Alternative Splicing of Heat Shock Transcription Factor 2 Regulates Expression of the Laccase Gene Family in Response to Copper in *Trametes trogii*

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ABSTRACT White-rot fungi, especially *Trametes* strains, are the primary source of industrial laccases in bioenergy and bioremediation. *Trametes* strains express members of the laccase gene family with different physicochemical properties and expression patterns. However, the literature on the expression pattern of the laccase gene family in *Trametes trogii* S0301 and the response mechanism to Cu²⁺, a key laccase inducer, in white-rot fungal strains is scarce. In the present study, we found that Cu²⁺ could induce the mRNAs and proteins of the two alternative splicing variants of heat shock transcription factor 2 (*TtHSF2*). Furthermore, the overexpression of alternative splicing variants *TtHSF2α* and *TtHSF2β-I* in the homokaryotic *T. trogii* S0301 strain showed opposite effects on the extracellular total laccase activity, with maximum laccase activities of approximately 0.6 and 3.0 U ml⁻¹, respectively, on day 8, which are 0.4 and 2.3 times that of the wild-type strain. Similarly, *TtHSF2α* and *TtHSF2β-I* play opposite roles in the oxidation tolerance to H₂O₂. In addition, the direct binding of *TtHSF2α* to the promoter regions of the representative laccase isoenzymes (*TtLac1* and *TtLac13*) and protein-protein interactions between *TtHSF2α* and *TtHSF2β-I* were detected. Our results demonstrate the crucial roles of *TtHSF2* and its alternative splicing variants in response to Cu²⁺. We believe that these findings will deepen our understanding of alternative splicing of heat shock transcription factors (HSFs) and their regulatory mechanism of the laccase gene family in white-rot fungi.

IMPORTANCE The members of laccase gene family in *Trametes* strains are the primary source of industrial laccase and have gained widespread attention. Increasing the yield and enzymatic properties of laccase through various methods has always been a topic worthy of attention, and there is no report on the regulation of laccase expression through HSF transcription factor engineering. Here, we found that two alternative splicing variants of *TtHSF2* functioned oppositely in regulating the expression of laccase genes, and copper can induce the expression of almost all members of the laccase gene family. Most importantly, our study suggested that *TtHSF2* and its alternative splicing variants are vital for copper-induced production of laccases in *T. trogii* S0301.

KEYWORDS white-rot fungi, *Trametes trogii*, laccase gene family, heat shock transcription factor, alternative splicing

Laccases (benzenediol: oxygen oxidoreductases, Lac, EC 1.10.3.2) belong to the family of copper-containing polyphenol oxidases and can catalyze the oxidation of diverse aromatic substrates concomitantly with the reduction of molecular oxygen to water (1, 2). Laccases display remarkably broad substrate selectivity and are able to

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oxidize *ortho*- and *para*-diphenols, aminophenols, polyphenols, polyamines, and aryl diamines, as well as some inorganic ions (2–4). Especially, the discovery of redox mediators represented by diammonium salt of 2,2'-azine-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) further expanded the substrate range and also the potential of laccase application (5–7). Alone or in concert with the redox mediators, laccases are considered to be ideal “green catalysts” and widely used in diverse industries including pulping and paper, textile, food, biosynthesis, bioenergy, biological detection, and bioremediation (1, 2, 8).

Laccases are distributed in several species, including plants, insects, bacteria, and fungi (1, 9). Fungal laccases, especially those present in white-rot fungi, have gained considerable attention because of abundant laccase-producing strains in nature, easy cultivation and fermentation, secretion of metabolites into the fermentation broth, high laccase activity, and excellent laccase property (2, 8, 10). Usually, most white-rot fungi produce multiple laccase isozymes with different kinetic and physicochemical features (11). Moreover, the laccase gene family was identified in the whole-genome sequences of *Coprinopsis cinerea* (12, 13), *Pleurotus ostreatus* (3, 14, 15), and *Auricularia auricula-judae* (16, 17) consisting of 17, 11, and 7 laccase isoenzymes, respectively. The primary source of industrial laccase, strains belonging to the *Trametes* genus, also possess members of the laccase gene family, with five members in *Trametes pubescens* (18), seven members in *Trametes trogii* (19), and four members in *Trametes versicolor* (18, 20).

Laccase isoenzymes usually show different expression patterns responsive to a wide range of inducers and/or physicochemical parameters (such as nutrient level, developmental stage, culture conditions, concentration of Cu^{2+} , lignin, and xenobiotics, pH, temperature) (2, 15, 21). In addition to being a key part of the laccase active site, Cu^{2+} is also the most effective inducer (15, 22). For example, Cu^{2+} has been reported to increase laccase production by 50-fold in *P. ostreatus* (22). Moreover, laccase induction by Cu^{2+} has been seen as a simple and straight forward process (Cu^{2+} added at a certain concentration leads to higher laccase production) (15, 23). Furthermore, Cu^{2+} can enhance laccase activity in almost all fungal strains, such as *Corioloropsis rigida* (24), *Cerrena unicolor* (25), *T. versicolor* (26), and *T. pubescens* (27). Thus, copper is the most commonly used metal ion for laccase induction (2). The expression of laccase isoenzymes has been demonstrated to mainly occur at the level of gene transcription (15, 22). However, the mechanism by which Cu^{2+} regulates the expression of different laccase isoenzymes in white-rot fungal strains is not well understood.

The experimental evidence regarding copper-induced production of laccase by transcription factors (TFs) has mainly been based on a copper-binding cysteine-rich transcription factor (*ACE1*) and copper-sensing transcription factors (such as *Cuf1* and *Mac1*). *ACE1* can bind to the ACE element (5'-NTNNHGCTG-3') in the promoter regions of laccase and other multicopper oxidases (Mcos), which has been confirmed in *Phanerochaete chrysosporium* (28) and *Ceriporiopsis subvermispora* (29) using electrophoretic mobility shift assay (EMSA). As a major virulence factor of *Cryptococcus neoformans* (30) and *Aspergillus fumigatus* (31), the activity and expression of laccase can be mediated by *Cuf1* and *Mac1* with lower laccase activity in mutant strains. In addition, a helix-turn-helix (HTH) DNA-binding domain transcription factor, *Ltf4*, in *P. ostreatus* is significantly upregulated by Cu^{2+} and can bind to metal response elements in the promoter region of laccase isoenzyme POXc (32). These studies expand our understanding of laccase gene regulation, but the exact mechanism by which those TFs affect laccase isozymes in white-rot fungi still needs more support from experimental evidence.

