Research Article

ScMT2-1-3, a Metallothionein Gene of Sugarcane, Plays an Important Role in the Regulation of Heavy Metal Tolerance/Accumulation

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Plant metallothioneins (MTs), which are cysteine-rich, low-molecular-weight, and metal-binding proteins, play important roles in detoxification, metal ion homeostasis, and metal transport adjustment. In this study, a novel metallothionein gene, designated as ScMT2-1-3 (GenBank Accession number JQ627644), was identified from sugarcane. ScMT2-1-3 was 700bp long, including a 240bp open reading frame (ORF) encoding 79 amino acid residues. A His-tagged ScMT2-1-3 protein was successfully expressed in Escherichia coli system which had increased the host cell's tolerance to Cd$^{2+}$, Cu$^{2+}$, PEG, and NaCl. The expression of ScMT2-1-3 was upregulated under Cu$^{2+}$ stress but downregulated under Cd$^{2+}$ stress. Real-time qPCR demonstrated that the expression levels of ScMT2-1-3 in bud and root were over 14 times higher than those in stem and leaf, respectively. Thus, both the E. coli assay and sugarcane plantlets assay suggested that ScMT2-1-3 is significantly involved in the copper detoxification and storage in the cell, but its functional mechanism in cadmium detoxification and storage in sugarcane cells needs more testification though its expressed protein could obviously increase the host E. coli cell's tolerance to Cd$^{2+}$. ScMT2-1-3 constitutes thus a new interesting candidate for elucidating the molecular mechanisms of MTs-implied plant heavy metal tolerance/accumulation and for developing sugarcane phytoremediator varieties.

1. Introduction

Along with the population growth and the rapid development of industrialization and urbanization, our planet is constantly subjected to various kinds of pollution damage. The heavy metal-contaminated farmland in China had already topped 20 million hectares in 2003, accounting for about 1/5 of the total cultivated area [1]. Due to the heavy metal pollution, China’s annual grain production cuts in more than 1,000 million tons, and this caused a direct economic loss of about 200 billion yuan [1]. The increasing trend of pollution continued from that time and is likely to continue over the next few decades if significant remedial measures are not implemented in China.

The concept of phytoremediation was first proposed by Chaney [2] and involved the use of plants to remove pollutants from the environment or to render them harmless [3]. Phytoremediation consists of mitigating pollutant concentrations in contaminated water, soils, or air, with the ability of plants to contain, degrade, or eliminate those materials of metals, pesticides, solvents, crude oil and its derivatives, explosives and various other contaminants from the media that contain them [4]. The plant-based remediation technologies have the potential to be low cost, low impact, visually benign, and environmentally sound [5]. In recent years, there has been an increasing interest in studying the molecular mechanisms of metal accumulation and tolerance in plants [6, 7].

Sugarcane (Saccharum spp. L.), a major sucrose accumulator and biomass producer, is one of the most important field crops grown in the tropics and subtropics. It accounts for more than 90% of China’s total sugar output at present.
Due to its outstanding biomass production and economic importance, sugarcane offers the potential to be a phytoremediator species, while its prospective metal accumulation and tolerance have not been fully characterized. A research on this topic was carried out by Sereno et al. [9], which showed that sugarcane could be a copper (Cu) or cadmium (Cd) phytoremediator as its plantlets were able to tolerate up to 100 μM of Cu^{2+} or 500 μM of Cd^{2+} in nutrient solution for 33 days without symptoms of toxicity while accumulating 45 mg Cu kg^{-1} or 451 mg Cd kg^{-1} shoot dry weight, respectively, without significant reduction in fresh weight.

Metallothioneins (MTs) are cysteine-rich, low-molecular-weight, and metal-binding proteins, which have been found in a wide variety of organisms including animals, plants, cyanobacteria, and fungi [10]. Plant MTs are extremely diverse [11] and can be classified into four subfamilies (MT1 to MT4), based on the arrangement of Cys residues [10]. Due to their ability to reversibly bind both toxic and essential metal ions, plant MTs play important roles in detoxification, metal ion homeostasis, and metal transport adjustment [10]. Consequently, the role of plant MT genes in heavy metal tolerance mechanism and phytoremediation has attracted more and more attention in recent years [7], and their ability to metal accumulation and tolerance has been demonstrated in several plants. It was shown in *Arabidopsis thaliana* that expression of *AtMT4a* gene in vegetative tissues at different developmental stages conferred increased tolerance towards Cu and Zn [12]. Transgenic *Avicennia marina* that expresses AmMT2 have been scored for enhanced tolerance to Zn, Cd, Cu, and Pb [13].

