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Potent hydroxyl radical-scavenging activity of drought-induced type-2 metallothionein in wild watermelon☆,☆☆

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Abstract

Wild watermelon (*Citrullus lanatus* sp.) has the ability to tolerate severe drought/high light stress conditions despite carrying out normal C_3-type photosynthesis. Here, mRNA differential display was employed to isolate drought-responsive genes in the leaves of wild watermelon. One of the isolated genes, *CLMT2*, shared significant homology with type-2 metallothionein (MT) sequences from other plants. The second-order rate constant for the reaction between a recombinant CLMT2 protein and hydroxyl radicals was estimated to be 1.2×10^{11} M^{-1} s^{-1}, demonstrating that CLMT2 had an extraordinary high activity for detoxifying hydroxyl radicals. Moreover, hydroxyl radical-catalyzed degradation of watermelon genomic DNA was effectively suppressed by CLMT2 in vitro. This is the first demonstration of a plant MT with antioxidant properties. The results suggest that CLMT2 induction contributes to the survival of wild watermelon under severe drought/high light stress conditions.

Keywords: Metallothionein; Wild watermelon; Drought tolerance; Hydroxyl radical; Oxidative stress

Water deficit is one of the major factors limiting plant productivity [1]. Water deficit-induced damage in plants is closely associated with reactive oxygen species (ROS) [2,3]. The production of ROS, such as superoxide radicals and hydrogen peroxide, is significantly enhanced under water stress conditions where the light energy captured by the leaves is far in excess of that required for photosynthetic assimilation. If these ROS are not decomposed safely, reactive toxic hydroxyl radicals are generated, and these give rise to severe damage in plant cells [4,5].

Plants are equipped with multiple enzymes and non-enzymatic molecules that are involved in the decomposition of ROS [5,6]. Superoxide radicals are rapidly converted to hydrogen peroxide and dioxygen by the action of superoxide dismutase, and the hydrogen peroxide produced is then converted to water and dioxygen by ascorbate peroxidase and catalase. In contrast, no enzymes are known to degrade hydroxyl radicals in organisms. Antioxidants such as ascorbate, glutathione, and α-tocopherol are thought to be involved in scavenging of toxic radicals, such as hydroxyl radicals and singlet oxygen [7]. The levels of antioxidant enzymes are known to be elevated during drought/high light stress conditions in the leaves of many domesticated plant species [8,9]. However, these plants do not withstand severe environmental stress conditions, and suffer irreversible damage and/or necrotic disorders due to oxidative injuries.

☆ The nucleotide sequences reported in this paper have been registered in the DDBJ/GenBank/EMBL databases with Accession Nos. AB182918 for *CLMT2*, and AB182919–AB182938 for the wadi003b to wadi154-5 cDNA fragments, respectively.

☆☆ *Abbreviations:* MT, metallothionein; ROS, reactive oxygen species; MALDI-TOF MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; ICP-AES, inductively coupled plasma-atomic emission spectrometry; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DHBA, dihydroxybenzoic acid.

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Wild watermelon (*Citrullus lanatus* sp.) offers unique opportunities for investigating how wild C_3-plants survive harsh drought/high light stress conditions [10]. One of the unique metabolic responses of this plant under such stress conditions is the massive accumulation of a novel compatible solute, citrulline [11], which was found to be one of the most potent hydroxyl radical scavengers [12]. However, this citrulline accumulation occurs at the later stages of the stress response [11], suggesting that this plant may possess other undiscovered mechanisms for protecting cellular components from oxidative damage.

In an effort to elucidate the molecular mechanisms of stress-tolerance in wild watermelon, we set out to examine the genes induced by drought/high light stress conditions. One of the isolated genes shared significant sequence homology with that of plant type-2 metallothionein (MT). Biochemical analysis of the gene product demonstrated, for the first time, that this plant MT has an extraordinarily potent activity for scavenging hydroxyl radicals. These data raise interesting questions regarding the possible roles of MTs in oxidative stress-tolerance in plants.

