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Research paper

Genome-wide identification and analysis of *MICU* genes in land plants and their potential role in calcium stress

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ABSTRACT

Mitochondrial calcium uptake (*MICU*) plays a vital role in the regulation of mitochondrial calcium homeostasis, and, consequently, influences calcium signaling transduction. Although genes involved in mitochondrial calcium uptake have been well studied in animals, less is known about their ubiquity and function in plants. In this study, we identified 96 *MICU* genes in land plants. On the basis of phylogenetic analysis of MICU proteins, they were classified into three clades: MICU from eudicots (Clade I), from monocots (Clade II), and from a basal angiosperm, a bryophyte, and a lycophyte (Clade III). Pairwise identity analysis across all MICU proteins showed that they are highly conserved among land plants at the protein level. Conserved motif analysis showed that most MICU proteins contained three EF-hands, and an additional EF-hand motif first identified in the MICU of *Arabidopsis thaliana* but not mammals was found in all 96 putative MICU proteins. This suggests that a cellular pathway of calcium uptake and signaling that requires three EF-hand motifs is evolutionarily conserved in plants. In addition, we discovered that *MICU*-defective mutants of *Arabidopsis thaliana* exhibited longer roots than wild-type under high calcium stress. Concurrently, the mRNA transcription levels of *MICU* were decreased under high calcium conditions. These results suggest that loss-of-function mutations of *MICU* may have potential roles in helping plants resist high calcium stress. This study provides clues to the possible role of plant MICU in mitochondrial calcium uptake, as well as useful information to support further studies on MICU function in plants.

1. Introduction

Calcium ions (Ca^{2+}) have been recognized as important second messengers in intracellular signal transduction (Berridge et al., 2000; Hetherington and Brownlee, 2004; Sanders et al., 2002). Changes in intercellular free Ca^{2+} concentration are involved in a number of physiological processes (Simeunovic et al., 2016), such as abiotic and biotic stress responses (Cao et al., 2017; Jiang et al., 2013; Ranty et al., 2016; Schulz et al., 2013; Yuan et al., 2014), developmental regulation (Foreman et al., 2003; Monshausen et al., 2008), and control of stomatal dynamics (Kim et al., 2010; Pei et al., 2000). Ca^{2+} concentration is differentially regulated between the cytosol, plastid stoma, and mitochondrial matrix (Stael et al., 2012). Among intracellular organelles, the mitochondria are the central conductors, as they are able to influence cytosolic signaling due to their Ca^{2+} buffering ability (Jouaville et al., 1995; Hajnóczky et al., 1995; Kaftan et al., 2000).

Ca^{2+} influx into mitochondria acts as a calcium pool that modulates intracellular calcium signaling transduction (Clapham, 2007; Laude and Simpson, 2009). Primary routes for mitochondrial Ca^{2+} uptake are through the voltage-dependent anion-selective channels in the outer mitochondrial membrane, and through the mitochondrial calcium uniporter complex in the inner membrane (Wagner et al., 2016). In mammals, this complex comprises the pore-forming subunit, mitochondrial calcium uniporter (*MCU*) (Baughman et al., 2011; Chaudhuri et al., 2013; De Stefani et al., 2011), which regulates Ca^{2+} uptake along with the Ca^{2+}-sensing regulator, mitochondrial calcium uptake 1 (*MICU1*) (Mallilankaraman et al., 2012; Perocchi et al., 2010; Wang et al., 2014) or its homolog MICU2 (Kamer and Mootha, 2014). MICU1 is a membrane protein with two EF-hand domains (Perocchi et al., 2010). The EF-hand domains are a critical part of MICU1's ability to sense and modify calcium signaling (Mallilankaraman et al., 2012; Wang et al., 2014). MICU1 regulates Ca^{2+} uptake based on cytoplasmic Ca^{2+} concentration, closing MCU at low concentrations and opening it at high concentrations (Mallilankaraman et al., 2012; Wang et al., 2014), thereby enabling it to control important cellular functions such as tissue regeneration (Antony et al., 2016).

