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Research article

Expression of a *Medicago falcata* small GTPase gene, *MfARL1* enhanced tolerance to salt stress in *Arabidopsis thaliana*

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ABSTRACT

To understand the role of small GTPases in response to abiotic stress, we isolated a gene encoding a small GTPase, designated *MfARL1*, from a subtracted cDNA library in *Medicago falcata*, a native legume species in semi-arid grassland in northern China. The function of *MfARL1* in response to salt stress was studied by expressing *MfARL1* in *Arabidopsis*. Wild-type (WT) and transgenic plants constitutively expressing *MfARL1* showed comparable phenotype when grown under control conditions. Germination of seeds expressing *MfARL1* was less suppressed by salt stress than that of WT seeds. Transgenic seedlings had higher survival rate than WT seedlings under salt stress, suggesting that expression of *MfARL1* confers tolerance to salt stress. The physiological and molecular mechanisms underlying these phenomena were elucidated. Salt stress led to a significant decrease in chlorophyll contents in WT plants, but not in transgenic plants. Transgenic plants accumulated less amounts of H_2O_2 and malondialdehyde than their WT counterparts under salt stress, which can be accounted for by the higher catalase activities, lower activities of superoxide dismutase, and peroxidase in transgenic plants than in WT plants. Transgenic plants displayed lower Na^+/K^+ ratio due to less accumulation of Na^+ than wild-type under salt stress conditions. The lower Na^+/K^+ ratio may result from less accumulation of Na^+ due to reduced expression of *AtHKT1* that encodes Na^+ transporter in transgenic plants under salt stress. These findings demonstrate that *MfARL1* encodes a novel stress-responsive small GTPase that is involved in tolerance to salt stress.

1. Introduction

Being sessile organism, plants are frequently exposed to changing environment that results in holdback of growth and development. Plants have evolved various mechanisms to avoid and/or alleviate the adverse effects of abiotic stress. Upon exposure of plants to a stressed environment, numerous molecular and physiological processes are altered [1]. Environmental signals are often sensed by plants and transduced to activate downstream targets through intricate signaling network, in which transcription factors and signal-transducing GTPases play key roles. Small GTPase proteins are monomeric G proteins that are related to the subunit of heterotrimeric G proteins with molecular masses of 20–40 kDa. Similar to heterotrimeric G proteins, small GTP-binding proteins can cycle between GTP and GDP-bound states [2]. Once stimulated by upstream signals, the GDP-bound inactive form can be converted into the GTP-bound active form by guanine nucleotide exchange factors (GEF), thus activating downstream targets. Conversely, GTPase-activating proteins (GAP) can catalyze the GTP form to the GDP form [3].

Members of small GTPase share several common structural features, including four guanine nucleotide-binding domains and an effector-binding domain [2]. However, small GTPases also exhibit a remarkable diversity in both structure and function. They are further divided into four main subfamilies in plants: Rab, Rop, Ran and Arf [4]. A diverse function of plant small GTPases has been reported in the literature. For instance, the Rab and Arf GTPase families have been suggested to regulate distinct steps of membrane trafficking [5,6]. Ran GTPases are involved in mediation of transport of proteins and RNA across the nuclear envelope [7].

Abbreviations: ABA, abscisic acid; Arf, ADP-ribosylation factor; Arl, Arf-like; CaMV, cauliflower mosaic virus; CAT, catalase; GAP, GTPase-activating proteins; GEF, guanine nucleotide exchange factors; GFP, green fluorescent protein; GUS, β-glucuronidase; MDA, malondialdehyde; MS, Murashige and Skoog; ORF, open reading frame; POD, peroxidase; RACE, rapid-amplification of cDNA end; ROS, reactive oxygen species; RT-qRCR, real-time quantitative PCR; SOD, superoxide dismutase; SSH, suppression subtractive hybridization; WT, wild-type.

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Rop GTPases regulate several physiological processes, ranging from pollen growth and root hair development to abscisic acid (ABA) response [8]. These findings highlight that small GTPases may act as important molecular switches associated with plant signaling.

In recent years, the involvements of small GTPases in response of plants to abiotic stresses have been investigated. Several genes in the Rab GTPase family have been shown to be responsive to abiotic stress, including response of *SsRab2* to water stress [9], *OsRab7* to cold stress [10] and *AtRabG3e* to salt/osmotic stress [11]. Among them, *OsRab7* is differentially regulated by several environmental stimuli including cold, salt, dehydration and phytohormone ABA [10]. In addition, *OsRab7* is localized to the vacuolar membrane, suggesting that *OsRab7* may be implicated in a vesicular transport to the vacuole in plant cells [10]. In *Arabidopsis thaliana*, transgenic plants of overexpressing *AtRabG3e* exhibited accelerated endocytosis in roots, leaves and protoplasts. In addition, the transgenic plants also showed increased tolerance to salt stress due to reduced accumulation of reactive oxygen species [11]. These results imply that vesicle trafficking may play an important role in plant adaptation to stress. Furthermore, expression of *AtRop2* renders seed germination less sensitive to ABA [8], suggesting that Rop GTPases negatively regulate ABA signaling. A recent study demonstrated that overexpression of *OsRAN2* in rice confers tolerance to cold stress by regulating cell cycle [12]. In contrast to cold stress, overexpression *OsRAN2* in rice renders transgenic plants hypersensitive to salinity and osmotic stress [13].

