INTRODUCTION

Fungi are capable of producing a variety of secondary metabolites (SMs), which have played pivotal roles in drug discovery and development derived from their diverse bioactivities [1]. However, a number of natural products remain to be discovered because most biosynthetic gene clusters (BGCs) are silent under lab conditions. Genetic engineering offers a practical route to activate those BGCs. For example, many cryptic gene clusters have been activated by heterologous expression approaches and manipulations of global transcription factors, epigenetic regulators or regulators involved in fungal development [2–8]. Therefore, an efficient genetic transformation system is required for studying or finding novel natural products in fungi.

Species in the Trichoderma genus inhabit diverse environments and are prolific producers of SMs. Trichoderma spp. are widely used as commercial biocontrol reagents in agriculture [9]. They produce a diverse range of metabolites that not only contribute to signalling but also interact with other organisms in various ways. For example, viridepyrone, massolactone and decanolactone exhibited good biocontrol activities [10–12], and trichocaranes and harzianolide acted as growth regulators [13, 14]. In addition, Trichoderma spp. are also used for cellulose production in industry [15]. In search of rich metabolite producing fungi, we characterized a species, T. hypoxylon, which was previously isolated from the surface of the stroma of Hypoxylon anthochroum [16]. Chemical analysis of this strain identified six trichothecene polyunsaturated octadioic acid esters, named trichodermates A–F [17]. Recently, trichothecenes have attracted attention because they are sesquiterpenoid mycotoxins with detrimental effects on plants and animals [18]. Considering the rich metabolites produced by T. hypoxylon, we sequenced this strain and found 62 BGCs in the genome, indicating its potential ability to produce SMs. However, no genetic transformation method is available for T. hypoxylon. Currently, the reports regarding the Trichoderma transformation strategy mostly focus on the cellulolytic fungus T. reesei. In common cases, the targeted genes are integrated...
randomly into the genome of *Trichoderma* spp., which complicates gene deletion by homologous recombination [19].

Ongoing attempts have been made to increase the number of available marker genes, e.g. *hph* (hygromycin B resistance) and neomycin phosphotransferase gene (*neo*) (G418 resistance). It is worth noting that uridine/uracil (UU) auxotrophy has been widely exploited and proved to be efficient [20–22]. *pyrG* in *Aspergillus* (*ura3* in *Mortierella*) encodes an orotidine 5’-monophosphate (OMP) decarboxylase, which can convert exogenous 5’-fluoroorotic acid (5-FOA) to the toxic intermediate. Therefore, the mutants with loss of *pyrG* can survive on 5-FOA-containing media supplemented with exogenous UU. In other words, *pyrG* can be used as a marker gene for complementation of UU auxotrophy. In addition, efforts have been made to block non-homologous end-joining (NHEJ) at the homologous site. Ku70/Ku80 and Lig4, the two factors involved in the NHEJ, have been well-studied. In *Neurospora crassa*, the orthologues of human Ku70 and Ku80, MUS-51 and MUS-52, have been deleted, which led to nearly 90% of transformants exhibiting successful homologous recombination [23]. Another enzyme, MUS-53 (homologue of human Lig4), was reported to be involved in the final step of NHEJ. This indicated that Lig4 was an alternative to MUS-51 and MUS-52 in some species [24, 25].

In this study, we reported the construction of a transformation system using a UU auxotrophic strain and an exogenous *AfpyrG* gene. To further improve the homologous integration efficiency, an NHEJ gene, *thlig4*, was deleted and proved to act as the key function in this process. Finally, the harzianum B biosynthetic cluster was identified in *T. hypoxylon* using this system. These data indicate that efficient genetic vehicles have been established for *T. hypoxylon*. The establishment of a highly efficient genetic system in *T. hypoxylon* will provide a convenient method for mining the biosynthetic diversity in this fungus.