In eukaryotes, heat shock transcription factors (HSFs) are the major regulators responsive to heat stress, developmental processes, and chemical stimuli (33–35). Under stress conditions, HSFs form a trimer and bind to the DNA-responsive element, the heat shock element (HSE), in the promoters of stress-inducible genes, resulting in activation of their target proteins such as molecular chaperones and other stress proteins

(36, 37). Putative HSEs are abundantly present in the promoter regions of several laccase genes in white rot fungi (38). Furthermore, it has been reported that heat shock treatment can increase the laccase activity in *Trametes* strains, and an increase in one of the laccase isoenzyme mRNA levels was detected (39). In addition, the effects of HSFs on the laccase activity have been found in pathogenic fungi *Phytophthora sojae* and *C. neoformans* (40–42). In *P. sojae*, the silencing of *PsHSF1* reduced the extracellular laccase activity, with significant downregulation of two laccase genes (*PsLAC4* and *PsLAC5*) (41). Similarly, HSF and Ssa1 (an Hsp70 homolog) can form a regulatory complex in *C. neoformans*. Moreover, *Ssa1* knockout strains showed lower laccase activity, along with reduced *Lac1* mRNA levels (40, 42). However, the mechanisms by which HSF regulates the expression of laccase genes in white-rot fungi are not well understood. For example, it is unclear whether HSFs are involved in copper-induced laccase gene expression and activity. The regulation of expression of laccase isoenzymes by HSF engineering has not been reported yet.

In addition, alternative splicing (AS) is a critical posttranscriptional event and plays an important role in stress responses in eukaryotes (43). Multiple alternative splicing forms of HSFs have been obtained from plants (*Populus*, rice, lily, etc.) and animals (human, mouse, *Schistosoma mansoni*, etc.) (44–46). The transcriptional activity of HSFs regulated by alternative splicing has been documented (47, 48). Different alternative splicing variants may perform different physiological functions (49, 50). However, no study is available on HSF alternative splicing in fungal species.

Among white-rot fungi, strains belonging to the *Trametes* genus are the main laccase producers in nature, and the potential laccase-producing strains can be utilized in industrial applications (10). In our previous studies, Cu^{2+} was shown to induce a thermotolerant *T. trogii* S0301 strain to produce high levels of laccase with high thermal stability through liquid fermentation processes (23, 51), indicating the potential applications of *T. trogii* S0301 in laccase production. Furthermore, the approximately complete genome of *T. trogii* S0301 from high coverage Sequel platform sequencing reads has revealed the diversity of the laccase gene family in this strain (19). Here, we find the existence of two splicing isoforms of *TtHSF2* (*TtHSF2 α* and *TtHSF2 β*), along with the increase of laccase activity induced by Cu^{2+} in *T. trogii* S0301. To further explore whether *TtHSF2* splicing isoforms are involved in the transcriptional regulation of the laccase gene family in *T. trogii* S0301 (*TtLacs*), we sought to (i) analyze the expression patterns of *TtHSF2* splicing isoforms under Cu^{2+} treatments, (ii) construct *TtHSF2* splicing isoforms overexpressing strains under the genetic background of *T. trogii* S0301 homokaryotic strain, and (iii) explore the effects and the possible role of *TtHSF2* splicing isoforms on laccase gene expression and laccase activity.

RESULTS

Temperature and Cu^{2+} induced the expression of two alternative splicing variants of *TtHSF2*. *TtHSF2* was composed of five exons interrupted by four introns, and all sequences at exon-intron junctions followed the GT-AG rule (Fig. 1A). Two alternative splicing variants of *TtHSF2* (designated *TtHSF2 α* and *TtHSF2 β*) were obtained from the mycelium of *T. trogii* S0301 by heat shock treatment (Fig. 2A). *TtHSF2 α* and *TtHSF2 β* were composed of all five exons; however, *TtHSF2 α* retained intron 3, a single 68-nucleotide intron (Fig. 1A). The results of reverse transcription-PCR (RT-PCR) revealed that both *TtHSF2 α* and *TtHSF2 β* were induced by Cu^{2+} (0.02 and 2 mM) and/or higher temperature (35°C). *TtHSF2 α* was the predominant subtype at the mRNA level, whereas that of *TtHSF2 β* was relatively low (Fig. 2A).

TtHSF2 α was predicted to encode a protein with 607 amino acids (*TtHSF2 α*), whereas *TtHSF2 β* contained a premature stop codon in the sequences and generated two potential open reading frames (ORFs). These ORFs were predicted to encode proteins of 81 (*TtHSF2 β -I*) and 358 (*TtHSF2 β -II*) amino acids (Fig. 1B). The deduced amino acid sequences of *TtHSF2 β -I* and *TtHSF2 β -II* were the same as the N- and C termini of *TtHSF2 α* , respectively. However, there was a slight difference in *TtHSF2 β -I*, an additional 13 amino acids (DLRSPAGSTARGQ) at its C terminus (Fig. 1B). Amino acid

FIG 1 Sequence analysis of *TtHSF2*. (A) Gene and two cDNA isoform structure analyses of *TtHSF2*. The Gene Structure Display Server 2.0 was used to analyze the intron and exon distributions of *TtHSF2*. Both the alternative splicing site (GT-AG) and the *TtHSF2β* cDNA putative early translation termination sequence (TGA) are marked in red. (B) Protein domain analysis of *TtHSF2* based on the deduced amino acid sequences of *TtHSF2β-I* and *TtHSF2β-II*. The domains of HSFs included the N-terminal DBD, the oligomerization domain (HR-A/B), an NLS, an NES, and the C-terminal transcriptional activation domain (AHA motifs).

sequence alignment and phylogenetic analysis revealed that *TtHSF2α* contained typical domains of HSFs, including an N-terminal DNA-binding domain (DBD), an oligomerization domain carrying two adjacent hydrophobic heptad repeats (HR-A/B), a nuclear localization signal (NLS), nuclear export signal (NES), and a C-terminal transcriptional activation domain (AHA motifs) (Fig. 1B; see also Fig. S1 and S2 in the supplemental material). However, *TtHSF2β-I* contained a part of the DBD domain, and *TtHSF2β-II* had typical domains in the C terminus, including NLS, NES, and AHA motifs (Fig. 1B).

We further explored which proteins of *TtHSF2* were expressed *in vivo* and their expression patterns. Western blotting results revealed three protein bands (ca. 65.4, 37, and 8.7 kDa) using antiserum raised against the full length of *TtHSF2α* and *TtHSF2β-I* that matched to the length of the deduced *TtHSF2α*, *TtHSF2β-I*, and *TtHSF2β-II*, respectively (Fig. 2B). Temperature and Cu^{2+} caused the accumulation of *TtHSF2α* and *TtHSF2β-I* proteins (Fig. 2B), which was consistent with their expression pattern at the mRNA level (Fig. 2A). There was no regularity in the effect of Cu^{2+} and temperature on protein accumulation of *TtHSF2β-II*. Both *TtHSF2β-I* and *TtHSF2β-II* were more predominant in *T. troglia* S0301 than *TtHSF2α* under the tested conditions (Fig. 2B).

