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Molecular characterization and phylogenetic analysis of *ZmMCUs* in maize*

Qingchang MENG[1]**, Yuanyuan CHEN[2], Meijing ZHANG[1], Yanping CHEN[1], Jianhua YUAN[1] & Seth C. MURRAY[2]

[1] *Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, 50 Zhongling Street, Nanjing 210014, People's Republic of China; e-mail: qcmeng01@gmail.com*
[2] *Department of Soil and Crop Science, Texas A&M University, College Station, Texas 77843, USA*

Abstract: The mitochondrial calcium uniporter (MCU), located in the organelle's inner membrane of eukaryotic organisms, is a highly selective ion channel which plays a unique role in the calcium signaling. Six *MCU*-like genes (assigned as *ZmMCU1*∼*ZmMCU6*) were identified in the maize genome. Genomic analysis revealed that these six genes were located on chromosome 1, 3, 8 and 9. Sequence identity percent between *ZmMCU1* and *ZmMCU2*, or between *ZmMCU4* and *ZmMCU5* was relatively high at both the nucleic acid and amino acid levels. Sequence alignment of ZmMCU indicated that for plants, there was "DVME" motif in the deduced protein sequences. Digital expression analysis showed the *ZmMCU6* was strongly expressed in all of the studied organs in maize, while the other five *ZmMCU* genes had unique expression patterns. In the developing seeds of maize, the six *ZmMCU* genes had three divergent expression patterns. Finally, the phylogenetic analysis demonstrated that MCU homologs in plants could be grouped into two types and each type could be further classified into two subtypes along the monocot and dicot division. Moreover, MCU homologs in both the dicot and monocot had been independently duplicated and then those in maize had undergone an extra duplication for two subclades. Based on genomic structure, sequence similarity, digital expression profile, and phylogenetic analysis, our results clearly suggested that *ZmMCU1* and *ZmMCU2* as well as *ZmMCU4* and *ZmMCU5* are functionally redundant in maize genome.

Key words: mitochondrial calcium uniporter; phylogenetic inference; maize (*Zea mays*); gene duplication.

Abbreviations: MCU, mitochondrial calcium uniporter; MYA, million years ago.

Introduction

In eukaryotic organisms the mitochondria play a key role in decoding the highly pleiotropic calcium (Ca^{2+}) signals, which act as vital intracellular second messengers and govern a large array of cellular processes (Raffaello et al. 2013). A change in the cytosolic concentration of the Ca^{2+} in the plant is an important component of the signaling network evoked by diverse physiological and pathological stimuli (Hetherington & Brownlee 2004; Lecourieux et al. 2006; McAinsh & Pittman 2009; Marchi and Pinton 2014; Pendin et al. 2014). This signaling pathway is trigged through a highly selective channel called the calcium uniporter, which moves calcium ions across the mitochondrial inner membrane. Although much research had elucidated the biophysical properties of uniporter in animals and plants during the past decades (McAinsh & Pittman 2009), the molecular identity was unclear until the mitochondrial calcium uniporter (MCU) in mammals was recently identified (Baughman et al. 2011; De Stefani et al. 2011).

MCU, known as the uniporter pore, is an essential and integral membrane protein for the electrophysiologically defined uniporter current (Chaudhuri et al. 2013); it has two transmembrane domains and orients both its N- and C-termini into the matrix (Baughman et al. 2011; Martell et al. 2012). MCU, together with its regulatory components, MICU1 and MICU2, provide new molecular tools to investigate the calcium signaling process.

The *MCU* homologs exist extensively across metazoan and plants (Bick et al. 2012). Prole et al. (2012) examined the genome of some fungi and found that even the genomes of *Aspergillus* spp. and *Cryptococcus* spp. encoded homologues of *MCU*, but no further molecular information was provided. The vast anatomic and/or physiological differences of MCU in animals and fungi, have received more attention but, in plants still little information on *MCU* is known. Here we report

