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 Cu\(^{2+}\), a key laccase inducer, in white-rot fungal strains is scarce. In the present study, we found that Cu\(^{2+}\) 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 H\(_2\)O\(_2\). 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 Cu\(^{2+}\). 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
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 bio-remediation (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). 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 Cu₂⁺, lignin, and xenobiotics, pH, temperature) (2, 15, 21). In addition to being a key part of the laccase active site, Cu²⁺ is also the most effective inducer (15, 22). For example, Cu²⁺ has been reported to increase laccase production by 50-fold in P. ostreatus (22). Moreover, laccase induction by Cu²⁺ has been seen as a simple and straightforward process (Cu²⁺ added at a certain concentration leads to higher laccase production) (15, 23). Furthermore, Cu²⁺ can enhance laccase activity in almost all fungal strains, such as Coriolopsis 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 Cu²⁺ 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 Cu²⁺ and can bind to metal response elements in the promoter region of laccase isozyme 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.
Putative HSEs are abundantly present in the promoter regions of several laccase genes in white rot fungi. 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. In addition, the effects of HSFs on the laccase activity have been found in pathogenic fungi _Phytophthora sojae_ and _C. neoformans_. In _P. sojae_, the silencing of _PoHSF1_ reduced the extracellular laccase activity, with significant downregulation of two laccase genes (_PsLAC4_ and _PsLACS_) (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. 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. In our previous studies, Cu²⁺ 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. Here, we find the existence of two splicing isoforms of _TtHSF2_ (_TtHSF2α_ and _TtHSF2β_), along with the increase of laccase activity induced by Cu²⁺ 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, we sought to (i) analyze the expression patterns of _TtHSF2_ splicing isoforms under Cu²⁺ 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 Cu²⁺ 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 _TtHSF2α_ and _TtHSF2β_) were obtained from the mycelium of _T. trogii_ S0301 by heat shock treatment (Fig. 2A). _TtHSF2α_ and _TtHSF2β_ were composed of all five exons; however, _TtHSF2α_ retained intron 3, a single 68-nucleotide intron (Fig. 1A). The results of reverse transcription-PCR (RT-PCR) revealed that both _TtHSF2α_ and _TtHSF2β_ were induced by Cu²⁺ (0.02 and 2 mM) and/or higher temperature (35°C). _TtHSF2α_ was the predominant subtype at the mRNA level, whereas that of _TtHSF2β_ was relatively low (Fig. 2A).

_TtHSF2α_ was predicted to encode a protein with 607 amino acids (_TtHSF2α_), whereas _TtHSF2β_ 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 (_TtHSF2β-I_) and 358 (_TtHSF2β-II_) amino acids (Fig. 1B). The deduced amino acid sequences of _TtHSF2β-I_ and _TtHSF2β-II_ were the same as the N-and C termini of _TtHSF2α_, respectively. However, there was a slight difference in _TtHSF2β-I_, an additional 13 amino acids (DLRSPAGSTARQ) at its C terminus (Fig. 1B). Amino acid
sequence alignment and phylogenetic analysis revealed that ThSF2α 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, ThSF2β-I contained a part of the DBD domain, and ThSF2β-II had typical domains in the C terminus, including NLS, NES, and AHA motifs (Fig. 1B).

We further explored which proteins of ThSF2 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 ThSF2α and ThSF2β-I that matched to the length of the deduced ThSF2α, ThSF2β-I, and ThSF2β-II, respectively (Fig. 2B). Temperature and Cu²⁺ caused the accumulation of ThSF2α and ThSF2β-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 Cu²⁺ and temperature on protein accumulation of ThSF2β-II. Both ThSF2β-I and ThSF2β-II were more predominant in T. trogii S0301 than ThSF2α under the tested conditions (Fig. 2B).