Overexpression of *TtHSF2α* and *TtHSF2β-I* showed opposite effects on laccase activity in homokaryotic strain of *T. troglia* S0301. To explore the effect of *TtHSF2* on the laccase activity *in vivo*, homokaryotic *T. troglia* S0301 strains overexpressing *TtHSF2α* and *TtHSF2β-I* were constructed (Fig. 3; see also Fig. S3). No laccase activity was detected in the culture filtrates of overexpressed or wild-type (WT) strains, whereas laccase activity was induced in the culture grown on GYP liquid medium (see Materials and Methods) containing 2 mM Cu^{2+} after 2 days (Fig. 4A and B). Overexpression of *TtHSF2α* reduced the laccase activity to approximately 0.3 and 0.6 U ml⁻¹ on days 6 and 8, respectively (Fig. 4A). The enzymatic activity of strain overexpressing *TtHSF2α* was approximately 33.2 and 36.2% of the control, indicating that the overexpression of *TtHSF2α* strains significantly inhibited the enzyme activity (Fig. 4A and C). In contrast,

FIG 2 Expression pattern analysis of *TtHSF2*. (A) Analysis of expression pattern of *TtHSF2α* and *TtHSF2β* by RT-PCR in GYP medium with Cu^{2+} (0, 0.02, and 2 mM) and/or temperatures (28 or 35°C). (B) Western blot analysis of the expression patterns of *TtHSF2* in *T. troglia* S0301 cultured in GYP medium with Cu^{2+} at different concentrations (0, 0.02, and 2 mM) at 28 or 35°C. β -Actin served as the loading control. Lanes 1 to 5 in panels A and B represent 28°C with 0 mM Cu^{2+} , 28°C with 2 mM Cu^{2+} , 35°C with 0 mM Cu^{2+} , 35°C with 2 mM Cu^{2+} , and 35°C with 0.02 mM Cu^{2+} , respectively.

increased extracellular laccase activity was observed in strains overexpressing *TtHSF2β-I*, with laccase activities of approximately 1.0 and 3.0 U ml⁻¹ on days 6 and 8 (Fig. 4B), respectively, whereas laccase activities of the control were 0.6 and 1.3 U ml⁻¹ on days 6 and 8 (Fig. 4B), respectively. The enzyme activities of strains overexpressing *TtHSF2β-I* were 1.6 and 2.3 times higher than those of the control on days 6 and 8, respectively (Fig. 4B and D).

In addition to the effects on the total extracellular laccase activity, thermostability assay revealed that the crude laccases of strains overexpressing *TtHSF2β-I* were more stable than those of strains overexpressing *TtHSF2α*, with the residual average activity of 86.6 and 57.3% after incubation at 50°C for 6 h, respectively, whereas the wild-type strain *T. troglia* S0301 had a residual average activity of 73.0% (Fig. 4E). In addition, thermoactivation was observed in the crude laccases from strains overexpressing *TtHSF2β-I* at 50°C (Fig. 4F). These results indicated that *TtHSF2* played a dual regulatory function in laccase production through different alternative splicing variants and is likely to cause the expression of different laccase isoenzymes.

Dual role of *TtHSF2* in oxidation tolerance. A previous study indicated that PsHSF1 is essential for the proper growth of *P. sojae* under oxidative stress (41). To explore whether the deregulation of *TtHSF2* effect the tolerance to the oxidative stress in this strain, the WT strain, the blank load transformation group (control), the strains overexpressing *TtHSF2α* and *TtHSF2β-I* were exposed to H₂O₂. The growth of all those strains was normal on GYP medium for 6 days at 28°C (Fig. 5A and B). Compared to the GYP medium without H₂O₂, treatment with 13 mM H₂O₂ led to an average of 38.9% reduction of mycelial growth in the WT and control strains, with 77.8 and 6.3% reductions in strains overexpressing *TtHSF2α* and *TtHSF2β-I*, respectively (Fig. 5C). In contrast to the WT and control, the mycelial growth was increased by an average of 32.6% in strains overexpressing *TtHSF2β-I* under treatment with 13 mM H₂O₂ (Fig. 5C). These observations support that HSFs are required for tolerance against oxidative stresses in

FIG 3 Detection of strains overexpressing *TtHSF2α* and *TtHSF2β-I*. (A and B) Detection of hygromycin resistance genes in strains overexpressing *TtHSF2α* (A) and *TtHSF2β-I* (B). Lanes WT, (+), and (-) represent the wild-type strain of *T. troglia* S0301, pRH2304 vector (positive control), and negative control, respectively. Lanes 1 to 7 represent independent strains overexpressing *TtHSF2α* and *TtHSF2β-I*. (C and D) Detection of gene expression at the transcription level in three representative strains overexpressing *TtHSF2α* (C) and *TtHSF2β-I* (D). The data are from three independent measurements and represent means $\pm$ the standard deviations (SD). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (E and F) Gene expression detected at protein levels by Western blotting in three representative strains overexpressing *TtHSF2α* (E) and *TtHSF2β-I* (F).

fungi and that *TtHSF2α* and *TtHSF2β-I* play opposite effects on oxidation tolerance (Fig. 4 and 5).

***TtHSF2β-I* overexpression activated the expression of *TtLacs*.** To reveal whether the increase in the laccase activity and change in enzyme characterization was due to the change in the expression pattern of different laccase isoenzymes, the transcriptional analysis of *TtLacs* was performed using qRT-PCR. The overexpression of *TtHSF2β-I* upregulated the expression of all laccase isoenzyme genes except *TtLac53* (Fig. 6; see also Table S1). In *TtHSF2β-I* transformants, the transcription level of *TtLac13* was affected by *TtHSF2β-I* up to 5.9 times (highest) compared to the control in the GYP medium without Cu^{2+} and *TtLac16* up to 1.4 times (lowest) with no significance ($P > 0.05$) (Fig. 6; see also Table S1). The transcription of *TtLacs* was upregulated to various degrees by 2 mM Cu^{2+} , and the laccase isoenzyme genes with the highest upregulation multiples were *TtLac2-2* (6.3 times), *TtLac50* (5.3 times), and *TtLac16* (4.3 times), whereas the transcription of *TtLac13* was suppressed to 60 and 67% in the GYP medium containing 2 mM Cu^{2+} compared to the GYP medium in control and *TtHSF2β-I* transformants, respectively (Fig. 6; see also Table S1). Interestingly, the addition of 2 mM Cu^{2+} to strains overexpressing *TtHSF2β-I* induced a major increase of up to 10.6 times in the laccase isoenzyme transcription (*TtLac50*), except *TtLac53* (1.1 times) (Fig. 6). In addition, the transcription of two other multicopper oxidases (Mcos) of *T. troglia*, *TtMco2-5*, and *TtMco2-8*, was affected by *TtHSF2β-I* up to 1.8 and 1.7 times compared to the control in the GYP medium without Cu^{2+} (Fig. 6; see also Table S1). These results confirmed that *TtHSF2* played a key role in regulating the expression of *TtLacs* and other multicopper oxidases.