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** Corresponding author

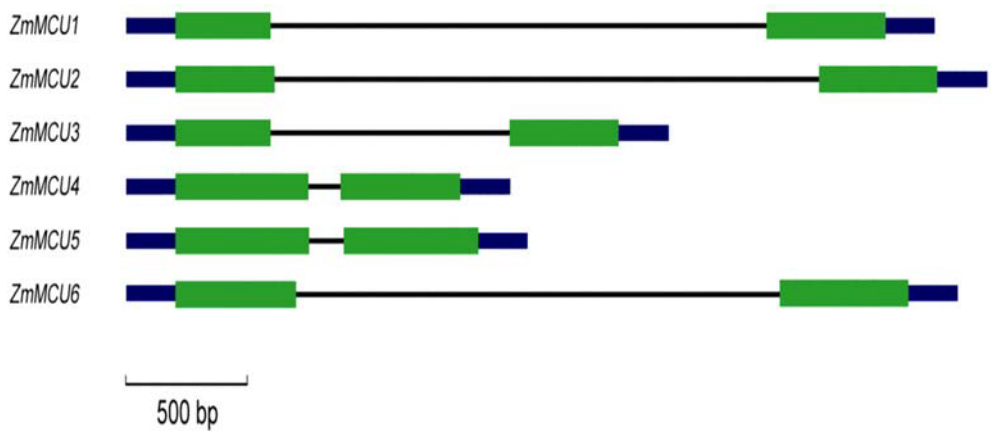

Fig. 1. Graphic genomic structure of the six *ZmMCU* genes. Boxed shapes refer to exons. Two hundred base pair DNA at the both 5' and 3' UTRs are included in blue. Intron is shown as thin black line.

the molecular identification of *MCU* homologs in maize (*Zea mays* L.), one of the most important cereals in the world.

Material and methods

Plant materials

The maize inbred line B73, the source of one of the maize genome-sequences (Schnable et al. 2009) was used throughout the bioinformatics experiments described here.

Sequence similarity searching

The highly conserved region (sites from 225 to 280) of MCU protein (NP_001028431.2; De Stefani et al. 2011) in mouse (*Mus musculus*) was selected as a query for a PSI-BLASTP (Altschul et al. 1997) with the expected threshold value of 10^{-5} against the non-redundant *Zea mays* protein sequence database in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The genomic sequences of gene models (B73 RefGen_V2) were downloaded and intron sites were compared with manual inspection. The genic structure was then drawn with GSDS online software (Guo et al. 2007). Protein sequences were predicted as described by Meng et al. (2007).

Gene expression analysis

For gene expression, data of robust multi-array average-normalized linear expression intensity for every gene was publicly available from the maize eFP browser (http://bar.utoronto.ca/; Sekhon et al. 2011) as well as that of the Ubiquitin-conjugating enzyme-encoding gene (GRMZM2G027378), which has shown to be stably and constitutively expressed in maize (Sekhon et al. 2011) and served as a positive control.

Phylogenetic analysis

We searched the homologs of the MCU protein in plants and then further validated the homologs in cereals using PGDD tool (Lee et al. 2013). All homologs, as well as some representative proteins in non-plant kingdoms, were used to infer phylogenetic relationships. At first, deduced amino acid sequences were aligned using the ClustalX2 (Larkin et al. 2007) with the default alignment parameters. Both the alignments of the whole protein sequences and of the conserved region from site 109 to 327 (Fig. 1) were further analyzed with the PHYLIP version 3.695 (Felsenstein 1993). Bootstrapping was performed 1,000 times with SEQBOOT subroutine to form the bootstrapped samples; the protein distance matrix was computed using the program PROTDIST. Then neighbor-joining and UPGMA trees were calculated using the program NEIGHBOR. A consensus unrooted phylogeny tree was obtained using CONSENSE using ordinary parsimony. Finally, the reconstructed phylogenies were displayed with the program FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Homologs in maize genome

To identify *MCU* homologs in maize, we selected one highly conserved region from the *Mus musculus* MCU protein as the query. After three cycles of iteration using PSI-BLASTP subroutine, there were no new accessions retrieved. In total, 15 protein accessions (Table 1) were identified in *Zea mays*, which showed high sequence similarity to the MCU protein. After amino acid sequence alignment and redundant sequences were manually removed, six non-redundant protein sequences were finally identified.

To obtain further evidence and to confirm there were no other MCU-homologs missing in maize, we used these identified protein sequences in maize as queries to search against B73 RefGen_V2 database in the maizeGDB. These same genomic loci were retrieved exactly and there were no other genomic region showing sequence similarity to the known MCU homologs in maize.

