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A mongolian pine specific endoplasmic reticulum localized CALMODULIN-LIKE calcium binding protein enhances arabidopsis growth

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ABSTRACT

Stress-adapted wild plants are natural sources of novel genes for molecular breeding. Here, we conducted a transcriptional analysis of *Pinus sylvestris* var. *mongolica* Litv, an evergreen pine in northeastern China, to identify a novel CALMODULIN-LIKE protein-encoding gene, *PsCML1*, no significant homologs found in other plant species. *PsCML1* encodes a protein predicted to have a single *trans*-membrane domain at its N-terminal. Four EF-hand motifs (calcium [Ca]-binding structures) are located at its C-terminal and showed Ca^{2+}-specific affinity in isothermal titration calorimetric analysis. Transient expression of *PsCML1* in *Nicotiana benthamiana* showed that the PsCML1 localizes to the endoplasmic reticulum (ER). Heterologous expression of *PsCML1* in Arabidopsis significantly promoted seedling growth, and increased resistance to stress from NaCl and Ca^{2+} deficiency. The roots of the transgenic seedlings had higher contents of cellulose and pectin, but less hemicellulose than those of the wild type (WT). The biosynthesis of cell wall components is linked with protein glycosylation in the ER and reactive oxygen species (ROS) homeostasis. No significant difference was found in the extent of protein glycosylation between the transgenic and WT plants. However, the transgenic roots had higher steady-state levels of ROS, NADPH oxidase activity, and *endo*-membrane dynamics than those of the WT. A working model was proposed to delineate the interaction among Ca^{2+}, ROS homeostasis, and cell wall loosening-dependent cell division.

1. Introduction

Ca^{2+}-mediated signaling cascades play an essential role in plant cellular sensing and responses to a variety of environmental stresses. Ca^{2+} exists throughout plant cells, including in the cell wall, apoplast, plasma membrane, cytoplasm, and all organelles. Fluctuations in Ca^{2+} concentration or Ca^{2+} redistribution are linked with modulation of the activities of Ca^{2+} binding proteins, which in turn modify cellular activity at multiple levels. Ca^{2+} redistribution in plants depends on Ca^{2+} transporters and channels located in different membranes, primarily (Li et al., 2013; Wang et al., 2017a).

Locally changing Ca^{2+} concentrations are sensed by proteins with different affinities for Ca^{2+}, which in most cases depends on the presence of EF-hand motifs, highly conserved helix-loop-helix Ca^{2+} binding structures (Nakayama and Murayama, 2000; Day et al., 2002). Ca^{2+} binding proteins with different numbers of EF-hand motifs are largely categorized into two groups: (1) simple Ca-signaling transducers with no known functional domains that interact with downstream target proteins after binding to Ca^{2+}, which primarily includes Calmodulin (CaM), CaM-like proteins (CMLs), and Calcineurin B-like proteins (CBLs); (2) proteins having both Ca-binding domains, like EF-hand motifs, and another functional domain, typically calcium-dependent protein kinases (CDPK). CaM exists in all eukaryotes (Snedden and Fromm, 1998; Yang and Poovaiah, 2003; McCormack et al., 2005; Kim et al., 2009; Du et al., 2011). It is a small acidic protein composed of two pairs of EF-hands located at both the N- and C-terminus.

CMLs are plant-specific Ca^{2+} sensors, usually with four EF-hand motifs, that show an overall low similarity to CaMs (McCormack and Braam, 2003; Boonburapong and Buaboocha, 2007; Perochon et al., 2011). Arabidopsis has seven CaM isoforms and 50 CML-encoding genes (McCormack et al., 2005; Zhu et al., 2015). Arabidopsis CML39 mediates seed germination (Ubaid et al., 2018) and seedling

Abbreviations: APX, Ascorbate Peroxidase; CAT, Catalase; CaM, Calmodulin; CBL, Calcineurin B-Like Protein; CDPK, Calcium-Dependent Protein Kinases; CIPK, CBL-Interacting Protein Kinase; CML, Calmodulin-Like Protein; ConA, Concanavalin A; DHR, Dihydrorhodamine; ER, Endoplasmic Reticulum; FM4-64, N-(3-Triethylammoniumpropyl)-4-(6-(4-(Diethylamino) Phenyl) Hexatrienyl) Pyridinium Dibromide); GFP, Green Fluorescent Protein; NOX, NADPH Oxidase; PI, Propidium Iodide; POD, Peroxidase; ROS, Reactive Oxygen Species; SOD, Superoxide Dismutase; WT, Wild Type