### METHODS

#### Accession number

Sequences used in this study have been deposited in the GenBank database. Accession numbers of the *T. hypoxylon* genes are as follows: MF504019.1 for *thlig4*, MF504018.1 for *thku70*, MF504025 for *thtri5*, MF600466 for *thtri4*, MF600467 for *thtri6*, MF600469 for *thtri10*, MF600470 for *thtri11*, MF600471 for *thtri12* and MF600472 for *thtri14*.

#### Strains, media and culture conditions

*Trichoderma hypoxylon*, deposited in the China General Microbiological Culture Collection Center with the strain number CGMCC 3.17906, was used for genetic system creation [16]. *T. hypoxylon* and its transformants were routinely maintained at 25°C on potato dextrose agar (PDA) or potato dextrose broth (PDB) in the presence of appropriate antibiotics as required (Table 1). *Escherichia coli* DH5α was propagated in Luria-Bertani (LB) medium with appropriate antibiotics for plasmid DNA isolation.

#### Fungal sensitivity test

G418 sensitivity was determined by plating the strain on PDA medium supplemented with different concentrations of G418 (FG401-01, TransGen Biotech) (20, 30, 40, 50, 60 and 700 μg ml⁻¹). Hygromycin B sensitivity was also determined on PDA medium with different concentrations of hygromycin B (K547, Amresco) (20, 30, 40, 50, 60 and 70 μg ml⁻¹). Control plates without antibiotics were prepared. Mycelia from the tested strain were inoculated on each plate and incubated for 7 days at 25°C.

#### Deletion cassette construction and genetic manipulation

The plasmids used in this study are listed in Table 1. The oligonucleotide sequences for PCR amplification primers are given in Table 2. For the creation of deletion strains

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichoderma hypoxylon</em></td>
<td>WT (CGMCC 3.17906)</td>
<td>[16]</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>WT</td>
<td>[51]</td>
</tr>
<tr>
<td>TYHL26</td>
<td>Δthtri5::neo</td>
<td>This study</td>
</tr>
<tr>
<td>TYHL44</td>
<td>Δthlig4::hph</td>
<td>This study</td>
</tr>
<tr>
<td>TYHL45</td>
<td>Δthtri5::neo Δthlig4::hph</td>
<td>This study</td>
</tr>
<tr>
<td>TYWG2</td>
<td>Δpyr4</td>
<td>This study</td>
</tr>
<tr>
<td>TYWG3</td>
<td>Δku70::AfpyrG*</td>
<td>This study</td>
</tr>
<tr>
<td>TYWG4</td>
<td>Δthlig4::hph Δpyr4</td>
<td>This study</td>
</tr>
<tr>
<td>TYHL46</td>
<td>Δthtri5::neo Δku70::pyr4*</td>
<td>This study</td>
</tr>
<tr>
<td>pYW142</td>
<td>neo in pEasy-Blunt</td>
<td>[7]</td>
</tr>
<tr>
<td>pUCH2-8</td>
<td>hph gene</td>
<td>[52]</td>
</tr>
<tr>
<td>pYHL41</td>
<td><em>thtri5</em> deletion cassette containing the <em>neo</em> selectable marker</td>
<td>This study</td>
</tr>
<tr>
<td>pYHL68</td>
<td><em>thlig4</em> deletion cassette containing the <em>neo</em> selectable marker</td>
<td>This study</td>
</tr>
</tbody>
</table>