Mitochondrial control of Ca^{2+} concentration via the calcium uniporter complex has been well studied in animals, particularly mammals,

Abbreviations list: Ca^{2+}, calcium ions; Col, Columbia; qPCR, quantitative real-time PCR; GDH, glutamate dehydrogenase

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but less is known about MICU in plants. It has been shown that changes in mitochondrial Ca^{2+} concentration in *Arabidopsis thaliana* occur in response to various stimuli, such as touch and hydrogen peroxide (Logan and Knight, 2003). In addition, a homolog to the mammalian MICU1 has been recently identified in *Arabidopsis thaliana* (Wagner et al., 2015), supporting the idea that the mitochondrial uniporter system, and MICU specifically, may be broadly conserved across evolutionary lineages (Bick et al., 2012). It has also been shown that there is an additional Ca^{2+} binding EF-hand motif in the MICU of *Arabidopsis thaliana* (Wagner et al., 2015) in addition to the two EF-hand motifs that are present in mammalian MICU1 (Perocchi et al., 2010). This suggests that a distinctive mechanism from that in the mammalian system is involved in moderating Ca^{2+} uptake and preserving Ca^{2+} homeostasis in the mitochondria of this plant, and perhaps other plants. It is therefore important to conduct a specific analysis of MICU in land plants with the available genome data for various plants.

In this study, we aimed to identify MICU homologs and EF-hand domains across available land plant genomes in order to determine whether MICU and its Ca^{2+} binding or sensing ability are broadly conserved. Additionally, we tried to test plant phenotype responses to calcium or other abiotic stresses using *MICU*-defective mutants of *Arabidopsis thaliana*. Furthermore, we attempted to investigate the expression levels of *MICU* in *Arabidopsis thaliana* seedlings grown under different calcium levels. Our results not only provide clues to the possible role of plant MICU in their mitochondrial calcium uptake, but also contribute to better understanding of MICU function in plants.

2. Materials and methods

2.1. Identification of MICU homologs in land plants

To identify MICU homologs in land plants, the MICU amino acid sequence from *Arabidopsis thaliana* (main transcript AT4G32060.1) was used for a TBLASTN search, with the default algorithm parameters. The TBLASTN search was performed for all land plant species in the Phytozome v12.0 database (Goodstein et al., 2012), a total of 56 species comprised of 3 bryophytes, 1 lycophyte, and 52 angiosperms (1 basal, 14 monocots, and 37 eudicots) (see Table 1). Thresholds was set to E-values $< 1E^{-5}$ and identity $> 50\%$ (Chambaud et al., 2001). Next, a self-BLAST of the obtained sequences was performed to remove redundant sequences. Finally, all of the retrieved sequences were submitted to the NCBI Batch CD search (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi; Marchler-Bauer et al., 2017) and the PAFM database (http://pfam.xfam.org; Finn et al., 2016) to confirm the presence of one or more EF-hand domains. A gene was labeled with the abbreviation of the species name plus MICU as a candidate if it was found among the complete ORFs.

2.2. Gene structure analysis

The exon/intron organizations were visualized by using the Gene Structure Display Server 2.0 (GSDS, http://gsds.cbi.pku.edu.cn) (Hu et al., 2015).

2.3. Multiple sequence alignments and phylogenetic analysis

The MICU protein sequences were aligned using the MUSCLE program implemented in MEGA7.0 software (Kumar et al., 2016). A phylogenetic tree was then constructed using the maximum likelihood (ML) method with one thousand bootstrapped replicates. The best fitting amino acid substitution model (JTT) plus Gap was selected.

2.4. Pairwise identity analysis

A pairwise identity matrix for the MICU full-length protein sequences and EF-Hand motifs was constructed using SDT (Muhire et al.,

Table 1
Copy number and intron number of *MICU* genes in 55 land plant genomes.

