Plant Biotechnology 30, 417–422 (2013)
DOI: 10.5511/plantbiotechnology.13.0517a

Note

Functional expression of an animal type-Na^+-ATPase gene from a marine red seaweed *Porphyra yezoensis* increases salinity tolerance in rice plants

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Received March 10, 2013; accepted May 17, 2013 (Edited by K. Kato)

Abstract Despite lacking Na^+-ATPase as a sodium pump in vascular plants, a gene encoding KPA (K^+ P-type ATPase), a putative animal type-Na^+/K^+-ATPase, has been isolated from the marine red alga *Porphyra yezoensis* and designated PyKPA1. To characterize the properties of PyKPA1 and also to confirm its ability to confer salinity tolerance in land plants, transgenic rice plants were produced that expressed the full-length *PyKPA1* cDNA under the control of cauliflower mosaic virus *35S RNA* promoter. We observed transcriptional activation of the transgene, plasma membrane-localization of the gene product fused with green fluorescent protein in onion epidermal cells, and Na^+-ATPase activity in the plasma membrane fraction from transgenic rice plants, indicating that PyKPA1 was functionally expressed in rice plants. Transgenic lines were examined in terms of growth in salinity stress conditions, resulting in protection from a decrease in biomass, although growth of control rice plants was repressed. These results demonstrate the utility of a red algal animal type-sodium pump for conferring salinity tolerance to land plants.

Key words: Na^+-ATPase, *Porphyra yezoensis*, rice, salinity tolerance, transformation.

Eukaryotes possess a P-type ATPase as a primary pump on the plasma membrane, which can play essential roles in the homeostasis of intracellular Na^+ concentrations (Palmgren and Nissen 2011; Pedersen et al. 2012). There are three kinds of P-type ATPase related to Na^+ efflux across the plasma membrane; Na^+/K^+-ATPase in animal cells, H^+-ATPase in plant and fungal cells and ENA ATPase found in bryophytes and protozoa. Among these, H^+-ATPases produce an electrical membrane potential by producing a H^+-gradient (ΔpH), which activates the secondary Na^+/H^+ antiporter for an electroneutral exchange of extracellular H^+ and intracellular Na^+ in vascular plants. With its electroneutral function, a Na^+/H^+ antiporter cannot perform Na^+ efflux under high pH conditions, whereas Na^+/K^+-ATPase and ENA ATPase are ΔpH-independent and thus they can mediate Na^+ efflux under alkaline pH conditions (Garciadeblas et al. 2001; Rodríguez-Navarro and Benito 2010). Genome sequence information has revealed the loss of Na^+-ATPase in vascular plants during evolution in fresh water environments (Graciadeblas et al. 2001; Pedersen et al. 2012), and the existence of Na^+/K^+-ATPase and/or ENA ATPase in eukaryotes living in highly alkaline and saline environments such as the sea was proposed.

Following on from the identification of ENA ATPase in yeast, the existence of ENA ATPase was confirmed in eukaryotes other than animals (Garciadeblas et al. 2001; Rodríguez-Navarro and Benito 2010). Although Na^+-ATPase activity has been detected in the plasma membranes of marine unicellular algae *Heterosigma akashiwo*, *Platynomas viridis* and *Dunaliella parva* (Balnokin and Popova 1994; Gimmler et al. 1989; Shono et al. 1995; Shono et al. 1996), the identification of a gene from *H. akashiwo* encoding an Na^+-ATPase with high homology to animal-type Na^+/K^+-ATPases was unexpected (Shono et al. 2001), indicating the unexpected importance of animal-type Na^+/K^+-ATPases in adaptation of marine algae to high salinity environments (Gimmler 2000).