pXX, plasmid. TXX, original transformant.
of thtri5 and thlig4, two steps were carried out. First, the deletion cassettes of thtri5 and thlig4 were constructed using a modified quick-change method [26]. The plasmids pYW42 and pUCH2-8, which contained the neomycin phosphotransferase gene (neo) and hygromycin phosphotransferase gene (hph), respectively, were used. Approximately 1.2 kb fragments upstream and downstream of the target genes thtri5 and thlig4 were amplified from genomic DNA of T. hypoxylon using designated primers (Table 2). These four amplified PCR fragments were purified with an EasyPure Quick Gel Extraction Kit (Transgene Biotech), quantified, and assembled to yield the plasmids pYHL41 and pYHL68 (Table 1). Second, the split marker fragments were amplified from pYHL41 and pYHL68 using the primers in Table 2. All plasmids were confirmed by restriction enzyme digestion. Deletion of thpyr4 was accomplished by a double homologous gene replacement cassette containing the upstream and downstream fragments of the target gene thpyr4 generated by the Fusion PCR method described previously [27]. Fusion PCR was also used to quickly obtain the deletion cassette for thku70 containing the AfpyrG marker. Genomic DNA was extracted according to the protocol described previously [28]. The selected mutants were tested by diagnostic PCR with primers inside and outside the corresponding gene, as shown in Fig. 2.

Table 2. PCR primer sets utilized in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide sequence (5’–3’)</th>
<th>Uses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lig4_up-F</td>
<td>CTATAGGCGGAAATTTGGAGCTCCACCGGcacatgcccacatctcaac</td>
<td>Up flanks' amplification for quick change</td>
</tr>
<tr>
<td>Lig4_up-R</td>
<td>GATCCACTAGTCTAGGACGCGCCACCGGctgctgctcaccacaaatg</td>
<td>Down flanks' amplification for quick change</td>
</tr>
<tr>
<td>Lig4_down-F</td>
<td>GAGCCGGAAGCATATAAGTGAAGCTGgcctgccaggtataatcagg</td>
<td>Down flanks' amplification for quick change</td>
</tr>
<tr>
<td>Lig4_down-R</td>
<td>GAGGTTAGTCATCATTAGGCAACCGgctcaggaaccaactacg</td>
<td>Up flanks' amplification</td>
</tr>
<tr>
<td>Lig4_target-F</td>
<td>CCATCTTTCAGCATGCAGGC</td>
<td>Δthlig4 transformant screening</td>
</tr>
<tr>
<td>Lig4_target-R</td>
<td>GAAGCGGAGATCATGTACG</td>
<td>Δthlig4 transformant up screening</td>
</tr>
<tr>
<td>Hyg_ch-F</td>
<td>CCATTAGATCCTATCAGTCAGGC</td>
<td>Δthlig4 transformant down screening</td>
</tr>
<tr>
<td>Hyg_ch-R</td>
<td>GCCTATGCAGCTACAGGC</td>
<td>Δthlig4 transformant up screening</td>
</tr>
<tr>
<td>Thri5_up-F</td>
<td>GCTATGACCATGATTACGCCAAGCCGcgcattcctccgaactgtc</td>
<td>Deletion pyr4 fragment amplification</td>
</tr>
<tr>
<td>Thri5_up-R</td>
<td>CGTTACTAGTGGACGCTCCGTAACG</td>
<td>Down flanks' amplification for fusion PCR</td>
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<td>Thri5_down-F</td>
<td>GTTCTATTCTCTATTGAGTAGC</td>
<td>Up flanks' amplification</td>
</tr>
<tr>
<td>Thri5_down-R</td>
<td>GCAGCTCCGCCAGGAACAGGAAG</td>
<td>Up flanks' amplification</td>
</tr>
<tr>
<td>Thri5_target-F</td>
<td>CCATCAAGACCATTTGGTCG</td>
<td>Δthtri5 transformant target screening</td>
</tr>
<tr>
<td>Thri5_target-R</td>
<td>GCCAGGCAAATGAGATGGG</td>
<td>Δthtri5 transformant up screening</td>
</tr>
<tr>
<td>Thri5_ch-F</td>
<td>GCGGACCATGAGATGGG</td>
<td>Δthtri5 transformant down screening</td>
</tr>
<tr>
<td>Thri5_ch-R</td>
<td>CGTTACAGGAAATGGAGG</td>
<td>Δthtri5 transformant up screening</td>
</tr>
<tr>
<td>Pyr4-check-F</td>
<td>TTCAACTGCGAGATGGG</td>
<td>Up flanks' amplification for fusion PCR</td>
</tr>
<tr>
<td>Pyr4-up-R</td>
<td>AGAAGTAAAAGGGACACACCATGGAAGGCTGAGAAAAAAAGAACACAAT</td>
<td>Down flanks' amplification for fusion PCR</td>
</tr>
<tr>
<td>Pyr4-down-F</td>
<td>ATTTTCGTCGTCACATGCTCCTCCCGAGTGTGCGCTTTAGCGTCT</td>
<td>Deletion pyr4 fragment amplification</td>
</tr>
<tr>
<td>Pyr4-check-R</td>
<td>CTTCCGAGATGGAGATGGG</td>
<td>Up flanks' amplification</td>
</tr>
<tr>
<td>Pyr4-ch-F</td>
<td>CGATCCGAGATGGAGATGGG</td>
<td>Up flanks' amplification</td>
</tr>
<tr>
<td>Pyr4-ch-R</td>
<td>CAGCTCCGAGATGGAGATGGG</td>
<td>Deletion thku70 fragment amplification</td>
</tr>
<tr>
<td>Ku70_up-F</td>
<td>ATTTCGTCATGACGAGAGG</td>
<td>Δthku70 transformant up screening</td>
</tr>
<tr>
<td>Ku70_down-F</td>
<td>TCTCGAGATGGAGATGGG</td>
<td>Δthku70 transformant down screening</td>
</tr>
<tr>
<td>Ku70_target-F</td>
<td>CCATCAAGACCATTTGGTCG</td>
<td>Δthku70 transformant target screening</td>
</tr>
<tr>
<td>Ku70_target-R</td>
<td>GCCAGGAAATGAGATGGG</td>
<td>Δthku70 transformant target screening</td>
</tr>
</tbody>
</table>