Sugarcane is one of the few species that contain genes encoding all four types of MTs [10] and one of the most potential phytoremediation species for its outstanding biomass production and high metal enrichment capability. Undoubtedly, isolation and characterization of sugarcane MT genes from sugarcane are the basis for better understanding MT gene function in heavy metal tolerance mechanism and phytoremediation. In this study, an MT2 gene, termed *ScMT2-1-3*, was successfully isolated based on large sequencing and bioinformatics analysis of the sugarcane stem full-length cDNA library. *ScMT2-1-3* protein was expressed in the *E. coli* Rosetta strain, and the transgenic bacteria showed an increased tolerance both to Cd and Cu. The expression patterns of *ScMT2-1-3* in sugarcane plant were characterized in response to CdCl\(_2\) and CuCl\(_2\), and its expression levels in different sugarcane tissues were determined by real-time quantitative polymerase chain reaction (real-time qPCR).

## 2. Materials and Methods

### 2.1. Plant Materials and Treatment.

Sugarcane varieties used in this study were provided kindly by the Key Laboratory of Sugarcane Biology and Genetic Breeding, Ministry of Agriculture (Fuzhou, China). Uniform tissue culture plantlets of an elite sugarcane variety FN39 were grown in 1/2 Hoagland nutrient solution for one week and then subjected to two different treatments: 500 μM CdCl\(_2\) or 100 μM CuCl\(_2\). The sampling times were 0 h, 3 h, 12 h, 48 h, and 72 h after the start of each treatment. All samples collected were immediately fixed in liquid nitrogen and stored in a refrigerator at −85°C until RNA extraction.

Nine healthy and consistent growing plants were randomly chosen and dug up with roots from sugarcane variety FN39 grown for 10 months. For each plant, the young root, maturing stem (internodes 4–6, 10–12, and 16–18), the leaf (+1), and all of the buds were sampled and fixed in liquid nitrogen. The collected materials were then stored in a refrigerator at −85°C until RNA extraction.

### 2.2. Molecular Cloning, Sequencing, and Bioinformatics Analysis.

The sugarcane stem full-length cDNA library was provided by the Key Laboratory of Sugarcane Biology and Genetic Breeding, Ministry of Agriculture (Fuzhou, China). *E. coli* Rosetta (DE3) and the prokaryotic expression vector pET28a were purchased from Abmart, Inc. (Shanghai, China). The restriction enzymes EcoR I, Xho I, T4 DNA ligase, *Ex-Taq* enzyme, PrimeScript RT-PCR Kit, TaKaRa LA PCR *in vitro* Cloning Kit, DNA, and protein molecular weight (MW) markers were purchased from TaKaRa (Dalian, China). HisTrap HP column was purchased from GE Healthcare Life Sciences. RQ1 RNase-Free DNase was obtained from Promega Corporation (USA), SYBR Green PCR Master Mix Kit was purchased from Applied Biosystems (USA), and the instrument used in the real-time qPCR analysis was the ABI PRISM7500 real-time PCR system.

