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Full Length Research Paper

Expression and subcellular localization of antiporter regulating protein OsARP in rice induced by submergence, salt and drought stresses

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We examined the expression and subcellular localization of antiporter regulating protein OsARP in a submergence tolerant rice (*Oryza sativa* L.) cultivar FR13A. In the public databases, this protein was designated as putative Os02g0465900 protein. The cDNA containing the full-length sequence of *OsARP* gene was present in Gene Bank Accession no. AK071205 and this encoded 216 amino acids which had molecular mass of 25 kD. The *OsARP* gene was first expressed into *E. coli* and antibody was produced by using purified recombinant protein. The expression of OsARP protein was detected under submergence, salt and drought stresses. This protein was widely expressed in roots, shoots and leaves of rice under salt stress. To get an insight into the functional role of OsARP protein, subcellular localization was done using cell fractionation of rice leaves. Immuno-blotting of 3-day submergence rice leaves cell fractions detected the presence of OsARP protein in plasma-membrane fraction only. This indicates that OsARP is a membrane bound protein of rice which is expressed under submergence, salt and drought stresses.

Key words: Drought, antiporter regulating protein, immuno-localization, rice, salinity, submergence.

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food of about 50% of the world population. The production of rice is greatly hampered due to some abiotic stresses like salinity, drought, and submergence. In contrast to other crop species, rice is well known for its ability to grow in flooded soil. But most rice cultivars cannot survive if the plants are completely submerged for more than 7 days. Two important factors, the limitation of gas diffusion under water and reduced irradiance, which impair photo-synthesis; and efficient utilization of carbohydrates and metabolites, influence rice plant survival during submer-gence (Ram et al., 2002). Accordingly, submergence tolerance of rice is an important trait for agricultural productivity. Many genes regulated during submergence are suspected to be involved in the physiological mechanisms of submergence tolerance of rice. Among these, *OsEXP* (Huang et al., 2000; Cho and Kende, 1997), *OsUSP* (Sauter et al., 2002), and *OsDD3*,(Van Der Knaap et al., 1998) were reported to have specific functions as cell expansion, universal stress protein, and putative type1a plasma membrane receptor, respectively. In addition to *OsARP* (*O. sativa* antiporter regulating protein), two other submergence related genes *OsMGD* (*O. sativa* monogalactosyldiacylglycerol synthase) and *OsGGT* (*O. sativa* glycogenin glucosyltransferase) were

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Abbreviations: IPTG, Isopropyl-β-D-thiogalactopyranoside; ***OsARP*,** *Oryza sativa* antiporter regulating protein; ***OsMGD*,** *Oryza sativa* monogalactosyldiacylglycerol synthase; ***OsGGT*,** *Oryza sativa* glycogenin glucosyltransferase; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis.

isolated from the submergence tolerant cultivar FR13A (Qi et al., 2004, 2005a, b). Salinity is generally defined as the presence of excessive amount of soluble salt that affects the normal functions needed for plant growth (Abrol, 1986; Szabolcs, 1994). Plant adaptation to salt stress requires alterations of various cellular, physiological and metabolic mechanisms that are controlled by specific gene expression. These specific genes could encode for proteins implicated in Na^+ sequestration (H^+-ATPase, NHX-type transporters) (Zhang and Blumwald, 2001) synthesis of specific osmolytes (proline, glycine-betaine, polyols), detoxification of toxic compounds (ROS scavenging enzymes (Yancy et al., 1982). The regulation of cellular ion concentrations is an essential process in all organisms necessary to sustain a multitude of physiological process which is performed mainly through ion transporters. Ion transporters and their regulatory systems accomplish several crucial physiological roles. They adjust the intracellular ion concentrations within the optimal range of cellular systems (Serrano et al., 1999). Drought, on the other hand, defined as a period of no rainfall or no irrigation that affects crop growth, has long been recognized as the primary constraint to rainfed rice production (Fukai et al., 1995). Tolerance to drought stress usually involves the development of low osmotic potentials, which characterize many plant species found in arid environments (Morgan, 1984). Osmotic adjustment, or accumulation of solutes by cells, is a process by which water potential can be decreased without an accompanying decrease in turgor (Taiz and Zeiger, 1991). According to them, most of the osmotic adjustment can usually be accounted for increases in concentration of a variety of common solutes, including sugars, organic acids and ions. However, a high concentration of ions can be severely inhibitory to enzymes so that this occurs mainly with the vacuoles where the ions are kept out of contact with enzymes in the cytosol or subcellular organelles.