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Protoplast preparation
To select an efficient enzyme for protoplast preparation, we treated *T. hypoxylon* mycelia with lysing enzymes (Sigma), Yatalase (Takara) and Vinotaste (Novozymes) and found that lysing enzymes can yield the maximum amount of protoplasts. While investigating the effect of lysing enzyme concentration on the yield of protoplasts, 15 mg ml\(^{-1}\) was found to be the best.

*T. hypoxylon* grown on PDA medium with glass paper for 3 days was easily harvested and ground by Tissuelyser with 700 µl of PDB media and two steel beads and then transferred into 100 ml of PDB medium in 250 ml flasks and further incubated at 28 °C for 24 h (200 r.p.m. min\(^{-1}\)). The mycelia were harvested and ground one more time, then transferred into 100 ml of TG medium (tryptone 10 g l\(^{-1}\), glucose 100 g l\(^{-1}\)) in 250 ml flasks and further incubated at 28 °C for up to 12 h (200 r.p.m. min\(^{-1}\)). The mycelia were harvested by filtration and washed with N-M solution (0.3 M NaCl, 0.3 M MgSO\(_4\), 10 mM Tris-HCl, pH=7.0). The harvested mycelia were transferred to a sterile 50 ml flask. Lysing solution (lysing enzymes 15 mg ml\(^{-1}\), dissolved in 10 ml N-M solution) was added to the flask, and the mixture was gently shaken at 28 °C for 8 h (100 r.p.m. min\(^{-1}\)). The quality of the protoplasts was examined under a microscope every 1 h. Protoplasts were purified by filtration through a sterile Miracloth (Merck), collected at 4000 g for 15 min at 4 °C and washed twice with STC solution (1.2 M sorbitol, 10 mM Tris-HCl, pH=7.5, 50 mM CaCl\(_2\)). Finally, the protoplasts were suspended in STC buffer at a concentration of 10\(^7\)–10\(^8\) ml\(^{-1}\).