When phylogenetic trees were inferred, the gene model GRMZM2G425863 (thereafter renamed as ZmMCU6) was found to be very similar to OsMCU1 in rice at the amino acid level but was truncated at the N-terminal of protein compared to OsMCU1. After searching both the genomic and EST databases of maize in NCBI we found that there was an error read of 5' cDNA sequences of GRMZM2G425863 in B73, which led to a translation frame shift and incorrectly predicted the translation start point in this gene model. Based on experimental confirmation of the RNAseq data (data not shown) we corrected this sequence error and annotation error for GRMZM2G425863.

Table 1. Partial information of the six *ZmMCU* genes identified in this research.

Gene	Gene model[a]	Protein accession No.[b]	cDNA	Chr Loc[c]
ZmMCU1	GRMZM2G130764	NP_001144428.1 ACG40465.1 ACR34458.1 AFW84769.1	NM_001150956.1 BT084105.1	8S(+)
ZmMCU2	GRMZM2G021881	NP_001168074.1 ACN27015.1 DAA57110.1	NM_001174603.1 EU971573.1	3S(+)
ZmMCU3	GRMZM2G043843	DAA59228.1 DAA59229.1	EU975375.1	3S(–)
ZmMCU4	GRMZM2G091742	AFW89117.1	EU946729.1	9S(+)
ZmMCU5	GRMZM2G180044	DAA44182.1	EU970976.1	1L(–)
ZmMCU6	GRMZM2G425863	NP_001132588.1 ACF81500.1 DAA49409.1 DAA49410.1	NM_001139116.1 BT084551.2 EU961828.1 BT066105.1 BT036495.1	1S(–)

[a] According to B73 RefGen V2 gene model in MaizeGDB (http://www.maizeGDB.org/).
[b] Supporting protein accession number in the NCBI (http://www.ncbi.nlm.nih.gov/).
[c] Chromosome location; letters followed chromosome number mean short arm (S) or long arm (L) of chromosome; letters in parentheses mean positive (+) or negative (–) chain of chromosome.