The gene expression of *OsARP* (*OsCTP*) was reported earlier (Qi et al., 2005b) in FR13A under submergence stress and large number of *OsARP* mRNA accumulated after submergence for 3 to 7 days. In addition, *OsARP* expression was also enhanced by salt, drought and abscisic acid stresses. In public databases, this protein was annotated as putative Os02g0465900 protein from *O. sativa* subsp. *japonica*. It has been reported that most of the transporters/ transporter regulators is localized in the membrane and several plant cation transporters mediate transport of sodium (Na^+), which is toxic at high concentrations leading to salinity stress (Maser et al., 2001). In rice, plasma membrane bound Na^+/H^+ transporter is *OsSOS1* (Martinez-Atienza et al., 2007) and another is tonoplast bound Na^+/H^+ antiporter *OsNHX1* (Fukuda et al., 2004). The expression of *OsARP* gene have great physiological role in plant defense mechanisms under various abiotic stresses. It was found that *OsARP* overexpressed tobacco plants had better growth and vigor under salt stress than wild type plants. Transgenic plants accumulated more Na^+ in their leaf tissue. Increased solute accumulation and water retention could confer salt tolerance in overexpressed tobacco plants (Uddin et al., 2008). Here, we report the cloning, expression and subcellular localization of antiporter regulating protein OsARP in rice cultivar FR13A in relation to submergence, salinity and drought stresses.

MATERIALS AND METHODS

Construction of *E. coli* expression vector using gateway technology

The cDNA containing *OsARP* gene (Accession no. AK071205; Rice Genome Project of the National Institute of Agrobiological Sciences, Japan) was amplified by PCR using Platinum Pfx DNA polymerase (Invitrogen) with gene specific primers (5'-GGCTT-CACCATGGTGCTCTGGGTCTTCGGCTATGG-3') and (5'-GAAAGCTGGGTCTCTTGAGTCCACAACAGTGCCCT-3'). The PCR condition was: 94°C for 1 min, 94°C for 30 s, 55°C for 45 s, 72°C for 1 min, 72°C for 10 min, 30 cycles (Takara Thermal Cycler, TP600, and Japan). Second PCR was performed using the same Pfx polymerase with primers (5'-GGGGACAAGTTTGTACA-AAAAAGCAC-3') and (5'-AGATTGGGGACCACTTTGTACAAGAA-3') which containing *att*B sites (Invitrogen), and the PCR products from the first reactions as templates and the PCR condition was the same as aforementioned. The linear fragments flanked by *att*B sequences were subjected to site-specific recombination with entry vector pDONR207 (Invitrogen), containing the *ccd*B gene, flanked by *att*P sites and catalyzed by BP Clonase yielding entry clones that were used to transform *Escherichia coli* competent DH5α cells. Transformants were grown on LB agar plates containing 50 μg mL^{-1} gentamycin. Colonies were picked from each plate for colony PCR using *Taq* polymerase and outer pDONR primers (Invitrogen) and grown in liquid culture for subsequent plasmid preparation. The entry clones were subjected to another round of site-specific recombination catalyzed by the LR Clonase enzyme mix in order to subclone the genes of interest into a destination vector, that is, *Escherichia coli* expression vector pDEST17 containing 6xHis-tag and T7 promoter, the *ccd*B gene flanked by *att*R sites, to generate expression clones. The resulting expression construct was used to transform *E. coli* DH5α strains. Transformants were selected on LB Agar plates containing 50 μg mL^{-1} kanamycin. The construct (pDEST17-*OsARP*) was then transformed into *E. coli* strain Nova Blue by heat shock method and insert was confirmed by colony PCR and restriction enzyme digestion.