Transformation of *T. hypoxylon*
Approximately 1.0×10\(^7\) protoplasts in 100 µl STC buffer were mixed with 1 µg of purified DNA fragment in a 15 ml Falcon tube and chilled on ice for 50 min. Then, 1.25 ml of 60 % PEG (PEG 6000 0.6 g ml\(^{-1}\), CaCl\(_2\)-2H\(_2\)O 7.35 mg ml\(^{-1}\), 10 mM Tris-HCl, pH=7.5) was added to the mixture and swirled gently, followed by incubation at room temperature for 30 min. Approximately 5 ml of STC solution was added to the transformation mixture and mixed gently; 1 ml aliquots were spread on every bottom PDAS (PDA supported with 1.2 M sorbitol) plate containing 60 µg ml\(^{-1}\) hygromycin B. Then, 5 ml of TOP PDAS medium (PDB supported with 1.2 M sorbitol and 0.8 % agar) containing half the amount of hygromycin B was used to overlay the bottom plate [29]. Transformants were transferred to PDA plates containing hygromycin B (60 µg ml\(^{-1}\)) and subcultured to ensure hereditary stability. A 0.5 cm mycelial plug was transferred to a 60 mm plate containing 4 ml of PDB liquid medium for genomic DNA extraction.

Secondary metabolite analysis
All strains were cultivated on solid PDA media at 25 °C for 7 days. The PDA culture was cut into pieces and then extracted with ethyl acetate. The extracts were evaporated under reduced pressure to yield residues, which were suspended in 1 ml of MeOH. Analytical HPLC analysis was conducted with a Waters HPLC system (Waters e2695, Waters 2998, Photodiode Array Detector) using an ODS column (C18, 250×4.6 mm, waters Pak, 5 µm) with a flow rate of 1 ml min\(^{-1}\). Fresh extracts from WT and mutants were analysed for 30 min using a linear gradient of 20–100 % MeOH (0–20 min), 100 % MeOH (20–25 min) and 20 % MeOH (25–30 min).

RESULTS
Maker gene selection and protoplast preparation
Hygromycin B and G418 were used to evaluate the sensitivity of *T. hypoxylon*. The concentrations for complete growth inhibition of *T. hypoxylon* were found at 60 µg ml\(^{-1}\) hygromycin B and 500 µg ml\(^{-1}\) G418, respectively (Fig. 1). To construct the UU auxotrophic strains, *T. hypoxylon* sensitivity was tested on 5-FOA, and the mycelia were completely inhibited at a concentration of 200 µg ml\(^{-1}\). The strain could be restored for growth when UU (0.5 mg ml\(^{-1}\), respectively) was added to the media (Fig. 1). Based on the sensitivities to hygromycin B, G418 and 5-FOA, the *hph* gene and *neo* gene were chosen as the marker genes, and 5-FOA can be used to construct UU auxotrophy. To prepare protoplasts, mycelia were cultivated at different times (10, 14, 18, 20 and 24 h) and treated with lysing enzymes (1–10 h) for digestion. The optimal mycelial age was found to be 20 h, which yielded approximately 5×10\(^7\) ml\(^{-1}\) protoplasts. For the enzymatic digestion time, 8 h was observed to produce the highest quality of protoplasts for all culture ages.

Identification of *thpyr4*, *thku70* and *thlig4* homologous genes in *T. hypoxylon*
To find the homologous genes of *pyr4*, *ku70* and *lig4* from the *T. hypoxylon* genome, the protein sequences of *Pyrg*, *Ku70* and *Lig4* from model fungi, including *Aspergillus nidulans*, *A. oryzae* and *Neurospora crassa*, were used as queries for BLAST searches. The predicted *T. hypoxylon* THPYR4 protein consists of 377 amino acids and shares high sequence identities to *pyr4* from other ascomycetes (32 % in *A. nidulans*, 92 % in *T. harzianum*). The *T. hypoxylon* THKU70 sequence shares 97.7 % identity with *A. oryzae* and *N. crassa* Ku70. The search revealed that *T. hypoxylon* possesses a single THLIG4 homologous sequence consisting of 1107 amino acid residues that shows 26 % identity to *Saccharomyces cerevisiae* Dnl4, 60 % to *N. crassa* Mus-53 and 53 % to *A. oryzae* LigD at the amino acid level [21, 30, 31].