The red seaweed *Porphyra yezoensis* is one of the most important algae in Japan for the production of nori (thin, dried sheets of seaweed used in Japanese cuisine) and has become a model organism for molecular biological studies of seaweeds. Expressed sequence tag

Abbreviations: CaMV, cauliflower mosaic virus; GFP, green fluorescent protein; MS, Murashige and Skoog; ORF, open reading frame.
This article can be found at http://www.jspcmb.jp/
Published online September 5, 2013

(EST) analysis using gametophytes and sporophytes of *P. yezoensis* suggested the presence of an animal-type Na^+/K^+-ATPase especially expressed in sporophytes (Asamizu et al. 2003), and the full-length cDNA encoding this P-type ATPase, designated PyKPA1 (*P. yezoensis* K^+ P-type ATPase 1), was isolated and functionally characterized in *Escherichia coli* and *Saccharomyces cerevisiae* (Barrero-Gil et al. 2005). Recently, it was demonstrated that transcriptional expression of the *PyKPA1* gene was transiently stimulated by alkali and cold stresses in gametophytes in addition to preferential expression in sporophytes; however, salinity stress did not alter the expression pattern of this gene (Uji et al. 2012). Therefore, the function of PyKPA1 in salinity stress tolerance is still unclear.

To date, there is no way to analyze the function of PyKPA1 using *P. yezoensis*, because of the lack of genetic manipulation systems in any seaweed for the inactivation or knockdown of genes (Mikami 2012; Mikami et al. 2011). Therefore, heterologous expression is only means of functional analysis of *PyKPA1*. Indeed, it has been reported that the expression of yeast Na^+-ATPase in tobacco cultured cells and transgenic *Arabidopsis thaliana* and also moss Na^+-ATPase in transgenic rice was performed successfully, all of which demonstrated an increase in salt tolerance (Jacobs et al. 2011; Kong et al. 2008; Nakayama et al. 2004). Therefore, we generated transgenic rice plants to analyze the enzymatic activity, subcellular localization and effects on plant growth of red algal PyKPA1. This is the first report of the successful expression of an animal-type Na^+/K^+-ATPase in land plants.

A plasmid carrying the *PyKPA1* cDNA (accession no. AJ972674), kindly provided from Dr. Begoña Benito (Universidad Politécnica de Madrid, Madrid, Spain), was used as a template for PCR-amplification of a full-length open reading frame (ORF) of 3,507 bp (1,169 amino acids) using prime STAR HS DNA polymerase plus GC buffer (TAKARA, Kyoto, Japan) with primer set 5′-CAC CAT GGC GGG TGG GGA TGA TGT C-3′, 5′-CCA GTA GGT GTT GTC GTA AAG-3′ under the conditions of initial denaturation at 98°C for 1 min, followed by 35 cycles of 98°C for 10 s, 60°C for 5 s and 72°C for 4 min and then final extension at 72°C for 7 min. Two plant expression plasmids were constructed using the Gateway system (Invitrogen, Carlsbad, CA, USA). In brief, a PCR product was inserted into pENTR/D-TOP (Invitrogen) to construct an entry plasmid following introduction of the *PyKPA1* ORF into a binary destination vector pGWB2 or pGWB5 (Nakagawa et al. 2007), the latter of which was employed for the transient expression of a green fluorescent protein (GFP) fusion protein, by LR Clonase reaction. The T-DNA regions of the two resultant plasmids containing *PyKPA1* and *PyKPA1::GFP* gene are shown in Figure 1.

Using expression plasmid *PyKPA1* introduced into *Agrobacterium* strain EHA105 (Hood et al. 1993) by electroporation, rice cv. Nipponbare plants were transformed in accordance with Hiei et al. (1994). Calli harboring this plasmid were selected on N6D medium supplemented with hygromycin (50 mg l^{-1}), yielding 57 transformant clones. Of these, 15 independent shoots were regenerated from calli. To obtain plant lines in which the *PyKPA1* genes was highly expressed, total RNA was isolated from leaf samples using Sepasol-RNA I Super (Nacalai Tesque, Japan), followed by synthesis of first-strand cDNA using Rever Tra Ace (Toyobo, Osaka, Japan) and an oligo-dT primer. The resulting cDNAs were used as PCR templates with a primer set of PyKPA1-F: 5′- GGA CAA GAC CGG TAC TCT TAC G-3′ and PyKPA1-R: 5′-GAT TCT TGA AGG TCG GTG AGT C-3′. PCR was conducted using the following conditions: 27 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s. Five lines were isolated for *PyKPA1*. These transformants were planted in soil in order to harvest T_1 seeds. It was confirmed that seeds from T_1 plants grew well on hygromycin-containing Murashige and Skoog (MS) agar medium (data not shown). Seed weight and free amino acid composition were compared among control and transformed lines, and there was no significant differences (data not shown).

