Hydrophobin HFBII-4 from *Trichoderma asperellum* induces antifungal resistance in poplar

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Abstract

Herein, the class II hydrophobin gene *HFBII-4* was cloned from the biocontrol agent *Trichoderma asperellum* ACCC30536 and recombinant rHFBII-4 was expressed in *Pichia pastoris* GS115. Treatment of *Populus davidiana* × *P. alba* var. *pyramidalis* (PdPap poplar) with rHFBII-4 altered the expression levels of genes in the auxin, salicylic acid (SA), and jasmonic acid (JA) signal transduction pathways. Polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) enzyme activities were induced with rHFBII-4. Evans Blue and nitro blue tetrazolium (NBT) staining indicated that cell membrane permeability and reactive oxygen species were lower in the leaves of plants treated with rHFBII-4. The chlorophyll content was higher than that of control at 2–5 days after treatment. Furthermore, poplar seedlings were inoculated with *Alternaria alternata*, disease symptoms were observed. The diseased area was smaller in leaves induced with rHFBII-4 compared with control. In summary, rHFBII-4 enhances resistance to *A. alternata*.

Keywords Biocontrol · *Trichoderma* sp. · Yeast expression · Hydrophobin · *Populus davidiana* × *P. alba* var. *pyramidalis*

Introduction

Hydrophobins are surface-active proteins produced exclusively by filamentous fungi that are characterized by the presence of eight conserved cysteine (Cys) residues that form four disulfide bonds [1–3]. Hydrophobins are small secreted hydrophobic proteins that self-assemble into amphipathic layers on the outer surface of fungal cell walls [1–3]. Based on differences in hydropathy patterns, sequence characteristics, and protein solubility, they can be divided into Class I and Class II hydrophobins. Hydrophobins perform a variety of biological functions during different phases of the fungal life cycle, including formation of a protective layer surrounding the hyphae and sexual structures, development of aerial hyphae, sporulation, spore dispersal, and fruiting body formation [4, 5]. For example, Sc3p from *Schizophyllum commune* is a small, hydrophobic, cysteine-rich protein involved in the

formation of aerial hyphae [5]. In *Cladosporium fulvum*, the class I hydrophobin Hcf-1 is involved in aerial hyphae development and dissemination of conidia [6]. In addition, hydrophobins can also mediate contact and communication between fungi and their environment, including recognition and adhesion to host surfaces, and development of penetration structures during pathogenic and symbiotic interactions. In *Cryphonectria parasitica*, the class II hydrophobin cryparin is required for fruiting bodies to erupt through host bark tissue [7]. The class I hydrophobin MPG1 from the rice blast fungus *Magnaporthe grisea* is important for conidiation and may act as a developmental sensor for appressorium formation [8]. The transcription of class II hydrophobin gene *MHP1* from *M. grisea* is highly induced during plant colonization and conidiation, and *mhp1* mutants exhibit a reduced ability to infect and colonize a susceptible rice cultivar, indicating that *MHP1* is required for fungal development and plant colonization [9]. In *Trichoderma reesei*, the null mutant of *Trhfb3* presents diminished conidial production compared with the parent strain, indicating that this class II hydrophobin plays a role in asexual development [10]. In *T. virens*, expression of class II hydrophobin gene *hfb7* is upregulated during formation of aerial mycelia in a standing liquid culture [11].

Among biocontrol fungi, some studies revealed that the participation of hydrophobins in biocontrol processes could

