Melatonin Confers Heavy Metal Tolerance of a Dark Septate Endophyte (DSE) Exophiala Pisciphila Via Lowering Oxidative Stress and Heavy Metal Accumulation

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Research article

Keywords: melatonin, dark septate endophytes, heavy metals accumulation, oxidative stress, tryptophan decarboxylase (TDC), N-acetylserotonin O-methyltransferase (ASMT)

DOI: https://doi.org/10.21203/rs.3.rs-54820/v1

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Abstract

**Background:** The high antioxidant capacity of melatonin contributing to heavy metal tolerance for plants and animals is widely studied, while researches on microorganisms especially in filamentous fungi are rare. One typical dark septate endophyte (DSE), *Exophiala pisciphila*, showed significant resistance to heavy metals.

**Results:** In this study, exogenous melatonin was verified to reduce heavy metal damage via relieving oxidative stress, activating antioxidant systems, and decreasing heavy metal accumulation in *E. pisciphila*. Melatonin biosynthesis enzyme genes were upregulated under heavy metal stress. Furthermore, the overexpression of *E. pisciphila TDC1* (*EpTDC1*) and *E. pisciphila ASMT1* (*EpASMT1*) responsible for melatonin biosynthesis in *Escherichia coli* and *Arabidopsis thaliana*, enhanced heavy metal stress tolerance for the two organisms by lowering the oxidative stress and reducing the Cd accumulation in the whole plants, especially in the roots.

**Conclusions:** Our results indicate that melatonin confers heavy metal resistance in *E. pisciphila* by lowering oxidative stress and heavy metal accumulation.

**Background**

Melatonin is an ancient indole molecule that dates back to 2.5 billion years ago, and exists in almost all organisms include animals, plants, and microorganisms [1–3]. The original function of melatonin is scavenging the excess reactive oxygen (ROS) and reactive nitrogen species (RNS) produced in the original respiration and photosynthesis [4, 5].

Under heavy metal stress conditions, the levels of ROS and RNS significantly increase, then cause serious oxidative stress damage to the organism [6, 7]. Melatonin levels were raised by various heavy metals such as cadmium (Cd), vanadium (V), and Zinc (Zn) treatment [7–9]. It is imperative for organisms to utilize elevated melatonin levels to improve their heavy metal stress tolerance by clearing excessive ROS and RNS. Consistent with this hypothesis, melatonin production was promoted through upregulating the expression of biosynthesis enzyme genes containing tryptophan decarboxylase (TDC), tryptophan hydroxylase (TPH), serotonin N-acetyltransferase (SNAT), and N-acetylserotonin O-methyltransferase (ASMT), and then enhanced heavy metal stress resistance for plants [3, 9, 12, 10–12].

Although various functions of melatonin in varied animals and plants, and partial microorganisms have been studied via regulating endogenous melatonin levels, little investigation in filamentous fungi is available [13]. Melatonin originated from the primitive bacteria (cyanobacteria and α-proteobacteria) and has been retained throughout the evolution of all organisms [14, 15]. Therefore, it can't be ignored that filamentous fungi have a significant position in the melatonin evolution process [5].

Dark septate endophytes (DSEs), a group of dark pigmented and septate hyphae fungi, widely colonize in the plant’s roots [16]. Notably, these fungi wildly inhabit in most roots of plants that grow in the high
concentrations of Pb, Zn, and Cd amine smelting region [17, 18]. We isolated one highly heavy metal tolerant DSE strain, *Exophiala pisciphila*, from the roots of *Arundinella bengalensis* living an old mine smelting site in Yunnan province, southwest China. The plumbum (Pb), Cd, and Zn concentrations in the dry weight of *E. pisciphila* hyphae reach over 25%, 4.9%, and 16.0% respectively [16]. Significant adaption to the high heavy metal environment for the isolate suggested its particular metal tolerance mechanisms. Ground on the primary anti-oxidation function of melatonin, the compound possibly participates in heavy metal tolerance formation of *E. pisciphila*. Moreover, our transcriptome data of *E. pisciphila* [19] showed that melatonin biosynthetic enzyme genes (*TDC*, *SANT*, and *ASMT*) were upregulated under Cd stress, which indicated the involvement of melatonin in heavy metal tolerance.