Materials and methods

Plant materials. Wild watermelon (*C. lanatus* L. sp. No. 101117-1) plants were grown essentially as described previously [11] in a growth chamber with a light intensity of 700 μmol photons m^{-2} s^{-1}, a daily light period of 16 h, day/night temperatures of 35/25 °C, and a relative humidity of 50/60%. Plants were supplied with a nutrient solution daily at 2 h after the start of the light period. Two-week-old plants with a fully expanded fourth leaf were used for experiments in this study. The plants were subjected to drought/high light stress conditions by withholding irrigation. The stomatal conductance of the attached leaves was measured 8 h after the start of the light period with an AP4 Porometer (Delta-T Devices, UK).

mRNA differential display. The fourth leaves were harvested 8 h after the start of the light period and immediately frozen in liquid nitrogen. Total RNAs were prepared from frozen leaves using a CsCl ultracentrifugation technique as described previously [13]. The mRNA differential display technique was performed essentially as described previously [14] with the following modifications. First-strand cDNAs were synthesized from 2 μg of each total RNA and rhodamine-labeled dT primers differing in their 3′ dinucleotide adaptor sequences (Fluorescence Differential Display Kit; Takara, Tokyo) using a First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ). A subset of the cDNA fragments was then amplified by PCR using combinations of dT primers and arbitrary 10-mer primers (Fluorescence Differential Display Kit; Takara). The conditions for PCR were as follows: 94 °C for 3 min, 40 °C for 5 min, 72 °C for 5 min, followed by 25 cycles of 94 °C for 30 s, 40 °C for 2 min, and 72 °C for 2 min, and a final extension step at 72 °C for 5 min. The amplified cDNAs were separated by electrophoresis in a 4% polyacrylamide gel containing 7 M urea on a 200×450 mm glass plate. Fluorescent images were scanned with an FMBIO II Multi-View image analyzer (Takara). Northern blotting analysis was performed as described previously [13] using the cDNA as a template for the ^{32}P-labeled probe.

Cloning and sequencing of cDNAs. Gel slices containing a cDNA band of interest were excised, and the gel was scanned again with the image analyzer to ensure accurate cutting. The cDNA was eluted from each gel slice by boiling in 50 μl TE buffer for 10 min, and then re-amplified by PCR using the dT-primer and arbitrary primers, and cloned into the pT7 Blue vector (Novagen, Darmstadt, Germany). Isolation of the full-length cDNA clone from a watermelon cDNA library was performed as described previously [11], using a radioactive probe prepared from the cloned fragment according to the manufacturer's instructions (StripAble PCR Probe Synthesis and Removal Kit; Ambion, Austin, TX). DNA sequencing was performed using the dye-terminator sequencing procedure (BigDye Terminator Cycle Sequencing Kit; Applied Biosystems, Foster City, CA) and a model 3100 sequencer (Applied Biosystems).

Production of a recombinant CLMT2 protein. The cDNA fragment representing the full-length CLMT2 protein-encoding region was amplified by PCR, and cloned into the *Nco*I–*Xba*I restriction sites of the pGEX4T-3 expression vector (Amersham Biosciences). *Escherichia coli* BL21(Lys3) containing the plasmid was cultured in liquid LB medium until the OD_{600} reached 0.5, and then $ZnSO_4$ was added to a final concentration of 0.5 mM and incubated for 15 min at 18 °C. The GST–CLMT2 fusion protein was induced by adding isopropyl-thio-β-galactopyranoside (IPTG) to a final concentration of 0.2 mM, and the culture was incubated at 18 °C overnight. The cells were harvested by centrifugation, and disrupted by sonication in a buffer containing phosphate-buffered saline (PBS) and 50 mM dithiothreitol. The fusion protein was purified by GSH-affinity chromatography (Glutathione–Sepharose 4B; Amersham Biosciences) according to the manufacturer's instructions. Glutathione was removed by gel filtration through Sephadex G-50 (PD-10; Amersham Biosciences) equilibrated with PBS and 50 mM DTT. The fusion protein was digested by thrombin (Amersham Biosciences) at 22 °C overnight, and GST was removed by another round of GSH-affinity chromatography. To eliminate residual GST, the solution containing CLMT2 was heat-treated at 70 °C for 20 min and centrifuged at 10,000*g* for 10 min. The solution was then applied to a Sephadex G-50 (PD-10) column equilibrated with nitrogen gas-purged 50 mM potassium phosphate buffer, pH 7.4, and 150 mM NaCl in an anaerobic chamber (Bactron IV; Sheldon Manufacturing, Cornelius OR). The purified CLMT2 protein was analyzed by 15% Tricine–SDS–PAGE as described previously [15].