***TtHSF2α* bound directly to the laccase isoenzyme promoter.** From the consistent relationship between *TtHSF2* expression and laccase activity under high temperature and Cu^{2+} treatments, we inferred that *TtHSF2* might directly regulate the expression of laccase isoenzymes. To investigate whether *TtHSF2* was directly bound to the promoter regions of laccase genes and also whether there was a significant difference in

FIG 4 Analysis of enzymatic properties of laccases from strains overexpressing *TtHSF2α* and *TtHSF2β-I*. (A and B) Extracellular laccase activity of strains overexpressing *TtHSF2α* (A) and *TtHSF2β-I* (B). All data were gathered using ABTS as a substrate. The data represent means $\pm$ the SD. Error bars, SD of three replicate samples and the enzyme activity calculated three times for each sample. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (C and D) Native-PAGE analysis of laccase from strains overexpressing *TtHSF2α* (C) and *TtHSF2β-I* (D). The protein content of each lane was 20 μ g and was dyed by ABTS for 5 min after electrophoresis was completed. (E and F) Thermostability analysis of laccase from strains overexpressing *TtHSF2α* (E) and *TtHSF2β-I* (F). The activity is represented as the percentage relative to the value without heat treatment, which was assigned as 100%. Data points are averages of triplicate measurements, and error bars represent the SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

the binding ability among three protein forms of TtHSF2, EMSA was performed with soluble recombinant TtHSF2 α , TtHSF2 β -I, and TtHSF2 β -II proteins expressed in *Escherichia coli*. The promoter regions of two representative laccase isoenzymes (*TtLac1* and *TtLac13*) containing GAANN TTC box elements were selected for EMSA (Fig. 7A). TtHSF2 α was found to retard the migration of both DNA fragments of *TtLac1* and *TtLac13*, and TtHSF2 α produced progressively slower DNA migration when the protein concentration increased (Fig. 7B and C). No shift in the band was observed for *TtLac1* and *TtLac13* when the biotin-labeled probes were mixed with TtHSF2 β -I or TtHSF2 β -II protein (Fig. 7D). This dependency on TtHSF2 α suggested that TtHSF2 α specifically bound and also exhibited the possibility of TtHSF2 functioning as a direct regulator of laccase activity.

Interactions between alternative isoforms of TtHSF2. The overexpression of *TtHSF2β-I* changed the expression pattern of several laccase isoenzymes (Fig. 6). However, the EMSA results showed that TtHSF2 β -I could not directly bind to the promoter regions of laccase genes (Fig. 7). Moreover, previous studies have shown that different alternative isoforms of HSFs can interact with each other (52). Therefore, Y2H was performed to explore whether alternative isoforms of TtHSF2 interacted with each other. It was observed that TtHSF2 α could interact with TtHSF2 β -I. Similarly, TtHSF2 α interacted with TtHSF2 β -I-Δ13 (Fig. 8), indicating that the 13 amino acids of TtHSF2 β -I had no effect on the interaction between them. Meanwhile, no obvious interaction

FIG 5 Analysis of the role of *TtHSF2α* and *TtHSF2β-I* in oxidative stress. (A and B) Mycelial growth of the wild-type strain (WT), the blank load transformation group (control). Strains overexpressing *TtHSF2α* and *TtHSF2β-I* were inoculated on GYP medium with 0 (A) or 13 (B) mM H_2O_2 and cultured at 28°C for 7 days, respectively. (C) The colony diameters of the testing strains were measured and subjected to statistical analysis. The growth inhibition rate is relative to the growth rate of GYP medium with 0 mM H_2O_2 (inhibition rate = [the diameter of untreated strain – the diameter of 13 mM H_2O_2 -treated strain]/[the diameter of untreated strain × 100%]) (70). Three repeats were performed, and similar results were obtained. Data points are averages of triplicate measurements, error bars represent the SD, and asterisks represent significant differences ($P < 0.001$).

was observed between *TtHSF2β-II* and the other two proteins of *TtHSF2* (*TtHSF2β-I* and *TtHSF2α*) (Fig. 8).

DISCUSSION

White-rot fungi usually produce multiple laccase isozymes with different kinetic and physicochemical features and expression patterns, and copper is the most commonly used inducer for laccase production (22, 38). However, little work has been done to study the mechanism by which Cu^{2+} regulates the expression of different

FIG 6 Analysis of *TtLac* expression pattern in strains overexpressing *TtHSF2* β -I. The expression of *TtLac* mRNA was detected by real-time PCR under different concentrations of Cu²⁺ (0 or 2 mM) at 28°C. Panels A to I show the transcription levels of different laccase isoenzymes. The WT strain (*T. troglis* S0301) was set as the control group. Data points are averages of triplicate measurements and represent the means $\pm$ the SD. The experiment was repeated three times.

laccase isoenzymes in white-rot fungi. In the present study, we found that *TtHSF2* is a Cu²⁺-induced transcription factor in *T. troglis* S0301, and *TtHSF2* overexpressing shows effects on the expression and activity of laccase in fungi (Fig. 4 and 6), which supports the studies in the pathogenic fungi *Phytophthora sojae* and *C. neoformans* (40, 41). We also made two novel observations. First, we discovered alternative splicing of *TtHSF2* and its protein products by RT-PCR and Western blotting, which extends our observation of HSF alternative splicing to fungal species. Second, our results indicated that *TtHSF2* α and *TtHSF2* β -I showed opposite effects on the laccase activity when overexpressed in a homokaryotic strain of *T. troglis* S0301 (Fig. 4). Furthermore, the direct binding of *TtHSF2* α to the promoter regions of the representative laccase isoenzymes and protein-protein interactions between *TtHSF2* α and *TtHSF2* β -I were detected (Fig. 7 and 8), which expands our understanding of the molecular mechanisms by which HSF and its alternative splicing variants regulate laccase gene expression and activity. Most importantly, our study suggested that *TtHSF2* and its alternative splicing variants are vital for copper-induced production of laccases in *T. troglis* S0301.

***TtHSF2*, another copper-responsive TF involved in laccase activity regulation.**

The expression of laccase isoenzymes in white-rot fungi mainly occurs at the level of gene transcription (2, 15, 22). The number and location of putative *cis*-acting elements (such as metal response element, heat shock response element, oxidative stress response element, and nitrogen suppression response element) in the upstream promoter regions of laccase isoenzymes suggest a complex picture of laccase expression and regulation (38), which provides us with the opportunity to use putative *cis*-acting elements as a probe for the identification of TFs in laccase gene regulation, especially

FIG 7 EMSA of TtHSF2 α binding to the promoter regions of *TtLac1* and *TtLac13*. (A) Probe sequences and mutant probe sequences extracted from *TtLac1* and *TtLac13* promoters used in the EMSA. The HSE *cis*-acting elements are marked in yellow (GAA) and green (TTC). (B and C) Mutated probes in which the perfect HSE motif 5'-GAANNNTC-3' is replaced with 5'-CAANNAAT-3' and TtHSF2 α is bound to the HSE elements of the *TtLac1* (B) and *TtLac13* (C) promoters, respectively. Biotin probe, 3' end was labeled with biotin; cold probes, probes not labeled with biotin; cold competition probes, mutant probes not labeled with biotin; antibody, anti-TtHSF2 α polyclonal antibody. (D) Comparison of the abilities of HSF2 α , HSF2 β -I, and HSF2 β -II proteins to bind to HSE elements.

by Cu²⁺. Thus, copper-induced laccase transcriptional regulation is mainly based on copper-binding and copper-responsive transcription factors, such as ACE1 in white-rot fungi and Cuf1 in *C. neoformans* (28, 29, 53).