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contribute to plant growth and elicit plant defense reactions. *T. asperellum* mutants that lack the hydrophobin gene *TasHyd1* are severely impaired in terms of root attachment and colonization, and these phenotypes are recovered by complementation of *TasHyd1*, indicating that this protein contributes to the interactions between *Trichoderma* and its host plant [12]. In *Clonostachys rosea*, the class II hydrophobin Hyd3 can influence the root colonization ability [13]. Similarly, the class II hydrophobin HYTLO1 in *T. longibrachiatum* can directly inhibit both spore germination and hyphal elongation of *Botrytis cinerea* and *Alternaria alternata* in vitro, and enhance tomato plantlet development [14]. The class II hydrophobin gene *HFB2-6* from the biocontrol agent *T. asperellum* probably can promote the growth of *Populus davidiana* × *P. alba* var. *pyramidalis* (PdPap poplar), and plays an important role in interactions with the phytopathogenic fungus *A. alternata* [3]. The class II hydrophobin gene *hfb7* from *T. virens* stimulates interactions between tomato roots and fungi, indicating a role in fungus–plant interactions [11]. The novel effector protein TVHYDIII1, a class II hydrophobin from *T. virens*, plays a role in plant root colonization and participates in antagonistic activity against *Rhizoctonia solani* [15].

Plant growth and response to environmental cues are largely governed by phytohormones. Plant hormones have important roles in the regulation of immune responses to beneficial microbes, phytopathogens, and insect herbivores. Salicylic acid (SA) and jasmonic acid (JA) are recognized as major defense hormones [16, 17]. The essential roles of SA- and JA-mediated signaling pathways in resistance to pathogens are well described [18]. Auxins are involved in regulating many aspects of plant development and function as modulators of the plant immune signaling network as well [19]. Moreover, plant hormones also play a role in induced resistance, which increases plant basal resistance against future pathogens. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two main forms of induced resistance wherein plant defenses are preconditioned by prior infection or treatment that results in resistance against subsequent challenge by pathogens [20].

In the present study, the small class II hydrophobin gene *HFBII-4* was cloned from *T. asperellum* ACCC30536 and its sequence characteristics were analyzed. *HFBII-4* was also heterologously expressed in *Pichia pastoris* GS115 and recombinant rHFBII-4 protein was assessed by Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE). Treatment of PdPap seedlings with rHFBII-4 upregulated the transcription of genes related to hormone signal transduction according to the results of RT-qPCR, and resistance of PdPap seedlings to the *A. alternata* pathogen was enhanced. Our results provide theoretical support and a practical reference for the development of biological small protein elicitors from *T. asperellum* ACCC30536.

Materials and methods

Strains, plasmids, and plant materials

T. asperellum ACCC30536 was obtained from the Agricultural Culture Collection of China (ACCC). *Escherichia coli* strain TOP10 was obtained from TaKaRa Biotechnology Co., Ltd. (Dalian China). *P. pastoris* GS115 and vector pPIC9K (Invitrogen, USA) were used for eukaryotic expression. The phytopathogenic fungus *A. alternata* CFCC82114 (poplar leaf blight) was obtained from the China Forestry Culture Collection Center. Aseptic *Populus davidiana* × *P. alba* var. *pyramidalis* (PdPap poplar) seedlings were cultured in liquid woody plant medium (WPM) [21].

Cloning of the *HFBII-4* gene from *T. asperellum*

Using the “hydrophobin” as a key word, the hydrophobin gene was obtained from *T. asperellum* CBS 433.97 at the JGI website (<http://genome.jgi-psf.org/>), following a BLASTx search. Two Primers, H-1 (5'-ATGAAGTTCTTCACTGCCG-3') and H-2 (5'-TTAGGCGGCACCGA CAGC-3'), were designed to amplify the *HFBII-4* cDNA sequence. PCR conditions for amplification were 95 °C for 5 min, followed by 35 cycles at 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 40 s and a final extension at 72 °C for 7 min. The amplified fragment was purified and ligated into the vector pPIC9K and then sequenced (Shanghai Sangon Co., China).

Construction of vector pPIC9K-HFBII-4 and *P. pastoris* transformation

To further investigate the functions of *HFBII-4*, the gene was amplified by PCR using primer HFBIIa (5'-ATCGGAATTCGGCCCTGTTGAAGTTCGCACCG-3') containing the *EcoRI* restriction enzyme site and primer HFBIIb (5'-CGATGCGGCCGCGCGGCACCGACAGCGGCCTGG-3') containing the *NotI* site. Recombinant vector pPIC9K-HFBII-4 was constructed and transformed into competent *E. coli* TOP10 cells, and a positive clone was identified by colony PCR and plasmid restriction analysis. Recombinant vector pPIC9K-HFBII-4 was then transferred into *P. pastoris* GS115 to obtain expression strain GS115-HFBII-4.

