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Isolation and characterization of the *Agmt2* gene and its response to abiotic and metalstress in *Apium graveolens*

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ABSTRACT

Metallothioneins (MTs) are defined as small cysteine-rich, metal-binding proteins. In this study, the *Agmt2* genes were cloned from three celery cultivars 'Liuhe Huangxinqin', 'Jinnan Shiqin' and 'Ventura', respectively. The *Agmt2* gene showed a length of 231 bp open reading frame (ORF) and encoded with 76 amino acid residues, with two introns of 385 bp. Sequence analysis indicated that AgMT2 belonged to the Metallothio_2 superfamily. The predicted molecular weight and pI value of the AgMT2 protein were 7.5 kD and 4.95 respectively. There was one change in nucleotide acid site in 'Liuhe Huangxinqin' compared to 'Jinnan Shiqin' and 'Ventura'. The amino acid sequences of AgMT2 in three cultivars were completely identical. Multiple sequence alignment showed that domain of MT2-like proteins was highly conserved. AgMT2 further demonstrated a very close evolutionary relationship with MT2 from *Nicotiana tomentosiformis*, *Nicotiana sylvestris* and *Nicotiana Plumbaginifolia* in *Solanaceae.* Quantitative RT-PCR revealed that *Agmt2* gene expression profiles exhibited tissue specificity in celery. *Agmt2* gene expression responsed more significant in 'Ventura' than in 'Liuhe Huangxinqin' and 'Jinnan Shiqin' under eight metal ions (Co^{2+}, Ni^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+}) and four abiotic stress treatments (hot, cold, salt and drought). Our findings served as a valuable resource for AgMT2 protein in celery.

1. Introduction

Metallothioneins (MTs) are present in almost all living creatures, such as bacteria, yeast, fungi, animals and plants (Palacios et al., 2011; Robinson et al., 1993; Zhou and Goldsbrough, 1994). The classification system of MT proteins is based on the number and arrangement of cysteine residues. Class I of MTs contain 20 conserved Cysteine residues and are prevalent in vertebrates. All plant and fungi MTs as well as nonvertebrate lacking this arrangement of residues are referred to as class II (Chaturvedi et al., 2014; Cozza et al., 2013; Murphy et al., 1997; Zhang et al., 2014; Zhou and Goldsbrough, 1995).

Cobbet integrated predecessors' research; the Class II members of plant MT proteins are further classified into four different types based on their amino acid sequences (Cobbett and Goldsbrough, 2002). Type 1 MTs have two cysteine-rich domains and their transcripts are mainly found in subterranean tissues (Ma et al., 2003; Yang et al., 2009). Each domain contains a total of three Cys-Xaa-Cys motifs (Xaa represents another amino acid). The two domains are separated by a large spacer with ~40 amino acid residues that include aromatic amino acids. The spacer of less than 10 amino acids can't carry aromatic residues. Type 2 MTs also have two cysteine-rich domains separated by a spacer region of ~40 amino acid residues and are abundant in aerial organs (Cozza et al., 2013; Domènech et al., 2006). However, the sequences of the N-terminal domain are highly conserved (MSCCGGNCGCG). The C-terminal domain contains three Cys-Xaa-Cys motifs. Type 3 MTs are mainly identified from ripening fruits or developing embryos and known to have four and six cysteine residues at the N- and C-terminals (Freisinger, 2007; Jordan et al., 2005). They contain only four Cys residues in motif (Cys-Gly-Asn-Cys-Asp-Cys) and motif (Gln-Cys-Xaa-Lys-Lys-Gly) in the N-terminal domain. The C-terminal cysteine-rich domains include six Cys residues arranged in Cys-Xaa-Cys motifs. The two domains are as well separated from each other by ~40 amino acid residues. Differ from other three types plant MTs, type 4 MTs have three cysteine-rich domains and are abundant in seeds (Rodríguez-Llorente et al., 2010). Each domain includes 5 or 6 conserved cysteine residues (most Cys-Xaa-Cys motifs) and are separated by a spacer of 10–15 residues. Type 4 MT was exemplified in higher plant by the wheat Ec protein, the first characterized plant MT protein (Lane et al., 1987).

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MTs were first isolated in the equine kidney (Margoshes and Vallee, 1957), as low molecular-mass (<10 kD), cysteine-rich proteins that participate in various protective stress responses. The two most important functions of MTs were as scavengers of free radicals and for metal detoxication and homeostasis (Carpenè et al., 2007).

Molecular biological research on the Apiaceae family is limited compared to research on model plants and major crop plants. The Apiaceae family has distinct characteristics and diverse species and exhibits varied evolution and tolerance to different environmental factors. Celery is a typical species of the genus Apium of Apiaceae. 'Liuhe Huangxinqin' is a local cultivar of celery with yellow-green leaves; it comes from Nanjing Liuhe District in east China. 'Jinnan Shiqin' is a cultivar of celery from Tianjin in north China, it is a tall plant with green leaves. 'Ventura' is a cultivar of celery introduced from the United States and has thick green stalks (Li et al., 2014a,b; Jiang et al., 2014). In this study, we cloned the *Agmt2* genes in three celery cultivars 'Liuhe Huangxinqin', 'Jinnan Shiqin', and 'Ventura' for detailed sequence analysis, evolutionary analysis. For function study, we focused on the abiotic stress and metal tolerance research (e.g., metal ions, cold, heat, salt and drought).

2. Materials and methods

2.1. Plant materials and treatments

Three celery cultivars (*Apium graveolens* cvs. 'Liuhe Huangxinqin', 'Jinnan Shiqin' and 'Ventura') were selected for the experiments. The seedlings were grown in a soil/vermiculite mixture (3:1) kept in a controlled environment growth chamber programmed with a 16/8 h (day/night) photo period. Temperatures of 25 °C for day and 16 °C for night were set.