In the presence of copper, ACE1 can bind to ACE sequence elements in the promoter of several genes, such as the *sod1* gene encoding superoxide dismutase (54, 55). To date, two ACE1 transcription factors have been identified from *P. chrysosporium* (28) and *Ceriporiopsis subvermispora* (29). *P. chrysosporium* produces a different type of multicopper oxidase (Mco) other than laccase. Copper increases the transcription from genes *mco1* and *mco2*. However, the transcription activity analysis by EMSA and a cell-free transcription system showed that only *mco1* is the potential target gene of PcACE1, rather than *mco2* (53). Similarly, Álvarez et al. (29) confirmed the binding of CsACE1 to the promoter regions of the laccase gene by EMSA. Those results provide evidence that activation of transcription of laccase genes by copper is mediated by an ACE1-like transcription factor. However, PcACE1 did not show the transcription activity toward the copper-induced PcMCO2 (53), which provides additional clues to understand the complex molecular mechanisms underlying copper-driven transcriptional regulation by other kinds of fungal transcription factors.

In addition, copper-responsive transcription factor Cuf1 is a homolog of the metallo-transcription factor (Mac1), which acts as a copper-sensing transcription factor and maintains copper homeostasis (30, 31). As a major virulence factor of *C. neoformans*, laccase activity and laccase expression were induced by high concentrations of exogenous copper, which was mediated by Cuf1 (30). Similarly, the laccase activity decreased dramatically when *mac1* was deleted in *Aspergillus fumigatus* (31). These results demonstrate the role of copper-responsive transcription factors in the expression of laccase in *C. neoformans*. Evidence is still lacking, however, as to their occurrence in white-rot fungi.

The HSF1 silencing in *P. sojae* and knockout of the interacting protein of HSF in *C. neoformans* can reduce the extracellular laccase activity, with significant downregulation of

FIG 8 Analysis of the interaction between TtHSF2α and TtHSF2β-I in yeast systems. Yeast cells with serial dilutions (1, 1/20, and 1/100) were present in synthetic dropout (SD) medium lacking Leu and Trp (left) or SD medium lacking Leu, Trp, His, and adenine (right) and photographed 3 days after plating. Yeast cells harboring AD-T and BD-53 or pGADT7 and pGBKT7 vectors were used as a positive or a negative control, respectively.

laccase genes (40–42). These data suggest that HSF may play a role in the regulation of laccase expression and activity. However, those studies aim to reveal the roles of laccase activity in the pathogenicity of pathogenic fungi. Thus, it is still unclear whether HSFs involve the regulation of laccase expression and activity by Cu^{2+} , especially in white-rot fungi. In the present study, we found that *TtHSF2* overexpression affects the expression and activity of laccase in fungi (Fig. 4 and 6), supporting the idea that HSFs are regulators of laccase expression and activity in fungi. Moreover, we also demonstrate that *TtHSF2* is a copper responsive transcription factors (Fig. 2) and can enhance the transcripts of several laccase isoenzymes under Cu^{2+} treatment (Fig. 6). Combined with the expression of *TtLacs* in *TtHSF2β-I* overexpression strains (Fig. 6) and the binding activity by EMSA (Fig. 7), we infer that several laccase isoenzyme genes (such as *TtLac1* and *TtLac13*) are potential target genes of *TtHSF2* in *T. troglia*. To further explore the relationship between copper and the expression of laccase gene family and also other potential target genes in the view of *TtHSF2*, the detailed function of *TtHSF2* is currently under investigation in our laboratory.

Alternative splicing of fungal HSF. HSFs in human, mouse, zebrafish, and *A. thaliana* exist as alternatively spliced isoforms—for example, two subtypes of human *HSF1*, *HSF2*, and *HSF4* (48, 56); four subtypes of *Drosophila* *HSF1* (47); two subtypes of rice *HSFA2d* (44); and *HSFA2*, *HsfA4c*, *HsfA7b*, *HsfB1*, and *HsfB2a* in *A. thaliana* (36)—suggesting that alternative splicing is a common feature among *HSF* genes in most eukaryotic cells. Our present study supports this idea with the finding of two alternative splicing subtypes, *TtHSF2α* and *TtHSF2β*, in fungal species (Fig. 1 and 2).

Typical HSFs have a modular structure containing N-terminal DBD, oligomerization domain (OD or HR-A/B region), NLS, NES, and activator motifs (AHA motifs) (57). The isoforms are formed by intron retention and exon skipping, leading to protein products containing partial domains, recombinant domains, or loss of certain domains (43, 57). The alternative splicing of HSFs is usual in the downstream of HR-A/B domain in plants and animals, including *Drosophila* (47), mice (50), *Medicago sativa* (58), *Bombyx mori* Diapause (59), and Pacific oyster *Crassostrea gigas* (60). We found both *TtHSF2α* (arising from exon 4 retention) and *TtHSF2β* (arising from exon 4 skipping) isoforms by

alternative splicing in the coding region of DBD (Fig. 1), and similar observations have been detected mainly in plants, such as rice *HSFA2d*, *A. thaliana* *HSFA2*, lily *HSFA3B*, *Sedum alfredii* Hance *HSF4c*, and *Medicago sativa* *MsHSF1* (44, 52, 56, 58, 61). These results suggest that HSFs of plants and animals adopt different strategies to generate alternative splicing isoforms, and fungal HSFs are closer to those of plants.

Alternative splicing in the coding region of N-terminal DBD usually introduces new stop codons to form truncated small-molecule proteins of HSFs. Small molecular weight proteins of S-HSFA2, with molecular weights of 14 and 16 kDa, were detected in *A. thaliana* and LiHSFA3B-III in lily, respectively (52, 56). However, truncated small-molecule proteins OsHSFA2dII in rice were not found (44). Another reason for this is that new HSF splice products with premature stop codons are degraded through the nonsense-mediated mRNA decay pathway (58, 62, 63). We found that *TtHSF2 α* encodes a 607-amino-acid protein (TtHSF2 α) that contains all conserved domains exhibiting a characteristic of class A HSFs, whereas *TtHSF2 β* transcripts encode a truncated small-molecule protein of 81 amino acids (TtHSF2 β -I) that contains a partial N-terminal DBD (Fig. 1). In addition, another protein (TtHSF2 β -II) with a molecular weight of $\sim$ 37 kDa was detected (Fig. 2), which is consistent with the deduced protein encoded by ORF2 of *TtHSF2 β* . Most interestingly, this protein precisely contains N-terminal NLS and NES domains of TtHSF2. Posttranscriptional mechanisms, including alternative splicing (AS) and alternative translation initiation, may explain the diversity of proteins involved in plant development and stress responses (49).