Creation and characterization of the UU auxotrophic mutant
To generate UU auxotrophic mutants, the *thpyr4* deletion cassette was constructed by the Fusion PCR method and transformed into the protoplasts of *T. hypoxylon* according to the homologous recombination strategy (Fig. 2). As expected, the WT strain was sensitive to 5-FOA, whereas the Δ*thpyr4* strain was resistant to 5-FOA in the presence of UU. The transformants were selected on selective PDA medium supplemented with 0.5 % uridine, 0.5 % uracil and 200 µg ml\(^{-1}\) of 5-FOA (Fig. 1). To verify these mutants, genomic DNA was extracted,
and diagnostic PCR was performed using the designated primer. The correct mutants showed a band of 2.5 kb, implying that they carried a 1.7 kb fragment of the thpyr4 gene, while the WT strain showed a 4.2 kb band (Fig. 2c). The results demonstrated that the UU auxotrophic mutant deletion of thpyr4 in T. hypoxylon was successfully created. The efficiency of homologous recombination was approximately 30%. Then, we examined the growth rates of deletion mutants, and no significant growth differences were found between the WT strain and the mutant. By HPLC (high-performance liquid chromatography) analysis of the extracts from the WT strain and mutant, the results showed that the chemical profiles were similar, indicating that these mutants can be used for the following study (Fig. 4).

**Disruption of the NHEJ factors thku70 and thlig4 in T. hypoxylon**

To improve the transformation efficiency, the gene thku70 involved in the NHEJ pathway was deleted in the T. hypoxylon Δthpyr4 strain. To use pyrG as the auxotrophic marker, exogenous AfpyrG encoding OMP decarboxylase was amplified from the genomic DNA of A. fumigatus by PCR. The Δthku70 deletion cassette was then constructed, where the entire ORF was replaced by the AfpyrG cassette with thku70 (Fig. 2b). Three transformants were selected on plates without adding UU. To confirm the correct integration, the mutants were screened by PCR as shown in Fig. 2(c). The results indicated that the Δthku70 strains were successfully created and possessed a restriction pattern that is consistent with replacement of thku70 by AfpyrG at the genomic locus. However, the efficiency of homologous recombination was approximately 30% and the same as the WT strain, indicating that thku70 is not working here. Exogenous AfpyrG functions as an auxotrophic marker in transformation.

Next, we selected another gene coding for DNA ligase IV for deletion. Lig4 has been reported to improve homologous integration in N. crassa, Magnaporthe grisea and Penicillium marneffei [25, 32, 33]. To delete thlig4 in T. hypoxylon, a Δthlig4 :: hph deletion plasmid was constructed, where the entire thlig4 ORF was replaced by the hph gene (Fig. 2). The transformants were selected with hygromycin B, and diagnostic PCR was conducted for transformant confirmation (Fig. 2). Nine transformants were screened, and eight transformants showed that the thlig4 gene was disrupted, and the gene target efficiency was 89%. These results suggested that deletion of the thlig4 gene greatly improved the

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*Fig. 1. Fungal sensitivity test of T. hypoxylon WT and mutants. All strains were cultivated on PDA for 4 days at 25 °C.*
transformation efficiency. The mutants thku70 and thlig4 showed no notable changes in growth rate and chemical profiles compared with the WT.

**Transformation rate estimation of the Δthku70 and Δthlig4 strains**

To evaluate the effect of thku70 and thlig4 on homologous recombination, the thtri5 gene was selected for deletion in the WT, Δthku70 and Δthlig4 backgrounds. The G418 resistance gene was chosen as a selection marker. The Tri5 gene encodes trichodiene synthase, which catalyses the first step in trichothecene biosynthesis. After transformation, the transformants were screened by diagnostic PCR. The frequency of gene targeting was found at 35 % and 28 % in the Δthku70 strain and the WT, while the transformation rate was 89 % in the Δthlig4 strains (Fig. 3). These results indicate that deletion of the thlig4 gene in *T. hypoxylon* significantly increases gene targeting frequency.

