Environmental and Experimental Botany 155 (2018) 21–30

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Role of salt-induced RING finger protein 3 (OsSIRP3), a negative regulator of salinity stress response by modulating the level of its target proteins

Yong Chan Park, Jun-Cheol Moon, Sandeep Chapagain, Dae Gyeom Oh, Jung Ju Kim, Cheol Seong Jang*

Plant Genomics Lab, Department of Applied Plant Sciences, Kangwon National University, Chuncheon 24341, Republic of Korea

ARTICLE INFO

Keywords:
RING
Rice
Salinity stress
Negative regulator
Ubiquitination

ABSTRACT

As sessile organisms, plants are always exposed to various abiotic stresses, and therefore, they have developed defense mechanisms against abiotic stresses. Ubiquitin-mediated proteasomal degradation is an important mechanism that regulates the level of proteins in plants. The present study describes *Oryza sativa* salt-induced RING finger protein 3 (OsSIRP3), a functional RING E3 ligase, that is possibly involved in salt-stress response. The transcript of *OsSIRP3* gene was highly expressed in whole rice samples, such as root and shoot, after exposure to high salinity stress. Furthermore, *in vitro* ubiquitination assay demonstrated that OsSIRP3 has E3 ligase activity due to RING H2 domain. The results revealed the interaction of OsSIRP3 with both salt-induced and non-induced proteins, leading to their degradation *via* ubiquitin (Ub)/26S proteasome-mediated system. Overexpression of *OsSIRP3* in *Arabidopsis* resulted in hypersensitivity phenotypes under salinity stress during seed germination and root growth. These findings suggest that OsSIRP3 negatively regulates salinity stress response by modulating the level of its target proteins.

1. Introduction

Abiotic stresses, such as high salinity, extreme temperature, drought, and heavy metals, are recognized as limiting factors of agricultural productivity (Cavanagh et al., 2008; Grayson, 2013). In particular, salinity is the most devastating environmental stress that affects plants in several ways, including nutritional disorder, ion toxicity, oxidative stress, reduction in cell division and expansion, and alternation of metabolic processes (Munns, 2002; Zhu, 2001). As sessile organism, plants cannot avoid severe stresses; therefore, they have evolved diverse biological defense mechanisms against the damage or injury caused by adverse environmental conditions. Plants adopt various metabolisms to tolerate salinity, such as tolerance to osmotic stress, sodium (Na^+) exclusion from shoot, and accumulation of ions (James et al., 2008; Munns and Tester, 2008).Globally, more than 800 million hectares (6%) of total agricultural area is affected by high salinity. All soil types contain water soluble salts; therefore, Plants absorb their essential nutrients in the form of soluble salts. However, excessive absorption of salts significantly retards the growth of plants. Furthermore, when plants are exposed to salinity for a long time they face ionic stress, which leads to premature senescence, chlorosis, and necrosis in leaves, ultimately affecting plants by disrupting protein synthesis (Munns, 2005).

Rice is one of the important cereal crops in the world, and it is extremely sensitive to salinity. Its sensitivity varies with the growth stage, such as emergence, early seedling growth, and reproductive growth stages (Munns and Tester, 2008). Currently, around 6.5% of total area of the world is affected by soil salinity, which covers 20% of the cultivated area (Das et al., 2015). Furthermore, rice yield has reduced by 12% for every unit by salinity stress, leading to losses in production by over 50% (Zeng and Shannon, 2000). Therefore, numerous salt-induced genes have been identified and studied. However, the data on the biological functions of such genes are limited. Therefore, it is essential to study the role of genes that are regulated by salinity in order to boost the adaptation of plants to salt stress.

The regulation of protein degradation plays crucial roles in various aspects of development and defense mechanisms against abiotic and biotic stresses in plants (Vierstra, 2003). The covalent attachment of a small protein (76 amino acids) ubiquitin (Ub), which is highly conserved and ubiquitously expressed, is known to be involved in the degradation pathway of target proteins *via* ubiquitination proteasome system (UPS) in eukaryotic organisms, including plants (Smalle and Viestra, 2004). In *Arabidopsis thaliana*, a model research plant, almost 6% of the genome is associated with the UPS, and more than 1300 genes have been reported to encode ubiquitin ligases (Hua and Vierstra, 2011; Stone et al., 2005). A conjugation cascade of 3 classes of enzymes

* Corresponding author.
E-mail address: csjang@kangwon.ac.kr (C.S. Jang).

https://doi.org/10.1016/j.envexpbot.2018.06.017
Received 9 March 2018; Received in revised form 15 June 2018; Accepted 15 June 2018
Available online 22 June 2018
0098-8472/

is required, *viz.* ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3) are required in UPS pathway (Vierstra, 2009).