Sequencing the clones

The positive clones are sequenced using vector specific primers. The PCR was done separately using forward (pDONR207F: TCGCGTTAACGCTAGCATGGATCTC) and reverse (pDONR207R: TGTAACATCAGAGATTTGAGACAC) primers. Ten micro liters PCR reaction consisted of template DNA 1 μL, primer (0.8 μM) 4 μL, Dye terminator V1.1- 8 μL and dH_2O 7 μL. The PCR condition was: 96°C for 1 min, 96°C for 10 s, 50°C for 5 s, 60°C for 4 min, 60°C for 7 min, 25 cycles (Takara Thermal Cycler, TP600, and Japan) and sequencing was done in Prism ABI 3700 sequencer machine.

Expression and purification of *E. coli* His-tagged protein

The transformed *E. coli* host strain Nova Blue was cultured in LB

medium containing ampicillin (50 μg mL^{-1}) at 37°C with shaking until OD_{600} = 0.53 to 0.8. One milliliter (1 ml) of that sample was used as the control. Expression of protein was induced by adding IPTG to a final concentration of 0.5 mM and then incubated the culture at 37°C for 4 h with shaking. The culture was placed into ice to stop incubation and centrifuged in 10,000 rpm at 4°C for 2 min. After centrifugation, the supernatant was discarded and pellet was resuspended in binding buffer by vortex. The suspension was sonicated on ice followed by centrifugation (10,000 rpm for 10 min, at 4°C). The supernatant was transferred to new tubes. Ni-ATA agarose (Amersham Bioscience, UK) column was equilibrated by passing it through binding buffer. After that the series of imidazole solutions (1, 2.5, 5, 10, 15, 25, 50, 100, 200, 500 mM) were passed through the column. The target protein was separated from 500 mM imidazole solution. The protein solution was mixed with SDS sample buffer and boiled for 5 min. After SDS-PAGE (12%), the gel was washed with Mili-Q water; intended band was cut into pieces and mixed with 1 × SDS sample buffer to dissolve the protein at room temperature. The dissolved protein solution was concentrated by passing through filter centrifugal tube with a speed of 3000 rpm. The refined protein was collected from solution by substituting with physiological brine.

Plant culture

Seeds of rice (*O. sativa* L.) FR13A (submergence tolerant cultivar, *indica*) were obtained from the International Rice Research Institute (IRRI) and germinated by soaking in water at 30°C for 3 days and then transplanted into vermiculite. The seedlings were irrigated at 1-week intervals using Hyponex nutrient solution diluted 1: 1000 in water. Three week-old seedlings were submerged completely (approximately 20 cm under the water surface) for 3 to 7days at 25 to 30°C. Other seedlings of the same age were irrigated with 0.2 M NaCl, and exposed to drought by withholding water for 7 days. The rice root, shoot and leaves were harvested, frozen in liquid nitrogen and stored at -80°C until use. Rice seedlings cultivated under normal conditions served as controls for Western blotting.

Protein extraction

Frozen samples (0.3 g) were ground into a fine powder in liquid nitrogen with a chilled mortar and pestle. The powder of each sample was homogenized with potassium phosphate (K-P) buffer (1 mL) consisting of 50 mM potassium phosphate, 1 mM EDTA and 5 mM DTT, pH 7.8. The slurry was transferred to centrifuge tube and centrifuged for 20 min (at 20,000 × *g*, 4°C). Protein quantity was determined according to the method described by Bradford (1976).

Isolation of cell fractions

Cell fractionation was done according to the method described by Boyes et al. (1998) with slight modifications. We used sucrose buffer as grinding medium which contains 330 mM sucrose, 50 mM Tris-HCl of pH 7.5 to 8.0, 5 mM DTT and 1 × protein inhibitor cocktail. Fresh rice leaves were cut into 1 cm long pieces and put into 50 mL tube. About 10 to 15 times sucrose buffer was added into it and crushed the tissues by Poly-tron (Micro Tissue Grinder) on ice. After crushing, liquid was filtered by 4-layer Miracloth and collected in 2 mL tubes. The liquid was centrifuged in 100 × *g* for 10 min at 4°C and supernatant was transferred to others tubes. The supernatant was centrifuged by 500 × *g* for 10 min. Its deposit was used as nuclear fraction and its supernatant was centrifuged by 3000 × *g* for 10 min. Likewise, chloroplast fraction was separated by 3000 × *g*; chloroplast (fragmentized) portion at 8000 × *g*; mitochondrion portion at 10000 × *g*; endoplasmic reticulum and ribosome portion at 20000 × *g*, and the pellet at 100000 × *g* separated plasma-membrane portion. The remaining supernatant was transferred to other tubes and this contained cytoplasm portion. Total protein was estimated and 30 μg protein from each portion was used for Western blot analysis.