**Overexpression of ThSF2α and ThSF2β-I showed opposite effects on laccase activity in homokaryotic strain of T. trogii S0301.** To explore the effect of ThSF2 on the laccase activity in vivo, homokaryotic T. trogii S0301 strains overexpressing ThSF2α and ThSF2β-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 Cu²⁺ after 2 days (Fig. 4A and B). Overexpression of ThSF2α 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 ThSF2α was approximately 33.2 and 36.2% of the control, indicating that the overexpression of ThSF2α strains significantly inhibited the enzyme activity (Fig. 4A and C). In contrast,
increased extracellular laccase activity was observed in strains overexpressing TtHSF2\(\beta\)-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 TtHSF2\(\beta\)-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 TtHSF2\(\beta\)-I were more stable than those of strains overexpressing TtHSF2\(\alpha\), 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. trogii S0301 had a residual average activity of 73.0% (Fig. 4E). In addition, thermostabilization was observed in the crude laccases from strains overexpressing TtHSF2\(\beta\)-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 affect the tolerance to the oxidative stress in this strain, the WT strain, the blank load transformation group (control), the strains overexpressing TtHSF2\(\alpha\) and TtHSF2\(\beta\)-I were exposed to \(\text{H}_2\text{O}_2\). 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 \(\text{H}_2\text{O}_2\), treatment with 13 mM \(\text{H}_2\text{O}_2\) 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 TtHSF2\(\alpha\) and TtHSF2\(\beta\)-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 TtHSF2\(\beta\)-I under treatment with 13 mM \(\text{H}_2\text{O}_2\) (Fig. 5C). These observations suggest that HSFs are required for tolerance against oxidative stresses in...
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 Cu²⁺ 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 Cu²⁺, 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 Cu²⁺ compared to the GYP medium in control and TtHSF2β-I transformants, respectively (Fig. 6; see also Table S1). Interestingly, the addition of 2 mM Cu²⁺ 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. trogii, 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 Cu²⁺ (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 Cu²⁺ 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

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**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. trogii 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 ± 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).
the binding ability among three protein forms of TtHSF2, EMSA was performed with soluble recombinant TtHSF2\textsubscript{a}, TtHSF2\textsubscript{b}-I, and TtHSF2\textsubscript{b}-II proteins expressed in Escherichia coli. The promoter regions of two representative laccase isoenzymes (TtLac1 and TtLac13) containing GAANNTTC box elements were selected for EMSA (Fig. 7A). TtHSF2\textsubscript{a} was found to retard the migration of both DNA fragments of TtLac1 and TtLac13, and TtHSF2\textsubscript{a} 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\textsubscript{b}-I or TtHSF2\textsubscript{b}-II protein (Fig. 7D). This dependency on TtHSF2\textsubscript{a} suggested that TtHSF2\textsubscript{a} 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 TtHSF2\textsubscript{β-I} changed the expression pattern of several laccase isoenzymes (Fig. 6). However, the EMSA results showed that TtHSF2\textsubscript{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\textsubscript{a} could interact with TtHSF2\textsubscript{β-I}. Similarly, TtHSF2\textsubscript{a} interacted with TtHSF2\textsubscript{β-I-D\textsubscript{13}} (Fig. 8), indicating that the 13 amino acids of TtHSF2\textsubscript{β-I} had no effect on the interaction between them. Meanwhile, no obvious interaction

**FIG 4 Analysis of enzymatic properties of laccases from strains overexpressing TtHSF2\textsubscript{α} and TtHSF2\textsubscript{β-I}.** (A and B) Extracellular laccase activity of strains overexpressing TtHSF2\textsubscript{α} (A) and TtHSF2\textsubscript{β-I} (B). All data were gathered using ABTS as a substrate. The data represent means ± 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 TtHSF2\textsubscript{α} (C) and TtHSF2\textsubscript{β-I} (D). The protein content of each lane was 20 μg and was dyed by ABTS for 5 min after electrophoresis was completed. (E and F) Thermostability analysis of laccase from strains overexpressing TtHSF2\textsubscript{α} (E) and TtHSF2\textsubscript{β-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.
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 isoforms 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
laccase isoenzymes in white-rot fungi. In the present study, we found that TtHSF2 is a Cu$^{2+}$-induced transcription factor in T. trogii 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 TtHSF2α and TtHSF2β-I showed opposite effects on the laccase activity when overexpressed in a homokaryotic strain of T. trogii S0301 (Fig. 4). Furthermore, the direct binding of TtHSF2α to the promoter regions of the representative laccase isoenzymes and protein-protein interactions between TtHSF2α and TtHSF2β-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. trogii 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...
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...
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 Cu²⁺, 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 Cu²⁺ 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. trogii. 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 HSF2a/2d (44); and HSF2α, 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 TtHSF2α encodes a 607-amino-acid protein (TtHSF2α) that contains all conserved domains exhibiting a characteristic of class A HSFs, whereas TtHSF2β transcripts encode a truncated small-molecule protein of 81 amino acids (TtHSF2β-I) that contains a partial N-terminal DBD (Fig. 1). In addition, another protein (TtHSF2β-II) with a molecular weight of ~37 kDa was detected (Fig. 2), which is consistent with the deduced protein encoded by ORF2 of TtHSF2β. 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).