The objective of this study was to investigate the effects of melatonin in improving various heavy metals tolerance, genes controlling melatonin biosynthesis, and the behind mechanisms in terms of relieving oxidative stress and reducing heavy metal accumulation.

**Results**

**Exogenous melatonin alleviated oxidative stress under heavy metal stresses**

Under heavy metals stress, oxidative stress is one of the most serious damages for organisms [1]. In this study, we investigated the influence of different doses melatonin on oxidative stress caused by assessing the increases in malondialdehyde (MDA) and oxygen free radical (OFR) content in *E. pisciphila*. The MDA content of the isolate gradually decreased as the exogenous melatonin concentration application increased under Cd, Zn, and Pb stresses (Fig. 1A, B, and C). For instance, compared to the control, 200.0 µM melatonin application significantly reduced MDA production by 19.85% and 23.42% under Cd and Zn conditions respectively (Fig. 1A and C). Inconsistently, melatonin did not lower the MDA level under Cu stress (Fig. 1B).

The application of melatonin decreased the OFR amount under Cd, Zn, and Pb stress. (Fig. 1E, G and H). The pretreatment with 200.0 µM exogenous melatonin significantly reduced 33.31%, 27.64%, and 60.91% of OFR under Cd, Zn, and Pb stress respectively (Fig. 1E, G and H). Unlike Cd, Zn, and Pb, melatonin showed no mitigating effect on OFR production under Cu stress. It seems that the application of melatonin relieved the oxidative stress caused by Cd, Zn, and Pb in *E. pisciphila*.

**Exogenous melatonin increased superoxide dismutase (SOD) activity and decreased heavy metals accumulation**

Given that melatonin relieved the oxidative stress, the activity of superoxide dismutase (SOD), one of the antioxidant enzymes, was examined. The application of melatonin increased the activity of SOD under Zn and Pb stresses, and significantly increased by 30.01% and 33.45% respectively pretreated with
200.0 µM melatonin (Fig. 2C and D). However, melatonin did not significantly influence the SOD activity under Cd and Cu stresses (Fig. 2A and B).

Preventing excessive heavy metal accumulation is an important way to limit the deleterious impact on organisms [1]. Thus, we explored whether melatonin changed the Cd, Cu, Zn, and Pb contents in *E. pisciphila*. Intriguingly, the heavy metal accumulation in *E. pisciphila* gradually decreased with elevated melatonin levels (Fig. 2E, F, G, and H). Pretreated with 200.0 µM melatonin, the content of Cd, Zn, and Pb significantly reduced by 32.24%, 46.7%, and 31.55% respectively (Fig. 2E, G, and H). In summary, exogenous melatonin enhanced heavy metal tolerance by increasing SOD activity and reducing heavy metals accumulation in *E. pisciphila*.

EpTDC1, EpSNAT1, and EpASMT1 differentiated from that of plants and animals

Based upon the transcriptome data of *E. pisciphila* (Zhao et al., 2015), three melatonin biosynthetic enzymes cDNA sequences (*EpTDC1*, *EpSNAT1*, and *EpASMT1*) were obtained. According to amino acid BLAST search, the EpTDC1, EpSNAT1, and EpASMT1 homologs were found in various animals and plants. The phylogenetic tree indicated that EpTDC1, EpSNAT1, and EpASMT1 all gathered in the fungi cluster which formed a clade separately from the animals and plants (Fig. 3; Supplementary Fig. S1 and S2).

Heavy metals stresses upregulated EpTDC1 and EpSNAT1, and downregulated EPASMT1 expression, and promoted melatonin biosynthesis in *E. pisciphila*

To investigate the effects of heavy metal on melatonin biosynthesis, we measured melatonin levels and the expression of *EpTDC1*, *EpSNAT1*, and *EpASMT1* in *E. pisciphila*. At the early treatment period (2d), Cd, Cu, and Zn induced melatonin production (Fig. 4D). Simultaneously, *EpTDC1* (Fig. 4A) and *EpSNAT1* (Fig. 4B) were upregulated, and *EpASMT1* (Fig. 4C) expression was downregulated compared to the control. The expression of *EpTDC1*, *EpSNAT1*, and *EpASMT1*, and melatonin levels lowered compared to the control at 10 days (Fig. 4A B C and D). To the end, heavy metals rapidly (2d) induced melatonin biosynthesis which was strictly correlated to the upregulation of *EpTDC1* and *EpSNAT1* expression.