Western-blot analysis

Protein samples (30 μg) were separated in 12% SDS- PAGE and transferred to Hybond ECL Nitrocellulose membrane (Amersham Biosciences, UK) by ATTO semidry transfer cell (Atto Corporation, Tokyo, Japan). Immuno-detection (Eltayeb et al., 2006) was performed using diluted (1: 2000) guinea-pig antibodies raised against His-tagged OsARP protein as the primary antibody and a diluted (1: 5000) horseradish peroxidase-conjugated anti-guinea pig IgG (Sigma, St. Louis, MI, USA) as the second antibody.

RESULTS AND DISCUSSION

The aim of the study was to investigate the expression and localization of putative rice vacuolar antiporter regulating protein OsARP in relation to different abiotic stresses. We used the cDNA (Accession no. AK071205) for cloning and protein expression in *E. coli* as required for the production of OsARP antibody. The cloning was done using Gateway cloning technique. The *OsARP* insert was cloned from entry vector to expression vector by LR reaction. The construct pDEST17-*OsARP* was first introduced into *E. coli* strain DH5α by heat shock method. The cloning of *OsARP* insert was confirmed by *Nhe*I restriction enzyme digestion. Both *OsARP* and expression vector pDEST17 had the *Nhe*I restriction sites. Restriction digestion gave the DNA fragment of 5.3 kb and 430 bp was detected by electrophoresis, if the *OsARP* was not introduced into the vector, the DNA fragment of 6.3 kb could be detected. So, this confirmed the cloning of *OsARP* into the *E. coli* expression vector pDEST17.

Plasmid containing *OsARP* insert isolated from *E. coli* strain DH5α was used to transform *E. coli* strain Nova Blue. The transgenic *E. coli* (Nova Blue) culture was treated with IPTG (isopropyl-β-D-thiogalactopyranoside) to induce the expression of protein. The expressed fusion protein, OsARP with 6xHis-tagged was united with Ni-ATA column and washed by imidazole of a series of concentration as described in materials and methods. The purified target protein was separated from 500 mM imidazole solution and the molecular weight expressed His-tagged protein was 30 kD as determined by SDS-PAGE (Figure 1). The purified His-tagged protein (1.18 mg) was injected to guinea pig for OsARP antibody production. We tested the activity of OsARP antibody by Western blotting of IPTG induced 6xHis-tagged protein along with control non-IPTG protein. The positive signal of about 30 kD protein was detected from IPTG induced His-tagged OsARP protein (Figure 2).

We tested the expression level of *OsARP* gene in FR13A rice variety under submergence of 3-day and 7-