	Family	Species	Copy number	Intron number[b]
Bryophytes	Funariaceae	*Physcomitrella patens*	2[a]	7, 7
	Marchantiaceae	*Marchantia polymorpha*	0	
	Sphagnaceae	*Sphagnum fallax*	0	
Lycophyte	Selaginellaceae	*Selaginella moellendorffii*	1	7
Basal angiosperm	Amborellaceae	*Amborella trichopoda*	1	8
Monocots	Araceae	*Spirodela polyrhiza*	1	7
	Zosteraceae	*Zostera marina*	2	7, 7
	Bromeliaceae	*Ananas comosus*	1	7
	Poaceae	*Brachypodium distachyon*	2	7, 7
	Poaceae	*Brachypodium stacei*	2	7, 7
	Poaceae	*Oropetium thomaeum*	2	7, 8
	Poaceae	*Oryza sativa*	2[a]	7, 7
	Poaceae	*Panicum hallii*	2	7, 7
	Poaceae	*Panicum virgatum*	3	7, 7, 7
	Poaceae	*Setaria italica*	2	7, 5
	Poaceae	*Setaria viridis*	2	7, 7
	Poaceae	*Sorghum bicolor*	2[a]	7, 7
	Poaceae	*Zea mays*	3	7, 7, 7
	Musaceae	*Musa acuminata*	2	7, 7
Eudicots	Ranunculaceae	*Aquilegia coerulea*	1	6
	Crassulaceae	*Kalanchoe fedtschenkoi*	2	6, 6
	Crassulaceae	*Kalanchoe laxiflora*	4	6, 6, 6
	Vitaceae	*Vitis vinifera genoscope*	2[a]	6, 5
	Fabaceae	*Glycine max*	5	6, 6, 6, 7, 4
	Fabaceae	*Medicago truncatula*	3	6, 6, 6
	Fabaceae	*Phaseolus vulgaris*	3	6, 6, 6
	Fabaceae	*Trifolium pratense*	1	6
	Rosaceae	*Fragaria vesca*	1	8
	Rosaceae	*Malus domestica*	2	6, 6
	Rosaceae	*Prunus persica*	2	6, 6
	Cucurbitaceae	*Cucumis sativus*	2	6, 5
	Euphorbiaceae	*Manihot esculenta*	2	6, 6
	Euphorbiaceae	*Ricinus communis*	2	7, 7
	Linaceae	*Linum usitatissimum*	3	6, 5, 5
	Salicaceae	*Populus trichocarpa*	1	6
	Salicaceae	*Salix purpurea*	1	6
	Myrtaceae	*Eucalyptus grandis*	1	5
	Rutaceae	*Citrus clementina*	2	6, 6
	Rutaceae	*Citrus sinensis*	1	6
	Brassicaceae	*Arabidopsis halleri*	1	5
	Brassicaceae	*Arabidopsis lyrata*	1	5
	Brassicaceae	*Arabidopsis thaliana*	1[a]	5
	Brassicaceae	*Boechera stricta*	1	5
	Brassicaceae	*Brassica oleracea capitata*	1	6
	Brassicaceae	*Brassica rapa FPsc*	1	6
	Brassicaceae	*Capsella grandiflora*	1	5
	Brassicaceae	*Capsella rubella*	1[a]	5
	Brassicaceae	*Eutrema salsugineum*	1	6
	Caricaceae	*Carica papaya*	1	6
	Malvaceae	*Gossypium raimondii*	3	6, 6,6
	Malvaceae	*Theobroma cacao*	2	6, 6
	Amaranthaceae	*Amaranthus hypochondriacus*	1	6
	Apiaceae	*Daucus carota*	2	6, 7
	Phrymaceae	*Mimulus guttatus*	2[a]	6, 6
	Solanaceae	*Solanum lycopersicum*	2	6, 6
	Solanaceae	*Solanum tuberosum*	1	6

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