Large-scale sequencing and bioinformatics analysis of the full-length cDNA library of sugarcane stems were conducted as described by Guo et al. [14]. A full-length metallothionein homolog gene of sugarcane (named *ScMT2-1-3*) was identified by BLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with a metallothio-2 superfAMILY domain (pfam01539). The open reading frame (ORF) of the full-length cDNA sequence of *ScMT2-1-3* was predicted using the ORF finder online tool from NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The accession numbers of the chosen proteins were AhMT2a (*Arachis hypogaea*) ABA08414, AhMT2b (*A. hypogaea*) AB05520, AhMT2a (*Arabidopsis thaliana*) NP187550, AtMT2b (*A. thaliana*) NP195858 and AAA22212, NcMT2a (*Noccaea caerulescens*) ACR46970, NcMT2b (*N. caerulescens*) ACR46962, OsMT2a (*Oryza sativa*) P94029 and AAC49627, OsMT2b (*O. sativa*) A3AZ88 and AAB18814, OsMT2c (*O. sativa*) Q5JM82 and BAA19661, PMT2a (*Populus trichocarpa × P. deltoids*) AAT02524, PMT2b (*P. trichocarpa × P. deltoids*) AAT02525, PoMT2a (*Posidonia oceanica*) CAB96155, PoMT2b (*P. oceanica*) CAB96154, SbMT2 (*Sorghum bicolor*) XP002455197, SbMT2c (*S. bicolor*) XP002439147, SmMT2a (*Salix matsudana*) ABM21761, SmMT2b (*S. matsudana*) ABM21762, SnMT2a (*Solanum nigrum*) ACFI0395, SnMT2b (*S. nigrum*) ACFI0396, ScMT2-1-1 (*Saccharum complex*) (the deduced amino acid sequence of CA232620/SCRULF3063A10.g), ScMT2-1-2 (S. complex) AA50043 and ABP37784, ScMT2-1-3 (S. complex) AFJ44425, ZmMT2-1 (Zea mays) NP001150795, and ZmMT2-2 (Z. mays) NP001147309. Alignment of putative ScMT2-1-3 protein sequence to MT2 proteins from *A. hypogaea*, *A. thaliana*, *N. caerulescens*, *O. sativa*, *P. trichocarpa × P. deltoids*, *P. oceanica*, *S. matsudana*, *S. nigrum,*
2.3. **Plasmid Constructs.** To study the function of *ScMT2-1-3* in prokaryotes, the *ScMT2-1-3* ORF with matched sites was amplified by PCR from the identified cDNA clone of the full-length cDNA library. The used PCR primer sequences were MT F: 5′-CGGCGATCCATGCTGTCGGAGGC- AAATG-3′ and MT R: 5′-CCGCTCGAGCTGGCAGAGT- CCGGTTGACG-3′ (*BamH* I and *Xho* I sites are underlined). PCR was performed in a reaction volume of 50 µL containing 5.0 µL PCR buffer, 4.0 µL deoxynucleotide triphosphates (dNTPs) (2.5 mM), 2.0 µL each of forward and reverse primers (10 µM), 0.25 µL Ex-Taq enzyme (5 U/µL), and ddH₂O as supplement. The PCR amplification program consisted of predenaturation for 5 min at 94°C, denaturation for 30 s at 94°C, annealing for 30 s at 55°C, extension for 30 s at 72°C for 30 cycles; and final extension for 10 min at 72°C. The *ScMT2-1-3* ORF with *BamH* I and *Xho* I sites was subcloned into pET28a (+) (*BamH* I-*Xho* I sites) in the *E. coli* strain Rosetta to generate the putative recombinants. A bacterial clone containing the desired recombinant plasmid was identified and validated by PCR amplification, double digestion, and sequencing, and the clone was named as pET28a-MT2.

2.4. **SDS-PAGE and MALDI-TOF-TOF-MS Analysis of Prokaryotic Expression Products.** The pET28a-MT2 and empty pET28a (+) were both transformed into *E. coli* Rosetta (DE3). The single colony was inoculated into an LB medium (20 mL) containing kanamycin (50 µg·mL⁻¹) and chloramphenicol (170 µg·mL⁻¹) and incubated with 150 rpm shaking overnight at 37°C. The following day, a dilution of 1% of this overnight cultured medium was inoculated into a fresh LB medium (20 mL) containing the same concentration of kanamycin and chloramphenicol and then shake-cultured in the same conditions. When OD₆₀₀ of the medium reached 0.4–0.6, a pair of real-time qPCR primers was designed to generate the putative recombinants. A bacterial clone containing the desired recombinant plasmid was identified and validated by PCR amplification, double digestion, and sequencing, and the clone was named as pET28a-MT2.

2.5. **Study on the Response of *E. coli* Cells Containing Recombinant ScMT2-1-3 Gene to Abiotic Stresses.** Spot assay was performed to ascertain the response of *E. coli* Rosetta (DE3) cells transformed with recombinant plasmid (pET28a-MT2) or vector alone (pET28a) to Cd²⁺, Cu²⁺, PEG, and NaCl stresses. When cells grew to 0.6 (OD₆₀₀) in LB medium, IPTG was added up to a final concentration of 1.0 mM, and then the cells were grown for further 12 h at 37°C. The cultures were diluted to 0.6 (OD₆₀₀) and then to 10⁻³ and to 10⁻⁴ [15]. In group one, 10 µL from each dilution was spotted on LB plates containing 100 µM, 250 µM, 500 µM, and 750 µM CdCl₂. In group two, 10 µL from each dilution was spotted on LB plates containing 50 µM, 100 µM, 250 µM, and 500 µM CuCl₂. In group three, 10 µL from each dilution was spotted on LB plates containing 250 mM, 500 mM, 750 mM, and 1000 mM NaCl. In group four, 10 µL from each dilution was spotted on LB plates infiltrated with 15.0%, 20.0%, 25.0%, 30.0%, and 35.0% PEG8000 [16, 17]. All the LB plates contained 50 µg·mL⁻¹ kanamycin and 170 µg·mL⁻¹ chloramphenicol.