The concentration of CLMT2 protein was determined by analyzing the sulfur content of the protein using inductively coupled plasma-atomic emission spectrometry (ICP-AES; Optima2000; Perkin–Elmer, Wellesley, MA). The method of Bradford [16] was used for further experiments, with BSA as the standard, and gave an estimated protein concentration 75% higher than that determined by ICP-AES.

The reduced form of the thiol group (SH) was measured by the method of Ellman [17] in a buffer containing 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), 6.4 M urea, 10 mM Tris–HCl buffer, pH 8.0, and 1 mM EDTA. The amount of SH was calculated using the molar absorption coefficient of the thionitrophenylate anion at 412 nm (E_{mol} = 13,600).

MALDI-TOF MS. One microliter of purified CLMT2 (1 mg/ml) was mixed with 1 μl of a matrix solution containing 10 mg/ml α-cyano-4-hydroxycinnamic acid and 50% (v/v) acetonitrile. The mixture was loaded on a sample plate and the solvent was removed by evaporation. The molecular mass of CLMT2 was determined using an Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA). The instrument was calibrated using insulin (mol. mass 5734 Da; Sigma, St. Louis, MO), cytochrome *c* (12,361 Da; Sigma), myoglobin (16,952 and 8476 Da; Sigma), and ubiquitin (8566 Da; Sigma) as standard proteins.

Metal analyses. Reconstitution of CLMT2 with either cupric or zinc ions was performed in an anaerobic chamber essentially as described previously [18]. A solution containing purified CLMT2 (200 μg) was acidified to pH 2 by adding trifluoroacetic acid, and apo-CLMT2 was then prepared by gel filtration through Sephadex G-50 (PD-10; Amersham Biosciences) equilibrated with 20 mM HCl. Either CuCl or $ZnSO_4$ solution was added to the apo-CLMT2 to a

final concentration of 0.33 mM, and then the metal–protein mixture was neutralized by slowly adding Tris–HCl buffer, pH 8.0, to a final concentration of 100 mM. The excess metal ions were removed by Sephadex G-50 column chromatography. The metal content of the reconstituted CLMT2 was determined by ICP-AES.

Hydroxyl radical-scavenging activity. The hydroxyl radical-scavenging activity of the zinc-bound CLMT2 protein was examined using a competitive trapping assay as described previously [12]. The second-order rate constant for the reaction between CLMT2 and hydroxyl radicals was calculated as described previously [19], using the constant for salicylate, $1.2 \times 10^{10}\ M^{-1}\ s^{-1}$ [20], as the reference.

Cleavage of DNA by hydroxyl radicals. Genomic DNA was isolated from the leaves of wild watermelon as described previously [21]. The DNA (200 ng) was treated with 133 mM H_2O_2, 0.1 mM EDTA-Na-Fe(III), and 0.1 mM ascorbate in 50 mM KH_2PO_4–KOH buffer at pH 7.4, in the presence or absence of CLMT2. The reaction was carried out at 25 °C for 60 min, and quenched by adding citrulline to a final concentration of 62.5 mM. The samples were then separated by electrophoresis in a 0.8% agarose gel, stained with ethidium bromide, and visualized under UV light.

Results

Screening for drought/high light stress-responsive genes in wild watermelon leaves

Wild watermelon plants were grown in the growth chamber with a light intensity of 700 μmol photons $m^{-2}\ s^{-1}$ and subjected to drought/high light stress by withholding water. The stomatal conductance of the fourth leaves was 305 ± 47 mmol $m^{-2}\ s^{-1}$ ($n = 3$) at 6 h after the final watering (referred to as 'stressed for 0 days') and decreased to 98 ± 32 mmol $m^{-2}\ s^{-1}$ over the next 24 h (stressed for 1 day). The stomatal conductance dropped to 61 ± 31 mmol $m^{-2}\ s^{-1}$ and 32 ± 14 mmol $m^{-2}\ s^{-1}$ at 2 and 3 days after the final watering, respectively.