After linearization with *SacI*, plasmids were transformed into *P. pastoris* GS115 cells by electroporation using a gene pulser apparatus (Bio-Rad Laboratories GmbH, Munich, Germany) with a 2-mm electroporation cuvette, sample volume of 40 µL, charging voltage of 1150 V, capacitance of 25 µF, resistance of 200 Ω, field strength of 575 V/cm, and a pulse length of ~5 ms. Integration of recombinant plasmids into the gap locus of the genome was achieved by homologous recombination [22]. Transformed colonies were selected on RDB plates and incubated at 30 °C for 3 days and a

multicopy recombinant *Pichia* strain was selected based on G418 resistance. Positive transformants were further analyzed by PCR with primers 5'AOX1 (5'-GACTGGTTCCAATTGACAAGC-3') and 3'AOX1 (5'-GCAATGGCATTCTGACATCC-3'; Invitrogen).

Tricine-SDS-PAGE analysis

Recombinant transformant GS115-HFBII-4 and control transformant GS115-pPIC9K were separately cultured in 20 mL BMGY medium at 28 °C. To induce recombinant protein production, strains were harvested by centrifugation at 5000×g for 10 min at room temperature and resuspended to a cell density (OD_{600}) of ~1 in BMMY, and 100% methanol was added to a final concentration of 0.5% every 24 h to maintain induction. After 72 h, supernatants were collected for extraction and analysis of recombinant proteins, which were precipitated with saturated ammonium sulfate and dissolved in 2 M urea. After adding 1× SDS-PAGE loading buffer, supernatants were boiled for 10 min, centrifuged for 10 min at 12000×g, and loaded into a slab gel for 16.5% Tricine-SDS-PAGE [18]. Recombinant rHFBII-4 was purified using the method described previously [23].

Differential expression of hormone signal transduction genes in PdPap poplar seedlings induced with recombinant rHFBII-4

PdPap poplar seedlings were cultured in WPM medium containing 100 mg/L rHFBII-4 (The inactivated recombinant rHFBII-4 was used as a control). Leaves were collected at 0, 2, 4, 8, 24, and 72 h and stored at –80 °C. Total RNA was extracted using a Plant RNA Kit (Omega Bio-Tek, Norcross, GA, USA), digested with DNase I, and reverse-transcribed into cDNA. The transcription of the auxin signal transduction genes (*AUX1*, *LAX2*, *TIR1*, *IAA8*, *MP*, and *GH3.17*), SA signal transduction genes (*PR1* and *NPR1*), and JA signal transduction (*JAR1*, *COI*, *JAZ6751*, *MYC2*, and *ORCA3*) were detected by RT-qPCR. All experiments were performed in triplicate. Primers for RT-qPCR were as described in our previous study [3] and RT-qPCR was performed as described previously [24].

Measurement of enzyme activity, chlorophyll content, and soluble protein content in PdPap poplar seedlings induced with recombinant rHFBII-4

Treatment of PdPap poplar seedlings with rHFBII-4 was performed as described above. Leaves were harvested after 0, 1, 2, 3, 5, and 7 days, and stored at –80 °C. Fresh tissue was weighed from the above samples in each treatment with three replicates. Enzymatic activity of polyphenol oxidase (PPO), peroxidase (POD), and phenylalanine ammonia lyase (PAL)

was measured as described previously [25, 26]. The chlorophyll and soluble protein content was measured using the methods of Lu et al. [27] and Sedmak et al. [28] as previously described.

