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Calmodulin-like gene *MtCML40* is involved in salt tolerance by regulating MtHKTs transporters in *Medicago truncatula*

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ABSTRACT

Calcium (Ca^{2+}) is a universal messenger mediating numerous physiological processes in responses to developmental and environmental cues in plant cells. Calmodulin (CaM) and calmodulin-like proteins (CMLs) are important plant Ca^{2+} sensors involved in decoding Ca^{2+} signatures to execute downstream physiological responses. Despite the involvements of CML proteins in the regulation of developmental processes, little is known about the function of CMLs in response to abiotic stresses in plants. To characterize CML proteins, we isolated and functionally characterized a gene encoding a CML protein from legume model plant *Medicago truncatula,* referred to as *MtCML40.* The MtCML40 belonged to subgroup VI of CML family. Expression of *MtCML40* was up-regulated by salt, cold and osmotic stress as well as ABA treatment, suggesting a role of *MtCML40* in abiotic stress. To test this hypothesis, we generated *MtCML40* overexpressing transgenic lines in *M. truncatula.* Overexpression of *MtCML40* rendered seed germination more sensitive to salt stress as evidenced by greater inhibition of seed germination of transgenic lines than wild-type seeds when exposed to NaCl, while seed germination of WT and transgenic lines was comparable under control conditions. In addition to seed germination, exposure to salt stress led to greater inhibition of shoot and root growth, reduction in chlorophyll and carotenoid concentrations and photosynthetic rates in the transgenic lines than WT plants, suggesting a negative regulation of salt tolerance by MtCML40. The greater accumulation of Na^+ in shoots of transgenic lines may account for the greater sensitivity to salt stress. We further found that overexpression of *MtCML40* resulted in down-regulation of *MtHKT1;1* and *MtHKT1;2* that encoded proteins associated with removal of Na^+ from shoots. Taken together, our results demonstrate that MtCML40 is involved in the regulation of salt tolerance by targeting MtHKT-dependent Na^+ accumulation in *M. truncatula.*

1. Introduction

Calcium (Ca) is one of the mineral nutrients essential for plant growth and development. In addition, Ca ion (Ca^{2+}) is also an ubiquitous signaling molecule involved in the moderation of numerous developmental and environmental cues by evoking a transient elevation of cytosolic Ca^{2+} activity ($[Ca^{2+}]_{cyt}$) in plant cells (Dodd et al., 2010; Kudla et al., 2010). Membrane transporters, including channels, carriers and pumps are invovled in the generation of specific Ca^{2+} signals in response to specific stimuli (Dodd et al., 2010; Kudla et al., 2010). Most Ca^{2+} sensors bind to Ca^{2+} via the EF-hand motif, a helix-loop-helix structure, leading to a conformational change (Gifford et al., 2007). EF-hand proteins act as transducers of Ca^{2+} signals in plants, and include calmodulin (CaM) and calmodulin-like proteins (CMLs), calcineurin B-like proteins (CBLs) and Ca^{2+} dependent protein kinases (CDPKs) (Hrabak et al., 2003; Ranty et al., 2006; Weini and Kudla, 2009). The involvements of SOS pathway in the salt stress has been clearly documented. Salt stress elicits an increase in $[Ca^{2+}]_{cyt}$ sensed by a calcineurin B-like protein CBL4 (SOS3), which subsequently interacts with a CBL-interacting protein kinase CIPK24 (SOS2). The SOS3/SOS2 complex then targets to the plasma membrane activating the membrane-bound Na^+/H^+ antiporter (SOS1) via phosphorylation (Shi et al., 2002; Qiu et al., 2003).