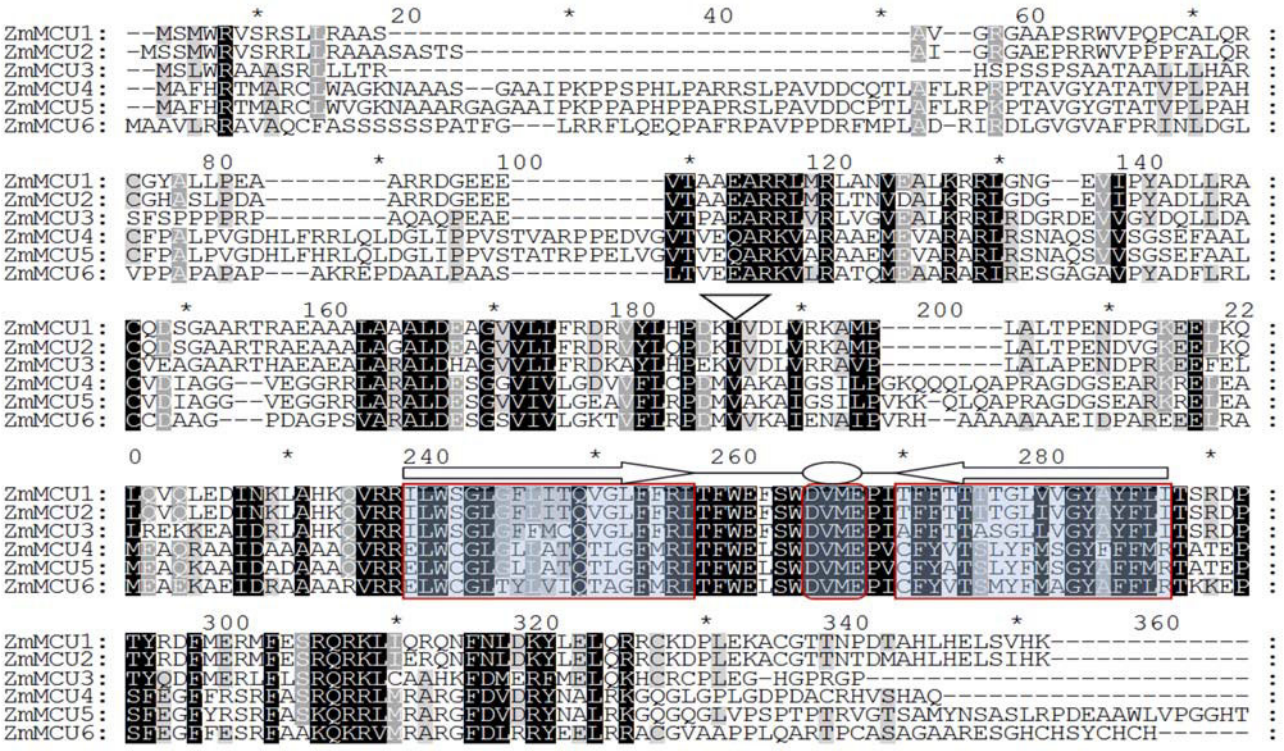

Fig. 2. Sequence alignment of *ZmMCU* deduced proteins. Identical amino acids are shaded in black. The downward triangle indicates insertion sites of introns in genomic sequences. Two highly conserved transmembrane domains flanking the plant DVME motif (oval shaped) are boxed. The horizontal right or left arrow shows the putative direction of two transmembrane domains from inner mitochondrial membrane to intermembrane space.

Finally based on the previous analysis, we obtained the gene information of these six MCU-like proteins in the maize genome and renamed them as*Zea mays* MCU1 (ZmMCU1) sequentially through *Zea mays* MCU6 (ZmMCU6) (Table 1). At the nuclear level, *ZmMCU1* and *ZmMCU2* shared 92% of sequence identity of cDNA encoding region; *ZmMCU4* and *ZmMCU5* also showed high sequence identity (88%).

Genic organization of ZmMCU genes

The genomic organization of the six *ZmMCU*-like genes places them across four different chromosomes (Table 1). *ZmMCU2* and *ZmMCU3* were located on the same chromosome (chromosome 3S), as it was for *ZmMCU5* and *ZmMCU6* (chromosome 1L and 1S). Comparably, *ZmMCU1* and *ZmMCU4* were located on chromosome 8S and 9S, respectively.

All of these genes contained one intron with varying lengths in the B73 genome (Fig. 1). The intron sizes of *ZmMCU4* and *ZmMCU5* were 141 bp and 150 bp, respectively. Comparably, the intron sizes of *ZmMCU1*, *ZmMCU2* and *ZmMCU6* were much bigger (∼2kb).

All introns of the six *ZmMCU*-like genes contained the canonical GT-AG bi-nucleotide splice site junctions (data not shown). Moreover, the occurring site of the intron was obviously conservative (Fig. 2), suggesting the evolutionary relevance of a possible correlation between exons and subdomains (Meng et al. 2007). Interestingly, the intron of *ZmMCU3* contained an extra "GT" bi-nucleotide alternative splice site, which caused another enlarged expressed transcript but did not affect the reading frame of the protein.

Characterization of ZmMCU proteins

The six ZmMCU proteins shared a more highly conserved part at their C-terminal side than N-terminal side based on the protein sequence alignment (Fig. 2). The most highly conserved region was located from amino acid 253 to amino acid 266 in the aligned consensus sites, which contained a DVME motif (Fig. 2) rather than a DIME motif as described by Bick et al. (2012). This DVME motif was specific within the plant kingdom. There were also two conserved transmembrane regions flanking the DVME motif (Fig. 2).

Among the six ZmMCU proteins, ZmMCU1 and ZmMCU2 shared 90% sequence identity at the amino acid level; ZmMCU4 and ZmMCU5 shared 83% sequence identity. However, ZmMCU2 and ZmMCU5 were highly divergent, sharing the lowest sequence identity (22%) at the amino acid level (Fig. 3).

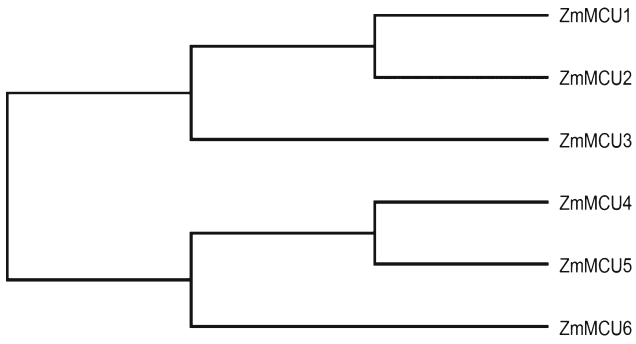

Fig. 3. The phylogenetic tree of *ZmMCU* deduced proteins, as created using the ClustalX2 program.