TtHSF2 α and TtHSF2 β -I showed opposite effects on laccase activity. Previous studies demonstrate that the alternative splicing of HSFs can be induced when cells are exposed to heat shock and oxidative stress and so on (47, 56). Expression patterns of HSF alternative splicing variants and their products are associated with specific biological roles by means of the deregulation of the target genes, including heat shock protein chaperones (HSPs) such as HSP70 and HSP90 and other genes beyond HSPs (33, 58, 64, 65). For example, in rice, *OsHSFA2d* was alternatively spliced to encode two different proteins with different expression profiles, subcellular localizations, and functions (44). *A. thaliana* *HSFA3* transcription is induced by oxidative stress, which can further upregulate the expression of antioxidant gene *APX2*, thus increasing *APX2* activity and reducing the accumulation of H₂O₂ (65). In this study, we found both *TtHSF2 α* and *TtHSF2 β* were induced by Cu²⁺ treatment at mRNA and protein levels (Fig. 2), and overexpressing *TtHSF2 β -I* transformants showed higher extracellular laccase activity along with the upregulation of laccase isozymes mRNA than in the WT strain under control growth conditions, whereas the extracellular laccase activity of *TtHSF2 α* -overexpressing *T. troglit* S0301 strains decreased (Fig. 4). These results indicated a dual regulatory function of *TtHSF2* alternative splicing variants in laccase production.

Such antagonistic functions of different alternative splicing variants have been previously described for *HSF4* isoforms (*HSF4a* and *HSF4b*) in mice (50), *HSFA2d* in rice (44), and other transcription factors, e.g., bZIP transcription factor *ABI5* (66) and *WRKY62* in rice (67). In addition, the ratio of different alternative splicing variants is believed to play important roles in regulating the expression of the HSP gene (50). Thus, future studies should analyze whether the ratio of alternative splicing variants of TtHSF2 regulates the activity of laccases.

Alternative splicing may generate premature termination codons in reading frames, thus generating truncated protein products with smaller molecular weights and partial domains. The functions of these truncated proteins are not identical to those of their full-length counterparts (44, 56, 61). TtHSF2 β -I, a small protein of TtHSF2 β with a molecular weight of $\sim$ 8 kDa, was detected; it exhibited promotive effects on laccase expression and extracellular laccase activity, which were opposite to those of TtHSF2 α (Fig. 4). The truncated protein products of different alternative splicing variants that act as a new functional HSF to affect the gene expression of its own or target genes have been found in *A. thaliana* and lily (52, 56). In *A. thaliana*, the small-molecular-weight (14 kDa) subtype (S-HSFA2) can function as a functional HSF and bind to the

TATA box-proximal clusters of HSE in the *HsfA2* promoter to activate its own transcription (56). In lily, LIHSFA3B-III can specifically disturb the protein interactions of LIHSFA3A-I and LIHSFA3B-I, and the heterologous expression of LIHSFA3B-III increases the tolerance of salt and heat in *A. thaliana* and *Nicotiana benthamiana* (52). Furthermore, EMSA analysis showed that TtHSF2 α directly bound to the promoter regions of *TtLac1* and *TtLac13* (Fig. 7), which indicates that genes encoding laccase isozymes could be target genes that are directly regulated by TtHSF2 and its alternative splicing variants. However, our study was constrained by the lack of TtHSF2 knockout strains. Therefore, further studies are required to confirm the function of HSF2 β -I and other proteins encoded by TtHSF2 in regulating the laccase expression under HSF knockout.

The results of the domain analysis showed that TtHSF2 β -I lacked a typical DBD and C-terminal transcriptional activation domains (AHA domains). Moreover, EMSA (Fig. 7) and H2Y analysis (Fig. 8) did not reveal any binding and transcriptional activation activity, suggesting that other functional proteins could cooperate with TtHSF2 β -I to regulate the expression of laccase isoenzymes. The protein interactions between different protein products encoded by alternative splicing variants can regulate the expression and function of their own genes (67, 68). In mouse embryonic fibroblasts, the interaction between HSF2 β and HSF1 β inhibited the transcription activity of HSF1 β (68). LIHSF3B-III interacted with LIHSFA3A-I, affecting its homologous interaction or heterologous interaction with LIHSFA3B-I. In our study, the protein interaction between TtHSF2 β -I and TtHSF2 α was detected (Fig. 8). Based on these results, we infer that the interaction between TtHSF2 β -I and TtHSF2 α could regulate the expression of laccases in the *T. troglia* S0301 strain.

Conclusion. In summary, we showed the existence of two novel TtHSF2 isoforms and their roles in laccase gene expression and the extracellular laccase activity in fungi. Our data provide evidence that TtHSF2 is a Cu²⁺-responsive gene, and alternative splicing variants of TtHSF2 play a dual role in laccase gene expression and the extracellular laccase activity. These findings are important to understand the molecular regulatory mechanism of the laccase gene family members in *T. troglia* S0301 and other white-rot fungi responsive to Cu²⁺, the most important laccase inducer.

MATERIALS AND METHODS

Fungal strains and chemicals. The homokaryotic and heterokaryotic strains of *T. troglia* S0301 were maintained on the GYP medium (2% glucose, 0.5% yeast extract, 0.5% tryptone, 0.1% MgSO₄·7H₂O) at 28°C. All strains were stored at the strain collection center of Biotechnology Research Center of Life Science and Technology College, Kunming University of Science and Technology. ABTS was purchased from Sigma-Aldrich (USA).

Culture conditions. Homogenized inocula of *T. troglia* S0301 strains were prepared according to the methods described in our previous studies (69). Aliquots of the mycelial suspension (5% [vol/vol]) were used as inoculum and added to 250-ml Erlenmeyer flask containing 50 ml of GYP, followed by incubation at 28 or 35°C at 200 rpm. For enzyme production, and RNA and protein preparations, CuSO₄ was added to get the final concentrations of 0.02, 0.5, 1, and 2 mM. The liquid cultures were sampled every 2 days, and the supernatants or mycelia were obtained by centrifugation at 9,000 rpm for 5 min at 4°C for further studies. In order to observe the mycelial growth under oxidative stress conditions (70), H₂O₂ (Aldrich, 323381, 30 wt%) was mixed into GYP solid medium and cultured at 28°C for 7 days.

DNA and RNA isolation and gene cloning. Total genomic DNA was extracted from 6-day cultured fresh fungal hyphae using the CTAB (cetyltrimethylammonium bromide) method. Extraction of total RNA and first-strand cDNA synthesis were performed using TRIzol RNA isolation reagents (Promega) and HiScript II Q Select RT SuperMix for qPCR (Vazyme Biotech, Nanjing, China), respectively. Total genomic DNA or cDNA was then subjected to PCR analysis using gene-specific primers (Table 1) to clone the gene coding or promoter regions. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Plasmid construction and transformation of homokaryotic *T. troglia* S0301 strains. The cDNA fragments of TtHSF2 α and TtHSF2 β -I were amplified using primers harboring BamHI and NcoI sites and subsequently cloned into vector pRH2304-TtGPD (see Fig. S3) using a ClonExpress II OneStep cloning kit (Vazyme, Nanjing, China). The pRH2304-TtGPD:TtHSF2 α and pRH2304-TtGPD:TtHSF2 β -I plasmids were extracted in large quantity using the Qiagen plasmid plus maxi kit (Qiagen, Germany), and protoplasts of the homokaryotic *T. troglia* S0301 strain were prepared as described previously (73). These were used for polyethylene glycol-mediated protoplast transformation (71) to obtain TtHSF2 α and TtHSF2 β -I over-expressed homokaryotic *T. troglia* S0301 strains. Resistant colonies were randomly selected from the

