Prokaryotic RT-qPCR Available 13 (Article Received Rongshu disulfide have at Wösten, et al., 2005, December 2005; 2004, October 2004; pistil 2011; Kim et al., 2005, Cladosporium ful- luum et al., 2005, Whiteford et al., 2004), the class I hydrophobins Hyd1 and Hyd2 of the corn pathogen Fusarium verticilloides (Fuchs et al., 2004) and hydrophobin MPG1 from the rice blast fungus Magnaporthe grisea are required for the dispersal of conidia (Whiteford and Spanu, 2002; Elliot and Talbot, 2004), and MPG1 also plays a role in the interaction of rice with M. grisea (Sunde et al., 2008; Mosbach et al., 2011; Kim et al., 2005). Among biocontrol fungi, the class I hydrophobin Tashyd1 of Trichoderma asperellum plays a role in interactions with cucumber (Viterbo and Chet, 2006) and the class II hydrophobin Hyd3 of Clonostachys rosea is involved in interactions with barley roots (Dubele et al., 2014).

The genus Trichoderma/Hypocrea is well known for its biocon- trol function against many fungal phytopathogens (Harman et al., 2004; Olson and Michael Benson, 2007; Hanada et al., 2009). One of the biocontrol mechanisms of Trichoderma is the secretion of many kinds of small molecular secreted cysteine-rich proteins (SSCPs) to induce plant resistance and elicit plant growth (Druzhinina et al., 2012). These SSCP sizes include class I and class II hydrophobins. Using gene deletion and complementation methods, the hydrophobin Tashyd1 was shown to be involved in the attachment of Trichoderma...
hyphae to cucumber roots, indicating that Tashy1 plays a role in rhizosphere colonization (Viterbo and Chet, 2006). Compared with class I hydrophobins, Trichoderma species have an abundance of class II hydrophobins (Kubicek et al., 2008). In other biocontrol fungi, class II hydrophobins function in plant root colonization (Dube et al., 2014) and are also involved in virulence to insects (Sevim et al., 2012). However, studies on the class II hydrophobins in Trichoderma have focused on their functions in physiological growth (Askolin et al., 2005; Mikus et al., 2009) or protein family classification analysis (Kubicek et al., 2008; Seid-Seiboth et al., 2011; Espino-Rammer et al., 2013), and no reports about their biocontrol functions have been published.

We wondered whether class II hydrophobins in Trichoderma had functions in Trichoderma spp.–plant–pathogen interactions, especially during interactions between Trichoderma spp. and woody plants. Thus, we cloned the small molecular hydrophobin gene HFB2-6 from T. asperellum ACC30536 and analyzed its sequence characteristics. We also studied the transcription of HFB2-6 in T. asperellum with RT-qPCR under eight different conditions (including treatment with Shanzin poplar and its fungal host). HFB2-6 was also heterologously expressed in Escherichia coli BL21. The recombinant hydrophobin rHFB2-6 and its purified product were analyzed by SDS-PAGE. After poplar seedlings were induced with rHFB2-6, the transcription of genes related to hormone signal transduction was studied by RT-qPCR. Our results provide theoretical support and a practical reference for the development of biological small hydrophobins from T. asperellum ACC30536.

### Materials and methods

#### Strains, plasmids and plant materials

T. asperellum ACC30536 was obtained from the Agricultural Culture Collection of China. E. coli strain TOP10 and the vector pMD18-T were obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). E. coli BL21 and the vector pGEX-4T-2 (Novagen,
Table 2: Sequences of the class II hydrophobins and their promoters in the T. asperellum CBS 1343.97 genome.

<table>
<thead>
<tr>
<th>Gene name and chromosomal position</th>
<th>LOC</th>
<th>NCBI locus</th>
<th>AA</th>
<th>pl</th>
<th>M0/KDa</th>
<th>pI</th>
<th>MM/kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFB2-1</td>
<td>Scaffold:2.1366109–1366741 (+)</td>
<td>15% and 16% AA-AP</td>
<td>18–84</td>
<td>8.87</td>
<td>4.58</td>
<td>8.87</td>
<td>15</td>
</tr>
<tr>
<td>HFB2-2</td>
<td>Scaffold:1.2101661–1210978 (+)</td>
<td>15% and 16% AA-AP</td>
<td>18–84</td>
<td>8.95</td>
<td>5.54</td>
<td>8.95</td>
<td>15</td>
</tr>
<tr>
<td>HFB2-3</td>
<td>Scaffold:5.1210977–5.1210978 (+)</td>
<td>16% and 17% AA-AP</td>
<td>18–84</td>
<td>9.77</td>
<td>4.30</td>
<td>9.74</td>
<td>16</td>
</tr>
<tr>
<td>HFB2-4</td>
<td>Scaffold:2.1366109–1366741 (+)</td>
<td>15% and 16% AA-AP</td>
<td>18–84</td>
<td>9.75</td>
<td>4.41</td>
<td>9.75</td>
<td>16</td>
</tr>
<tr>
<td>HFB2-5</td>
<td>Scaffold:6.1792199–1792201 (+)</td>
<td>15% and 16% AA-AP</td>
<td>18–84</td>
<td>10.13</td>
<td>4.06</td>
<td>10.06</td>
<td>16</td>
</tr>
<tr>
<td>HFB2-6</td>
<td>Scaffold:1.2101661–1210978 (+)</td>
<td>15% and 16% AA-AP</td>
<td>18–84</td>
<td>10.38</td>
<td>5.22</td>
<td>9.82</td>
<td>16</td>
</tr>
<tr>
<td>HFB2-7</td>
<td>Scaffold:2.1366109–1366741 (+)</td>
<td>15% and 16% AA-AP</td>
<td>18–84</td>
<td>14.14</td>
<td>5.92</td>
<td>14.02</td>
<td>16</td>
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<tr>
<td>HFB2-8</td>
<td>Scaffold:5.1210977–5.1210978 (+)</td>
<td>16% and 17% AA-AP</td>
<td>18–84</td>
<td>14.38</td>
<td>6.42</td>
<td>14.42</td>
<td>16</td>
</tr>
<tr>
<td>HFB2-9</td>
<td>Scaffold:1.2101661–1210978 (+)</td>
<td>15% and 16% AA-AP</td>
<td>18–84</td>
<td>16.00</td>
<td>4.63</td>
<td>16.00</td>
<td>16</td>
</tr>
</tbody>
</table>