Really interesting new gene (RING) finger protein is one of the E3 ligases and is composed of consensus sequence motifs having cysteine and histidine residues that coordinate two zinc atoms (Freemont et al., 1991). The genome of *Arabidopsis* and rice is believed to possess ~470 and ~425 RING-type E3 ligases, respectively (Lim et al., 2010; Stone et al., 2005). Recently, E3 ligases have been reported to function as modulators (positive and negative) of responses of plants to various abiotic stresses, including salinity, cold, heat, drought, radiation, and toxic metalloid stress, through degradation of target proteins (Chapagain et al., 2017; Yee and Goring, 2009). Molecular regulation of responses of plants to abiotic stresses, including salinity, by RING-type E3 ligases have been reported. For instance, the overexpression of *Arabidopsis* RING E3 ligase SALT- AND DROUGHT-INDUCED RING FINGER 1 (SDIR1) resulted in salt hypersensitivity during germination and increased drought tolerance with enhanced abscisic acid (ABA)-induced stomatal enclosure (Zhang et al., 2007). Another RING E3ligase *Arabidopsis thaliana* ABA-insensitive RING protein 4 (AtAIRP4) induced by different abiotic stresses, including salt, mannitol, cold, hydrogen peroxide (H_2O_2), and ABA. Furthermore, the result of phenotypic evaluation of AtAIRP4-overexpressed plants indicated that AtAIRP4 might act as a positive regulator of drought tolerance and a negative regulator of salt tolerance. Similar findings have been reported in rice under different abiotic stress conditions. For example, rice RING E3 ligase *Oryza sativa* salt-induced RING E3 ligase 1 (OsSRFP1) is induced by salt, dehydration, cold, H_2O_2, and ABA, and overexpressing plants showed less tolerant to salt, cold and oxidative stresses then wild type plants (Fang et al., 2015). Furthermore, the overexpression of microtubule-associated rice RING finger E3 ligase (*OsRMT1*) has been reported to be involved in salt tolerance by modulating its substrate proteins (Lim et al., 2015a). The overexpression of salt-induced RING E3 ligase1 (*OsSIRP1*) resulted in hypersensitivity to salt stress (Hwang et al., 2016), whereas the overexpression of salt-induced RING E3 ligase 2 (*OsSIRP2*) resulted in tolerant phenotypes when compared with control plants (Chapagain et al., 2018).

Our previous study reported different expression patterns of 44 *OsRFP* genes, which were randomly selected based on Rice Micro Array Dataset in the root of salt-treated rice plants (Hwang et al., 2016; Lim et al., 2010). The present study reports the molecular characterization of another salt-induced RING E3 ligase (*Oryza sativa* salt-induced RING finger protein 3, OsSIRP3), suggesting that the RING E3 ligase acts as a negative regulator in response to salinity.

2. Material and methods

2.1. Plant material and growth conditions

The rice seeds (*O. sativa* L. 'Donganbyeo') were grown in mesh-supported plastic containers (Incu Tissue 72 mm × 72 mm × 100 mm (SPL Life Sciences, Gyeonggi-do, Korea) in a growth chamber (16:8 h light/dark photoperiod; 25 °C/23 °C; 70% relative humidity) for 2 weeks. To determine the expression pattern of genes under abiotic stress conditions, individual 2-wk-old rice seedlings were subjected to different stress treatments, including salinity, drought, and ABA. For salinity treatment, the rice seedlings were treated with 200 mM sodium chloride (NaCl)-containing nutrient solution. For drought treatment, the rice seedlings were grown under water deficit condition. For ABA treatment, the seedlings were treated with 0.1 mM ABA. The seedlings were sampled at different periods, 0, 1, 6, 12, and 24 h, post treatments. Similarly, heat and cold stress treatments were conducted by incubating 2-week-old seedlings at 45 °C and 4 °C, respectively, and then sampled at 0, 6, 12, and 24 h post treatments. The treated samples were ground in liquid nitrogen and immediately stored at −80 °C until further use.