TABLE 1 PCR primer sets used in this study

Target gene	Sequence ID	Primer Name	Sequence (5'-3') ^a	Use	T _m (°C)
<i>ThHSF2α</i>	T_trogii_03921	OE-ThHSF2α-for	gatgacgagtagccatgagATGTCACAAAGCGAATCAGGTG	Amplification of <i>ThHSF2α</i> for overexpression vector construct	62
<i>ThHSF2β-I</i>	T_trogii_03921	OE-ThHSF2α-rev	tcgcccctcgagaccgcatccACGTTTCTTCTTCGTGACGG	Amplification of <i>ThHSF2β-I</i> for overexpression vector construct	62
<i>ThHSF2α</i>	T_trogii_03921	OE-ThHSF2β-I-for	gatgacgagtagccatgagATGTCACAAAGCGAATCAGGTG	Amplification of <i>ThHSF2α</i> for prokaryotic expression vector construct	58
<i>ThHSF2β-I</i>	T_trogii_03921	PE-ThHSF2α-rev	tcgcccctcgagaccgcatccCAAGAGCTCGCTGCA	Amplification of <i>ThHSF2β-I</i> for prokaryotic expression vector construct	58
<i>ThHSF2α</i>	T_trogii_03921	PE-ThHSF2β-I-for	cacATGTCACAAAGCGAATCAGGTG	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	PE-ThHSF2α-rev	TCAACGTTTCTTCTTCGTG	RT-PCR	60
<i>ThHSF2α</i>	T_trogii_03921	PE-ThHSF2β-I-for	cacATGTCACAAAGCGAATCAGGTG	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	PE-ThHSF2α-rev	TCAATGTCGCGCGCAGTAG	RT-PCR	60
<i>ThHSF2α</i>	T_trogii_03921	RT-ThHSF2β-I-for	TCCCTCGTTTCTCTCTTAAGCT	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	RT-ThHSF2α-rev	CGGTAATGACAGTCAAGTCGG	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	RT-ThHSF2β-I-for	ACCCCGCCAATGAATCT	RT-PCR	60
<i>TtGpd</i>	T_trogii_08382	RT-ThHSF2β-I-rev	TCAACGTTTCTTCTTCGTG	RT-PCR	60
<i>TtLac1</i>	T_trogii_01690	TtGpd-A4	GGCATTCCTGGACTACACCGAGG	RT-PCR	60
<i>TtLac7</i>	T_trogii_07087	RT-TtLac1-for	GGCATGAGCTTCAGAAAGTCTTG	RT-PCR	60
<i>TtLac13</i>	T_trogii_03201	RT-TtLac1-rev	CTTCATTTTCATTATATACACCCAG	RT-PCR	60
<i>TtLac16</i>	T_trogii_10550	RT-TtLac7-for	TGCGGTGAGCGTATCG	RT-PCR	60
<i>TtLac50</i>	T_trogii_13594	RT-TtLac13-for	TGGCGTTGACTATGCCTGA	RT-PCR	60
<i>TtLac53</i>	T_trogii_01340	RT-TtLac7-rev	CTTGCGTTCATCGGGAAC	RT-PCR	60
<i>TtLac2-2</i>	T_trogii_01688	RT-TtLac16-rev	TGAAAGCGAAGGACGAG	RT-PCR	60
<i>TtMco2-5</i>	T_trogii_05747	RT-TtLac13-for	GAAGGAGGAGTAAATGTCT	RT-PCR	60
<i>TtMco2-8</i>	T_trogii_11554	RT-TtLac16-rev	CTGATGCGCGGTGAAT	RT-PCR	60
<i>ThHSF2α</i>	T_trogii_03921	RT-TtLac50-for	AGACAGGAGCACTGGGAGG	RT-PCR	60
<i>ThHSF2β-II</i>	T_trogii_03921	RT-TtLac50-rev	GTCCCTGTCCACCTGAATC	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	RT-TtLac53-for	CGAGCACGAAGGAATAGCG	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	RT-TtLac53-rev	GGCTCCAATTGTGCTTAT	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	RT-TtLac2-2-for	TCCCTGGGCGACCTT	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	RT-TtLac2-2-rev	CTACATCAGCTTTCCTCG	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	RT-TtMco2-5-for	TGGCATCTCGGGTGAAA	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	RT-TtMco2-5-rev	CCACCACCACCATGAC	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	RT-TtMco2-8-for	ACCTTTAGGATACCGCAT	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	RT-TtMco2-8-rev	CATCAGGCCCTACGAGTG	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	Y2H-ThHSF2α-for	GTCCGCTATTGGTG	RT-PCR	60
<i>ThHSF2β-I</i>	T_trogii_03921	Y2H-ThHSF2α-rev	tcagagagagcctcatatgATGTCACAAAGCGAATCAGGTG	Amplification of <i>ThHSF2α</i> fragment for <i>ThHSF2α</i> -pGBKT7 vector construct	63
<i>ThHSF2β-I</i>	T_trogii_03921	Y2H-ThHSF2β-II-BD-for	tcagagagcctccggaattcTCAACGTTTCTTCTTCGTGACG	Amplification of <i>ThHSF2β-II</i> -pGBKT7 vector construct	62
<i>ThHSF2β-I</i>	T_trogii_03921	Y2H-ThHSF2β-II-BD-rev	gtaccagattacgctcatatgATGTCGCGTATGGCGAC	Amplification of <i>ThHSF2β-II</i> -pGBKT7 vector construct	62
<i>ThHSF2β-I</i>	T_trogii_03921	Y2H-ThHSF2β-II-AD-for	atgccaccgggtggaattcTCAACGTTTCTTCTTCGTGACG	Amplification of <i>ThHSF2β-II</i> -pGADT7 vector construct	63
<i>ThHSF2β-I</i>	T_trogii_03921	Y2H-ThHSF2β-II-AD-rev	gtaccagattacgctcatatgATGTCGCGTATGGCGAC	Amplification of <i>ThHSF2β-II</i> -pGADT7 vector construct	63
<i>ThHSF2β-I</i>	T_trogii_03921	Y2H-ThHSF2β-I-AD-for	tcagagagcctccggaattcTCAACGTTTCTTCTTCGTGACG	Amplification of <i>ThHSF2β-I</i> -pGADT7 vector construct	62
<i>ThHSF2β-I</i>	T_trogii_03921	Y2H-ThHSF2β-I-AD-rev	gtaccagattacgctcatatgATGTCGCGTATGGCGAC	Amplification of <i>ThHSF2β-I</i> -pGADT7 vector construct	62
<i>ThHSF2β-I</i>	T_trogii_03921	Y2H-ThHSF2β-I-Δ13-AD-for	atgccaccgggtggaattcTCAATGTCGCGCGCAGT	Amplification of <i>ThHSF2β-I</i> -Δ13-pGADT7 vector construct	62
<i>ThHSF2β-I</i>	T_trogii_03921	Y2H-ThHSF2β-I-Δ13-AD-rev	gtaccagattacgctcatatgATGTCGCGTATGGCGAC	Amplification of <i>ThHSF2β-I</i> -Δ13-pGADT7 vector construct	62

^aThe primer sequence consistent with the vector is indicated by lowercase letters.

screening medium (0.1% yeast extract and peptone, 1% sucrose, $200\text{ }\mu\text{g ml}^{-1}$ hygromycin B). Subsequently, the hygromycin B resistance gene was detected by PCR and further confirmed by checking the mRNA and protein expression by qPCR and Western blotting, respectively.