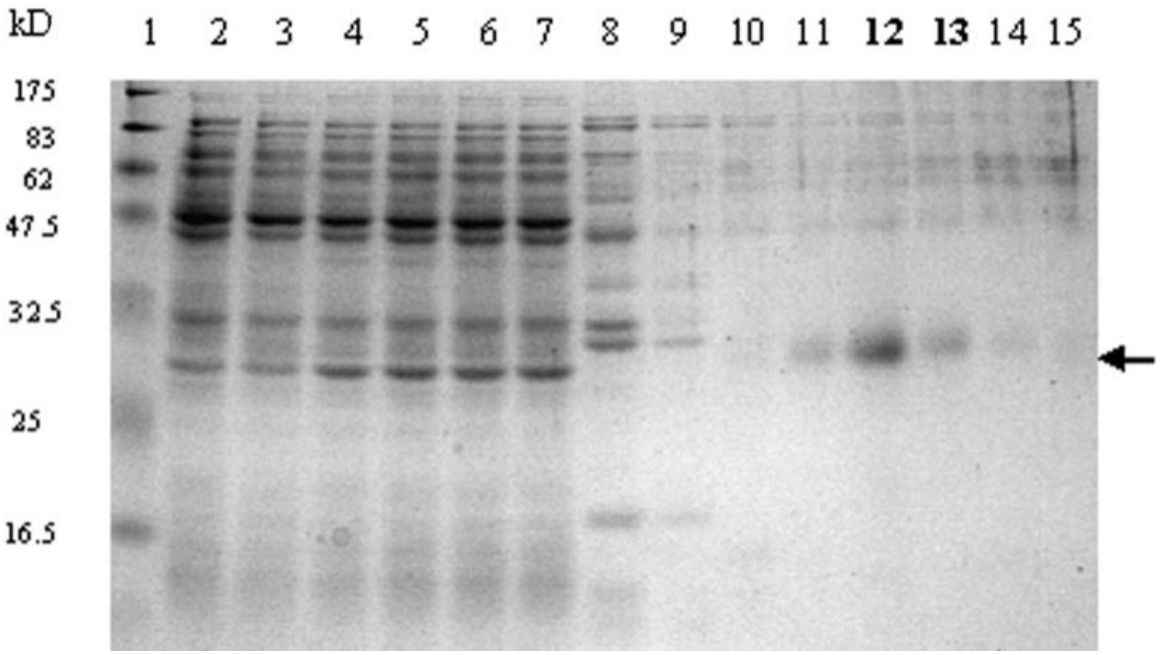

Figure 1. Purification of recombinant *OsARP* protein. SDS-PAGE of successive fractions from a nickel affinity column. Lane 1: Marker; lane 2: control (non IPTG); lane 3: crude (+IPTG); lane 4 to 7: flow through (+IPTG); lane 8: 15 mM imidazole (+IPTG); lane 9: 25 mM imidazole (+IPTG); lanes 10 to 11: 100 mM imidazole (+IPTG); lanes 12 to 15: 500 mM imidazole (+IPTG). Arrowhead indicates target recombinant protein (lanes 12 and 13) separated from 500 mM imidazole solution.

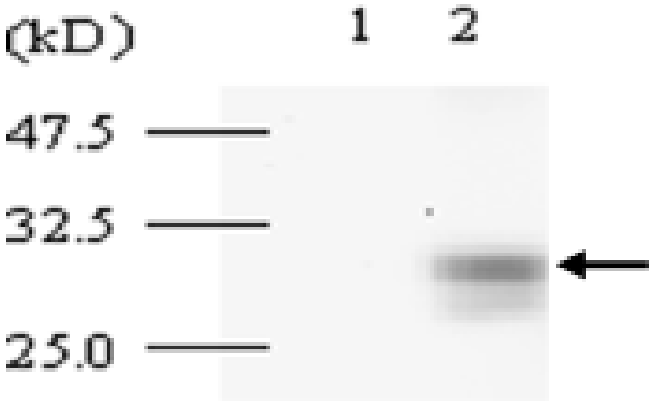

Figure 2. Verification of OsARP antibody by Western-blot analysis. 6xHis-tagged *OsARP* protein induced in *E. coli* (Nova Blue) by IPTG with 500 mM imidazole (lane 2) along with non-IPTG protein (lane 1). Arrowhead indicates 6xHis-tagged OsARP protein. Twenty-five microgram (25 µg) proteins were used for 12% SDS-PAGE and subsequent transfer to Hybond ECL Nitrocellulose membrane. Immuno-detection was performed using diluted (1: 2000) guinea pig antibody raised against 6xHis-tagged OsARP protein as the first antibody and a diluted (1: 5000) horseradish conjugated anti-guinea pig IgG as secondary antibody.

day. The OsARP protein was expressed in leaves from 3-day submergence to 7-day submergence as detected by Western-blot analysis (Figure 3A). The expression of OsARP protein was also detected under salt (0.2 M NaCl) and drought (Figure 3B) stresses. In addition to leaves, the *OsARP* gene expression was also confirmed in roots and shoots of FR13A rice plants under salt stress by Western blotting (Figure 3C). This indicated that the expression of this protein was widespread in rice plants. In other study, overexpression of *OsARP* gene in tobacco (*Nicotiana tabacum* L.) showed enhanced tolerance to salt, drought and submergence stresses. Transgenic tobacco plants showed better growth and vigor than wild type plants under salt stress. Net photosynthesis and chlorophyll fluorescence were significantly higher in OsARP transgenic tobacco plants than those of wild type (Uddin et al., 2008). These results indicated the functional role of OsARP protein in relation to the tolerance of different abiotic stresses. The study of abiotic stress response has advanced considerably in recent years. Analyzing of a single stress in plants can be very different from the conditions encountered by plants in the field where several stresses may be occurred simultaneously (Mittler et al., 2001). This can alter plant metabolism in a novel manner that may be different from that caused by each of the different stresses applied individually, and may require a new type of response that would not have been induced by each of the individual stresses (Rizhsky et al., 2002). Therefore, some genes that can be expressed by specific stress might be expressed in other stress as well. The expression of putative vacuolar antiporter regulator OsARP was wide spread in all the plant parts