EpTDC1 and EpASMT1 conferred heavy metals tolerance for *E. coli* and *A. thaliana*

The expression of *EpTDC1*, *EpSNAT1*, and *EpASMT1* in response to heavy metal stress suggested that it is involved in the physiological process of conferring heavy metal resistance. We transferred *EpTDC1* and *EpASMT1* into *E. coli* and *A. thaliana* to further investigate the role of melatonin in the heavy metal stress resistance. The abundance of *E. coli* in liquid culture was measured by the optical density at 600 nm (OD$_{600}$), which is a widely used method in bacteria [20]. The OD$_{600}$ of the *E. coli* overexpressing *EpTDC1* and *EpASMT1* were significantly enhanced under Cd Cu, Zn, and Pb stresses (Fig. 5). Hence, both *EpTDC1* and *EpASMT1* conferred *E. coli* heavy metal stress resistance.
**Figure 5.** *EpTDC1* and *EpAMST1* increased the optical density at 600 nm (OD$_{600}$) of the transgenic *E. coli* strains. A total of 1.0 mM IPTG was added to each culture to induce the expression of the recombinant protein. 50.0 mg L$^{-1}$ Cd$^{2+}$ (A, E), 100.0 mg L$^{-1}$ Pb$^{2+}$ (B, F), 70.0 mg L$^{-1}$ Cu$^{2+}$ (C, G), and 100.0 mg L$^{-1}$ Zn$^{2+}$ (D, H). Data are means ± SD (n = 4). Asterisks indicate significant differences ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$) compared to the control according to Independent-Samples T test.

For transgenic *Arabidopsis*, *EpTDC1* and *EpAMST1* were used for investigating the heavy metal tolerance. Under 10.0 µM Cd$^{2+}$, the root length and fresh weight significantly increased by 19.2% 29.6% in *EpTDC1* and 14.0% 15.5% in *EpTDC2* in comparison with the wild-type plants (Fig. 6B and C). The overexpression of *EpAMST1* also enhanced the growth of *Arabidopsis* under Cu stress (Fig. 6D). The root length and fresh weight significantly increased by 31.46% and 78.82% in *EpAMST1*, and 36.52% and 63.53% in *EpAMST2* under 10.0 µM Cu$^{2+}$, respectively (Fig. 6E and F). In this section, it has been indicated that the overexpression of *EpTDC1* and *EpAMST1* relieved heavy metal stresses for transgenic *Arabidopsis*.

**Figure 6.** The overexpression of *EpTDC1* and *EpAMST1* enhanced the heavy metal tolerance of *Arabidopsis*. Seeds of the transgenic *EpTDC1* (*EpTDC1* and *EpTDC2*) and *EpAMST1* (*EpAMST1* and *EpAMST2*) *Arabidopsis* grown on 1/2 MS medium plates contained 0, 5.0, and 10.0 µM Cd$^{2+}$, and 0, 10.0, and 30.0 µM Cu$^{2+}$ respectively for 10 days with the wild-type (WT) *Arabidopsis* as the control. The corresponding pictures were respectively taken (A, D), Scale bars = 1 cm. Meanwhile, the root length (B, E) and fresh weight (C, F) were measured. Data are means ± SD (n = 6). Columns with different letters denote significant differences at $P < 0.05$ according to Duncan's multiple range test.

*EpTDC1* and *EpAMST1* decreased Cd accumulation in *Arabidopsis* plants, especially in underground parts.