Cell membrane permeability and reactive oxygen species in PdPap poplar seedlings induced with recombinant rHFBII-4

Treatment of PdPap poplar seedlings with rHFBII-4 was performed as described above. The leaves were stained with 1 mg/mL Evans Blue overnight and destained in 95% ethanol in a boiling water bath for 5 min. Reactive oxygen species (ROS) levels in leaves were measured by staining with 1 mg/mL nitro blue tetrazolium (NBT) and the method was in accordance with Evans Blue staining.

Analysis of PdPap poplar seedlings induced with recombinant rHFBII-4 in response to *A. alternata*

A. alternata was cultured in PDA medium at 28 °C for 20 days and spores were collected and adjusted to 5×10^6 spores/mL in sterile water. PdPap poplar seedlings were cultured in WPM medium containing 100 mg/L purified rHFBII-4 (The inactivated recombinant rHFBII-4 was used as a control). Subsequently, leaves were infected by injection (two sites on either side of the main vein) with 5-μL spore suspension and cultured at 25 °C on wet filter papers in a petri dish for 4 days.

Results

Sequence analysis of hydrophobin gene *HFBII-4*

The DNA sequence of the hydrophobin gene *HFBII-4* was 429 bp in length, containing three exons and two introns. The cDNA sequence of *HFBII-4* was 294 bp in length, encoding a 97 amino acid (aa) with a calculated molecular weight of 9.74 kDa and a theoretical isoelectric point (pI) of 4.41. SignalP prediction indicated the presence of a 16 aa signal peptide at the N-terminus and a predicted cleavage site for signal peptidase was observed between residues A16 and G17 (A1A-GP), indicating that HFBII-4 is secreted. The HFBII-4 protein was a member of the “hydrophobin 2 superfamily” by sequencing analysis, which was shown in our previous publication [3]. The cDNA sequence of the *HFBII-4* gene was deposited into the GenBank database with accession number KY914555.

Tricine-SDS-PAGE analysis

Tricine-SDS-PAGE analysis was performed to determine whether *P. Pastoris* GS115-HFBII-4 could successfully

express recombinant rHFBII-4. Unlike the empty vector transformant GS115-pPIC9K, GS115-HFBII-4 yielded a clearly visible protein band with a molecular weight of ~9.74 kDa in the Tricine-SDS-PAGE gel (Fig. 1), indicating that hydrophobin HFBII-4 was successfully produced in the *P. pastoris* cells.

Differential expression of hormone signal transduction-related genes in PdPap poplar seedlings induced with recombinant rHFBII-4

To determine whether the recombinant rHFBII-4 could stimulate a response in PdPap seedlings, the transcription levels of genes related to plant hormone signaling were studied using RT-qPCR. The plant hormone auxin plays a role in most aspects of plant growth and development. *AUX1* and *LAX2* encode auxin influx carrier, which plays a major role in many aspects of plant growth and development [29]. The expression of *AUX1* was downregulated under rHFBII-4 induction and was higher than the control at 4 and 24 h. Protein transport inhibitor response 1 (TIR1) encoded by *TIR1* is an auxin receptor. *GH3.17* encodes Indole-3-acetic acid-amido synthetase, which can catalyze the synthesis of indole-3-acetic acid (IAA)-amino acid conjugates. The expression levels of *LAX2*, *TIR1*, *IAA8*, and *GH3.17* were initially decreased, then increased and at last decreased. The expression levels of *GH3.17* under rHFBII-4 induction were higher than the control. The peak transcription levels of *AUX1*, *LAX2*, *TIR1*, *IAA8*, and *GH3.17* genes under rHFBII-4 induction were 0.37-, 4.00-, 3.16-, 2.17-, and 3.14-fold at 24 h, respectively (Fig. 2a, b, c, d, f). However, expression level of the *MP* gene under rHFBII-4 induction was upregulated and higher than that of the control at 24 h (Fig. 2e), peaking at 2.57-fold at 24 h.