Expression specificity of ZmMCU genes

The publicly available digital expression atlas in maize at genomic level facilitated an investigation into the specific genes' expression. One of such data set comprised expression estimates in 60 different tissues/timepoints of the inbred line B73 from a NimbleGen array of 23,740 genes in the filter gene set (Sekhon et al. 2011). Generally, different *ZmMCU* genes had different expression patterns (Fig. 4). However *ZmMCU1* and *ZmMCU2* had similar expression patterns, both of which were strongly and predominantly expressed in anther (R1) and moderately expressed in root (V1), suggesting that they were functionally redundant genes in the maize genome; this was expected based on their sequence similarity and genomic organization models. *ZmMCU3* was moderately expressed in cobs (R1). *ZmMCU4* and *ZmMCU5* were weakly and constitutively expressed across multiple organs studied with an expression level below 200. Comparably, *ZmMCU6* was

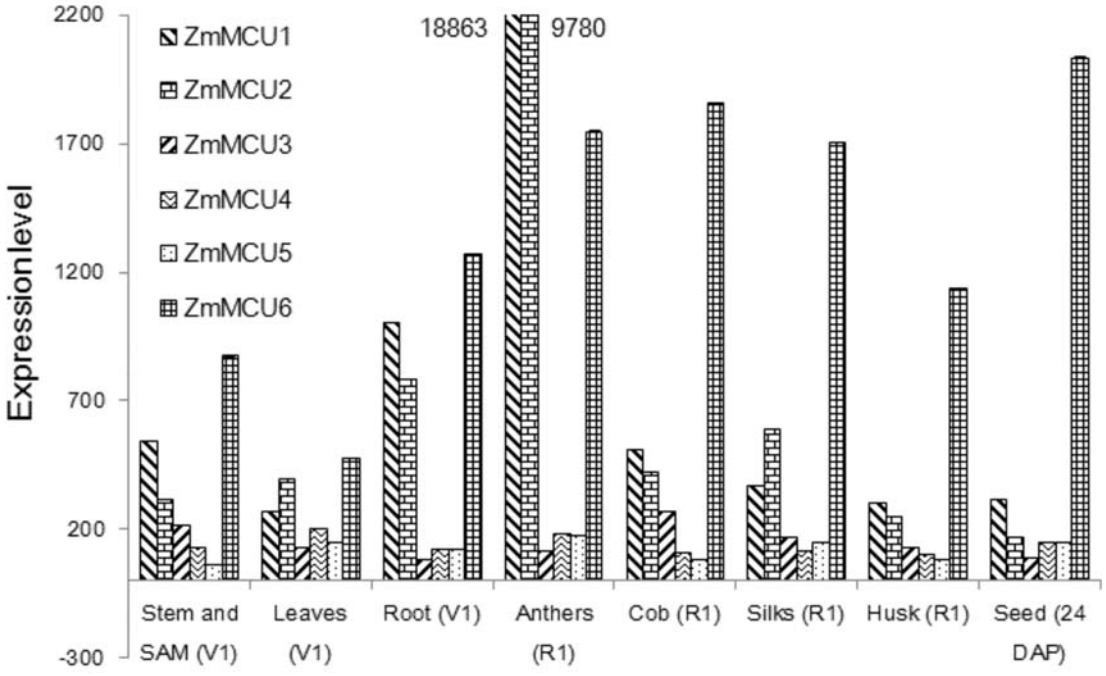

Fig. 4. Specific expression pattern for each of the six *ZmMCU* genes in maize across a variety of organs.