Madison, WI, USA) were used for prokaryotic expression experiments. The phytopathogenic fungus *Alternaria alternata* CFCC82114 (poplar leaf wither) was obtained from the China Forestry Culture Collection Center. Tissue cultured poplar seedlings *Populus davidiana* × *P. alba* var. *pyramidalis* (Shanxin poplar) which have similar height (about 5 cm), similar number of leaves (6–8 leaves) and similar growth status, were rooting for 15 days in liquid woody plant medium (WPM) at 25°C.

**Cloning of the HFB2-6 gene and bioinformatics analysis**

Primers for cloning of the HFB2-6 gene were designed as follows: HFB2a, 5′-ATGCGATTCCTCAGGCCTGCGC-3′, and HFB2b, 5′-TTAGTTGCGGCGGCGCCGG-3′. Genomic DNA was extracted from mycelia of *T. asperellum* according to procedures in the book “Molecular Cloning” (Sambrook, 2001). The PCR conditions were 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 40 s, 72°C for 30 s. The amplified fragment was purified and ligated into the vector pMD18-T (TaKaRa, D101A) according to the manufacturer’s instructions, and then sequenced (Shanghai Sangon Co., Shanghai, China).

Multiple sequence alignment was conducted with the Clustal X program (http://www.ebi.ac.uk/Tools/clustalw2/). A phylogenetic tree was constructed using the neighbor-joining method in the MEGA 5.10 program.

**Analysis of the class II hydrophobin family in the *T. asperellum* genome and their differential expression**

The sequence of HFB2-6 was used as a general BLASTP query in *T. asperellum* CBS 433.97 at the JGI website (http://genome.jgi-psf.org/). Bioinformatic analysis of the eight class II hydrophobin genes (HFB2-1 to HFB2-8) was carried out according to Tamura et al. (2011). Furthermore, the 1000 bp upstream fragments of these coding regions were obtained as predicted promoter regions. Regulatory motifs in these regions were predicted using the Promoter Database of PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and Sascharomyces cerevisiae (http://rulai.cshl.edu/SCPD/).

*T. asperellum* (1 × 10⁶ spores) was cultured for 2 days in PD. Then, the mycelia were treated with four different culture conditions including mineral medium (MM) (15 g/L NaH₂PO₄·5 g/L (NH₄)₂SO₄·600 mg/L CaCl₂·2H₂O·600 mg/L MgSO₄·7H₂O·5.0 mg/L FeSO₄·2.0 mg/L CoCl₂·1.6 mg/L MnSO₄·1.4 mg/L ZnSO₄) with 0.5% (w/v) glucose and 0.5% (w/v) ammonium sulfate, C-starvation (MM with 0.5% (w/v) ammonium sulfate), N-starvation (MM with 0.5% (w/v) glucose) and SXV (variable carbon source in MM as follows: 1% (w/v) root powder, 1% (w/v) stem powder, or 1% (w/v) leaf powder from Shanxin poplar). The hyphae were harvested after cultivating for 72 h and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Transcriptome profiles were constructed using Illumina Sequencing technology (Shentu et al., 2014). For each transcriptome, 4 Gb of data were obtained (unpublished). The expression amounts of the eight class II hydrophobin genes were totaled from the data of the four transcriptomes.

**Phylogenetic analysis of all class II hydrophobins from six sequenced Trichoderma genomes**

We further screened all class II hydrophobins from the other five sequenced *Trichoderma* species genomes: *Trichoderma longibrachiatum* ATCC18648, *Trichoderma reesei*, *Trichoderma atroviride* ATCC74058, *Trichoderma virens* Gv29-8 and *Trichoderma harzianum* CBS 226.95 (http://genome.jgi-psf.org/). Multiple sequence alignment of class II hydrophobins from the six sequenced *Trichoderma* genomes was conducted with the Clustal X
Differential expression of HFB2-6 in T. asperellum under different culture conditions

We studied the transcription of HFB-26 in response to eight conditions, namely MM with 0.5% (w/v) glucose and 0.5% (w/v) ammonium sulfate (Penttilä 1987), carbon starvation (0.5% (w/v) ammonium sulfate in MM without glucose), nitrogen starvation (0.5% (w/v) glucose in MM without ammonium sulfate), and different carbon sources in MM (0.5% (w/v) ammonium sulfate) as follows: 1% (w/v) root powder, 1% (w/v) stem powder, or 1% (w/v) leaf powder from Shanxin poplar, 1% (w/v) powdered cell walls or 5% (w/v) fermentation supernatant of A. alternata. Root, stem, and leaf powders were prepared as described by Fan et al. (2013). Cell wall fragment and fermentation liquid samples of the poplar pathogenic fungus A. alternata were prepared as described by Morisset et al. (2006).