2.6. **Expression Profile of ScMT2-1-3 under Heavy Metal Stresses.** Total RNA isolation was performed using the TRIzol Reagent (Invitrogen). The removal of DNA from RNA samples was realized by RQI RNase-Free DNase (Promega). The reverse transcription was realized by following the specifications of the PrimeScript RT reagent Kit (TaKaRa). Finally, the real-time qPCR reaction was realized by using the SYBR Green PCR Master Mix (ABI).

The 25S rRNA (BQ536525) and GAPDH (CA254672) genes were chosen as the internal control in the real-time qPCR analysis [18, 19], and the forward and reverse primers for 25S rRNA were 5′-CCAGGCGCAGGTCATA TC-3′ and 5′-CTTGTGTCGGTGAACATCC-3′ and for GAPDH were 5′-CCAGGCGACTGGAAGCA-3′ and 5′- TCCTCAGGTTCCCTGATGCC-3′ [18]. From the sequence of *ScMT2-1-3*, a pair of real-time qPCR primers was designed using the Primer Express 3.0 software, and the forward and reverse primers for *ScMT2-1-3* were 5′-ACCACCCAGGGCTTCATAT-3′ and 5′-CACCCAGGGTTCCCTGATGCC-3′, respectively.

The real-time qPCR reaction was realized with following conditions: 2 min at 50°C, 10 min at 95°C and then 40 cycles of 94°C for 15 s, and 60°C for 60 s. Each sample was repeated three times in the assay. When the reaction was completed, a melting curve was obtained. The 2⁻ΔΔCT method was adopted to analyze the real-time qPCR results [20].

3. Results and Analysis

3.1. **Cloning and Sequence Analysis of ScMT2-1-3.** A full-length cDNA sequence of a metallothionein-like gene designated as *ScMT2-1-3* (GenBank Accession number JQ627644) was obtained from sugarcane by large sequencing of a stem...
Table 1: Classification of MT2s of plants.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Feature of Cys-rich domains at amino- and carboxy-terminal regions</th>
</tr>
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<tbody>
<tr>
<td>MT2-1</td>
<td>CXXXXXXXXCCXXXXXCCXCCXXCXCX ⋯ ⋯ CXXXXXXXXCCXXXXXCCX</td>
</tr>
<tr>
<td>MT2-2</td>
<td>CXXXXXXXXCCXXXXXCCXCCXXCXXCXCX ⋯ ⋯ CXXXXXXXXCCXXXXXCCX</td>
</tr>
<tr>
<td>MT2-3</td>
<td>CXXXXXXXXCCXXXXXCCXCCXXCXXCXCX ⋯ ⋯ CXXXXXXXXCCXXXXXCCX</td>
</tr>
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Note: “C” represents a Cys residue, X represents an amino acid residue other than Cys, and “⋯⋯” represents the intermediate region between the two Cys-rich domains.

3.2. Expression of ScMT2-1-3 in E. coli Rosetta. The recombinant protein was specifically induced after 2 h of IPTG treatment and reached a maximum at 8 h (Figure 3). The expression products were purified by HisTrap HP column and showed a single band when checking an SDS gel (Figure 4). The MW of recombinant protein (His-tagged-ScMT2-1-3) was estimated to be 12.34 kDa and 13.98 kDa using the online software MS-Digest and protein molecular weight, respectively, but the protein gave a 19.01 kDa band in the SDS gel when calculated by Quantity One 4.5.0 software (Bio-Rad). We repeated the experiment and got the same results, even by changing the E. coli host cell for BL21 (data not shown).

Further validation of the recombinant protein was realized by using MALDI-TOF-TOF MS method, and the results were analyzed using online software Mascot and MS-Digest. Three mass peaks with value of 1083.514, 1535.647 and 1768.869 (Figure 5) were matched with the peptide sequences of "LEHHHHHHH," "GSHMAMSGGQQGMGR," and "GSSHHHHHSGLVPR," respectively, which were partial sequences of the recombinant protein (Figure 6).