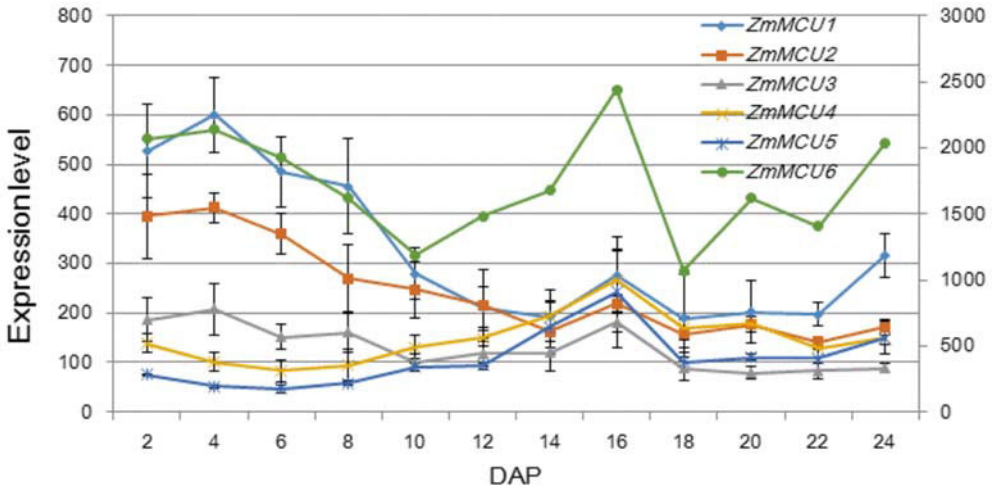

Fig. 5. Expression dynamics of *ZmMCU* genes in developing seeds of maize from 2 to 24 days after pollination (DAP). Bar shows expression error based on three replications. The right secondary axis is for *ZmMCU6*.

strongly expressed in multiple-organs, especially in the reproductive organs (anthers, cob, silks and seeds).

The expression level was very high in developing seeds for *ZmMCU6* (Fig. 5) as stated above, with an average robust multi-array average-normalized linear expression value of 1,721 when compared with those for other *ZmMCUs* (with an average from 108 to 327). There were three different expression patterns observed: *ZmMCU1*, *ZmMCU2* and *ZmMCU3* were expressed similarly in pattern I; *ZmMCU4* and *ZmMCU5* were expressed similarly in pattern II. *ZmMCU6* belongs to pattern III and was not similar to any other genes (Fig. 5). Interestingly, all surveyed genes were expressed at their local maximums at 16 days after pollination, and decreased sharply at 18 days after pollination. These data showed that the *ZmMCU* genes played diverse roles in the developing seeds of maize and some of them showed coordinated expression, or were functionally redundant.

Phylogenetic analysis of MCU-like protein in plants

The deduced protein sequences for all *MCU* genes in plants were determined and used for phylogenetic analysis (Fig. 6; Table S1), which showed that MCU proteins in flowering plants could be grouped into four large clades: Monocot_A, Dicot_A, Monocot_B, and Dicot_B. The former two clades formed the type A family; the other two clades belonged to the type B family. ZmMCU1, ZmMCU2 and ZmMCU3 belong to Monocot_A clade, whereas ZmMCU4, ZmMCU5 and ZmMCU6 belong to Monocot_B clade. Furthermore, each clade could also be separated into two sub-clades (I and II). For example, ZmMCU1 and ZmMCU2 were separated from ZmMCU3, while ZmMCU4 and ZmMCU5 were separated from ZmMCU6. Similar separation was also observed in Dicot_A and Dicot_B clades. It should be noted that even in some subclades, some MCU proteins were also duplicated for some species, for example, ZmMCU1 and ZmMCU2 or ZmMCU4 and ZmMCU5 were grouped into the same subclades in maize, respectively. This was also very common in dicots, especially for soybean (*Glycine max*), which is believed to be an ancient polyploid.

The MCU proteins in the clades Monocot_A and Dicot_A possess a distinct domain found in the flanking of the DVME motif, RLTFWEFSWDVMEP. The clades Monocot_B and Dicot_B share the common motif RLTFWELSWDVMEP. This suggests that the differentiation of these sites were retained for some certain, but yet unknown, function.