Spores ($1 \times 10^6$) of the biocontrol agent T. asperellum ACCC30536 were cultured in 1/4 PD medium at 25°C for 48 h under continuous shaking at 180 rpm. After the spores germinated into mycelia, the mycelia were transferred into MM medium for 2 h and then into one of the different induction media above at 25°C for 36 h. The mycelia were then harvested at 0, 4, 8, 12, 24, and 36 h for RNA extraction. The mycelia cultured for 0 h were used as the control.

Digestion and reverse transcription of total RNA and RT-qPCR were performed according to Fan et al. (2013). The expression level of HFB-26 was calculated from the threshold cycle according to the 2$^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The RT-qPCR primers are shown in Table 1. Vector construction and E. coli transformation

To express HFB-26 in E. coli BL21, the primers HFB2-6a (5'-ATCCGTTACGATGTCCACCATGTCG-3', containing a KpnI site) and HFB2-6b (5'-CGATGCAGATTACGTGGCGAAGAAGC-3', containing a SalI site) were used to amplify the coding region of the HFB-26 gene. The recombinant vector pGEX-HFB2-6 and the

Fig. 1. Protein characteristics analysis of the hydrophobin HFB2-6. (A) The protein family of HFB2-6 and its characteristics. (B) Multiple sequence alignment of the partial conserved domain of HFB2-6. Eight highly conserved catalytic Cys residues are boxed, these form four disulfide bonds. Asterisk (*), identity; colon (:), high similarity; period (.), low similarity. The sequences used were: ABSS59366 (Trichoderma atroviride); ABSS59365 (T. atroviride); ABSS59380 (T. virens); AAN76355 (Fusarium verticillioides); ABSS59377 (T. virens); EFQ36046 (Colletotrichum graminicol); AAB41284 (Ophiostoma ulmi); XP_382007 (F. graminearum); CBJ94531 (F. poae); AAO16870 (F. verticillioides); CAB02147 (C. glaucus); XP_382982 (Neurospora crassa); ABR15767 (O. quercus); CAD58939 (O. novolam); EFQ39490 (C. graminicol); XP_003041182 (Nectria haematococa); ABR15765 (O. quercus); XP_363347 (Magnaporthe oryzae); ABSS59378 (T. virens); XP_001907576 (Podospora anserina); CB155673 (Sordaria macrospora). (C) Three-dimensional structure of the hydrophobin HFB2-6; 1, 2, and 3 are the different sides of the HFB-26 protein. Disulfide bonds and Cys side chains are shown as yellow sticks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

program (http://www.ebi.ac.uk/Tools/clustalw2/). A phylogenetic tree was constructed using the neighbor-joining method in the MEGA 5.10 program with 1000 bootstrap replications.
transformant BL21-HFB2-6 were obtained using the method of Tu et al. (2005).

**SDS-PAGE analysis**

The recombinant transformant BL21-HFB2-6 and control transformant BL21-pGEX were each inoculated into 10 mL LB broth (containing ampicillin 50 µg/mL) and grown at 37 °C and 180 rpm for 12 h. Cultures of BL21-HFB2-6 and BL21-pGEX in the logarithmic phase (OD_{600} = 0.5) were induced for 4 h with 1.0 mM IPTG at 30 °C and 180 rpm. The *E. coli* cells were harvested by centrifugation at 12,000 × g for 10 min after culturing for 4 h. The collected cells were processed using *E. coli* Protein Extraction Solution (C2301, HaiGene, China) following the manufacturer’s instructions. After adding 1× loading buffer, the supernatants and cell pellets were boiled for 5 min, centrifuged for 10 min at 8000 × g, and loaded into a slab gel for 15% SDS-PAGE. The recombinant protein rHFB2-6 was reanimated in TGE buffer containing 50 mM Tris (pH 7.9), 0.5 mM EDTA, 50 mM NaCl and 5% glycerol (v/v). Then, the reanimated rHFB2-6 was purified as described in the Glutathione S-transferase (GST) Gene Fusion System Handbook (GE Healthcare UK Ltd, Buckinghamshire HP7 9NA, England).

**Differential expression of hormone responsive genes in Populus seedlings induced with recombinant rHFB2-6**

*Populus* seedlings were cultured in WPM medium containing 1 g purified rHFB2-6; 1 g inactivated recombinant rHFB2-6 was used as a control. The leaves were then harvested at 0, 2, 4, 8, 24 and 72 h for RNA extraction. Total RNA was extracted from the leaves using a Plant RNA Kit (Omega Bio-Tek, Norcross, GA, USA) and digested with DNase I. The elongation factor (EF), β-tubulin and actin genes were used as internal references to normalize the amount of total RNA present in each reaction. PCR primers for amplifying poplar cDNA fragments are listed in Table 1. Other methods were as described above.

