens* mediated transformation of the oomycete plant pathogen *Phytophthora infestans*. Mol Plant Pathol 4:459–467

Walkerpeach CR, Velten J (1994) *Agrobacterium*-mediated gene transfer to plant cells: cointegrate and binary vector systems. In: Gelvin SB, Schilperoort RA (eds) Plant molecular biology manual B1. Kluwer, Dordrecht, pp 1–19

Zwiers LH, De Waard MA (2001) Efficient *Agrobacterium tumefaciens*-mediated gene disruption in the phytopathogen *Mycosphaerella graminicola*. Curr Genet 39:388–393