The calmodulin-like proteins (CMLs) do not contain known functional motifs, but they do possess EF-hand motif (s), in which more than 16% of the amino acid sequences can match CaM (McCormack and

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Braam, 2003). In Arabidopsis, several CMLs have been proposed to be related with different physiological processes. The first characterized CML, AtCML24, has been shown to be involved in ionic homeostasis, photoperiod response, abscisic acid-mediated inhibition of germination and seedling growth (Delk et al., 2005). In addition, CML23 and CML24 are involved in flowering (Tsai et al., 2007), while CML42 plays a role in trichome branching (Dobney et al., 2009). Wang et al. (2015) reported the involvement of CML25 in pollen germination and pollen tube elongation. CML7 is associated with elongation of root hairs (Won et al., 2009; Lin et al., 2011). CML20 and CML39 are involved in microtubule organization (Azimzadeh et al., 2008), and seedling establishment (Bender et al., 2013), respectively. But few studies have functionally characterized CMLs in the context of responses to abiotic stresses in plants (Bender and Snedden, 2013). Genes encoding *AtCML8* (Park et al., 2010) and *AtCML24* (Delk et al., 2005) were found to be induced by salt and cold treatment respectively. Further studies suggested that *atcml9* mutants exhibited enhanced tolerance to salt and drought stress through ABA-mediated pathway (Magnan et al., 2008). Moreover, the rice and tuber mustard CML genes, *OsMSR2* and *BjAAR1* were also suggested to be involved in ABA-mediated salt and drought tolerance (Xu et al., 2011; Xiang et al., 2013). Yamaguchi et al. (2005) found that AtCML18 binding to AtNHX1 significantly lowered its Na^+/H^+ exchange activity, leading to a decrease in the Na^+/K^+ ratio. In addition to the above mentioned CMLs, *AtCML37*, *AtCML38*, and *AtCML39* are also responsive to various stimuli, including salt, drought, and ABA (Vanderbeld and Snedden, 2007). More recently, Yin et al. (2017) suggested a novel rice CML gene, *OsDSR-1*, played important roles in conferring tolerance to drought stress by decreasing the occurrence of oxidative damage. However, the roles of CML proteins in plants are still largely unknown.

Salinity is one of the major abiotic factors limiting crop yield worldwide (Julkowska and Testerink, 2015). Plants growing in saline soils have to cope with osmotic stress, sodium toxicity and the associated oxidative stress (Zhu, 2002; Munns and Tester, 2008). Upon exposure to salt stress, plants firstly encounter osmotic stress due to the presence of NaCl in soil solution (Julkowska and Testerink, 2015). As salt stress persists, accumulation of Na^+ in the cytosol of plant cells disrupts cellular ion homeostasis, exerting a toxic effect on plants. Plants have evolved mechanisms to counteract Na^+ toxicity (Munns and tester, 2008). Tonoplast-localized Na^+/H^+ exchanger1 (NHX1) (Blumwald and Poole, 1985) and plasma membrane-localized SALT OVERLY SENSITIVE 1 (SOS1) (Qiu et al., 2002; Yamaguchi et al., 2013) are two major players at the cellular level responsible for low cytoplasmic Na^+ concentrations in plants. Among them, NHXs mange Na^+ detoxification via sequestration of Na^+ within the vacuole, while the SOS pathways are involved in exporting Na^+ out of the cells (Deinlein et al., 2014). Regardless of the mechanisms mentioned above, HKT1 is an another major membrane transporter responsible for the Na^+ transportation in Arabidopsis and wheat (Rus et al., 2001; Laurie et al., 2002; Mäser et al., 2002; Rus et al., 2004). The common roles that AtHKT1;1 and its rice ortholog OsHKT1;5 play are transferring Na^+ from the xylem into the surrounding xylem parenchyma cells, thereby protecting plants from Na^+ toxicity (Ren et al., 2005; Sunarpi et al., 2005; Horie et al., 2006). Further studies showed that AtHKT1 may directly retrieve Na^+ from the xylem and unload Na^+ into the root vacuoles (Davenport et al., 2007). However, Berthomieu et al. (2003) performed genetic and molecular analyses of the *sas2* mutants and concluded that AtHKT1 is involved in recirculation Na^+ from shoots to roots, probably by mediating Na^+ loading into the phloem in shoots and then unloading in roots.