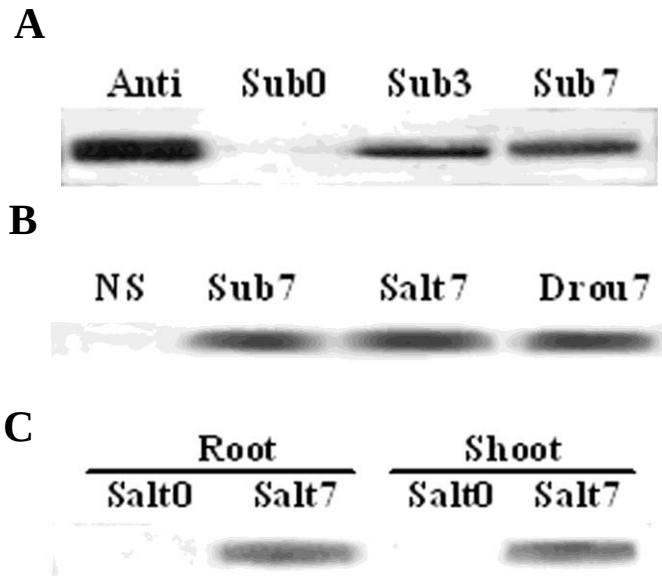

Figure 3. Expression of OsARP protein in rice cultivar FR13A. A. Expression was detected at 3-day (Sub3) and 7-day (Sub7) of submergence, Sub0 indicates no submergence stress. Leaves of FR13A harvested at 0, 3 and 7-day of submergence were used for total protein isolation. OsARP antigen was used as positive control (Anti). B. Expression under submergence, drought and salt stresses. The leaf samples of submerged (Sub7: for 7 days), salt stress (Salt 7: 0.2 M NaCl stress for 7days), drought (Drou7: withholding water for 7 days) and control (no stress: NS) were used for total protein isolation. C. Expression in roots and shoots. Total proteins from salt stress (Salt 7: 0.2 M NaCl for 7days) and control (Salt 0) root and shoot tissues were used for Western blotting. Thirty µg proteins were used for 12% SDS-PAGE and transfer to Hybond ECL Nitrocellulose membrane. Immuno-detection was performed using diluted (1: 2000) guinea pig antibody raised against His-tagged OsARP protein as the first antibody and a diluted (1: 5000) horseradish conjugated anti-guinea pig IgG as secondary antibody.

under salt stress. In addition to leaves, the expression of OsARP protein was also detected in roots and shoots (Figure 3C) of rice. The effect of salt stress on the expression of *OsNHX1*, a Na^+/H^+ antiporter was examined in rice plants. The salt stress (0.2 M NaCl) increased the transcripts levels of *OsNHX1* in roots and shoots indicating that ionic stress played major role for the expression of this gene in different plant parts (Fukuda et al., 2004).

The putative antiporter regulating protein OsARP is highly expressed in submergence, salt and drought stresses and has the homology in ChaC *E. coli* cation transport protein and it is thought to be related to the maintenance of ion balance inside the cell. As for salt stress tolerance, Na^+ ion adjustments are the most important factor for plants. This gene is equally expressed in NaCl and drought stresses, where antiporter/antiporter regulator protein might play an important role under those stresses. In different study, the cation contents of *OsARP* transgenic and wild type tobacco plants were estimated in 100 mM NaCl stress over 8 days. In general, transgenic tobacco plants retained more cations in their leaf tissues, and in particular, the Na^+ content was found to have more than two-fold higher in transgenic plants than wild type under salt stress. It was conceivable that the toxic effect of Na^+ in OsARP over-expressed tobacco plants was reduced by sequestration into vacuole (Uddin et al., 2008).