Prokaryotic expression and polyclonal antibody preparation. The cDNA fragments of *TtHSF2 α* and *TtHSF2 β -I* were cloned into the pDE1 vector using a pDE1 directional expression kit (TsingKE; Beijing, China) to produce pDE1-6 $\times$ His:*TtHSF2 α* and pDE1-6 $\times$ His:*TtHSF2 β -I*. These were transformed into *E. coli* strain Rosetta(DE3) for protein expression and purification according to the instructions from Invitrogen. Anti-TtHSF2 α and anti-TtHSF2 β -I polyclonal antibodies were generated by immunizing mice with purified bacterially expressed recombinant proteins.

Total protein extraction and Western blotting. Fresh fungal hyphae were collected and fully milled with liquid nitrogen. Next, 0.1 g of powder was added to 200 μl of protein extraction buffer containing 10 mM Tris-HCl (pH 8.0), 0.02% NaN_3 , and 0.001% phenylmethylsulfonyl fluoride. After centrifugation and subsequently freeze-drying at low temperature, crude proteins were obtained and used for Western blotting according to the standard protocol (72). The concentration of proteins was determined using the bicinchoninic acid (BCA) protein assay kit (Tiangen Biotech, Shanghai, China). The primary antibodies used were anti-TtHSF2 α polyclonal antibody at a 1:4,000 dilution and anti- β -actin (AC026; Abcam) at a 1:10,000 dilution. The secondary antibody used was horseradish peroxidase (HRP)-conjugated goat anti-rat IgG(H+L) (AS028; Abcam) at a 1:8,000 dilution. Finally, a chemiluminescent HRP substrate (TaKaRa, Dalian, China) was used to perform autoradiography on a polyvinylidene difluoride film.

Laccase activity and thermal stability assay. The laccase activity was determined under standard conditions with ABTS as the substrate as described previously (51, 69). In brief, a reaction mixture containing 0.5 ml of appropriately diluted crude enzyme and 1.1 ml of 2 mM ABTS in phosphate citrate buffer (100 mM [pH 4.0]) was evaluated at 30°C. The increase in the absorbance at 420 nm within 3 min was recorded. One unit of the enzyme activity was defined as the amount of enzyme that oxidized 1 μmol of the ABTS per min.

For thermostability analysis, the laccase supernatants were preincubated at given temperatures (50, 60, and 70°C) in phosphate citrate buffer (100 mM [pH 4.0]) for different time intervals and subsequently thoroughly cooled on ice for another 30 min. Next, the residual laccase activity was determined according to the standard laccase determination above. An identical amount of enzymes that were not heat treated but placed on ice was used as the positive control and set as 100%. All assays were performed in triplicate.

Electrophoretic mobility shift assay. DNA fragments in the upstream promoter regions of laccase isoenzymes *TtLac1* and *TtLac13* containing putative HSE (5'-GAANN TTC-3') element were cloned, and 3'-end biotin-labeled oligonucleotide probes were synthesized using an EMSA probe biotin labeling kit (Beyotime, Shanghai, China). EMSA was performed using a chemiluminescent EMSA kit (Beyotime), and chemiluminescent HRP substrate (TaKaRa) was used to perform autoradiographic detection on nylon membranes. Five reactions (a negative-control reaction, a sample reaction, a probe cold competition reaction, a mutant probe cold competition reaction, and an antibody-added supershift reaction) were set up. In each case, the experiment was performed three times, and representative blots are shown in the figures.

Real-time PCR. Quantitative RT-PCR was performed on an ABI Prism 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA) using gene-specific primer pairs (Table 1). The amplification conditions were 94°C for 5 s and 60°C for 30 s for 40 cycles. The glyceraldehyde-3-phosphate dehydrogenase (*TtGpd*) gene of *T. trogii* S0301 was used as a constitutively expressed endogenous control, and the $\Delta\Delta C_T$ method was used to calculate the relative transcription level.

Yeast two-hybrid assay. A yeast two-hybrid assay (H2Y) was performed according to the manufacturer's instructions (Clontech). In brief, the cDNA fragments of alternative splicing variants of *TtHSF2* were inserted into the pGBKT7 plasmid to generate bait vectors (*TtHSF2 α* -BD and *TtHSF2 β -II*-BD), whereas the *TtHSF2 β -I*, *TtHSF2 β -II*, and *TtHSF2 β -I- Δ 13* cDNA fragments (the nucleotides corresponding to the 13 amino acids at the end of *TtHSF2 β -I* were deleted to test the function of these 13 amino acids) were inserted into the pGADT7 plasmid to generate prey vectors *TtHSF2 β -I*-AD, *TtHSF2 β -II*-AD, and *TtHSF2 β -I- Δ 13*-AD, respectively. All of these plasmids were transformed into Y2HGold and Y187 yeast cells by electrotransformation, plating on synthetic dropout medium lacking Trp (SD/-Trp) and SD/-Leu, and incubation for 3 days at 30°C. The positive strains were selected for hybridization and generation of progeny cells grown on SD/-Trp/-Leu media. Subsequently, hybrid cells containing pairs of bait and prey vectors were transferred to SD/-Trp/-Leu/-His/-Ade media to determine protein interactions between different pairs. Yeast cells with BD-53 and AD-T vector pair were used as positive controls, whereas yeast cells with empty BD and AD vectors were used as negative controls (67).

Sequence and phylogenetic analysis. The gene structure was analyzed by the online software Gene Structure Display Server (<http://gsds.cbi.pku.edu.cn/>). The ORFs of *TtHSF2* were analyzed using the ORF Finder of NCBI (<https://www.ncbi.nlm.nih.gov/>). Based on the genome sequence, the laccase isozyme gene family sequence was extracted and analyzed from the *T. trogii* S0301 genomic data. The alignment was performed using Bioedit software, and phylogenetic analysis was performed with the neighbor-joining method (p-distance substitution model, complete deletion, and 1,000 bootstrap tests) using the software package MEGA 6.

Statistical analyses. Statistical analyses were performed using GraphPad Prism v8.3. One-way analysis of variance, followed by Tukey's test, was performed to determine the significance, and the significance level was set at 0.05 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Data availability. The genome and putative mRNA sequencing data presented here are associated with NCBI BioProject [PRJNA480364](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA480364) and BioSample [SAMN09635320](https://www.ncbi.nlm.nih.gov/biosample/SAMN09635320). The data sets generated/analyzed

during the present study are available and included either within this article or in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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We declare that there are no conflicts of interest.

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