**Results**

**Cloning and bioinformatics analysis of the hydrophobin HFB2-6**

The DNA sequence of the class II hydrophobin gene HFB2-6 was 819 bp in length, containing three exons and two introns. The splice sites between the exons and introns followed the GT-AG splicing rule. The cDNA sequence of HFB2-6 was 340 bp in length, encoding 106 amino acids with a calculated molecular weight of 10.8 kDa and a pI of 5.92. SignalP prediction showed that there was a signal peptide (16 amino acids) in the N-terminal region of HFB2-6. The cleavage site of signal peptidase was between positions A16 and A17 (ASA-AP), indicating that HFB2-6 was secreted extracellularly. The GenBank accession numbers of the DNA and cDNA sequences are JX185070 and JX014433, respectively.

The HFB2-6 protein was a member of the “hydrophobin_2 superfamily” (pfam06766) (Fig. 1A). Multiple sequence alignment of 22 class II hydrophobins from different fungal species was conducted using the ClustalX program, revealing that the amino acid sequences of these hydrophobins were conserved, with identities ranging from 50% to 87%. Furthermore, the eight cysteine residues that form four disulfide bridges were highly conserved in all 22 hydrophobins (Fig. 1B). The spacing of the HFB-26 protein sequence, C–C–X9–C–X11–C–X16–C–X8–C–X10–C– (C indicates cysteine and X indicates any other amino acid), was in accordance with the class II hydrophobin consensus.

The three-dimensional structure of hydrophobin HFB2-6 was predicted by the SWISS-MODEL program to be a compact globule, consisting of two adjacent beta hairpins at the core of the β-barrels.
Fig. 3. Regulatory element-binding motifs in the promoters of the class II hydrophobins of *Trichoderma asperellum.*

One α-helix was connected with the β-barrel surface through a disulfide bond, and the other disulfide bond formed two strands crosslinked with each beta hairpin. Disulfide bonds were bound to the barrel structure at both ends, so the structure of hydrophobin HFB2-6 was very stable (Fig. 1C).

To obtain insight into the mechanisms driving the evolution of small hydrophobic proteins in different fungi, we constructed a phylogenetic tree based on the multiple sequence alignment of HFB2-6. The resulting tree is shown in Fig. 2. The proteins were divided into four groups. Interestingly, we found that five hydrophobins from *Ophiostoma* spp. had a close relationship in group 1, and most of the hydrophobins from *Fusarium* spp. also had a close relationship in group 3 (except the protein AAO16870), while hydrophobins from *Trichoderma* spp. were distributed in all four groups, showing their diversity. HFB2-6 (AFN88524) had a close relationship with Tahyd5a (AB593966) in group 2.

Analysis of the class II hydrophobin sequences in the *T. asperellum* CBS433.97 genome

Through screening of the *T. asperellum* CBS433.97 genome, eight putative hydrophobin ORF sequences were identified, all of which were predicted to be members of the class II hydrophobin superfamily (Table 2). According to their amino acid numbers, the hydrophobins were named HFB2-1 to HFB2-8. All eight hydrophobin sequences contained three exons and two introns, and comprised 88–160 amino acids with predicted molecular weights ranging from 8.87 to 15.68 kDa. Except for HFB2-8, all of the sequences contained signal peptide cleavage sites. Four hydrophobins had signal peptide cleavage sites between amino acid positions 16 and 17 and the other three had cleavage sites between positions 15 and 16. The conserved domains were mostly comprised of 28–66 amino acids. All eight proteins were acidic proteins according to their isoelectric points (pI) (Table 2).

Regulatory motifs were predicted in the promoter regions of the eight class II hydrophobins using the PLACE program, showing that they contained many kinds of stress response elements (Fig. 3). The stress response elements were involved in responses to anaerobic (ANAERO1CONSENSUS, S000477), dehydration (MYB2CONSENSUSAT, S000409), low temperature (MYCCONSNSUSAT, S000407; LTR1HVT49, S000250), phosphate starvation (PIBS, S000459), pathogen- or salt-induced (GT1GSMCA4, S000453), cold- or drought-induced (LTRCOREATC15, S000153) and pathogenesis-related (WRKTV7105, S000447) stress. Moreover, regulatory motifs were also predicted using the Promoter Database of *S. cerevisiae* (Fig. 3). The motif GCR1, a transcriptional activator required for the expression of glycolytic and ribosomal genes, was found in the promoter regions of all eight class II hydrophobins; the HFB2-6 promoter in particular contained the highest number with 10 GCR1s. Alcohol Dehydrogenase Regulator (ADHR1, S000002624) is required for transcription of the glucose-repressed gene ADH2 (alcohol dehydrogenase II), and six ADR1s were found in the HFB2-6 promoter region.

The transcription of the eight hydrophobin genes under four different conditions showed that most of them were induced by woody plant extracts and nutritional stress conditions (Table 3). HFB2-8 was the only gene for which we could not detect a transcription signal under the four conditions and contained no signal peptide sequence, so we consider it likely to be a pseudogene. Among the other seven genes, HFB2-1 was not induced by starvation conditions, and HFB2-5 and HFB2-4 showed very high transcription levels after interacting with poplar. Though HFB2-6 and HFB2-7 both showed high transcription levels under all four conditions, HFB2-6 had a higher transcription level than HFB2-7.