In the metal treatment experiment, two-month old seedlings of the three celery cultivars were irrigated with aqueous solutions of 200 μmol L^{-1} $CuSO_4$, $ZnSO_4$, $MgSO_4$, $FeSO_4$, $CaCl_2$, $MnSO_4$, $CoCl_2$ and $NiCl_2$ for 24 h, respectively. Control plants were irrigated with ddH_2O. Plant samples (the whole plant) were collected, immediately frozen in liquid nitrogen and stored at −70 °C until use.

In the abiotic stress treatment experiment, two-month old seedlings were irrigated every 24 h with ddH_2O (control), 400 mmol L^{-1} NaCl (salt treatment), and 20% PEG6000 (drought treatment). Cold and heat treatments were performed by placing pots containing two-month old seedlings in two growth chambers (4 °C and 38 °C). Samples with young leaves were collected at 0, 1, 2, 4, 8 and 24 h after different treatments. Leaves from the two-month old plants were collected, immediately frozen in liquid nitrogen, and stored at −70 °C until use.

2.2. DNA extraction and cDNA synthesis

DNA extraction was used by a DNA secure Plant Kit (Tiangen, Beijing, China). Total RNA was extracted using a total RNA kit (RNA Simply, Tiangen, Beijing, China) according to the manufacturer's protocol. The cDNA was synthesized by using 5 μg of total RNA as the template of the reverse transcription system into a 20 μL reaction volume. Reverse transcription was performed using a Prime Script RT reagent kit (TaKaRa, Dalian, China).

2.3. Primer design and PCR amplification

The predicted *Agmt2* gene sequence was obtained based on the transcriptome sequence data of celery (Accession No. SRA109935), which sequenced and analyzed by our group (Li et al., 2014a,b). All the primers used in the study are listed in Supplementary Table 1. The *Agmt2* genes were amplified from three celery cultivars using DNA and cDNA as the template with AgMT2-Forward and AgMT2-Reverse Primers, respectively. The PCR condition were as follows: 95 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 54 °C for 15 s, and 72 °C for 60 s; and 72 °C for 10 min. The PCR products were recovered and inserted into plasmid pMD-18 T vector (TaKaRa, Dalian, China). The ligation mixture was then applied to transform the *E. coli* strain (DH5α). The bacteria (Ampicillin resistance) grown in medium were identified. Afterwards, two tubes of extracted plasmid DNA were sequenced by Gen-Script Inc. (Nanjing China).

2.4. Quantitative real-time PCR of the Agmt2 gene under metal ions and abiotic treatments

Quantitative real-time PCR (qPCR) was performed on an ABI 7500 (Applied Biosystems, USA) with SYBR Premix *Ex Taq* (TaKaRa, Dalian, China). mRNA expression levels of *Agmt2* were measured by qPCR using the primer pairs of target genes (AgMT2-qRT-Forward Primerand AgMT2-qRT-Reverse Primer). To establish reference points for *Agmt2* gene expression profiles, primers were also designed for *A. graveolens actin* gene (Actin-Forward primer and Actin-Reverse primer). The PCR conditions were as follows: 95 °C for 3 min; followed by 40 cycles of 95 °C for 10 s and 54 °C for 30 s; and 65 °C for 15 s. The primers were listed in Supplementary Table 1.

2.5. Data analysis

Nucleotide and amino acid sequences were analyzed using the BLAST program from NCBI website, DNAMAN 6.0 and Clustal W program. A molecular phylogenetic tree was created using MEGA 5 with the neighbor-joining (NJ) method (Tamura et al., 2011). The basic properties of the proteins were analyzed using a related software from http://www.expasy.org (Gasteiger et al., 2003). Two-dimensional structure prediction and analysis were conducted using SOPM Secondary Structure Prediction Model, which is available online at PBIL-IBCP http://npsa-pbil.ibcp.fr (Heymann et al., 2008). Signal peptides were predicted using an online program (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al., 2004). Transmembrane regions and orientations were predicted using TMpred method (Bertaccini and Trudell, 2002), and relative expression ratios were calculated using the ΔΔCT method (Pfaffl, 2001).

3. Results

3.1. Cloning of Agmt2 genes from three celery cultivars

The *Agmt2* genes were cloned from three celery cultivars 'Liuhe Huangxinqin', 'Jinnan Shiqin' and 'Ventura'. Sequence analysis indicated that *Agmt2* gene contained a 231 bp ORF, a 283 bp intron-1 and a 102 bp intron-2. The nucleotide and deduced amino acid sequences were shown in Supplementary Fig. 1. The ORF sequences of 'Jinnan Shiqin' and 'Ventura' showed 99.56% identical. A change in the ORF sequences was detected (G/A/A, 'Liuhe Huangxinqin'/'Jinnan Shiqin'/'Ventura'). Deduced amino acid sequences showed identity. Sequence analysis indicated that AgMT2 is a hydrophobic protein (Fig. 1). Prediction of the conserved domain of AgMT2 and composition of the domain indicated that AgMT2 protein belonged to type 2 MTs of Metallothio_2 superfamily (Supplementary Figs. 1 and 2). Compared to MTs in *Arabidopsis thaliana*, AgMT2 was also classified to plant type 2 MTs (Fig. 2).

3.2. Multiple sequence alignment analysis of AgMT2

Plants selected for multiple sequence alignment were based on their conserved domain of AgMT2 searching from BLAST program (Fig. 3). The predicted proteins from other plants were all belonged