2.2. Analysis of gene expression

To determine the expression pattern of genes, total RNA was extracted using the TRIzol® reagent according to the protocol of the manufacturer (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from total RNA (3 μg) using a cDNA synthesis Kit (Takara-Bio, Ohtsu, Japan). The cDNA synthesized was mixed with the TOPreal™ qPCR 2X Premix with SYBR green (Enzynomics, Daejeon, Korea), and then subjected to real-time quantitative polymerase chain reaction (PCR) using the CFX96 real-time PCR Detection System (BioRad, Laboratories, Hercules, CA, USA). *OsActinII* (Park et al., 2017) was used as an internal control.

2.3. Subcellular localization

To identify the subcellular localization of Oryza sativa genes, OsSIRP3, OsMADS70, OsABC1P11, OsCAF1 AL1 and OsRR4, isolation and transfection of rice protoplasts were performed as described by Park et al., 2018. The full-length each of genes were amplified using *pfu* Turbo DNA polymerase (Stratagene, La Jolla, C, USA). The PCR Products were digested and cloned into the enhanced yellow fluorescent protein (EYFP) vector. In addition, the full-length clone of *OsSIRP3* and each of the interacting genes were inserted into 35S:HA-SPYCE(M) and 35S:c-Myc-SPYNE(R) vectors for BiFC. Each plasmid DNA was transfected in to the protoplast using 40% polyethylene glycol (PEG) solution (40% PEG, 400 mM mannitol, 100 mM calcium nitrate) for 30 min at room temperature. Subsequently, W5 solution was added to dilute the PEG solution, and then decanted. The transfected protoplasts were then resuspended in W5 solution and incubated overnight at room temperature. The transfected protoplasts were observed using a confocal microscope16 h after incubation. Fluorescent images were obtained using a multiphoton confocal laser scanning microscope (model LSM 510 META; Carl Zeiss, Jena, Germany) at the Korea Basic Science Institute Chuncheon Center. Excitation/emission wavelengths were 514/534–590 nm for imaging the expression of EYFP constructs.

2.4. In vitro ubiquitination assay

To construct recombinant proteins, the full-length *OsSIRP3* gene was amplified and cloned into a pMAL–c5x vector (New England BioLabs, MA, USA). A single amino acid substitution ($OsSIRP3^{C245A}$) in the RING finger domain of OsSIRP3 was generated using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene). Each recombinant maltose binding protein (MBP)-fusion protein was expressed by *Escherichia coli* strain BL21 (DE3) pLysS (Promega, Madison, WI, USA), and then purified by affinity chromatography using amylose resin (New England BioLabs).

To identify the E3 ligase activity of OsSIRP3, an *in vitro* self-ubiquitination assay was performed as described by Park et al. (2017). The purified MBP-OsSIRP3 was mixed with human E1 (Sigma-Aldrich, St. Louis, MO, USA), 6X His-tagged AtUBC10, and bovine ubiquitin (Sigma-Aldrich), and then incubated at 30 °C for 3 h in ubiquitination reaction buffer (1 M Tris-hydrochloric acid, pH 7.5; 40 mM adenosine triphosphate; 100 mM magnesium chloride; 40 mM dithiothreitol). Immunoblot analysis was conducted using an anti-Ub antibody (Sigma-Aldrich) with a secondary goat anti-rabbit IgG peroxidase antibody (Sigma-Aldrich). The antibodies were detected using the chemiluminescent substrate SuperSignal® West Pico (ThermoScientific, Waltham, MA, USA) for horse-radish peroxidase (HRP). Photographs were obtained using the ChemiDoc™ XRS + (Bio-Rad).

2.5. Yeast two hybrid assay

To identify the interacting proteins of OsSIRP3, yeast two hybrid (Y2H) assay was carried out (Matchmaker Gold Yeast Two-Hybrid system; Clontech, Palo Alto, CA, USA), and then yeast library screening