![Fig. 4. Phylogenetic relationships of the eight hydrophobins from *Trichoderma asperellum.*](image-url)

**Table 3**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Location in chromosome</th>
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<th>MM</th>
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<th>N-hungry</th>
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</thead>
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<tr>
<td>HFB2-1</td>
<td>Scaffold2:1366109–1366741 (+)</td>
<td>18,787</td>
<td>7184</td>
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<td>23,741</td>
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<tr>
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<td>52</td>
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<tr>
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<td>Scaffold9:835346–836061 (+)</td>
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<tr>
<td>HFB2-4</td>
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<td>75</td>
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<td>1246</td>
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<tr>
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<tr>
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</tbody>
</table>

SXY, Shanxin poplar.
Fig. 5. Alignment of the class II hydrophobins from six sequenced Trichoderma genomes. The eight highly conserved catalytic Cys residues form four disulfide bonds. Asterisk (*), identity; colon (:) high similarity; period (.), low similarity. The sequence below the alignment is a suggested consensus sequence for the Trichoderma class II hydrophobins, as derived from this study. The cysteines are numbered in order of their appearance in the sequence. X denotes any amino acid, and the subscript indicates the number of residues.
The characteristic elements in its promoter region (Fig. 3) and its high expression under the four different conditions (Table 3) suggested that HFB2-6 may have multiple functions in *T. asperellum*.

A phylogenetic tree was also constructed, and the eight hydrophobins were divided into four groups (Fig. 4). The pseudogene HFB2-8 with no transcription (Table 3) was in group 4, and HFB2-2 with low transcript levels under all four conditions (Table 3) was in group 3. The other six highly transcribed hydrophobins (Table 3) were concentrated in groups 1 and 2.

**Phylogenetic analysis of all class II hydrophobins from the six sequenced Trichoderma genomes**

We screened all class II hydrophobins from the six sequenced *Trichoderma* species genomes, including 8 in *T. asperellum* CBS 433.97 (Table 2), 10 in *T. atroviride* ATCC74058, 9 in *T. virens* Gv29-8, 8 in *T. harzianum* CBS 226.95, 6 in *T. reesei* and 6 in *T. longibrachiatum* ATCC18648 (Supplemental Table S1). Alignment of the 47 class II hydrophobins across the area from the first to the eighth cysteine (Fig. 5) showed that the eight cysteine residues were highly conserved but the α-helix region between C4 and C5 showed little conservation. Kubicek et al. (2008) found that 32 of a total of 64 aa were conserved among hydrophobin sequences, while in our results, only 13 of a total of 64 aa (Fig. 5) were conserved at the genome level across the six *Trichoderma* genomes.

Supplemental Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2014.12.004.

A NJ phylogenetic tree based on the sequences from the first to eighth cysteine was also constructed (Fig. 6). We divided the 47 hydrophobins from the six *Trichoderma* genomes into seven groups. Twenty-three of the 47 hydrophobins were clustered in group 1. In particular, 6 of the 9 hydrophobins from *T. virens* Gv29-8 were in group 1. In group 2, three class II hydrophobins (HFB2-1, Tvi2a and Tat2a) had a glycine (G) insertion between C5 and C6, which was different from the other hydrophobins (Fig. 5). Two hydrophobins in group 3 were missing nine amino acids between C4 and C5 (Fig. 5). We also found that the class II hydrophobins of *T. longibrachiatum* ATCC18648 had a close relationship with those of *T. reesei*, with a one-to-one correspondence in the phylogenetic tree.

**Differential expression of HFB2-6 under eight different treatments**

To investigate the transcription of HFB2-6 in response to plants and fungal phytopathogens, RT-qPCR was performed. The results indicated that the HFB2-6 gene was differentially regulated in different culture conditions. The transcription of HFB2-6 was mainly up-regulated. HFB2-6 transcription was obviously up-regulated by 1% root and stem powder of Shaxin poplar, with peak transcription levels of 9.81 (2^3.29) and 32.05 (2^5.00) times, respectively, at 36 h (Fig. 7D and E). Interestingly, the transcription of HFB2-6 was down-regulated after induction by 1% leaf powder (Fig. 7F). HFB2-6 transcription was induced by 1% *A. alternata* cell wall and 5% *A. alternata* fermentation liquid treatments, with transcription maximums of 225.08 (2^7.81) and 7.43 (2^2.89) times the pretreatment level, respectively (Fig. 7G and H). In addition, the impact of carbon or nitrogen starvation on the transcription of HFB2-6 was also studied. HFB2-6 transcription was induced by MM (0.5% glucose and 0.5% ammonium sulfate), carbon starvation and nitrogen starvation. The peak transcription levels were 3.17 (2^1.66), 37.61 (2^5.23) and 80.40 (2^5.33) times, respectively, which were all observed at 36 h (Fig. 7A–C). In brief, transcription of the HFB2-6 gene was affected by all eight different treatments. Both plant and plant pathogenic fungus treatments could trigger HFB2-6 transcription.