Discussion

Putative diverse functions of MCU homologs in maize

With the molecular identification of *MCU* in mammals (Baughman et al. 2011; De Stefani et al. 2011), there is an increasing interest in the *MCU* homologs in plants, especially for agriculturally important crops suffering from various biotic/abiotic stresses. It has been shown that *MCU* are involved in the stress responses through expression and regulation (McAinsh & Pittman 2009). In this research, we have shown that six *ZmMCU* genes are present in B73, as well as several homologs in sorghum and rice, which spanned two subfamilies (Panicoideae and Ehrhartoideae) in the Poaceae. The fact that ZmMCU proteins are overall more closely related across these three species than they are to each other within each species suggests that gene family members may share paralog-specific roles in the plant; either in terms of gene regulation or with regard to different levels of Ca^{2+} signal stimuli.

Recent data have shown that miR-25 decreases mitochondrial Ca^{2+} uptake through selective *MCU* down-regulation, conferring resistance to apoptotic challenges in human (Marchi et al. 2013). In this study, we also identified that microRNAs zma-miR393d, zma-miR172m and zma-miR528a/b could regulate the expressions of *ZmMCU1*, *ZmMCU3* and *ZmMCU6*, respectively (data not shown), which suggested that this

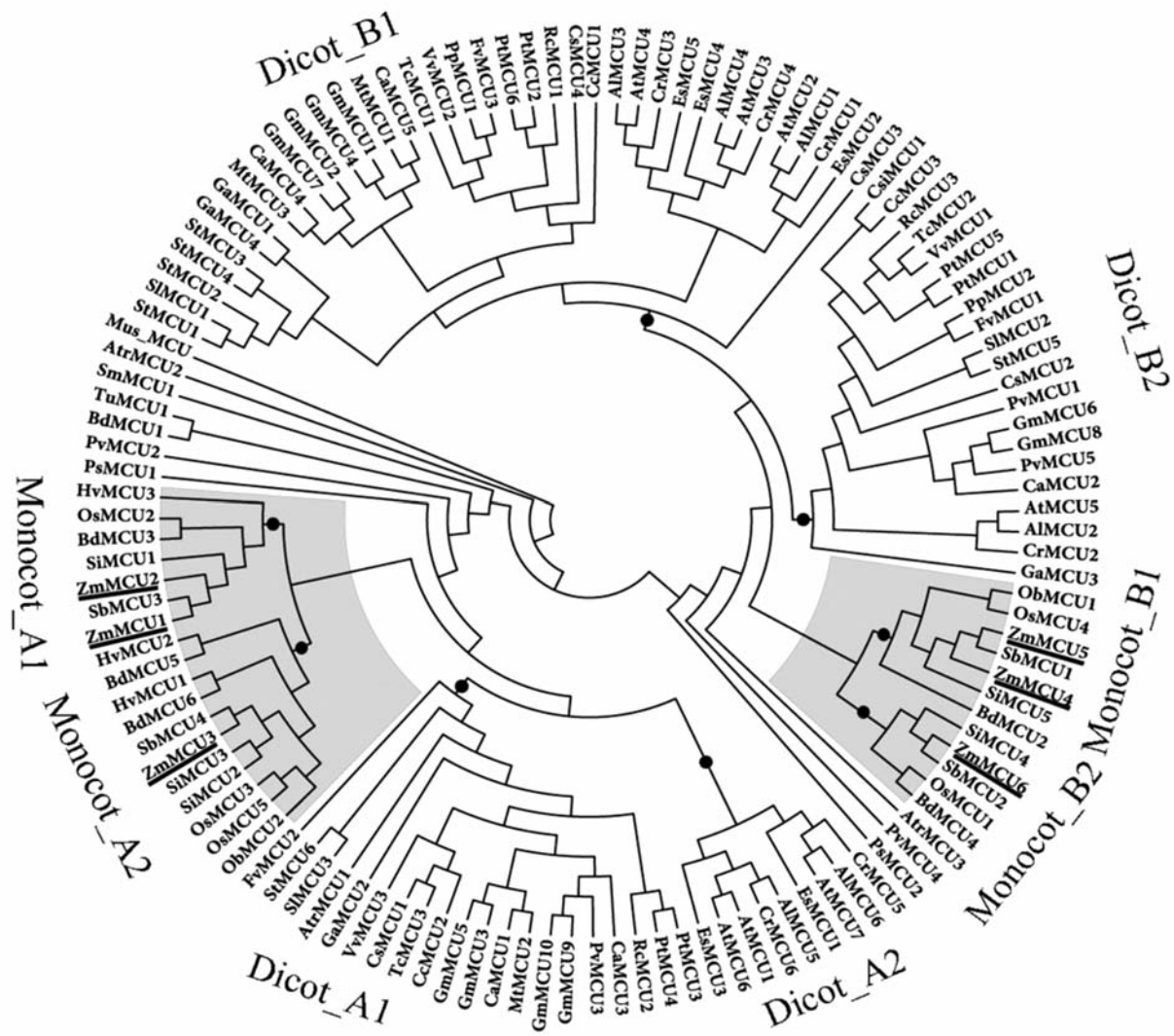

Fig. 6. Phylogenetic tree of deduced MCU proteins in plants. The *ZmMCU* deduced proteins were underlined. GenBank accessions corresponding to gene assignment could be found in Table S1. Monocot clades are highlighted.

kind of gene regulation play a key role in normal physiological life.

High sequence similarity of different genes implies a similar function. This was confirmed in the way that some of these *ZmMCU* genes appeared to be expressed in a similar manner (Figs 5, 6). However, how these genes cooperate, collocate or allocate remains still an open question.

Phylogenetic inference of ZmMCU homologs

Homologs of MCU are distributed widely across all major branches of eukaryotic life, present in nearly all plants and metazoan, but have had apparent loss events in certain protozoan and fungal lineages (Bick et al. 2012).

Examining the genome of the ciliate, *Tetrahymena thermophile*, which shared a last common ancestor with plants ~2 billion years ago (Hedges et al. 2004), revealed that there were more than two MCU-like proteins with relatively high sequence similarity, most likely the result of recent gene duplication. However, in a unicellular species of alga, such as *Chlamydomonas reinhardtii*, which diverged from plants ~955 million years ago (MYA; Hedges et al. 