To get an insight into the functional role of *OsARP* gene products, the localization of the proteins in the cell was carried out by biochemical cell fractionations in fructose buffer by changing the centrifugal gravity. Western-blot analysis was done using these fractions, showed that OsARP protein was only present in the plasma-membrane fraction collected from 100,000 × *g* (Figure 4). Membrane localization of OsARP protein was also investigated by immuno-gold labeling. Immuno-gold electron microscopy was conducted using leaves of FR13A rice plants submerged for 3 days. It demonstrated that gold particles were localized in vacuolar membrane

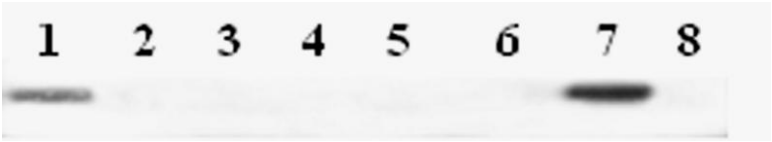

Figure 4. Localization of OsARP protein by cell fractionation. Western-blot analysis of different cell fractions (Lanes 1 to 8). Rice shoots and leaf tissues harvested after submerging (for 3 days) were used for total protein isolation. Then homogenized tissues were fractionated by changing the centrifugal gravity and cell fractions (lane1-total protein; lane 2- nuclear portion, g; lane 3-chloroplast portion; lane 4-chloroplast fragmentized portion; lane 5- mitochondrial portion, lane 6-endoplasmic reticulum portion; lane-7 plasma membrane portion; lane 8-cytoplasmic portion, were collected Hybond ECL Nitrocellulose membrane. Immuno-detection was performed using diluted (1: 2000) guinea pig antibody raised against His-tagged OsARP protein as the first antibody and a diluted (1:5000) horseradish conjugated anti-guinea pig IgG as secondary antibody.

(tonoplast) of the cell (Uddin et al., 2008), and no gold particles were detected in other organelles or in cell matrix. These results confirmed that OsARP protein was localized in plasma membrane as well as vacuolar membrane. Moreover, Western-blot analysis by OsARP antibody detected high level of OsARP protein in tonoplast fractions from transgenic tobacco plants (Uddin et al., 2008).

Membrane proteins have been evolved to control the movement of ions in and out of cells and their subcellular compartments. Membrane transporters/antiporters act to alter many aspects of plant growth and development. At the whole plant level, accumulation of ions in the leaf is the result of actions of transporters in individual membrane (Hall et al., 2006). Na^+/H^+ antiporters are ubiquitous membrane proteins that play major roles in cellular pH and Na^+ homeostasis throughout the biological kingdom. They catalyze the exchange of Na^+ for H^+ across membranes. Recently, some of Na^+/H^+ anitporters in *Arabidopsis* and rice were reported which functions were mainly to extrude Na^+ ion from cells or to sequester/compartmentalize Na^+ in vacuoles (Brini et al., 2007; Sottosanto et al., 2007; Zhang and Blumwald, 2001; Fukuda et al., 2004). It was assumed that the role of OsARP protein might take place into the vacuolar membrane for ion adjustments (specifically Na^+) in cells under salt stress. This hypothesis was confirmed by the Na^+/H^+ translocation study of OsARP protein enriched tonoplast fractions from transgenic tobacco plants. The Na^+/H^+ antiporter activity was three-fold higher in transgenic tobacco plants than wild type in presence of NaCl. It was concluded that OsARP on the tonoplast played an important role to enhance/regulate the antiporters to sequester Na^+ into vacuole (Uddin et al., 2008).

In conclusion, bio-informatics analysis of amino acids sequences indicated single transmembrane domain was present in OsARP protein. This single transmembrane domain was not enough to penetrate the membrane, because antiporters might have required more hydrophobic regions to span the membrane. This suggested that OsARP was likely to be a surface bound antiporter-regulating protein of rice localized both plasma-membrane and tonoplast (vacuolar membrane) of the cell. This is a new type protein which might have some regulatory functions to enhance the existing antiporters of plants required for ion homeostasis under salt or drought stresses.

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