Copy numbers of transgenes in the two transformed lines *PyKPA1#28* and *PyKPA1#115* from five ones, which showed high and stable expression level in the RT-PCR analysis (data not shown), was determined. Total DNA was isolated from leaves by the CTAB method (Murray et al. 1980), digested with *Hind*III or *Sac*I (see Figure 1), separated by 0.7% agarose gel electrophoresis, and finally transferred to a nylon membrane. DNA probes (neomycin phosphotransferase II or hygromycin phosphotransferase, see Figure 1) were labeled using a Gene Images AlkPhos Direct Labeling and Detection system (GE Healthcare, UK) and hybridized to genomic DNA on the nylon membrane. This Southern blotting analysis demonstrated the T-DNA copy number in the transgenic lines: 5 copies in *PyKPA1#28*, 4 copies in *PyKPA1#115*.

In order to examine transgene expression, T_2 plants were grown on MS medium for 2 weeks. Total RNA was isolated from roots and leaves. After synthesis of cDNAs using reverse transcriptase, PCR amplification was performed with specific primers (PyKPA1-F and PyKPA1-R) for the *PyKPA1* cDNA. As shown in Figure 2, two lines of transformed rice plants, *PyKPA1#28* and *#115*, displayed specific amplification of *PyKPA1* both in leaves and roots, whereas no amplification was detected in those from non-transformed Nipponbare. Thus, the gene introduced into the genome was expressed efficiently.

For evaluating the intracellular localization of

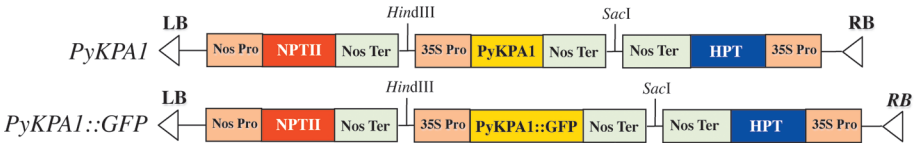

Figure 1. Schematic diagram of the *PyKPA1* gene cassettes in the T-DNA regions. *PyKPA1*: a full-length cDNA encoding the Na^+-ATPase gene from *P. yezoensis*, *PyKPA1::GFP*: a coding region of green fluorescent protein (S65T) fused to the 3′-end of the *PyKPA1* ORF in frame. 35S Pro: cauliflower mosaic virus *35S RNA* promoter, Nos Ter: nopaline synthase terminator, Nos Pro: nopaline synthase promoter, NPTII: neomycin phosphotransferase II, HPT: hygromycin phosphotransferase, LB/RB: left/right T-DNA border. Unique restriction enzyme sites *Hind*III and *Sac*I in the T-DNA were used for the digestion of genomic DNAs in Southern analysis.

PyKPA1, transient expression of the *PyKPA1::GFP* fusion gene in onion (*Allium cepa*) epidermal cells was performed. To this end, the fusion gene cassette (*35S Pro::PyKPA1::GFP:: Nos Ter*) shown in Figure 1 was PCR-amplified with an appropriate primer set and introduced into a pGEM-T Easy Vector (Promega, USA), and the resultant plasmid was transfected into onion cells by particle bombardment (IDERA-GIE-III, TANAKA, Japan) essentially as described by Shimajiri et al. (2013). The vector pTH-2 that contains the modified green fluorescent protein (sGFP-S65T) reporter gene was used as a control (Chiu et al. 1996). After bombardment, the epidermal tissues were incubated for 16 h at 23°C. To visualize plasma membranes, the tissues were immersed in MS liquid medium containing 5 μg ml^{-1} FM 4–64 (Invitrogen) to incubate for 5 min, followed by rinsing with distilled water three times. Fluorescent images were taken with excitation at 488 nm and emission at 500–530 nm for GFP or with excitation 543 nm and emission at 620–680 nm for red irradiation of FM 4–64 using a confocal laser-scanning microscope (Leica TCS SP5, Germany). Merged images were constructed using the Leica LAS Image Overlay System. As shown in Figure 3, in transient expression of *sGFP-S65T* gene green fluorescence was observed in the whole cell, while in case of transient expression of a *PyKPA1::GFP* fusion gene green fluorescence was observed in the plasma membrane, which was confirmed by the overlap of fluorescence with plasma membrane-marker FM 4–64. Therefore, PyKPA1 from red seaweed was correctly localized at the plasma membranes when it was expressed in onion-cells, suggesting the functional activity of its enzymatic properties by heterologous expression as reported for yeast and moss Na^+-ATPase (Jacobs et al. 2011; Kong et al. 2008; Nakayama et al. 2004).