3.3. Overexpression of ScMT2-1-3 in E. coli Enhances Its Growth under Abiotic Stresses. Both the ScMT2-1-3 transformed and the control cells could grow in the plates containing Cd²⁺, Cu²⁺, and PEG, respectively. However, the former formed more colonies compared with the latter (Figures 7, 8, and 9). The results show that the recombinant protein enhances its growth under Cd²⁺, Cu²⁺, and PEG stresses. The growth difference was observed with the NaCl-containing LB plates after overnight culture. The ScMT2-1-3 expressed cells were able to tolerate high salt concentrations of up to 500 mM NaCl. In contrast, the growth of the control cells was obviously inhibited at 250 mM NaCl and completely inhibited at 500 mM NaCl, a lethal level for the control cells (Figure 10).

3.4. Tissue-Specific Expression Analysis of ScMT2-1-3. For tissue-specific expression analysis of ScMT2-1-3, the sugarcane variety FN39 was used as experimental material, and the GAPDH gene was used as an internal control for real-time qPCR. The results showed that the ScMT2-1-3 is highly expressed in root and bud but very lowly expressed in stem and leaf (Figure II).

3.5. Expression Profile of ScMT2-1-3 under Different Heavy Metal Stresses. Real-time qPCR was used to examine the expression profile of ScMT2-1-3 on sugarcane plantlets of the variety FN39 under Cd²⁺ and Cu²⁺ stresses, respectively. The real-time qPCR results showed that the expression of ScMT2-1-3 was inhibited by Cd²⁺ stress which was visibly downregulated at 3 h following the treatment and maintained at a relatively lower level, compared to that of the control (Figure 12). In contrast, the expression of ScMT2-1-3 was upregulated after Cu²⁺ treatment: first slightly increased at 3 h then obviously upregulated and reached its highest level at 12 h (2.87 times higher than that of the control), and the expression was maintained to a relatively high level (more than two times higher than that of the control) during the following examined time points (Figure 13).

4. Discussion

Plant metallothionein was first discovered from soybean in 1977 [21]. Based on the sequence homology, this family of genes can be grouped into four subfamilies (Type 1 to Type 4 or MT1 to MT4) [10, 11]. Considering the large member of the plant MT family and the high sequence diversity, further subdivision should be necessary for plant MTs. In A. thaliana, Zhou and Goldsbrough [22] classified the MT2 proteins into two subgroups, MT2a and MT2b, according to the four codons in the central domain of AtMT2a (codons 30–33: GFSG) which are absent in AtMT2b (Figure 2). This region was shown to be highly variable among plant MTs [22]. Our analysis in Figure 2 suggests that plant MT2 proteins can be subdivided into at least 3 subgroups according to
Figure 1: Nucleotide sequence and deduced amino acid sequence of ScMT2-1-3. Note: the C shows the conservative cysteine residue contained in two domains of ScMT2-1-3 with metal-binding motifs in combination among CC, CXC, and CXXC.

The arrangement of Cys residues (Figure 2; Table 1). This is in agreement with Wong et al. [23] who classified the rice MT2s into three subgroups termed as OsMT2a, OsMT2b, and OsMT2c, respectively. In this study, the three subgroups were termed as MT2-1, MT2-2, and MT2-3, respectively.

From the alignment of sequences, we find that the sequences of the N-terminal domain of MT2 are highly conserved (MCCGGNCCGCS) (Figure 2). MT2-1 seems to be the most abundant class among the MT2 family with typical plant MT2 Cys-rich domains pattern characterized by Cobbett and Goldsbrough [10] (Figure 2). MT2-1 contains two cysteine-rich domains separated by a spacer of approximately 40 amino acid residues (Figure 2; Table 1).

The N-terminal domain contains four Cys-containing motifs. The first pair of cysteines is present as a Cys-Cys motif in amino acid positions 3 and 4 of these proteins. Two Cys-Xaa-Cys motifs are present in the center of the N-terminal cysteine-rich domain. A Cys-Xaa-Xaa-Cys motif is at the end of the N-terminal cysteine-rich domain. From the alignment of sequences, we find that the sequences of the N-terminal domain of MT2 are highly conserved (MCCGGNCCGCS) (Figure 2). MT2-1 seems to be the most abundant class among the MT2 family with typical plant MT2 Cys-rich domains pattern characterized by Cobbett and Goldsbrough [10] (Figure 2). MT2-1 contains two cysteine-rich domains separated by a spacer of approximately 40 amino acid residues (Figure 2; Table 1).