The JA and SA signal pathways play important roles in plant defense systems [18]. The pathogenesis-related protein

Fig. 1 Tricine-SDS-PAGE analysis of recombinant rHFBII-4 protein secreted by transgenic yeast strain GS115-HFBII-4. 1, protein markers; 2 and 4, supernatants from transformant GS115-HFBII-4 induced by methanol; 3, supernatant of empty vector transformant GS115-pPIC9K induced by methanol

gene (*PR1*) encodes pathogenesis-related protein PR-1, which is a major PR group induced by pathogens, SA, or ethylene and is commonly used as a marker for SAR. Non-expressor of pathogenesis-related gene (*NPR1*) encodes regulatory protein NPR1, which is a regulatory gene of the SA signal pathway. In the SA signal pathway, transcription levels of *PR1* and *NPR1* under rHFBII-4 induction were higher than the control, with peak transcription levels of 76.90- and 3.29-fold, respectively (Fig. 2g and h). The *JAR1* is a JA-amino synthetase gene that is required to activate JA for further transduction of plant hormone signal [30], the MYC2 protein functions as transcriptional activators in plants and can upregulate the expression of disease-resistant gene under stresses. The F-box protein CORONATINE INSENSITIVE1 (COI1) is a key regulator of the JA signaling pathway. Together with JASMONATE ZIM (JAZ) domain transcriptional repress or proteins, COI1 functions as a JA-Ile receptor in the E3 ubiquitin-ligase SKP1-Cullin-F-box complex SCF^{COI1} [31]. Octadecanoid derivative-responsive catharanthus AP2-domain protein gene *ORCA3* is a jasmonic acid-responsive gene and regulates jasmonate-responsive expression, which could induce systemic resistance in the host plant [32]. In our study, the transcription of *JAR1* was higher than the control at 8–72 h. The transcription levels of *COI* and *ORCA3* under rHFBII-4 induction were higher than the control. The peak transcription levels of *JAR1*, *COI*, *JAZ6751*, *MYC2*, and *ORCA3* were 2.87-, 72.50-, 749.61-, 14.42-, and 1.58-fold, respectively, at 24 h (Fig. 2i–m). In the control, expression of *ORCA3* was downregulated following treatment with denatured rHFBII-4 (Fig. 2m).

Enzymatic activities of PPO, POD, and PAL in PdPap poplar seedlings induced with recombinant rHFBII-4

Following treatment of PdPap poplar seedlings induced with recombinant rHFBII-4, PPO, POD, and PAL enzyme activities were measured. In plants, PPO activity has been related to defense mechanisms against pathogens. PPO catalyzes the oxygen-dependent oxidation of phenols to quinones, which is assumed to be active in plant defense against pests and pathogens. Compared to 0 day, PPO activity in PdPap seedlings induced with rHFBII-4 was lower at 1, 2, 3 and 7 days. However, PPO activity in PdPap poplar seedlings under rHFBII-4 induction was higher than the control, peaking at 513 U g⁻¹ min⁻¹. Also, the PPO activity in the control decreased with increasing processing time (Fig. 3a). PAL is a key enzyme of the phenylpropanoid pathway that catalyzes the deamination of phenylalanine to trans-cinnamic acid, a precursor for the lignin and flavonoid biosynthetic pathways. Studies of several different species of plants show that PAL activity increased with the biotic and abiotic stress [33]. Compared with the control, both PPO and PAL activities in PdPap seedlings under rHFBII-4 induction were higher at 1, 2, 3, 5, and 7 days after treatment (Fig. 3a and b). POD is associated

Fig. 2 Expression of genes related to PdPap poplar hormone signal transduction pathways after induction with recombinant rHFBII-4. PdPap poplar seedlings were cultured in WPM medium containing rHFBII-4 (The inactivated recombinant rHFBII-4 was used as a control). The leaves were harvested at 0, 2, 4, 8, 24, and 72 h for RNA extraction. Primers for RT-qPCR were as described in our previous publication [3]. X axis: time points, Y axis: expression level = $\log_2$ (fold change in expression). The graph shows gene expression in PdPap poplar following induction with recombinant rHFBII-4. In the auxin signal transduction pathway, **a–f** represent the expression of *AUX1*, *LAX2*, *TIR1*, *IAA8*, *MP*, and *GH3.17*, respectively; in the salicylic acid signal transduction pathway, **g** and **h** represent the expression of *PR1* and *NPR1*, respectively; in the jasmonic acid signal transduction pathway, **i–m** represent the expression of *JAR1*, *COI*, *JAZ6751*, *MYC2*, and *ORCA3*, respectively

with the active defense reactions in higher plants in response to foreign organisms. Additionally, the activity of the POD in PdPap seedlings under rHFBII-4 induction was higher than the control at 3 and 5 days and lower than the control at 1, 2, and 7 days (Fig. 3c).