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establishment (Bender et al., 2013); CML24 (Yang et al., 2014) mediates pollen tube growth; and CML25 (Wang et al., 2015) and CML42 (Dobney et al., 2009) mediate pollen germination and trichome branching, respectively. Arabidopsis CML8 (Zhu et al., 2017), CML9 (Leba et al., 2012), CML24 (Ma et al., 2008), and CML37 (Scholz et al., 2014) positively regulate plant immunity responses. Arabidopsis CML9 (Magnan et al., 2008), CML10 (Cho et al., 2016), CML20 (Wu et al., 2017), CML24 (Delk et al., 2005), CML37 (Scholz et al., 2015), and CML38 (Lokdarshi et al., 2016) play roles in the responses to abiotic stresses. The subcellular localization of CML30 and CML3 (Chigri et al., 2012), and the tissue-specific expression patterns of *CML37*, *CML38*, and *CML39* (Vanderbeld and Snedden, 2007) have been investigated. CML families are also found in Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) (Nie et al., 2017), soybean (*Glycine max*) (Zeng et al., 2017), *Lotus japonicus* (Liao et al., 2017), and many other plant species (Zhu et al., 2015; Mohanta et al., 2017).

Environmental stress-adapted wild plants are a natural library for resistance genes, and the identification of these genes has been greatly facilitated by high-throughput and low-cost DNA (genome) and RNA (transcriptome) sequencing technologies. *Pinus sylvestris* var. *mongolica* Litv, commonly known as Mongolian pine, is an evergreen coniferous tree belonging to the *Pinus* genera of the Pinaceae, a geographical variety of Scotch pine (*Pinus sylvestris* Linnaeus). Due to its adaptation to cold, drought, and nutrient deficient conditions, it has been widely used as a tree specie for forestation projects in desert and semi-desert regions in northern China (Zhao et al., 2007). Most studies on Mongolian pine have focused on characterizing environmental influences on population structure (Li et al., 2015; Wang et al., 2017b) and growth (Zhu et al., 2006; Tang et al., 2016; Zhang et al., 2017; Zhang et al., 2018). However, there is limited genetic information about Mongolian pine. Twenty-five expressed sequence tag-simple sequence repeat (EST-SSR) molecular markers have been developed for the *Pinus* genera (Fang et al., 2014). Proteomic analysis has been conducted with its bud tissues (Bi et al., 2011) and a natural mutant defective in apical dominance has been characterized (Ning et al., 2013). In this study, we used transcriptome analysis of the Mongolian pine to identify an endoplasmic reticulum (ER)-localized CML with a single transmembrane domain and four EF-hand Ca^{2+}-binding motifs. Ectopic expression of this *Pinus sylvestris* CML (*PsCML*) in Arabidopsis resulted in enhanced seedling growth and resistance to abiotic stresses through modulating reactive oxygen species (ROS) homeostasis and cell wall composition.

2. Results

2.1. Identification of a Pinus sylvestris var. mongolica litv.-specific CML-encoding transcript

To identify transcripts encoding Ca^{2+} binding proteins in *Pinus sylvestris* var. *mongolica* Litv, we conducted root RNA transcriptome analysis and identified 99 unique start codon-containing transcripts (Table S1) putatively encoding Ca^{2+} binding proteins. The putative proteins were categorized into 6 major families including: Ca^{2+}/cation exchangers, Ca^{2+} transporters/channels, Ca^{2+} transporting ATPases, Ca^{2+} binding proteins, Calmodulin-Like proteins (CMLs), mitochondrial Ca^{2+} uniporters, and Ca^{2+} dependent protein kinases, one Ca^{2+} sensing receptor, and one protein involved in endoplasmic reticulum (ER) Ca^{2+} homeostasis. PsCMLs were the largest family with 22 members (Table S1). Previous studies with Arabidopsis demonstrated that CMLs play various roles in plant adaption to environmental stresses, as detailed in the Introduction. Therefore, we focused on this group of genes.