Changes in the gene expression patterns in the leaves were analyzed using a mRNA differential display technique. A total of 26 primer combinations were used, and approximately 2700 cDNA bands were displayed by polyacrylamide gel electrophoresis (data not shown). Classification of the cDNA bands according to their expression patterns showed that 389 transcripts (14%) were up-regulated during drought/high light stress conditions, while 72 genes (2.7%) were down-regulated. The cDNAs up-regulated by the stress conditions were subjected to further analysis.

To gain more information on the up-regulated genes, 46 of the up-regulated cDNA bands were randomly chosen and cloned into plasmid vectors. The isolated cDNAs (designated "wadi" for watermelon drought-induced genes) were characterized by partial sequencing of the clones. The sequences were compared with those in public databases using BLASTX and TBLASTN searches. Of the 46 sequences determined, 32 (70%) were significantly homologous (E value $<10^4$) to sequences from other organisms, and 21 of them showed similarities to sequences with known functions (Table 1). These cDNAs encoded a broad spectrum of proteins with many different cellular functions, and some have been reported to be induced by abiotic stresses in other plants. Calcium-dependent protein kinase was reported to be induced by drought and salinity stress conditions

Table 1
Inventory of putative drought/high light stress-induced genes in the leaves of wild watermelon isolated by mRNA differential display

Clone	Accession number	Putative functional identity	Organism	E value
wadi003b	AAM62824	Cytochrome *b* 561	*A. thaliana*	4.00E−46
wadi005v	AAL59948	Calcium-dependent protein kinase	*A. thaliana*	5.00E−84
wadi006-2	AAB04057	Sterol 24-C-methyltransferase	*G. max*	1.00E−83
wadi019-7	BAB60721	Glucosyltransferase	*N. tabacum*	1.00E−32
wadi021-12	AAK31319	Transcriptional adaptor ADA2a	*A. thaliana*	8.00E−30
wadi022-5	AAC77857	CSN complex subunit 2	*A. thaliana*	3.00E−09
wadi032-2	AY065252	Argininosuccinate synthase	*A. thaliana*	1.00E−15
wadi035-5	BAA10929	Cytochrome P450-like TBP	*N. tabacum*	1.00E−34
wadi042-4	AAF40198	Translationally controlled tumor protein	*T. aestivum*	2.00E−19
wadi045-4	CAB81825	HD-ZIP protein	*L. esculentum*	4.00E−37
wadi046-23	CAB81587	Eukaryotic translation initiation factor 6	*A. thaliana*	2.00E−11
wadi048-26	AAB04675	Type 2 metallothionein	*L. esculentum*	5.00E−12
wadi065-1	AAF20221	RNA-binding protein	*A. thaliana*	8.00E−09
wadi078-3	AAD17230	FtsH-like protein Pftf precursor	*N. tabacum*	4.00E−04
wadi096-2	CAB87409	Leucine-rich repeat transmembrane protein kinase	*G. max*	3.00E−87
wadi098-8	AAL71857	Dehydroascorbate reductase	*O. sativa*	2.00E−11
wadi100-4	AAK82449	Phospholipase	*O. sativa*	3.00E−41
wadi117-1	BAA83469	Csf-1 protein	*C. sativus*	3.00E−43
wadi123-2	AAD27914	RING zinc finger protein	*A. thaliana*	4.00E−19
wadi145-2	AAF97974	SET-domain transcriptional regulator	*A. thaliana*	1.00E−32
wadi154-5	AAD15443	DnaJ protein	*A. thaliana*	7.00E−09

Accession number and organism refer to the accession number and source of the protein with the highest sequence similarity to the wild watermelon cDNA. E values for the BLAST searches are also shown.

in *Arabidopsis thaliana* [22] and *Mesembryanthemum crystallinum* [23], while *DnaJ* and *FtsH* were induced by a combination of drought and heat stress conditions in *A. thaliana* [24].