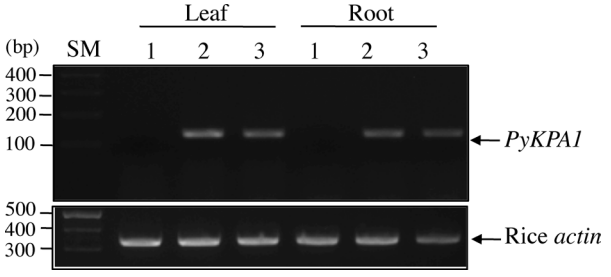

Figure 2. RT-PCR analysis of *PyKPA1* transgene expression in rice. Total RNA was isolated separately from leaf and root tissues and used for producing a cDNA mixture as the template for PCR reactions with primer sets PyKPA1-F and PyKPA1-R or for internal expression control of the rice *actin* gene (accession no. AK100267, 5′-TCCATCTTGGCATCTCTCAG-3′ and 5′-GTACCCGCATCAGGCATCTG-3′). Lane 1, non-transformed rice cv. Nipponbare; lanes 2 and 3, transformed lines *PyKPA1#28* and *#115*, respectively. SM: DNA size marker.

To check the above possibility, Na^+-ATPase activity was measured using transgenic rice plants. Because each plant material from two transgenic rice was little for isolation, they were mixed before preparation. Shoots and roots from about 5 g of fresh rice seedlings grown on MS medium for 2 weeks were cut into fine pieces using scissors and immersed directly in 50 ml of homogenizing medium (0.25 M sucrose, 75 mM MOPS-KOH (pH 7.6), 1.5% (w/v) PVP (MW 24,500), 5% (w/v) defatted bovine serum albumin, 5 mM EDTA, 5 mM EGTA, 1 mM phyenylmethylsulfonyl fluoride, 4 mM salicylhydroxamic acid, 10 mg ml^{-1} butylated hydroxytoluene, 5 mM

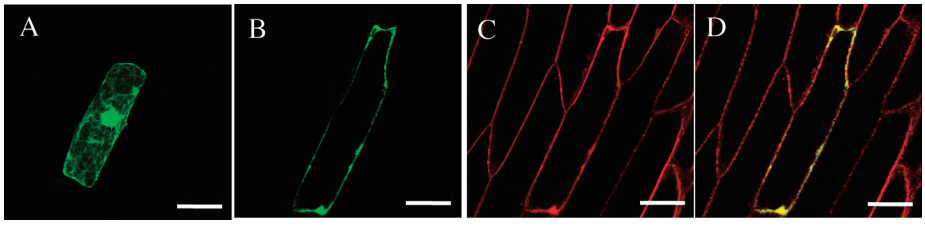

Figure 3. Subcellular localization of heterologously expressed PyKPA1 in onion epidermal cells. A and B: fluorescence from onion cells transiently transformed with an expression plasmids carrying the *sGFP-S65T* gene (as a control) and *PyKPA1::GFP* fusion gene by particle bombardment, respectively, C: cells stained with FM 4–64, D: merged image with B and C. scale bars = 100 μm.