Medicago truncatula has emerged as a model plant in legume genetics and genomics due to its small diploid genome, short life cycle, prolific seed production and easy transformation (Tang et al., 2014). Legumes are particularly important because of their symbiotic relationship with nitrogen-fixing bacteria. Like other crops, many leguminous crops are sensitive to salt stress (Kang et al., 2010). Efforts have been made to understand the molecular mechanisms by which *M. truncatula* responds and adapts to saline conditions. Merchan et al. (2007) identified several regulatory genes associated with root growth in response to salt stress using suppressive subtractive hybridizations (SSH) and the microarray technique. de Lorenzo et al. (2007) identified differentially expressed genes in salt-tolerant and salt-sensitive *M. truncatula* genotypes in response to salt stress. Li et al. (2011) conducted a detailed transcriptomic analysis of *M. truncatula* in response to salt stress. However, the mechanisms by which *M. truncatula* responds to salt are rarely explained. As for the SOS pathway, upregulation of SOS1 has been shown to play a role in salt tolerance in *M. truncatula* (Liu et al., 2015; Sandhu et al., 2017). On the other hand, Sandhu et al. (2018) identified six NHX genes in *M. truncatula* and confirmed their important roles in sequestering Na^+ into vacuoles.

Based on the results reported in the literature, few studies have linked the Ca^{2+} sensors to Na^+ transporters under salt stress in *M. truncatula*. Although the CML family has been extensively investigated in Arabidopsis, little is known about the CML members in *M. truncatula*. In the present study, we first isolated a CML gene, *MtCML40*, which encoded a calmodulin-like protein consisting of four predicted Ca^{2+} binding sites and overexpression of *MtCML40* leads to Na^+ accumulation, rendering the transgenic plants more sensitive to salt stress. We further dissected the signaling pathways associated with the function of *MtCML40* in response to salt stress.

2. Materials and methods

2.1. Plant materials, growth conditions, and stress treatments

M. truncatula ecotype R108-1 and three transgenic lines were used in this study. Plants were grown in petri dishes in the green house with 25 °C (day)/20 °C (night), 14 h – day/10 h – night periods with light intensity of 140 μmol m^{-2} s^{-1}. Sulfuric acid treated seeds were surface sterilized by incubation for 10 min in 10% (*v/v*) sodium hypochlorite, and then washed with sterile water. After stratification for 2 day at 4 °C in darkness, the seeds were placed on appropriate medium containing 0.8% (w/v) sugar, 0.8% (w/v) agar with or without NaCl. The composition of medium was as followed: 0.5 mM KH_2PO_4, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 0.1 mM Fe-Na_2-EDTA, 1 mM NH_4NO_3, 2.5 mM KNO_3, 30 μM H_3BO_3, 5 μM $MnSO_4$, 1 μM $ZnSO_4$, 1 μM $CuSO_4$ and 0.7 μM Na_2MoO_4. Seed germination is defined as the emergence of the radicles through the seed coat. Thirty-five seeds per sample in each treatment were tested for the determination of seed germination rate.

For the analysis of stress responses, roots growth was monitored in the same medium as mentioned before. Sulfuric acid treated seeds were surface-sterilized for 10 min in 10% (v/v) sodium hypochlorite, and then washed with sterile water. After washing with sterilized water, seeds were sown on 0.8% water-agar plates and stored for 2 days at 4 °C before incubating overnight at 25 °C in the dark to ensure uniform germination. Germinated seedlings were transferred to square plates containing appropriate medium and grown vertically in a growth chamber.

To analyze salt stress responses in mature plants, germinated seedlings with about 2-cm radicles were transferred to plastic buckets filled with tap water. Three days later, changed the water with fully aerated nutrient solution and then changed the nutrient solution every three days. Each bucket contained wild type (R108-1) and transgenic lines for 12 plants and repeated for three buckets. After grown in the culture solution for 3 weeks, half of the plants were transferred to culture solution with 100 mM NaCl. The pH of the hydroponic solution was adjusted to 6.0.

2.2. Constructs and transformation of M. truncatula

To obtain transgenic materials, the coding region of *MtCML40* was amplified using primers 5′-TGG CGC GCC ATG AAG AAT GCG GGA-3′