The deduced amino acid sequence of the wadi048-26 clone exhibited homology to the type-2 metallothionein (MT) sequences from other plants. Northern blotting analysis confirmed the induction of this gene in wild watermelon leaves under drought/high light stress conditions (data not shown). Aside from the established roles of MTs in Cd-detoxification and Cu/Zn-homeostasis [25], another function suggested in fungal and vertebrate MTs is to protect cells from oxidative injuries [26–28]. In plants, however, an antioxidant role for MTs has not been demonstrated. This prompted us to examine the properties of this gene product in more detail.

Structure of CLMT2 deduced from the cDNA structure

To reveal the molecular entity of wadi048-26 in more detail, the full-length cDNA clone was isolated and its nucleotide sequence was determined. The protein sequence deduced from the cDNA indicated a small protein of 77 amino acids, which showed 88%, 70%, 67%, and 60% homology to the type-2 MT sequences from cucumber, soybean, *Arabidopsis*, and rice, respectively (Fig. 1A). Notably, 14 of the 77 residues in the watermelon sequence were cysteines, comprising 18% of the total amino acid composition. The positions of the 14 cysteine residues at the N- and C-terminal domains were completely conserved with those in the plant type-2 MTs, suggesting the functional importance of these amino acid residues. As a result, we designated this protein CLMT2 (*Citrullus lanatus* metallothionein type-2).