As shown above, exogenous melatonin reduced heavy metals accumulation in *E. pisciphila*. To determine whether *EpTDC1* and *EpAMST1* suppress the heavy metal accumulation in transgenic *Arabidopsis* seedlings, the Cd level was measured. The overexpression of *EpTDC1* and *EpAMST1* showed no significant effect on the Cd content in the shoot, root, and the whole plant of *Arabidopsis* under 20.0 mg kg$^{-1}$ Cd. However, *EpTDC1* and *EpAMST1* decreased the Cd accumulation in the whole plant and root tissues when pretreated with 40.0 mg kg$^{-1}$ Cd$^{2+}$ (Fig. 7A and B). These data suggested that *EpTDC1* and *EpAMST1* enhanced Cd resistance was associated with decreasing Cd accumulation in root tissues of *Arabidopsis*.

**Discussion**

Excessive heavy metals lead organisms to produce excessive reactive oxygen species (ROS) via inhibiting the antioxidant system, disrupting the electron transport chain, and disturbing the metabolism of essential elements [21–23]. Organisms have evolved kinds of mechanisms to relieve oxidative stress caused by heavy metals [24, 25].
As one ancient antioxidant, melatonin plays key roles in decreasing oxidative stress [13, 26]. Therefore, melatonin could potentially confer heavy metal stress resistance. Consistent with this hypothesis, melatonin production was promoted to ease heavy metal stress in plants and animals [1, 2]. Significantly enhanced melatonin accumulation was also observed in *E. pisciphila* challenged with Cd, Cu, and Zn at 2 days (Fig. 3D). While Pb had no mitigating effect on melatonin production similar to that observed in rice [27], which indicated that *E. pisciphila* have distinct stress responses to the different type heavy metals [28]. The application of exogenous melatonin reduced OFR and MDA contents of *E. pisciphila* under Cd, Zn and Pb stresses (Fig. 1) suggested that melatonin may act as an antioxidant to relieve the deleterious impact [29].

Except to directly scavenged ROS, melatonin indirectly activated antioxidant enzymes and decreased heavy metal accumulation to relieve heavy metal stress [27, 30]. For instance, melatonin decreased excessive ROS caused by heavy metals via the activation of antioxidant enzymes such as SOD in rice, watermelon, and wheat [6, 8, 9]. Our findings identified that 200.0 µM melatonin significantly increased SOD activity under Zn and Pb stresses (Fig. 2C and D). Hence, melatonin lowered oxidative stress through enhanced the activity of SOD under heavy metal stresses in *E. pisciphila*.

Another important way to relieve heavy metal toxicity is by decreasing heavy metal accumulation [1]. Melatonin application significantly lowered vanadium (V) in *Citrullus lanatus*, and Cd in rice and *Arabidopsis* [9, 6, 12]. Given that 200.0 µM melatonin significantly reduced the Cd, Zn, and Pb content of *E. pisciphila* (Fig. 2), it is likely that melatonin decreased the heavy metal accumulation to enhance heavy metal tolerance for this DSE (Fig. 1). In general, melatonin enlaced heavy metal tolerance was closely related to increase SOD activity and decrease heavy metal accumulation in *E. pisciphila* (Figs. 1 and 2).

Establishing the precise roles of melatonin on heavy metal resistance in *E. pisciphila* requires an investigation of biosynthetic pathways [11]. Although the biosynthetic mechanisms of melatonin have been well characterized in animals and plants, little related research was carried out in microorganisms especially in filamentous fungi. In plants, the first committed step for melatonin biosynthesis is TDC, and SNAT and ASMT contribute to the two final catalyses [3]. In our previous transcriptome analysis of *E. pisciphila* [19], three genes annotated as *TDC*, *SNAT*, and *ASMT* expression were upregulated by Cd. The phylogenetic tree showed that EpTDC1, EpSNAT1, and EpASMT1 differentiated from that of plants and animals (Fig. 3S1 and S2). Under Cd, Cu, and Zn stresses (2d), *EpTDC1* and *EpSNAT1* were transcriptionally upregulated with elevated melatonin levels (Fig. 4). Similar to our findings, the upregulation of *TDC* and *SNAT* expression induced melatonin production with Cd treatment in rice [7]. Our results identified the essential role of *EpTDC1* and *EpSNAT1* at melatonin levels for heavy metal resistance in *E. pisciphila*. The reason for the downregulation of *EpASMT1* (Fig. 4C) is not clear, but maybe important for the induction of melatonin production. Under various scenarios, transcript levels are not sufficient to predict protein levels and to thus explain genotype-phenotype relationships [31]. Byeon et al. (2015) also found that the melatonin production did not consist with the expression of its biosynthesis enzyme genes under Cd stress [27].
Furthermore, we overexpressed EpTDC1 and EpASMT1 in *E. coli* and *A. thaliana*, which rescued the growth inhibition caused by heavy metal (Figs. 5 and 6). Consistent with the finding of alfalfa SNAT overexpressed in *Arabidopsis* [12], EpTDC1 and EpASMT1 decreased Cd content both the total and roots of *Arabidopsis* under 40 mg kg\(^{-1}\) Cd\(^{2+}\) (Fig. 7). These data indicated that EpTDC1 and EpASMT1 played key roles in decreasing excessive heavy metals accumulation.