**SDS-PAGE analysis**

We used SDS-PAGE analysis to determine whether the *E. coli* BL21-HFB2-6 could synthesize the recombinant hydrophobin rhHFB2-6. Compared with the control transformant BL21-pGEX, the recombinant transformant BL21-HFB2-6 showed a clearly visible
Fig. 7. Expression of HFB2-6 in T. asperellum in response to eight different treatments. X-axis: time points, Y-axis: expression level = log2 (fold change in expression), namely gene expression in T. asperellum under different conditions; (A) HFB2-6 expression under MM conditions; (B and C) HFB2-6 expression under C and N starvation conditions, respectively; (D–F) HFB2-6 expression under 1% root, stem and leaf powder of Shanxin poplar, respectively; (G and H) HFB2-6 expression under 1% mycelia powder and 5% zymotic supernatant of A. alternata grown in 1/4 PD for 10 d. All experiments were performed three times.

protein band with a molecular weight of approximately 36.8 kDa (10.8 kDa HFB2-6 and 26 kDa GST tag protein) in the SDS-PAGE gel (Fig. 8A), indicating that the recombinant hydrophobin rHFB2-6 was successfully synthesized in the E. coli cells. We also renatured and purified the rHFB2-6 from E. coli BL21 cells (Fig. 8B).

Hormone signal transduction in Populus under induction with recombinant rHFB2-6

To determine whether the recombinant rHFB2-6 could promote Populus growth, the expression of genes related to plant hormone signal transduction was studied by RT-qPCR. Firstly, the transcription of auxin signal transduction-related genes was obviously induced by recombinant rHFB2-6. In the experimental group, the expression of the AUX1 (auxin), LAX2 (auxin), IAA8 (indole-3-acetic acid) and TIR1 (auxin receptor) genes showed the same trend as the control group but their transcription levels all exceeded the control group (Fig. 9H–K). The peak transcription levels of the genes in the experimental group were 10.83 (23.44), 13.40 (23.74), 4.77 (22.25) and 1.75 (20.80) times at 2, 4, 24 and 2 h, respectively, and they were all down-regulated at 8 h after interacting with rHFB2-6. However, the transcription of the GH3.17 (auxin original response) and MP (monopteros) genes showed clear differences between the experimental and control groups (Fig. 9L and M). The peak transcription levels of these two genes in the experimental group were 1.14 (20.20) and 393.40 (28.62) times, respectively, at 24 h.

Secondly, the transcription of jasmonic acid genes was obviously induced by recombinant rHFB2-6, with peak transcription levels of
by recombinant HFB2-6. The supernatant and E. coli cells of the control transformant BL21-pGEX induction by IPTG at 4 h; M: protein markers; 3, 4: the supernatant and E. coli cells of the recombinant transformant BL21-HFB2-6 induced by IPTG for 4 h; 5, 6: the supernatant and E. coli cells of the recombinant transformant BL21-HFB2-6 without IPTG induction at 4 h; B1: purified recombinant rHFB2-6. OD_{600} = 0.5. The concentration of IPTG was 1.0 mM.

5.72 (22^{25}), 18.98 (24^{25}), 9.37 (23^{23}), 4.48 (22^{16}) and 5.50 (22^{46}) times at 4, 24, 2, 2 and 4 h, respectively (Fig. 9A–E). In the control group, while the expression of MYC2 (a helix-loop-helix domain gene) was up-regulated after interaction with rHFB2-6 (Fig. 9C), JAR1 (jasmonic acid resistant gene), COI (coronatine insensitive protein gene), ORCA3 (octadecanoid-derivative responsive Catha ranthus AP2-domain) and JAZ6751 (jasmonate ZIM-domain gene) were down-regulated at 8, 8, 2 and 4 h, respectively (Fig. 9A, B, D and E).

Lastly, the transcription of the salicylic acid gene PR1 (pathogenesis-related protein gene) was induced by recombinant rHFB2-6, with peak transcription of 4.58 (22^{19}) times the pretreatment level (Fig. 9F), while the expression of NPR1 (non-expression of pathogenesis-related gene) was down-regulated as early as 2 h after induction (Fig. 9G). However, PR1 and NPR1 were both down-regulated in the control group, with minimum transcription levels observed at 4 and 2 h, respectively (Fig. 9F and G). In summary, the recombinant rHFB2-6 had an influence on poplar hormone signal transduction.

Discussion

Filamentous fungi contain hydrophobin genes that play important roles in fungal growth, development and environmental communication. In particular, Trichoderma spp. have an abundance of class II hydrophobins (Kubicek et al., 2011). This diversity of class II hydrophobins is possibly why Trichoderma spp. have multiple biocontrol functions compared with other biocontrol fungi (e.g. Dubey et al. (2014) only found three class II hydrophobins in the biocontrol fungus C. rosea), indicating that different mycoparasites may rely on different mechanisms of interaction. Kubicek et al. (2008) previously studied the phylogenetic relationships of class II hydrophobins from nine Trichoderma species, including all of the class II hydrophobins from three Trichoderma species genomes and partial class II hydrophobin sets from six Trichoderma species. Now, more genomes of Trichoderma species have been sequenced, so we further studied the phylogenetics relationship of class II hydrophobins at the genome level. Though we use the same tree construction method, our results (Figs. 5 and 6) differed from those of Kubicek et al. (2008). Our results (Table 2 and Supplementary Table S1) suggest that different species of Trichoderma have different numbers of class II hydrophobins. The biocontrol Trichoderma spp. (T. virens, T. atroviride, T. harzianum and T. asperellum) have more class II hydrophobins than the industrial one (T. reesei), which may explain why different Trichoderma species have different functions. From the phylogenetic relationships of the class II hydrophobins (Figs. 5 and 6), T. longibrachiatum is phylogenetically very close to T. reesei; they both belong to the Longibrachiatum section (http://www.isth.info/biodiversity/index.php). T. reesei is used for industrial enzyme production, and T. longibrachiatum is also fit for this purpose. Moreover, T. longibrachiatum can also be used for biocontrol, but little references to this function were found. Thus, from both the numbers and phylogenetic relationships of class II hydrophobins, we can preliminarily determine if a Trichoderma species has biocontrol function.