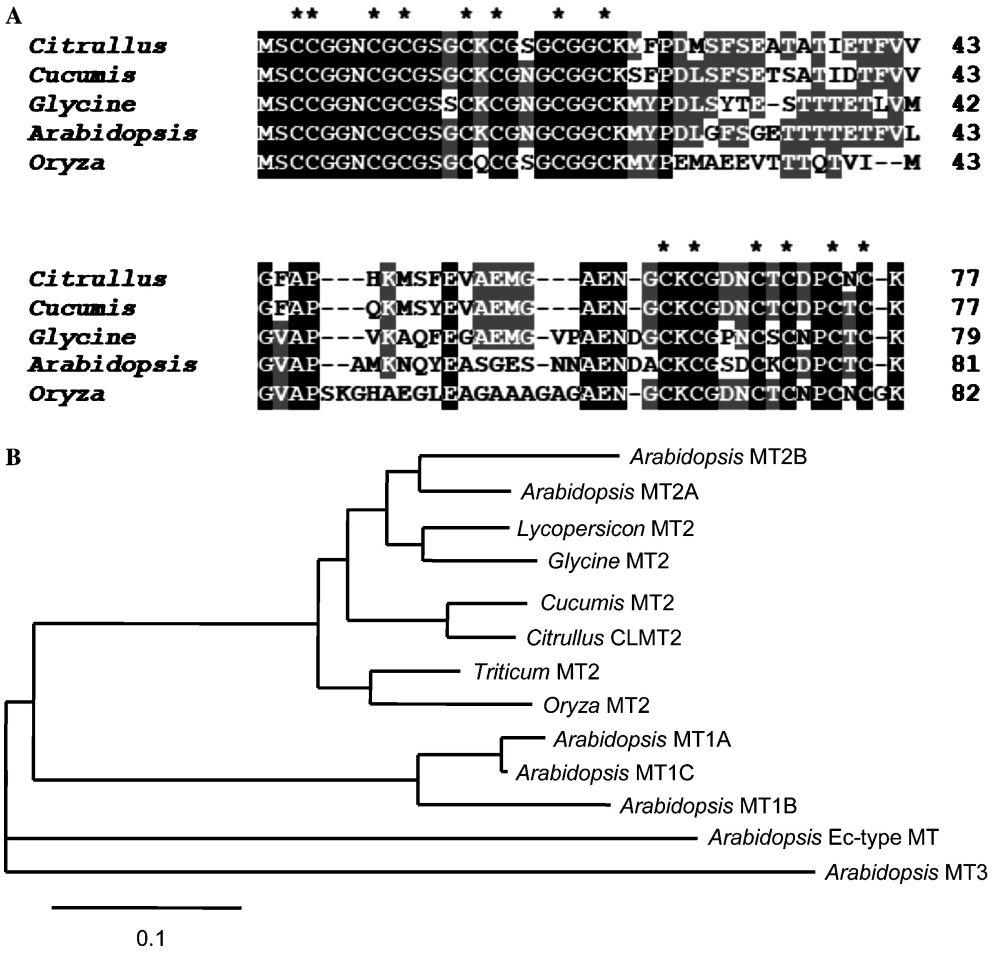

Fig. 1. Analysis of the deduced amino acid sequence for watermelon CLMT2. (A) Alignment of the watermelon sequence (*Citrullus*) with type-2 MT sequences from other plants. Black boxes indicate that the sequence is identical in all cases, and shaded boxes show where the majority of the genes (three or more) have the same amino acid residues. Asterisks indicate the conserved cysteine residues. (B) Phylogenetic analysis of MTs in plants. Sequences for type-2 MTs from representative plants and other MT types from *Arabidopsis* were used for ClustalW analysis (http://www.ddbj.nig.ac.jp) and visualized using the TREEVIEW program (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). The accession numbers for the sequences are as follows: *C. lanatus* CLMT2 (this study, AB182918), *Cucumis sativus* (CK700734), *Glycine max* (JQ2128), *Lycopersicon esculentum* (Q40158), *Triticum aestivum* (AF470355), *Oryza sativa* (P94029), *A. thaliana* MT2A (P25860), MT2B (Q38805), MT1A (P43392), MT1B (Q38803), MT1C (Q38804), MT3 (AAM64769), and E_c-type MT (Q42377).

Phylogenetic analysis showed that CLMT2 is situated in the cucurbitaceous family in the type-2 MT clade (Fig. 1B).

Production of recombinant CLMT2 protein

The cDNA fragment for CLMT2 was inserted into the pGEX-4T-3 expression vector and the recombinant protein was expressed in *E. coli*. The apparent molecular mass of the purified CLMT2 protein determined by SDS–PAGE analysis was 13 kDa (data not shown), which was larger than the expected mass (7.9 kDa) calculated from the deduced amino acid sequence. However, the MALDI-TOF MS detected a major peak at $m/z = 7944$ (Fig. 2), which correlated well with the expected mass for recombinant CLMT2 (mol. mass. 7943 Da), confirming that the recombinant CLMT2 had the expected molecular mass for its monomer. It has been reported that MTs show a broad band and a high apparent molecular mass in SDS–PAGE analysis [29].

Analysis of the thiol groups in the purified CLMT2 using DTNB showed that 83% of the cysteine residues were in a reduced state. ICP-AES analysis of the same CLMT2 preparation revealed that the molar ratio for zinc:CLMT2 was 3.4:1. To confirm the binding stoichiometry for metal ions to CLMT2, metal-CLMT2 complexes were reconstituted from the apo-form of CLMT2 protein in vitro. The procedure involved preparation of apo-CLMT2 by lowering the pH of the buffer followed by gel filtration chromatography. The metal-bound form of CLMT2 was then reconstituted by neutralizing the solution in the presence of cupric or zinc ions and removing the excess ions by gel filtration chromatography. ICP-AES analysis of the reconstituted samples revealed that the molar ratios of cupric and zinc ions to the CLMT2 polypeptide were 5.1:1 and 3.6:1, respectively.