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the C-terminal cysteine-rich domain, and one more Cys residue is randomly present between the first two Cys-Xaa-Cys motifs and the last two Cys-Xaa-Cys motifs. Moreover, the last two Cys-Xaa-Cys motifs are arranged in tandem at the end of C-terminal (Figure 2; Table 1).

Sugar cane is one of the few species which contain genes encoding all four types of MTs [10]. Of the 291, 689 ESTs in the sugarcane expressed sequence tag (SUCEST) database; a total of 849 reads (0.29%) were found to encode metallothionein-like proteins and give 55 clusters which were conceptually translated and contained the full-length protein [24]. Among the 55 clusters, 21 were related to MT2 proteins and represented 8 protein sequence variants with minor amino acid changes [24]. When ScMT2-1-1 (SCRUFL3063A10/g/CA232620) [9, 24], ScMT2-1-2 (AAV50043 and ABP37784), ScMT2-1-3 (AFJ44225), and other 7 ScMT2 proteins [24] were compared by sequence alignment, we conclude that all the MT2s in sugarcane belong to the MT2-1 subgroup and share over 93% identity in their amino acid sequences (data not shown). To date, MT2-2 and MT2-3 subgroup types have not yet been reported in sugarcane.

The expression profile of MT2-1 genes in different organs, such as root, stem, and leaf, has been studied in several plant species. As a general evidence, the expression level of MT2-1 genes tends to be higher in leaves than that in roots [10, 22, 25, 26]. Both AtMT2a (CAA44630) and AtMT2b (AAA82212) from A. thaliana were found to be highly expressed in leaves but lowly expressed in roots from mature plants [22, 25]. A similar result was obtained for OsMT-2 gene (AAC49627) in rice [27]. In Avicennia marina, the level of expression of the gene AmMT2 in leaves was found to be over 1 times higher than that in stems and 2.1 time higher than that in roots [13]. In Hevea brasiliensis, the gene HbMT2 was also strongly expressed in leaves and in latex, but weakly in roots and in barks [28]. AmMT2 and HbMT2 were both classified into the subgroup of MT2-1 and shared 61.73% and 65.00% identities with ScMT2-1-3, respectively (data not shown). Based on the large-scale EST sequencing databases, the expression patterns of four types of MTs in sugarcane were investigated using 13 different sources of cDNA libraries including shoot-root zone, root, lateral bud, stem bark, stem internode, leaf, leaf roll, apex, flower, seed, callus, in vitro plantlet infected with Herbaspirillum rubri ssp. Albicans, and in vitro plantlets infected with Gluconosucarcan diazotrophicans [24]. In general, the expression of MT2-1 in sugarcane tends to be lower in roots, higher in leaves, and so forth [9, 10, 22, 25, 26]. It is interesting to note that the expression level of ScMT2-1-3 in roots and in buds was significantly higher (over 14 times) than those in stems and in leaves (Figure II), never reported before.

Difficulties in identifying and isolating MTs in plants may arise from the instability of these proteins in the presence of oxygen [10]. There were few reports about expressing plant MTs in prokaryotic system, though the research on plant MTs has been carried out for decades. In some earlier studies, plant MTs have been expressed in E. coli as GST fusions to examine the metal-binding properties of these proteins [13, 29, 30]. Recombinant production of MTs helps to circumvent some of the problems associated with direct isolation, and expression as a GST fusion offers simple possibilities for purification, quantification, and detection [30]. GST is commonly used to create fusion proteins, and many commercially available sources of GST-tagged plasmids include a thrombin domain for cleavage of the GST tag during protein purification. GST tag has the size of 220 amino acids (roughly 26 kDa), which, compared to the low molecular mass target protein MT, is quite big. Thus, the small His-tag may be a better choice when the function of fusion protein was studied in vivo. In the present study, His-tag fusion protein of His-ScMT2-1-3 had successful expression in E. coli Rosetta (DE3), and the His-ScMT2-1-3 has an observed MW which was much greater

**Figure 3:** Protein expression of pET28a-MT2 in E. coli Rosetta strain. M, protein marker; 1, blank without induction; 2, blank induction for 8 h; 3, control without induction; 4, control induction for 8 h; 5, pET28a-MT2 without induction; 6 to 9, pET28a-MT2 induction for 2 h, 4 h, 6 h, and 8 h, respectively. IPTG-induced proteins shown by arrow.