**Conclusions**

In short, we first demonstrated melatonin alleviated heavy metal stresses in one filamentous fungi. Melatonin biosynthesis enzyme genes responsible for melatonin biosynthesis relieved oxidative stress via directly clearing and indirectly enhancing SOD activity, and decreased heavy metal accumulation in *E. pisciphila*.

**Methods**

**Fungal materials, growth conditions and treatments**

*Exophiala pisciphila* was isolated from the roots of *Arundinella bengalensis* (authenticated by Pro. Shugang Lu from Yunnan University, and the voucher specimen was preserved in Yunnan University), naturally growing in an old mine smelting site in Huize county, Yunnan province, southwest China (103°6'30" E, 26°55'0" N), and preserved in the Agricultural Culture Collection Center of China (accession number ACCC32496). The fungus was first incubated in Melin-Norkrans (MMN) liquid media at 28 °C, 180 rpm, for 7 days. Then, *E. pisciphila* pretreated with 0, 50.0, 100.0, 200.0 µM melatonin for one day. In the end, *E. pisciphila* were incubated in the media contained with or without of Cd\(^{2+}\) (111.2 mg L\(^{-1}\)), Cu\(^{2+}\) (100.0 mg L\(^{-1}\)), Zn\(^{2+}\) (1010.0 mg L\(^{-1}\)), and Pb\(^{2+}\) (800.0 mg L\(^{-1}\)) for 2 days.

**Determination of malondialdehyde (MDA), oxygen free radical (OFR), and Superoxide dismutase (SOD)**

A total of 0.5 g fresh hyphae were ground into fine powder with liquid nitrogen in a mortar. The detection of different parameters was performed by Malondialdehyde (MDA) Detection Kit (A003-1-2), Oxygen Free Radical (OFR) Detection Kit (A052-1-1), and Superoxide Dismutase (SOD) Detection Kit (A001-1-2) (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

**Determination of Cd, Cu, Zn, and Pb accumulation in *E. pisciphila***

Hyphae were collected and washed three times with deionized water. Subsequently, samples were oven-dried at 80 °C, then digested with HClO\(_4\) and HNO\(_3\) mixture (\(v/v = 1:4\)) at 260 °C. The Cd, Cu, Zn, and Pb concentrations were analyzed using an atomic absorption spectrophotometer (AA240; Shimadzu Co., Kyoto, Japan).
Quantification Of Melatonin By High-performance Liquid Chromatography (hplc)

Hyphae (0.1 g) were ground in liquid nitrogen and extracted with 1.0 mL chloroform for 1 hour at room temperature before melatonin quantification. Chloroform extracts (200.0 µL) were completely evaporated and dissolved in 0.1 mL 40% methanol, and 10.0 µL aliquots were subjected to HPLC using a fluorescence detector system (Waters, Milford, MA, USA). The samples were separated on a Sunfire C18 column (Waters; 4.6 x 150 mm) using the following gradient elution profile: from 42–50% methanol in 0.1% formic acid for 27 minutes, followed by isocratic elution with 50% methanol in 0.1% formic acid for 18 minutes at a flow rate of 0.15 mL min⁻¹. Melatonin was detected at 280 nm (excitation) and 348 nm (emission). All measurements were taken in triplicate.