$K_2S_2O_5$), followed by homogenized using a Polytron homogenizer at 0°C. Plasma membrane fractions were isolated in accordance with Hase (1993) and dissolved and washed in 250 to 500 μl of dilution buffer (10 mM Tris-HCl (pH 7.3), 0.25 M sorbitol, 2 mM DTT). Protein concentration was determined as described by Bradford (1976).

H^+-ATPase or Na^+-ATPase activity in the plasma membrane fraction was measured by determining the amount of released Pi at 30°C for 10 min essentially as described by Ames et al. (1966). Assay buffers (100 μl including about 10 μg of protein) were as follows: for the H^+-ATPase assay: 3 mM ATP, 3 mM $MgSO_4$, 25 mM HEPES-Bis-Tris-Propane (pH 6.5), 50 mM KCl, 1 mM $NaMoO_4$, 0.03% Triton X-100, ±0.1 mM vanadate (Na_3VO_4). For the Na^+-ATPase assay: 3 mM ATP, 3 mM $MgSO_4$, 25 mM HEPES-KOH (pH 8.5), 20 mM KCl, 100 mM NaCl, 0.015% Triton X-100, ±0.2 mM vanadate. The activities measured in the presence of vanadate, a specific inhibitor of P-type ATPases, were subtracted from that measured in its absence and thus vanadate-sensitive activity (mainly due to P-type ATPase) was determined. As shown in Table 1A, H^+-ATPase activity in the plasma membrane fraction showed almost the same level of vanadate-sensitive activity and vanadate inhibition rate (90% and 86%) for Nipponbare and the transformant, respectively. This result suggests the presence of the same endogenous H^+-ATPase activity among them. On the other hand, Na^+-ATPase activity in the plasma membrane fraction from control Nipponbare plants was slightly inhibited by vanadate (about 23%), whereas that from recombinant plants showed strong inhibition by vanadate under the same assay conditions (i.e., 90%, Table 1B), suggesting the presence of Na^+-ATPase activity in transformed rice. This interpretation was supported by positive control data from *Porphyra* sporophytes that has been suggested to contain Na^+-ATPase activity in the plasma membrane fraction (Barrero-Gil et al. 2005; Hase unpublished results), showing almost the same vanadate inhibition rate (81%; Table 1B).

Finally, in order to evaluate salinity tolerance, null transgenic rice and two transgenic rice lines (T_2; #28 and #115) at the third leaf stage were transferred to MS medium or MS medium containing 50 mM NaCl in glass tubes (18 cm in height), grown for 12 days, and then removed from to measure fresh weight ($n=12$). *P* values (control versus 50 mM NaCl in each plant line) were determined using Student's *t* test. As shown in Figure 4, cultivation under 50 mM NaCl stress, null transgenic plants exhibited a 20% loss in total biomass, indicating a significant decrease compared with that found in control (without NaCl) experiments. In contrast, *PyKPA1*-overexpressing rice plants #28 and #115 showed a slight increase (+8%) and a 10% loss, respectively, in total biomass, which was not statistically significantly different from that observed under the same experimental conditions. In addition, when the biomass of root tissues was compared, a 30% loss was observed in null transgenic plants, whereas transgenic lines #28 and #115 showed a 10% increase and a 5% decrease, respectively (data not shown). These results clearly demonstrated that the functional expression of *PyKPA1* conferred salt tolerance in rice plants, by which #28 and #115 transgenic lines maintained their biomass under high salinity conditions.

In this study we produced transgenic rice lines in which the *P. yezoensis Na⁺-ATPase* (*PyKPA1*) gene was stably integrated into the nuclear genome and expressed constitutively (Figure 2). Using these transgenic plants, we confirmed the transcriptional expression of the *PyKPA1* gene fusion protein, and *in vitro* Na^+-ATPase activity in the plasma membrane fraction from transformants but not from non-transformed rice. The transient expression of a *PyKPA1::GFP* in onion cells suggested the plasma membrane localization of it in the plant cells. Moreover, under the salt stress (50 mM NaCl) conditions, *PyKPA1*-overexpressing plants maintained

Table 1. ATPase activity in the plasma membrane fraction in plant cells.