The regulatory motifs in the promoter regions of the eight class II hydrophobins in T. asperellum CBS 433.97 were analyzed, indicating that many regulatory motifs were related to stress responses. In particular, the HFB2-6 promoter contains four kinds of stress response elements including MYC, MYB, ANAERO1CONSSENSUS and GT1GMSCAM4 (Fig. 3). This may be why the expression of HFB2-6 was up-regulated under A. alternata fermentation liquid (Fig. 7H) and carbon (Fig. 7B) or nitrogen starvation (Fig. 7C). Moreover, GCR1 and ADR1 motifs, which are required for glycolytic and glucose-repressed gene transcription, respectively, were also predicted in the HFB2-6 promoter region. In the RT-qPCR experiment, the expression of HFB2-6 was up-regulated under the A. alternata cell wall treatment, which contained chitin and glucan (Fig. 7G), but was inhibited by glucose in MM (Fig. 7A). The presence of these two motifs suggests that the expression of HFB-2 is inhibited by monosaccharides but induced by the amylase in fungal cell walls.

To understand the functions of the class II hydrophobin genes, we examined their transcription under different conditions. In Hypocrea atroviridis, the class II hydrophobin gene hfb-2b was induced under carbon starvation conditions in darkness, while all hydrophobin genes except hfb-22a were induced under carbon starvation conditions in the presence of light (Mikus et al., 2009). Among the class II hydrophobins Hyd1, Hyd2 and Hyd3 from the biocontrol fungus C. rosea, the expression of Hyd1 and Hyd2 was up-regulated in carbon limitation culture but changes in their expression were not clear in nitrogen limitation culture, while the expression of Hyd3 was increased in both cultures (Dubey et al., 2014). In this study, the expression of the class II hydrophobin gene HFB2-6 was induced by both carbon and nitrogen starvation. Thus, our results are similar to those of Dubey et al. (2014). Our results suggest that HFB2-6 plays an important role when the hyphae are under nutritional stress, allowing Trichoderma to adapt to a wide range of environments.

Hydrophobins are known to be involved in fungal interactions with plant leaves and roots, and are usually highly expressed under these conditions (Talbot et al., 1996; Kim et al., 2005). Piett et al. (2012) found that 12 class I hydrophobins from the mycorrhizal fungus Laccaria bicolor in mycorrhizal root tips were up-regulated during hyphal interaction with host plants, such as Douglas fir, Populus trichocarpa × Populus deltoides clone 545, P. trichocarpa and P. deltoides. Viterbo and Chet (2006) also showed that the hydrophobin TasHyd1 from T. asperellum T203 promoted root colonization in cucumber. For class II hydrophobins, Dubey et al. (2014) found that Hyd3 from C. rosea was needed for colonization of barley roots. However, there have been no reports on class II hydrophobin expression characteristics in response to woody plants. In this study, a high level of HFB2-6 expression was found during interaction with woody plant roots, indicating that the protein HFB2-6 may be necessary for root adhesion and colonization. However, HFB2-6 expression was down-regulated after interaction with woody plant leaves, which means HFB2-6 had little effect during interaction with woody plant leaves.

Viterbo and Chet (2006) also found that the hydrophobin TasHyd1 from T. asperellum had no function during interaction with the pathogen Rhizoctonia solani. Mikus et al. (2009) found that three class II hydrophobin genes (hfb-2h, hfb-2c, and hfb-6c) from H. atroviridis ATCC74058 appeared to be less strongly expressed during...
Fig. 9. Expression of genes related to *Populus* hormone signal transduction pathways after induction with recombinant rHFB2-6. X-axis: time points, Y-axis: expression level = log2 (fold change in expression), namely gene expression in *Populus* after induction by recombinant rHFB2-6. In the jasmonic acid signal transduction pathway (N), (A–E) represent the expression of JAR1, COI, MYC2, ORCA3 and JAZ6751, respectively; in the salicylic acid signal transduction pathway (O), (F and G) represent the expression of PR1 and NPR1, respectively; in the auxin signal transduction pathway (P), (H–M) represent the expression of AUX1, LAX2, TIR1, IAA8, MP and GH3.17, respectively. All experiments were performed three times.
interactions with the pathogens *Botrytis cinerea* and *R. solani*. The class II hydrophobins Hyd1, Hyd2 and Hyd3 from the biocontrol fungus *C. rosea* were also studied during pathogen interactions. These three genes showed no significant changes during interaction with the pathogen *Fusarium graminearum*, while only Hyd1 was significantly induced by interaction with *B. cinerea*. A significant reduction in *B. cinerea* necrotic lesion area was found on leaves after interaction with ΔHyd1, ΔHyd3 or ΔHyd1ΔHyd3 strains, suggesting that Hyd1, Hyd2 and Hyd3 are not involved in the recognition of hyphae by other organisms ([Dubeys et al., 2014]). In this study, we found that HFB2-6 transcription was up-regulated under induction with *A. alternata* cell walls; this result was different from the class II hydrophobins in *H. atroviridis*, but the same as for *Hyd1* in *C. rosea*. The reason for this needs further study. In addition, after *T. asperellum* interacted with fermentation liquid of *A. alternata*, the expression level of HFB2-6 increased up to 7.43 times. The possible reason for this is that under stress conditions *T. asperellum* can secrete a large amount of hydrophobin HFB2-6, forming an amphipathic membrane at the hyphal surface to protect itself against the infection of phytopathogenic fungi.