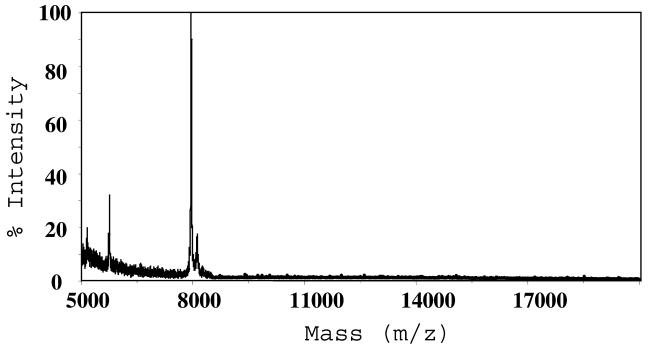

Fig. 2. Molecular mass of recombinant CLMT2 as determined by MALDI-TOF MS. A mass spectrum was recorded for a solution of purified CLMT2 in 25% acetonitrile and 5 mg/ml α-cyano-4-hydroxycinnamic acid. Insulin, cytochrome *c*, myoglobin, and ubiquitin were used as molecular weight standards for calibration of the molecular mass.

Hydroxyl radical-scavenging activity of CLMT2

The reactivity of the zinc-bound CLMT2 protein with hydroxyl radicals was examined in vitro, by allowing it to compete with salicylate for the radicals [12]. In this assay, the formation of dihydroxybenzoic acid (DHBA) by the reaction between salicylate and hydroxyl radicals is suppressed by a scavenger in a dose-dependent manner. As shown in Fig. 3, addition of the CLMT2 protein at concentrations of 10–40 μM effectively protected 200 μM salicylate from attack by hydroxyl radicals, suggesting that CLMT2 is a potent scavenger of hydroxyl radicals. In contrast, citrulline, a compatible solute in wild watermelon [12], was virtually ineffective at these low concentrations, and a concentration at least one order higher was required to achieve effective competition with salicylate (data not shown).

Using the data points in Fig. 3, the second-order rate constant for the reaction between CLMT2 and hydroxyl radicals was calculated. The rate constant for CLMT2 was estimated to be $1.2 \times 10^{11}\ M^{-1}\ s^{-1}$, which was one- or two orders higher than those reported for typical antioxidants, such as ascorbate and glutathione (7.2 and $8.8 \times 10^{9}\ M^{-1}\ s^{-1}$, respectively) [30], or citrulline ($3.9 \times 10^{9}\ M^{-1}\ s^{-1}$) [12].

CLMT2 protects genomic DNA from hydroxyl radicals

To examine whether CLMT2 effectively protects cellular components from oxidative injuries, the effect of CLMT2 on hydroxyl radical-induced DNA cleavage was investigated in vitro (Fig. 4). Genomic DNA was broken down into smaller fragments of 2–4 kbp by

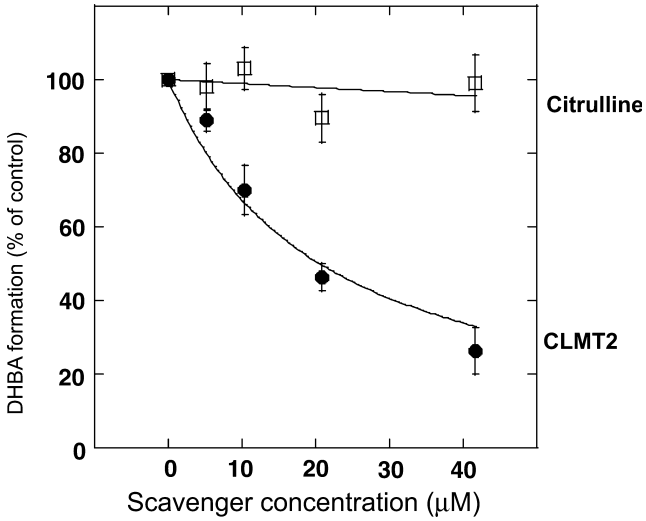

Fig. 3. Hydroxyl radical-scavenging activity of CLMT2. Competitive inhibition of radical-induced DHBA formation by adding increasing concentrations of CLMT2 (filled circles) or citrulline (open squares) was examined. Data are means ± SE ($n = 3$). The lines represent regression curves fitted to the plotted points.