By examining those *PsCMLs* transcript sequence integrity and conducting encoding protein structure predication, we selected a full-length transcript Locus23327 for further experiment and named it *PsCML1* (Fig. 1A). Proteins from different species with 80% residues identity and sequence coverage are considered as homolog (Yu et al., 2004). Under this standard, we did not identify *PsCML1* homolog in the NCBI protein database, instead five proteins from five different plant species were identified with approximate 75% query coverage and 45% identity as compared to that of PsCML1 (Fig. 1B). *PsCML1* encodes a protein predicated to have four EF-Hand Ca^{2+} binding motifs at its C-terminus, an trasmembrane domain and an non-cytoplasmic region at its N-terminus (Fig. 1B). Four of the five proteins are predicated to have similar structures of the PsCML1 (Fig. 1B).

2.2. PsCML1 localizes in the ER and has specific affinity for Ca^{2+}

To determine its subcellular localization, we transiently expressed *PsCML1* fused with *GFP* (green fluorescent protein) driven by the constitutive 35S promoter in epidermal leaf cells of tobacco. The PsCML1-GFP fusion protein showed strong co-localization with the ER marker ER-mCherry under confocal laser scanning microscopy (Fig. 2), indicating that PsCML1 is localized at the ER.

To determine the Ca^{2+} binding activity of the putative EF-Hand domain, we synthesized the peptide corresponding to its first EF-Hand motif at the N-terminus and quantitatively analyzed its Ca^{2+} affinity with isothermal titration calorimeter, which directly measures changes in heat or thermodynamics during the biochemical binding event between Ca^{2+} and the peptide. Titrating the peptide with Ca^{2+} caused large heating release (exothermic), which gradually saturated with continuous titration (Fig. 3A). However, no detectable thermodynamics changes were observed with titration of the peptide with Mg^{2+}, Mn^{2+} and Zn^{2+} (Fig. 3B). It demonstrated that PsCML1 is an ER-localized protein specifically binding to Ca^{2+}.

2.3. Heterologous expression of PsCML1 in Arabidopsis enhanced growth and resistance to abiotic stresses

To evaluate the physiological function of *PsCML1*, we heterologously expressed it in Arabidopsis. Homozygous transgenic lines with a single T-DNA insertion (*35S::PsCML1*) were grown along with the wild-type Col-0 on either control medium or medium supplied with 100 mM NaCl, or 10 μM Ca^{2+}. Three independent transgenic lines showed significantly enhanced growth on the control and the tested conditions compared to the wild type in terms of leaf fresh weight and root elongation (Fig. 4).

To investigate cellular mechanism underlying the enhancing growth phenotype of the transgenic lines, the apical root cellular patterns of the wild type and the *35S::PsCML1* transgenic lines on the control medium were examined (Fig. 5A). It was found that the root apical meristem (RAM) region of the transgenic is significantly longer and has more number of cells than those of wild type (Fig. 5B), but their individual cell length in the RAM were comparable (Fig. 5B). It implies that the *35S::PsCML1* transgenic has enhanced root cell division capacity.

2.4. Heterologous expression of PsCML1 altered the contents of cell wall components in Arabidopsis

Cell wall biosynthesis is often linked with cell division (Chebli and Geitmann, 2017; Barnes and Anderson, 2018). The *PsCML1* transgenic and wild type Arabidopsis roots were sequentially extracted for different cell wall components measurement. It was found that the transgenic roots had higher amounts of cellulose and pectin, but less hemicellulose than those of wild type (Fig. 6A). Xylan, arabinoxylan, and xyloglucan are the three main polysaccharides of hemicellulose (Pauly et al., 2013), which can be recognized by commercially available primary antibodies LM10, LM11, and LM15, respectively, which were further visualized with secondary antibody labeled with a green fluorescent reagent FITC (Fluorescein IsoThioCyanate). It was found that the transgenic roots had a lower fluorescent signal with the three probes than those of the wild type (Fig. 6B), indicating that the transgenic plants contained lower amounts of hemicellulose, which was consistent