2004), there was only a single MCU-like protein in the genome. This initially suggested that *MCU* genes in plants have evolved from a single common gene that was present in the common ancestor of algae and plants. After searching genomic sequences of Picea and Amborella, which belong to the ancient Gymnosperms and Angiosperms, respectively, two MCU homologs were identified and separated into type A and type B, suggesting the gene duplication event occurred before the divergence of Gymnosperms and Angiosperms ~700 MYA. Hence, we can infer that separation of type A and B of MCU in plants were the result of genomic duplication or polyploidy from ~955 MYA to ~700 MYA. Indirect evidence was that MCU proteins were not grouped within monocots or dicots for four individual clades in plants, strongly suggesting that gene duplication event that generated the MCU subclades occurred before the divergence of monocots and dicots. There was likely another independent event of gene or whole genomic duplication for both monocots and dicots after their divergence ~225–300+ MYA

and before the grass speciation, ∼60 MYA (Adams & Wendel 2005). There was then a recent gene duplication event in maize, which was shown as the *ZmMCU1* and *ZmMCU2*, *ZmMCU4* and *ZmMCU5* in the subclade Monocot_A I and Monocot_B I, respectively.

The simplest evolutionary model is that the *MCU*-like genes were derived from a very ancient duplication, and in plants the pair had undergone a further duplication. Of course, there are some other alternative possibilities. For example, the ancient form of only one of the *MCU* genes found in ciliate could be the progenitor of all MCU proteins found in eukaryotes and was duplicated before the divergence of plants and animals (Margis et al. 2006), but one of the copies was later lost.

We identified six *ZmMCU* genes in maize genome and studied their molecular characterization, expression patterns and phylogenetic inference. *ZmMCU6* and *ZmMCU3* were distinct from any other *ZmMCU* genes according to gene expression patterns and phylogenetic analysis. Other *ZmMCU* genes, two pairs (*ZmMCU1* and *ZmMCU2*; *ZmMCU4* and *ZmMCU5*) existed in maize in a functionally redundant manner based on the following evidence: resembled genomic organization (Fig. 1), high sequence similarity (Figs 2,3), similar expression pattern (Figs 4,5) and close phylogenetic relationship (Fig. 6). Indirect evidence is that genomic sequences of chromosome 3S and chromosome 8S showed co-linearity (Schnable et al. 2009), where *ZmMCU1* and *ZmMCU2* were located, respectively, doing thus chromosome 9S (*ZmMCU4*) and chromosome 1L (*ZmMCU5*).

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