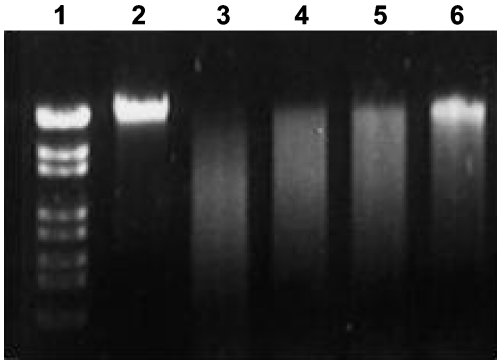

Fig. 4. Protective effect of CLMT2 on hydroxyl radical-induced DNA damage. Genomic DNA from wild watermelon was treated with hydroxyl radicals generated by the iron/H_2O_2 system for 60 min in the absence or presence of various concentrations of CLMT2. Lane 1, *Sty*I-digested lambda DNA markers; lane 2, untreated genomic DNA; lane 3, genomic DNA incubated in the absence of CLMT2; and lanes 4–6, genomic DNA incubated in the presence of 3, 6, and 12 μM CLMT2, respectively.

hydroxyl radicals generated by the iron/H_2O_2 system (Fig. 4, lane 3). The DNA degradation was significantly suppressed by CLMT2 in a dose-dependent manner (Fig. 4, lanes 4–6) and almost completely suppressed by 12 μM CLMT2.

Discussion

MTs are small polypeptides containing high percentages (15–30%) of cysteine residues, and play pivotal roles in heavy metal detoxification and homeostasis in eukaryotes and some cyanobacteria [25,31]. In vertebrates and fungi, however, accumulating evidence has suggested an alternative role for MTs as protectants from oxidative damage [26–28]. Although plant MTs share common characteristics with their vertebrate counterparts regarding the cysteine-rich amino acid composition, virtually no significant sequence similarities have been found other than the cysteine-rich tri- or tetra-polypeptide motifs CXC and CXXC [32–34]. Many studies have reported on the induction of plant MT genes in response to heavy metals [35], the metal-binding properties of plant MT [36], and the relationships between the expression levels of MTs and the accumulation of and tolerance to heavy metals in transgenic plants [37]. However, antioxidant properties of plant MTs have not been reported to date.

In this study, mRNA differential display and Northern blotting analyses revealed that the expression of the *CLMT2* encoding a type-2 MT was elevated under drought/high light stress conditions in the leaves of a C_3-type xerophyte, the wild watermelon. The biochemical analyses using the recombinant protein demonstrated that CLMT2 protein is an extraordinarily potent scavenger for hydroxyl radicals among known antioxidants from plant sources. The elevated expression of CLMT2 in wild watermelon leaves may therefore potentially strengthen the antioxidant defense capability of the cells and contribute to the tolerance of wild watermelon plants to oxidative stress brought about by drought/high light stress conditions. The results of this study provided the first evidence for a protective role of a plant MT against oxidative stress.

DNA is a very sensitive target of hydroxyl radicals [38]. Oxidative damage of DNA involves base modifications and strand cleavage, which lead to senescence and diseases in biological systems. The present results revealed that the CLMT2 protein efficiently protected watermelon genomic DNA from oxidative injuries caused by hydroxyl radicals in vitro. Although the intracellular localization of CLMT2 protein remains to be examined, the predicted CLMT2 polypeptide does not contain any recognizable amino acid motifs that would direct the protein to any particular subcellular compartments in the cells, indicating that CLMT2 may be localized in the cytosol and, due to its small molecular size, diffuse through nuclear pores and accumulate in the nucleus. In fact, vertebrate MTs are concentrated in the nucleus [27].

The findings in this study offer new implications regarding the functions of plant MTs in general. In addition to metals, expression of plant MT genes has been reported to be induced by various environmental and developmental stimuli, including heat shock [39], ethylene-promoted abscission [40], leaf senescence [41], and wounding and virus infection [42]. The common feature shared by these seemingly diverse stimuli is enhanced production of ROS. More recently, it has been described that treatment of *Arabidopsis* leaf tissue with a catalase inhibitor, 3-amino-1,2,4-triazole, resulted in enhanced expression of an MT gene [43], supporting the view that the expression of MT genes is correlated with the level of ROS. These observations, together with the findings in this study, suggest that the antioxidant function of MTs might be universal under various physiological conditions in diverse plant species.

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