**TtHSF2α and TtHSF2β-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 HSA3 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 TtHSF2α and TtHSF2β were induced by Cu2+ treatment at mRNA and protein levels (Fig. 2), and overexpressing TtHSF2β-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 TtHSF2α-overexpressing T. trogii 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 (HSF4α and HSF4β) in mice (50), HSFA2d in rice (44), and other transcription factors, e.g., bZIP transcription factor ABIs (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). TtHSF2β-I, a small protein of TtHSF2β with a molecular weight of ~8 kDa, was detected; it exhibited promotive effects on laccase expression and extracellular laccase activity, which were opposite to those of TtHSF2α (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 TtHSF2b directly bound to the promoter regions of TtLac1 and TtLac13 (Fig. 7), which indicates that genes encoding laccase isoenzymes 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 HSF2b-I and other proteins encoded by TtHSF2 in regulating the laccase expression under HSF knockout.

The results of the domain analysis showed that TtHSF2b-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 TtHSF2b-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 HSF2b and HSF1b inhibited the transcription activity of HSF1b (68). LIHSF3B-III interacted with LIHSFA3A-I, affecting its homologous interaction or heterologous interaction with LIHSFA3B-I. In our study, the protein interaction between TtHSF2b-I and TtHSF2a was detected (Fig. 8). Based on these results, we infer that the interaction between TtHSF2b-I and TtHSF2a could regulate the expression of laccases in the T. trogii 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. trogii 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. trogii 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. trogii 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 mycothalli 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 fragments of TtHSF2ogous interaction with LlHSFA3B-I. In our study, the protein interaction between LlHSF3B-III interacted with LlHSFA3A-I, affecting its homologous interaction or heterologous interaction with LlHSFA3B-I. In our study, the protein interaction between HSF2b-I and HSF2a was detected (Fig. 8). Based on these results, we infer that the interaction between HSF2b-I and HSF2a could regulate the expression of laccases in the T. trogii S0301 strain.

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Plasmid construction and transformation of homokaryotic T. trogii S0301 strains. The cDNA fragments of TtHSF2a and TtHSF2b-I were amplified using primers harboring BamHI and NcoI sites and subsequently cloned into vector pH2304-TgPD (see Fig. S3) using a ClonExpress II OneStep cloning kit (Vazyme, Nanjing, China). The pH2304-TgPD:TtHSF2a and pH2304-TgPD:TtHSF2b-I plasmids were extracted in large quantity using the Qiagen plasmid plus maxi kit (Qiagen, Germany), and protoplasts of the homokaryotic T. trogii S0301 strain were prepared as described previously (73). These were used for polyethylene glycol-mediated protoplast transformation (71) to obtain TtHSF2a and TtHSF2b-I over-expressed homokaryotic T. trogii S0301 strains. Resistant colonies were randomly selected from the
### TABLE 1 PCR primer sets used in this study

<table>
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<tr>
<th>Target gene</th>
<th>Sequence ID</th>
<th>Primer Name</th>
<th>Sequence (5’-3’)a</th>
<th>Use</th>
<th>Tm (°C)</th>
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<td>OE-TtHSF2α-for</td>
<td>tagacagcatgacgtctggtgATGTCAACAGCAATCAAGGTG</td>
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<tr>
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<td>Amplification of TtMco1 fragment for TtMco1 vector construct</td>
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</tr>
<tr>
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<td>Y2H-TtMco1-rev</td>
<td>CTACATCACGCTTTCCCTCG</td>
<td></td>
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</tr>
</tbody>
</table>
| aThe primer sequence consistent with the vector is indicated by lowercase letters.
and BioSample SAMN09635320 (Applied Biosystems, Foster City, CA) using gene-specific primers (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 (TgGpd) gene of T. trogii S0301 was used as a constitutively expressed endogenous control, and the ΔΔCT method was used to calculate the relative transcription level.

**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 isoform 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 and BioSample SAMN09635320. The data sets generated/analyzed during this study are deposited in NCBI under BioProject PRJNA480364.
SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

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Yu Zhang, Yuanyuan Wu, and Xulei Yang: methodology, data curation, writing—original draft; En Yang: visualization and investigation; Huini Chen: resources, investigation; Irbis Chagan: software; Jinping Yang: methodology, supervision, writing—review and editing.

We declare that there are no conflicts of interest.

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