Identification and phylogenic tree construction of EpTDC1, EpSNAT1, and EpASMT1

Based on the transcriptome database of *E. pisciphila* [19], the nucleic acid sequences of putative *EpTDC1*, *EpSNAT1*, and *EpASMT1* were obtained. The open reading frame nucleotide and amino acid sequences of these unigenes were predicted using Open Reading Frame Finder (ORF) in National Center for Biotechnology Information (NCBI). Then BLAST search was performed using the *EpTDC1*, *EpSNAT1*, and *EpASMT1* amino acid sequences. Phylogenetic trees were generated from various amino acid sequences after alignment with Clustal X (version 1.83), and phylograms were constructed using the Neighbor-joining method algorithm with branch length (MEGA 5.0).

Expression analysis of EpTDC1, EpSNAT1, and EpASMT1

Total RNA was extracted from hyphae using the RNAiso Plus (TaKaRa, Japan, 9108) according to the manufacturer's protocol. The isolated RNA (1.0 µg) was synthesized cDNA via the PrimeScriptII 1st Strand cDNA Synthesis Kit (TaKaRa, Japan, 6210A). The quantitative real-time PCR (qRT-PCR) analysis was performed with a LightCycler® 480 II Real-Time PCR Detection System (Roche, Basel, Swiss) using SYBR Premix Ex Taq (TaKaRa, Japan, RR820A), and *β-tubulin* gene as the internal control. Primers used in this study were listed in Table S1. All of the reactions were performed with three biological and technical replicates independently.

Overexpression of EpTDC1 and EpASMT1 in Escherichia coli and Arabidopsis thaliana

The ORF sequences of *EpTDC1* and *EpASMT1* were amplified by PCR using the specific primer set containing the restriction endonuclease enzyme sites. For *EpTDC1*, F: 5’-CGGGATCCATGGCTCTGAGAGGC-3’ (*BamH*I restriction site is underlined) and R: 5’-GGGTTCGAACATTTCATAGCCAGAGGC-3’ (*Hind*I restriction site is underlined) as the primer. For *EpASMT*, F: 5’-CGGAATTCCAATGGCTCTGAGAGGC-3’ (*EcoR*I restriction site is underlined) and R: 5’-GGGTTCGAACATTTCATAGCCAGAGGC-3’ (*Hind*I restriction site is underlined) as the primer. The purified PCR products were ligated to expression vector pET28a (Invitrogen, Carlsbad, CA, USA). Then the final
recombinant vectors (pET28a-EpTDC1 and pET28a-EpASMT1) were transformed into *Escherichia coli* BL21(DE3) cells (TaKaRa, Japan).

To obtain transgenic *Arabidopsis* plants, *EpTDC1* and *EpASMT1* were amplified by PCR with primers F: 5'-CGGGATCCATGCTCTGTTGAGAGGC-3' (*BamH*I restriction site is underlined)/R: 5'-GCTCTAGATTGGCATGGCCATTTC-3' (*Xba*I restriction site is underlined) and F: 5'-GGGTACCATGCTAGACAACAAAG-3' (*Kpn*I restriction site is underlined)/R: 5'-GCTCTAGAATTAAGACCCATCCGTC-3' (*Xba*I restriction sites is underlined) respectively. The PCR products were inserted into plant expression vector pCAMBIA1304 (Invitrogen, Carlsbad, CA, USA). The recombinant vectors pCAMBIA1304-35S::*EpTDC1* and pCAMBIA1304-35S::*EpASMT1* were introduced into *Agrobacterium tumefaciens* GV3101 (Invitrogen, Carlsbad, CA, USA) and then transformed into wild-type (WT) *Arabidopsis thaliana* Columbia-0 (Col-0) (stock no. CS70000, purchased from the Arabidopsis Biological Resource Center http://www.arabidopsis.org/abrc) through floral dip method. The seeds were screened on 1/2 Murashige-Skoog (MS) plates containing 25.0 mg L\(^{-1}\) kanamycin. The transgenic *Arabidopsis* plants were confirmed by PCR. The homologous T3 transgenic lines named *EpTDC1-1/*EpTDC1-2 and *EpASMT1-1/*EpASMT1-2 were selected for further analysis.