Chlorophyll and soluble protein content in PdPap poplar seedlings induced with recombinant rHFBII-4

Chlorophyll in leaves plays a vital role in plant growth and the chlorophyll content can be used as an index to diagnose

Fig. 3 Enzymatic activity of PPO, PAL, and POD in PdPap poplar induced with rHFBII-4. PdPap poplar seedlings were cultured in WPM medium containing rHFBII-4 (The inactivated recombinant rHFBII-4

was used as a control). Leaves were harvested at 0, 1, 2, 3, 5, and 7 days to determine PPO, PAL, and POD activity. **a** Activity of PPO. **b** Activity of PAL. **c** Activity of POD

nutrient status. Herein, the chlorophyll content under rHFBII-4 induction was higher than that the control at 2–5 days, peaking at 1.98 mg g^{-1} (Fig. 4a). Interestingly, the chlorophyll content under rHFBII-4 induction was lower than in control at 1 and 7 days.

Soluble protein is an important osmotic adjustment substance and nutrient. Soluble protein under rHFBII-4 induction was increased after 1–3 days, with peak content reaching 1.62 mg g^{-1} (Fig. 4b). However, soluble protein content under rHFBII-4 induction was lower than the control at 1 day and returned to control level after 7 days (Fig. 4b). Soluble protein showed a downward trend in the control at 3–7 days, which was similar to that under rHFBII-4 induction.

ROS content, cell permeability, and antifungal ability of PdPap poplar seedlings induced with recombinant rHFBII-4

Evans Blue staining revealed that the cell permeability of control PdPap poplar leaves (stained light blue; Fig. 5a: the upper pane) was higher than that of leaves under rHFBII-4 induction (unstained; Fig. 5a: the lower pane). In order to study plants

under rHFBII-4 induction, the ROS content was evaluated by staining leaves with NBT (Fig. 5b). The results revealed that the ROS content of leaves (the light color) under rHFBII-4 induction was lower compared with control leaves (the deep color).

Finally, PdPap poplar seedlings were infected by *A. alternata* and disease-related spots were apparent as regular circles on leaves under rHFBII-4 induction, but as irregular shapes covered with mycelia in control leaves (Fig. 5c). Thus, the disease area was smaller in leaves induced with rHFBII-4.

Discussion

Trichoderma can release small secreted cysteine-rich proteins (SSCPs) that stimulate plant growth and induce natural plant protection against pathogens [34]. Hydrophobins not only play important roles in the formation of a protective layer surrounding the hyphae and sexual structures, the development of aerial hyphae, sporulation, spore dispersal, and fruiting body formation [4, 5], but also mediate interactions between *Trichoderma* species with plants and pathogens in

Fig. 4 Chlorophyll and soluble protein content of PdPap poplar leaves. PdPap poplar seedlings were cultured in WPM medium containing rHFBII-4 (The inactivated recombinant rHFBII-4 was used as a

control). Leaves were harvested after 0, 1, 2, 3, 5, and 7 days to determine chlorophyll and soluble protein content. **a** Chlorophyll content. **b** Soluble protein content

Fig. 5 Cell membrane permeability, ROS content, and antifungal ability of PdPap poplar induced with recombinant rHFBII-4. PdPap poplar seedlings were cultured in WPM medium containing rHFBII-4 (The inactivated recombinant rHFBII-4 was used as a control). Leaves were stained with 1 mg/mL Evans Blue nitro blue tetrazolium (NBT) overnight, respectively, and destained in 95% ethanol in a boiling water bath for 5 min. After PdPap poplar induced with rHFBII-4, leaves were infected by injection with 5- μ L spore suspension and cultured at 25 °C on wet filter papers in a petri dish for