Thus, our results indicate that the hydrophobin HFB2-6 can be induced not only by nutritional stress conditions, but also plays as an important role during interactions with woody plants. We also found that HFB2-6 plays a role in interactions with the phytopathogenic fungus *A. alternata*, suggesting a wide range of roles for HFB2-6 during *T. asperellum*–plant–pathogen interactions.

Natural or heterologous recombinant hydrophobins have been purified previously. [Askolin et al. (2005)](doi:10.1007/s00253-005-0743-8) isolated the hydrophobins HBFI and HBBII from *T. reesi* QM9414, but no functions have been shown for these proteins in vitro. [Niu et al. (2012)](doi:10.1038/ncomms1292) expressed the hydrophobin HBFI in *Pichia pastoris* GS115, purified the recombinant protein, and showed that rHFB1 could be used as a novel emulsifying agent and a predictor of gushing risk. This research on hydrophobins was focused on the weak parasitic fungus *T. reesi* for the purpose of industrial manufacture. However, the functions of purified and recombinant hydrophobins in biocontrol fungi have not been researched. In this study, we constructed the *E. coli* recombinant-expressing strain BL21-HFB2-6 and the recombinant hydrophobin HFB2-6 was renatured using TGE buffer.

To study the functions of recombinant HFB2-6, we examined the transcript levels of genes related to poplar hormone signal transduction during induction with the recombinant protein. Firstly, compared with the control group, the expression of jasmonic acid transduction related genes was obviously up-regulated under induction with HFB2-6. Changes in the expression of these genes mainly occurred 2–8 h after interaction with HFB2-6. The jasmonic acid responsive gene ORCA3 (octadecanoid-derivative responsive CATHARANTHUS AP2-domain) regulates jasmonate-responsive expression via interaction with the JERE directly ([van der Fits and Memelink 2001; Vom Endt et al., 2007]). The transcript level of the poplar gene ORCA3 was basically consistent after induction with HFB2-6, indicating that HFB2-6 could cause ISR (induced systemic resistance) in woody plants at the early stage of induction. Secondly, in the plant salicylic acid signal transduction pathway (Fig. 9O), NPR1 plays an important role in both innate immunity and SAR (systemic acquired resistance). and PR1 is often associated with plant host resistance. Though NPR1 is a key component in the induction of SAR-related PR1 gene expression, its over-expression did not lead to PR1 gene expression in *Arabidopsis* ([Mou et al., 2003]). In this study, though the transcription trend of the poplar NPR1 gene was the same as in the control group, the transcription of poplar PR1 was up-regulated compared with the control group, which was consistent with the results of [Mou et al. (2003)]. The up-regulation of PR1 can cause SAR in woody plants, which gives poplar broad-spectrum resistance to pathogens. Thirdly, in the plant auxin signal transduction pathway (Fig. 9P), MP/ARF5 (monopteris) has a broad role in plant morphological development ([Weijers and Jürgens, 2004], and GH3.17 (auxin original response gene) is an early auxin induced expression gene ([Khan and Stone, 2007]). [Stasswick et al. (2005)](doi:10.1002/0471180115.a03_100.s9) found that GH3.17 could enhance sensitivity to IAA in root inhibition assays. The transcription of auxin signaling transduction related genes in poplar was up-regulated under induction with the recombinant hydrophobin HFB2-6, especially the genes GH3.17 and MP, indicating that HFB2-6 may promote poplar growth. The transcription trends of the other four (AUX1, TIR1, LAX2 and IAA8) genes were similar but all four were up-regulated compared with the control group. As [Tian et al. (2002)](doi:10.1002/0471180115.a03_100.s9) showed, auxin can regulate auxin-responsive genes, and our study found the same result. Moreover, the recombinant hydrophobin HFB2-6 could induce genes related to poplar auxin signal transduction, which may promote poplar growth. In summary, HFB2-6 had an influence on both the jasmonic and salicylic acid signal transduction pathways in poplar, which not only causes ISR but also SAR, giving poplar broad-spectrum resistance to pathogens. It also induced genes related to auxin signal transduction to promote poplar growth.

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**References**


Dubeys MK, Jensen DF, Karlsson M. Hydrophobins are required for conidial hydrophobicity and plant root colonization in the fungal biocontrol agent *Conostachys rosea*. *BMC Microbiol* 2014;14:1. 8.