ADP-ribosylation factor (Arf) is an important regulator of membrane-trafficking pathways, and was initially identified due to their ability to stimulate the ADP-ribosyltransferase activity of cholera toxin A [14]. The production of three types of vesicle coat proteins (COPI, COPII and clathrin) is related to Arf GTPases [15]. The protein sequences of Arf-like (Arl) GTPases are highly similar to that of Arf GTPases. Knockout of an Arl GTPase, *TITAN*, leads to dramatic alterations in mitosis and cell cycle control during seed development in Arabidopsis [16]. However, there has been no report to evaluate the role of Arf GTPases in response and adaptation of plants to abiotic stresses.

Medicago falcata is a native legume species that widely occurs in the areas of Russia, Mongolia, and northern China. *M. falcata* is distinguished by its outstanding capacity to tolerate abiotic stress, and has been widely used as a general source of germplasm for breeding forage of alfalfa. Several recent studies have investigated the physiological and molecular mechanisms by which *M. falcata* tolerates to cold [17,18], drought stress [19] and phosphorus deficiency [20,21].

In this study, we isolated an Arl GTPase gene, designated *MfARL1* from *M. falcata*, a native legume species exhibiting great tolerance to abiotic stress in Inner Mongolia in northern China using the method of suppression subtractive hybridization. We further functionally characterized *MfARL1* by expressing this gene in Arabidopsis. Our results demonstrated that expression of *MfARL1* in Arabidopsis led to an enhanced tolerance to salt stress due to less accumulation of Na^+ and H_2O_2 and MDA.

2. Results

2.1. Isolation and sequence analysis of MfARL1 cDNA

On the basis of the segment sequence of *MfARL1*, RACE was performed to obtain the full-length cDNA. The assembled results showed that *MfARL1* had a 618 bp open reading frame encoding a protein of 205 amino acid residues, with a calculated molecular mass of about 23 kDa. Sequence comparison revealed that the putative protein was highly homologous to the small GTP-binding proteins (Fig. 1, Supplemental Fig. S1), and accordingly was designated *MfARL1*. The sequence data have been deposited in GenBank (accession No. JX292786).

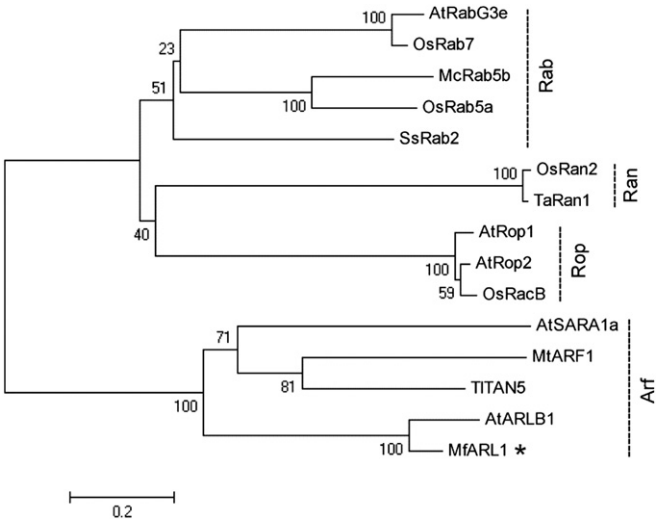

Fig. 1. Phylogenetic tree analysis of small G proteins. Phylogenetic analysis was performed using MEGA 5 software. Accession numbers are as follows: AtRabG3e (Q9XI98.1), OsRab5a (CAC19792.1), OsRab7 (AAO67728.1), McRab5b (CAA06922.1), SsRab2 (AAD30658.1), AtRop1 (AEE78776.1), AtRop2 (Q38919.1), AtRop5 (AEE86595.1), OsRacB (AAT84075.1), TaRan1 (AAM08320.1), OsRan2 (BAB82438.1), AtARLB1 (AAA87882.1), AtSARA1a (AEE28409.1), TITAN5 (AAM22961.1), MtARF1 (CAI29265.1), MfARL1 (JX292786).

MfARL1 protein contains 6 conserved functional domains. Similar domains have been found in many reported small GTP-binding proteins (Supplemental Fig. S1). Domains I–IV are involved in GTP-binding, and domain E is an effector region which can be recognized by GTPase-activation proteins (GAPs), and is essential for regulation of GTPases. Domain P, the C-terminal motif, is a prenylation site and important for attachment of small GTP-binding proteins to membrane [2,3].

Phylogenetic trees based on the full-length amino acid sequences of MfARL1 proteins were constructed using the MEGA 5 software (Fig. 1). The resulting trees contained four families, Rab, Ran, Rop and Arf. According to the phylogenetic trees, MfARL1 protein had the highest similarity with AtARLB1, a small GTP-binding protein of Arf family in *A. thaliana* with unknown function.

2.2. Expression pattern and subcellular localization of MfARL1

The *MfARL1* was isolated from the salt stress suppression subtractive hybridization library. To validate the response of *MfARL1* to various abiotic stresses, RT-qRCR was performed. As shown in Fig. 2A, transcripts of *MfARL1* were found to be accumulated after 2 h of exposure to salt stress, and peaked after 5 h of salt stress. Expression of *MfARL1* was also up-regulated by treatments with low temperature (4 °C) and osmotic stress (20% PEG6000) (Fig. 2B). Transcripts of *MfARL1* were detected in roots, stems, leaves, flowers and pods under non-stressed conditions, with the expression being greatest in leaves, followed by roots and stems, lowest in flowers (Fig. 2C).

To examine the subcellular localization of MfARL1, an open reading frame of MfARL1 was fused to the C-terminus of the GFP reporter gene of pEGAD [22]. The recombinant constructs of the MfARL1-GFP fusion gene and GFP alone were introduced into tobacco leaf epidermal cells by Agrobacterium injection. The results showed that the MfARL1–GFP fusion protein was specifically localized in the cell membrane, whereas GFP alone showed ubiquitous distribution in the whole cell (Fig. 3).