4 days. **a** and **b** Evans Blue and NBT staining of PdPap leaves, respectively. 1, Control; 2, rHFBII-4 induction. **c** Antifungal ability of PdPap leaves against *A. alternata*. 1, Control leaves not inoculated with *A. alternata* at 4 days after treatment; 2, control leaves induced with inactivated rHFBII-4 and inoculated with 5×10^6 spores/mL *A. alternata* at 4 days after treatment; 3, leaves induced with rHFBII-4 and inoculated with 5×10^6 spores/mL *A. alternata* at 4 days after treatment. The four repeats for each PdPap line are labeled **a**, **b**, **c**, and **d**

the environment [7–9, 13]. Our previous study demonstrated biocontrol functions of the class II hydrophobin HFB2-6 of *T. asperellum* ACCC30536 [3]. The hydrophobin gene *HFB2-6* was expressed in *E. coli* and the recombinant protein rHFB2-6 was found to affect the transcription of poplar defense-related genes. However, high-level expression of

recombinant protein in *E. coli* often results in aggregation and the formation of inclusion bodies [35]. This represents a great hurdle in the production and purification of recombinant proteins using *E. coli* as host. Inclusion bodies require extensive processing involving isolation from cells, solubilization, refolding, and purification to produce bioactive proteins.

Thus, in the present study, hydrophobin gene *HFBII-4* was expressed in *P. pastoris* and PdPap poplar seedlings were induced with the recombinant rHFBII-4.

Following treatment with rHFBII-4, transcription analysis of poplar hormone signal transduction genes was performed. The transcription trends of *AUX1*, *LAX2*, *TIR1*, and *IAA8* were similar and the expression levels of these genes were higher than these in the control, which was consistent with the results of our previous research [3], indicating that rHFBII-4 can also induce genes related to auxin signal transduction. In the salicylic acid signal transduction pathway related to disease resistance, the *NPR1* gene is an essential regulator of plant SAR and also plays an important role in JA/ET pathway [36], and SAR confers resistance to a broad spectrum of pathogens [37]. The *PR1* gene encodes an important defense protein reported to possess antifungal activity and play a role in host salicylic acid-mediated defense signaling [38]. In our current study, transcription of salicylic acid signal transduction-related gene *PR1* was induced by recombinant rHFBII-4 and *NPR1* expression also exceeded that in the control (Fig. 2g and h). The over-expression of *NPR1* did not lead to *PR1* gene expression in *Arabidopsis* [37]. In our study, the *PR1* in the control showed lower level at 24 h, which was consistent with the result of Mou et al. [37]. The transcription levels of *NPR1* and *PR1* in the present work were higher than those reported by Huang et al. [3]. This suggests that *PR1* and *NPR1* may respond more strongly to rHFBII-4 than to rHFB2-6.

The JA signal transduction pathway plays important roles in plants. The *JAR1* gene encodes jasmonate amino acid synthetase that activates JA for further transduction of plant hormone signals [31]. Meanwhile, COI1 is an E3 ubiquitin ligase that catalyzes the ubiquitination of proteins destined for degradation via the proteasome-mediated pathway. COI1 can perceive JA signals (JA-Ile) required for JA-mediated pathogen and wound responses, and activate a signal transduction pathway that culminates in the transcriptional activation or repression of JA-responsive genes [31]. JASMONATE ZIM-DOMAIN (JAZ) proteins could repress the expression of JA-response gene *MYC2*. However, the perception of bio-active JA by the F-box protein COI1 triggers the SCFCOI1/ubiquitin-dependent degradation of JAZ and further enhances the expression of transcription factor gene *MYC2*. The JA-responsive gene *ORCA3* regulates jasmonate-responsive expression, an important inducer of ISR in plants [32]. In our present study, transcription of *JAR1*, *COI*, *JAZ6751*, and *MYC2* was upregulated following treatment with rHFBII-4 (Fig. 2i–m). Thus, rHFBII-4 appears to play a role in jasmonic and salicylic acid signal transduction pathways, which are responsible for broad-spectrum resistance to pathogens in poplar.