**Thtri5 is responsible for harzianum B biosynthesis**

As described previously, *T. hypoxylon* can produce abundant SMs, harzianum B and trichoderamides as the major compounds [16, 17]. To identify the function of the thtri5 gene in trichothecene biosynthesis in *T. hypoxylon*, thtri5 disruption mutants and WT strains were cultured on PDA plates for 7 days at 25 °C for HPLC analysis. The metabolite profiles of Δthtri5 and Δthlig4Δthtri5 mutants showed significant changes with the complete abolishment of harzianum B compared to the WT. There were no clear differences in SMs between the Δthtri5 strain and the Δthlig4Δthtri5 strain (Fig. 4). Therefore, thtri5 encoding trichodiene synthase was confirmed to be involved in harzianum B biosynthesis.

**tri cluster analysis**

Compounds in the trichothecene family demonstrated a variety of structures because the gene organizations in the clusters are different. Comparative analysis of the *tri* cluster was conducted to further explore this biosynthetic pathway. A phylogenetic tree was constructed after alignment with other TRIs from ascomycetous fungi. All of the homologous proteins shared over 50 % identity with *T. hypoxylon*. As shown in Fig. 5(a), TRIs are mainly distributed in Hypocreales, Eurotiales (*Penicillium digitatum*, *A. niger*) and Glomerellales (*Colletotrichum simmondsii*). TRIs from *T. arundinaceum* and *T. brevicompactum* clustered most tightly with *T. hypoxylon*, which showed uniform organizations in the *tri* gene cluster. In *Trichoderma* species, the gene *thtri5* is located outside the cluster while it is located inside the cluster in *Fusarium*, *Stachybotrys* and *Cordyceps* (Fig. 5b). Some genes are conserved in the cluster, e.g. *thtri3*, *thtri4* and *thtri6* demonstrated similar patterns in most species, while most genes in the cluster have been rearranged in the process of evolution (Fig. 5b). The *tri* biosynthesis gene

**Fig. 2.** Schematic illustration of deletion and confirmation of *T. hypoxylon* genes. (a) Scheme of the destruction of the thpyr4 locus in the parental strain *T. hypoxylon* by homologous recombination yielding a thpyr4 deletion strain (Δthpyr4). (b) Strategy for homologous recombination of *T. hypoxylon* for thku70 gene disruption using the A. fumigatus AfpyrG gene as a selectable marker. hph and neo genes were chosen as selectable markers for deleting the thlig4 and thtri5 genes respectively. (c) Diagnostic PCR to identify (A) the Δthpyr4 deletion mutant with primer pairs pyr4-check F/pyr4-check R; (B) Δthku70 deletion mutants with primer pairs targetF/targetR (lanes 1 and 4), upcheckF/upcheckR (lanes 2 and 5) and downcheckF/downcheckR (lanes 3 and 6); (C) Δthlig4 deletion mutants with primer pairs targetF/targetR (lanes 1 and 4), upcheckF/upcheckR (lanes 2 and 5) and downcheckF/downcheckR (lanes 3 and 6); (D) Δthtri5 deletion mutants with primer pairs targetF/targetR (lanes 1 and 4), upcheckF/upcheckR (lanes 2 and 5) and downcheckF/downcheckR (lanes 3 and 6); (E) Δthpyr4Δthlig4 double deletion mutants with primer pairs targetF/targetR (lanes 1 and 4), upcheckF/upcheckR (lanes 2 and 5), downcheckF/downcheckR (lanes 3 and 6) and pyr4-check F/pyr4-check R (lanes 7 and 8); (F) Δthku70Δthtri5 double deletion mutants with primer pairs targetF/targetR (lanes 1 and 4; lanes 7 and 10), upcheckF/upcheckR (lanes 2 and 5; lanes 8 and 11), downcheckF/downcheckR (lanes 3 and 6; lanes 9 and 12).
cluster in *T. hypoxylon* has the same gene distributions as those in *T. arundinaceum* and *T. brevicompactum*, while their products are structurally different. The encoding product of the *tri* cluster in *T. hypoxylon* is *harzianum* B, while it is *harzianum* A in the other two species, which have a cis-trans isomerism in the long fat chain [17, 34, 35].