**Figure 4:** Protein purification of the recombinant protein. M, protein marker; 1, unpurified total protein; 2, Purified His-tagged-ScMT2-1-1 protein (shown by arrow).
(5.03 kDa–6.67 kDa) than that predicted by their sequences. It has been reported that the basic amino acid residues of His-tag may retard the mobility of the fusion protein bands in SDS-PAGE and cause deviation in MW determination [31]. This deviation was not observed on GST-tag fusion proteins [13, 29, 30]. Though the MW of GST-tag fusion protein GST-AtMT2a was consistent with its predicted value, the value of thrombin cleavage product after removal of GST by affinity purification was estimated at least 15 kDa in SDS-PAGE which was 3 kDa greater than the predicted one [29]. Thus, similarly, the electrophoretic mobility deviation was also observed in AtMT2a [29], and the deviation can be offset by the GST-tag for its 26 kDa MW which was much greater than AtMT2a (12 kDa). We infer that this deviation might be related to the characteristics of cysteine-rich.

AtMT2a (X62818) gene from A. thaliana has been shown to be able to complement the MT-deficiency in yeast (cup1Δ), conferring a high level of resistance to CuSO₄ and a moderate resistance to CdSO₄ [22]. Guo et al. [26] have demonstrated that all four types of A. thaliana MTs, including AtMT2a (X62818) and AtMT2b (u11256), can offer a metal tolerance when expressed in Saccharomyces cerevisiae. Expression of

**Figure 5:** MALDI-TOF-TOF MS results of ScMT2-His.
the MT2-1 gene PsMTa (Z23097) from *Pisum sativum* in *E. coli* led to increased tolerance to copper and cadmium [32, 33]. Overexpression of *AmMT2* in *E. coli* BL (DE3) led to increased metal tolerance towards Zn, Cu, Pb, and Cd [13]. In a similar way, the expression of *ScMT2-1* in *E. coli* Rosetta (DE3) enhances significantly the Cd and Cu tolerance in the present study. Furthermore, it leads to an increased tolerance to abiotic stresses of drought and salt.

Plant MTs exhibit beneficial metal-binding and induction properties which should protect these organisms from elevated levels of toxic heavy metals (such as Cd or Hg) and also affect, for example, the homeostasis of Cu and Zn, essential micronutrients for a range of plant physiological processes [10]. Some of the plant MTs’ biological function of metal tolerance has been demonstrated in nonplant systems; however, MTs’ in vivo function in plants has not yet been elucidated. We take the *MT2-1* homologous genes from various plants as samples in the following discussion. Using northern blotting technique, Zhou and Goldsbrough [22] had demonstrated that *AtMT2a* mRNA was present at a low level in *A. thaliana* 7-day-old seedlings, but the level of *AtMT2a* mRNA was increased in seedlings treated with CuSO$_4$ or CdSO$_4$ for 30 h. Moreover, this increase was positively correlated with metal concentration and exposure time [22]. Similarly, the regulation of *AmMT2* expression by Zn, Cu, or Pb was strongly dependent on the concentration and the time of exposure, as measured by real-time qPCR in seedlings of *A. marina* [13]. Conversely, the level of *OsMT2a* mRNA (u43530) from rice suspension cells was slightly reduced in the presence of excess Cd or Cu in the culture medium [27]. Exposures of 72 h to various concentrations of Cu, Cd, or Zn did not significantly affect the expression levels of *TcMT2* in shoots of 5-week-old *Thlaspi caerulescens* seedlings [11]. A subsequent study of *AtMT2* on 7-day-old *A. thaliana* seedlings had demonstrated that *AtMT2a* is strongly induced by CuSO$_4$ (50 μM), whereas *AtMT2b* remains insensitive to the same condition [22]. It seems that *TcMT2* and *AtMT2b* genes are expressed constitutively in
some plant organs or tissues [11, 22]. Further study by real-time qPCR showed that although copper treatment (40 μM CuCl₂) failed to cause a significant increase in the expression of AtMT2a in roots and in primary leaves of 6.5-day-old seedlings, the copper-induced increase in AtMT2a mRNA was restricted to the cotyledons and, to a lesser extent, the hypocotyl [34]. Consistent with the results of García-Hernández et al. [34], RNA blots showed that the levels of AtMT2a and AtMT2b RNA increased after Cu treatment, but not for every gene in every tissue [25]. The Cu treatment increased the mRNA expression of AtMT2b in roots and AtMT2a in leaves [25]. Thus, they suggested that the plant MTs have distinct functions in heavy metal homeostasis [25]. It should be stressed that although it is believed that plant MTs could play an important role in heavy metal tolerance mechanism and phytoremediation, the precise function of these MTs in plant tolerance to abiotic stresses is still not clear because of the lack of information.