In the process of enzymatic browning, PPO promotes POD activity by generating H_2O_2 from the oxidation of phenolic compounds. A number of studies indicate that PPO and PAL may participate in the defense reaction by inducing plant resistance against fungi [37, 38]. Herein, PdPap poplar seedlings were induced with rHFBII-4 and the enzymatic activities of PPO, PAL, and POD were measured. Both PPO and PAL activities were higher under rHFBII-4 induction than the control, which was consistent with the result of Yu et al. [39]. While the exact mechanisms have yet to be determined, POD is known to play a part in increasing a plant's defense against pathogens. However, the activity of the POD in PdPap seedlings was lower than in control at 1, 2, and 7 days, which was consistent with the result of Yu et al. [39]. It could be that the eliciting plant response protein Epl1 had a larger impact on the plant's defense. Chlorophyll in leaves plays a vital role in plant growth. In our study, the chlorophyll content under rHFBII-4 induction was higher than that in the control at 2–5 days after treatment (Fig. 4a). Interestingly, the chlorophyll content in leaves under rHFBII-4 induction was lower than the control at 1 and 7 days. However, there were no previous reports about the relationship between the hydrophobin and chlorophyll. Soluble protein is an important osmotic adjustment substance and nutrient. Soluble protein increased under rHFBII-4 induction after 1–3 days (Fig. 4a). However, soluble protein content returned to control levels after 7 days (Fig. 4b) and there were no reports about the relationship between the hydrophobin and soluble plant proteins before.

ROS such as the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO\cdot$) are formed by the partial reduction of oxygen. Many reports have shown that high levels of ROS are associated with damage to nucleic acids, proteins, and lipids. The ROS content can be measured by staining with NBT, which can reflect the resistance of plants to various stresses [40]. In our previous study, heat shock protein gene *Hsp24* and transcription factor gene *TasMYB36* from *Trichoderma* were transformed into PdPap poplar, and NBT staining revealed that transgenic PdPap-Hsp24s and PdPap-TasMYB36 plants possessed enhanced resistance to pathogens [24, 41]. In our present study, NBT staining revealed that the ROS content was lower under rHFBII-4 induction than the control (Fig. 5b). Moreover, ethidium bromide (EtBr) staining assays showed that transgenic PdPap-Hsp24s plants had lower cell permeability than PdPap-Con plants [24]. In our study, Evans Blue staining showed that the color of treated PdPap poplar leaves was deeper than the control, which revealed the cell permeability of treated PdPap poplar leaves was higher than that of control leaves (Fig. 5a). The results of NBT and Evans Blue staining indicate that transgenic and rHFBII-4-mediated induction in vitro can reduce damage to plants.

Furthermore, PdPap poplar seedlings were induced with rHFBII-4 and leaves were infected with *A. alternata*.

Interestingly, disease-related spots on leaves under rHFBII-4 induction were smaller than control leaves (Fig. 5c), indicating that *HFBII-4* from *T. asperellum* can enhance the resistance of PdPap poplar to *A. alternata*.

In summary, the class II hydrophobin gene *HFBII-4* upregulated the expression of growth-related, disease resistance and defense-response genes in PdPap poplar. Additionally, Evans Blue and NBT staining assays indicated that rHFBII-4 could reduce damage to plants. When PdPap seedlings induced with rHFBII-4 were infected with *A. alternata*, the diseased leaf area was significantly smaller than that of control leaves. Therefore, rHFBII-4 promotes PdPap by enhancing resistance to *A. alternata*.

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Compliance with ethical standards

Ethical approval The manuscript does not contain experiments using animal or human studies.

Conflict of interest The authors declare that they have no conflict of interest.

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