**DISCUSSION**

*Trichoderma* spp. are rich sources for producing SMs and are widely used in agriculture as biopesticides. They are also the sources of enzymes in industry [36–39]. *T. hypoxylon*, a recently reported species that exhibited abundant SM profiles, remains exploited extensively. Genetic manipulation has played a major role in the mining and development of natural products. However, most studies have focused on *T. virens* and *T. reesei* in the genus *Trichoderma* [19, 40, 41]. *Agrobacterium tumefaciens*-mediated transformation and PEG-mediated protoplast transformation are the two commonly used systems in fungi [42, 43]. Here, we developed an improved PEG-based method for the genetic transformation of *T. hypoxylon*. This system is technically simple and has common equipment requirements. Furthermore, we demonstrated that two selection maker genes, *hph* and *neo*, can be efficiently used for *T. hypoxylon*. In addition, we generated the UU auxotrophic strain by the PEG-mediated transformation method. *AfpyrG* can be recycled by 5-FOA selection, which can be used to disrupt multiple genes sequentially in *T. hypoxylon*.

To improve the transformation efficiency, we targeted the non-homologous recombination pathway. Proteins that are involved in NHEJ mainly include the Ku70–Ku80 heterodimer and the DNA ligase IV (Lig4)–Xrcc4 complex [44]. There are two pathways used when exogenous genes integrate into the chromosome: MUS-52 (ku 80)-dependent and MUS-52-independent non-homologous integration. MUS-53 (LIG4) was needed for both MUS-52-dependent and MUS-52-independent pathways [32]. The two genes *thku70* and *thlig4*, which are involved in this pathway, were deleted to create the mutants. The two strains were examined by knocking out the *thtri5* gene, which encodes a terpenoid cyclase. The homologous combination efficiency of *thtri5* deletion showed no obvious changes in the *Dthku70* strain compared to WT. This demonstrated that *thku70* cannot improve the transformation rates even though the deletion of the *ku70* gene has been widely used for increasing the homologous recombination frequency [45, 46]. Disruption of *thlig4* greatly improved the homologous integration rates up to 89 %. Therefore, the *thlig4* deletion mutant could be a very useful and convenient tool for genetic manipulation.

Mycotoxin trichotheccenes are mainly produced by *Trichoderma* and *Fusarium* [47, 48] and play important roles in...
fungal physiology [38, 49]. In T. hypoxylon, a gene cluster encoding trichothecene was found, including the genes encoding a range of functional enzymes similar to those encoded by the Fusarium cluster [50]. Biosynthetic genes (thtri3, thtri4), a P450 (thtri11), two regulatory proteins (thtri6 and thtri10), a transport protein (thtri12) and a virulence factor (thtri14) are included in this cluster (Fig. 5b). This gene cluster was also found in T. arundinaceum and T. brevicompactum. By gene deletion, we demonstrated that thtri5 is involved in the biosynthesis of harzianum B because the deletion mutant lost its production (Fig. 4).

In summary, we described the successful construction of a PEG-mediated transformation system in T. hypoxylon. The creation of UU auxotrophy in T. hypoxylon could be used to conduct multiple gene knockouts and complementation, which expand our ability to study the genetics and biology of Trichoderma. Deletion of the thlg4 gene remarkably increases homologous recombination rates. Using this system, we identified the BGC of harzianum B in T. hypoxylon. Our approach provided a general method for the establishment of genetic systems in fungi.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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