It has been reported that sugarcane plantlets were able to tolerate up to 100 mM of Cu or 500 mM of Cd in nutrient solution for 33 days while accumulating 45 mg Cu per kg or 451 mg Cd per kg shoot dry weight [9]. Using RNA blot, the expression patterns of sugarcane MT genes, including ScMT2-I-1, in shoots and in roots, were analyzed.
under increasing concentrations of copper and cadmium [9]. Increasing Cu concentration had little or no effect in modulating the expressions of MT genes, while an apparent minor modulation of some of the MT genes was detected in Cd treatments which presented a minor downregulation in 33 days Cd treatment samples. In this study, we showed that the level of ScMT2-1-3 expression in Cd-treated plantlets decreased steadily 3 h following the treatment and maintained a low expression level up to 72 h. This result was in agreement with Sereno et al. [9], who inferred that cadmium tolerance and accumulation in sugarcane might derive from other mechanisms. We infer that not ScMT2-1-3 but other member(s) of metallothionens or phytochelatins play a key role in cadmium detoxification and homeostasis in sugarcane, although ScMT2-1-3 has the ability of imparting Cd tolerance when expressed in E. coli. Clearly different from ScMT2-1-1 observed by Sereno et al. [9], steadily the increased expression level of ScMT2-1-3 began to be observed at 3 h after Cu treatment, and the expression maintained 2 times higher than the control during the time examined. Thus, both the E. coli assay and sugarcane plantlets assays suggested that ScMT2-1-3 is significantly involved in the copper detoxification and storage in the cell. The differential expression patterns of ScMT2-1 in response to Cd or Cu exposure, observed by Sereno et al. [9], and this study, suggested that the members of ScMT2-1 genes may have diverse roles or functions.

According to their chemical and physical properties, two different molecular mechanisms of heavy metal toxicity caused by copper and cadmium have been reported: (a) production of reactive oxygen species by autooxidation and Fenton reaction, which is typical for transition metal copper [35, 36]; (b) blocking of essential functional groups in biomolecules, which is well documented for nonredox-reactive heavy metal cadmium [37]. On the one hand, the
different expression pattern of ScMT2-1-3 may suggest different molecular mechanisms of heavy metal toxicity caused by Cd$^{2+}$ and Cu$^{2+}$ according to their chemical and physical properties. On the other hand, the up-regulation of ScMT2-1-3 under the stress of Cu$^{2+}$ indicated that this gene is significantly involved in the copper detoxification and storage in sugarcane cells, while the downregulation of ScMT2-1-3 under the stress of Cd$^{2+}$ implied that its functional mechanism in cadmium detoxification and storage in sugarcane cells needs more testification.

5. Conclusions

In conclusion, we reported here a new member of plant type 2 metallothionein subfamily, termed as ScMT2-1-3 identified in sugarcane. We demonstrated that the expression of ScMT2-1-3 in E. coli can significantly enhance the tolerance to abiotic stresses such as heavy metal (copper and cadmium), droughtly and salt stresses. In contrast with the previous, reported MTs in sugarcane, ScMT2-1-3 has a distinct expression pattern in response to copper and cadmium treatments: highly expressed in root and bud but lowly expressed in stem and leaf; more interestingly, its expression is clearly upregulated by copper and downregulated by cadmium in sugarcane. These results, taken together, showed that ScMT2-1-3 was involved in the response to copper stresses, while cadmium tolerance and accumulation in sugarcane might derive from other mechanisms, maybe compensation mechanisms though this deduction needs more testification. ScMT2-1-3 constitutes thus a new interesting candidate for elucidating the molecular mechanisms of MTs-implied plant heavy metal tolerance/accumulation and for developing sugarcane phytoremediator varieties.

Conflict of Interests

The authors declare no conflict of interests.

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References


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