**Heavy metal treatments of the transgenic** *Escherichia coli* and *Arabidopsis thaliana* for growth measurements

Control (pET28a) and transgenic (pET28a-EpTDC1/pET28a-EpASMT1) *Escherichia coli* BL21(DE3) strains were inoculated into 100.0 ml Luria Broth (LB) liquid media with 1.0 mM isopropyl β-D-thiogalactopyranoside (IPTG). The final concentrations of 50.0 mg L\(^{-1}\) Cd\(^{2+}\), 100.0 mg L\(^{-1}\) Zn\(^{2+}\), 70.0 mg L\(^{-1}\) Cu\(^{2+}\), and 100.0 mg L\(^{-1}\) Pb\(^{2+}\) were adjusted in the media separately. The optical density at 600 nm (OD\(_{600}\)) of the BL21 strains was measured at 4 h intervals for different times as described in the corresponding figure legends. Each treatment was conducted independently four times.

Seeds of transgenic *Arabidopsis* plants were sown on 1/2 MS medium contained 0, 5.0, 10.0 μM Cd\(^{2+}\) and 0, 10.0, 30.0 μM Cu\(^{2+}\) respectively with the wide-type *Arabidopsis* as the control. After one day of stratification at 4 °C, the seedlings grown for 10 days in a growth chamber at 25 °C with 16 h light (100 μmol m\(^{-2}\) s\(^{-1}\)) 8 h dark. The *Arabidopsis* plants were photographed, and the root length and fresh weight were measured immediately. Each treatment was conducted independently six times.

**Determination of Cd accumulation in** *Arabidopsis thaliana*

The transgenic *Arabidopsis* and wild-type plant seeds were germinated on 1/2MS plates. When the second true leaves were fully expanded, seedlings were transferred into the soil containing 0, 20.0, 40.0 mg kg\(^{-1}\) Cd\(^{2+}\). Plants grown in the soil for 30 days were collected and the Cd contents of the total, shoot, and root tissues were detected by the method in the determination of Cd accumulation in *E. pisciphila*. Each treatment was conducted independently four times.
Statistical Analysis

Independent-samples $t$-test and one-way ANOVA with Duncan's multiple range test (SPSS 16.0) were used to detect the significant differences between means, $P$-values $< 0.05$ were considered statistically significant.

Abbreviations

DSE
Dark septate endophyte; ROS: Excess reactive oxygen; RNS: Reactive nitrogen species; TDC: Tryptophan decarboxylase; ASMT: $N$-acetylserotonin $O$-methyltransferase; TPH: Tryptophan hydroxylase; SNAT: $N$-acetyltransferase; Cd: cadmium; Cu: copper; Zn: zinc; Pb: plumbum; V: vanadium; MDA: malondialdehyde; OFR: Oxygen free radical; SOD: Superoxide dismutase; $OD_{600}$: Optical density at 600 nm; MMN: Melin-Norkrans; ORF: Open reading frame finder; NCBI: National Center for Biotechnology Information; LB: Luria Broth; qRT-PCR: Quantitative real-time PCR

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Acknowledgements
Not applicable.

Authors’ contributions
DZ and ZZ, designed the experiments; YY, ZT and YL performed the experiments and analyzed the results; YY and ZT worked together to write the initial drafts of the manuscript. ZM, SC, and TL prepared figures for the article and read the final version. DZ and ZZ checked all drafts of the report. The authors agreed to this publication. The author(s) read and approved the final manuscript.

Funding: This work was financially supported by Yunnan Provincial Science and Technology Department-Yunnan University Joint Foundation, the key project [grant number 2018FY001-010]. The
funding bodies had no contribution in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**Figures**

**Figure 1**

Melatonin relieved the oxidative stress of *E. pisciphila* under Cd, Zn, and Pb stresses. *E. pisciphila* were pretreated with 0, 10.0, 50.0, or 200.0 μM melatonin (MT) for 1 day and then subjected to the 111.2 mg L⁻¹ Cd²⁺ (A, E), 100.0 mg L⁻¹ Cu²⁺ (B, F), 1010.0 mg L⁻¹ Zn²⁺ (C, G), or 800.0 mg L⁻¹ Pb²⁺ (D, H) stress for 2 days. The MDA (A, B, C, D) and OFR (E, F, G, H) contents of *E. pisciphila* were measured. Data are means ± SD (n=4). Columns with different letters denote significant differences at p < 0.05 according to Duncan’s multiple range test.
Melatonin increased SOD activity and decreased heavy metal accumulation. *E. pisciphila* were pretreated with 0, 10.0, 50.0, or 200.0 μM melatonin (MT) for 1 day and then subjected to the 111.2 mg L⁻¹ Cd²⁺ (A, E), 100.0 mg L⁻¹ Cu²⁺ (B, F), 1010.0 mg L⁻¹ Zn²⁺ (C, G), or 800.0 mg L⁻¹ Pb²⁺ (D, H) stress for 2 days. The SOD (A, B, C, D) activity and heavy metal (E, F, G, H) content of *E. pisciphila* were measured. Data are means ± SD (n=4). Columns with different letters denote significant differences at p < 0.05 according to Duncan's multiple range test.
Figure 3

A phylogenetic tree of TDCs based on amino acid sequences.
Figure 4

Heavy metal enhanced the expression of EpTDC1, EpSNAT1, EpASMT1, and the accumulation of melatonin in E. pisciphila. E. pisciphila were pretreated with 111.2 mg L⁻¹ Cd²⁺, 100.0 mg L⁻¹ Cu²⁺, 1010.0 mg L⁻¹ Zn²⁺, and 800.0 mg L⁻¹ Pb²⁺ respectively for 2 or 10 days. The relative expression of EpTDC1 (A), EpSNAT1 (B), and EpASMT1 (C) were analyzed by real-time RT-PCR, and the melatonin contents (D) were detected by HPLC. Data are means ± SD (n=3). Columns with different letters denote significant differences at p < 0.05 according to Duncan's multiple range test.
Figure 5

EpTDC1 and EpAMST1 increased the optical density at 600 nm (OD600) of the transgenic E. coli strains. A total of 1.0 mM IPTG was added to each culture to induce the expression of the recombinant protein. 50.0 mg L-1 Cd2+ (A, E), 100.0 mg L-1 Pb2+ (B, F), 70.0 mg L-1 Cu2+ (C, G), and 100.0 mg L-1 Zn2+ (D, H). Data are means ± SD (n=4). Asterisks indicate significant differences (p < 0.05*, p < 0.01**, p < 0.001***) compared to the control according to Independent-Samples T test.

Figure 6

The overexpression of EpTDC1 and EpASMT1 enhanced the heavy metal tolerance of Arabidopsis. Seeds of the transgenic EpTDC1 (EpTDC1-1 and EpTDC1-2) and EpASMT1 (EpASMT1-1 and EpASMT1-2) Arabidopsis grown on 1/2 MS medium plates contained 0, 5.0, and 10.0 μM Cd2+, and 0, 10.0, and 30.0...
μM Cu2+ respectively for 10 days with the wild-type (WT) Arabidopsis as the control. The corresponding pictures were respectively taken (A, D), Scale bars = 1 cm. Meanwhile, the root length (B, E) and fresh weight (C, F) were measured. Data are means ± SD (n=6). Columns with different letters denote significant differences at P < 0.05 according to Duncan's multiple range test.

### Figure 7

The overexpression of EpTDC1 and EpASMT1 decreased Cd accumulation in Arabidopsis. The Arabidopsis seedlings at the four-leaf stage of wild-type (WT), EpTDC1-1, EpTDC1-2, EpASMT1-1, and EpAMST1-2 were transferred to soil containing 0, 20.0, 40.0 mg kg⁻¹ Cd²⁺ for 30 days. The Cd contents in the shoot, root, and the whole plant of A. thaliana were measured. Data are means ± SD (n=4). Columns with different letters denote significant differences at p < 0.05 according to Duncan's multiple range test.
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