A. H^+-ATPase activity

Sample	Total ATPase activity	Vanadate-sensitive ATPase activity	Inhibition %
	μmol Pi mg protein^{-1} h^{-1}		
Nipponbare[a]	98.5±5.1	88.3±3.6	90
Recombinant[b]	104.5±8.7	89.6±7.6	86

B. Na^+-ATPase activity

Sample	Total ATPase activity	Vanadate-sensitive ATPase activity	Inhibition %
	μmol Pi mg protein^{-1} h^{-1}		
Nipponbare[a]	7.8±1.1	1.8±0.6	23
Recombinant[b]	8.9±2.1	8.0±1.6	90
Porphyra[c]	22.5±5.6	18.2±4.1	81

[a] Non-transformed rice plant, [b] mixed sample from rice plants transformed with *PyKPA1#28* and *PyKPA1#115*, [c] *Porphyra yezoensis* sporophytes.

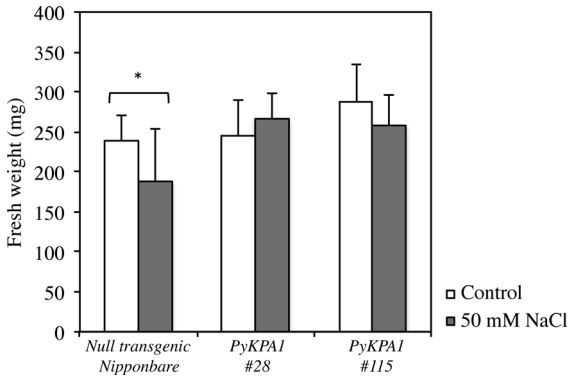

Figure 4. Comparison of biomass level between non-transformed and *PyKPA1*-overexpressing rice seedlings. Seedlings were germinated in MS agar-medium to grow for 1 week and transferred to the same MS agar medium (Control) or the same medium supplemented with 50 mM NaCl for culture of an additional 12 days. The fresh weight of the samples ($n=12$ for each experimental group) was measured. An asterisk indicates significant differences ($p<0.05$) between control and 50 mM NaCl in non-transformed rice.

their biomass in comparison with null transgenic plants showing significant decrease in biomass, although salinity tolerance of them were relatively low. Similarly, Barrero-Gil et al. (2005) reported that although the *PyKPA1* gene could complement alkaline cation transport mutants from *E. coli* and *S. cerevisiae*, these effects were relatively weak. In contrast, it has been reported that Na^+-ATPase is responsible for providing salt tolerance in the moss *Physcomitrella patens*, which confers growth at 100 mM NaCl and its ectopic expression in rice plants generated greater biomass at 50 mM NaCl (Jacobs et al. 2011; Lunde et al. 2007). Moreover, yeast Na^+-ATPase also confers salt tolerance in tobacco cultured cells and *A. thaliana* plants (Kong et al. 2008; Nakayama et al. 2004). It is worth noting that these P-type ATPases belong to the ENA type. Thus, it is possible that ENA-type ATPases are activated in land plant cells than PyKPA1 as an animal-type Na^+/K^+-ATPase, and this seems to be responsible for the weak effects of PyKPA1 overexpression in rice plants. Because the MS medium used for the growth transgenic rice plants was usually adjusted at pH 5.7, H^+-ATPase can act to produce an electronic membrane potential to activate Na^+/H^+ antiporter under high salinity conditions, which probably hid contribution of PyKPA1 for salinity tolerance. It is possible that the contribution of PyKPA1 for rice growth may be phenotypically visualized more readily under the high alkali conditions by inhibiting Na^+/H^+ antiporter activity. On the other hand, plasma membrane lipids in *P. yezoensis* are rich in long chains and highly unsaturated fatty acids, such as eicosapentaenoic acid and arachidonic acid, which are not observed in land plants and yeast (Araki et al. 1987). Thus, the physical properties of the membrane are different between *P. yezoensis* and rice, which may prevent the full activation of red algal PyKPA1 in rice plasma membranes.

Although there are some issues to be resolved, the present study is the first successful functional expression of an animal-type Na^+/K^+-ATPase in land plants. The results obtained here, therefore, reveal the availability of an algal sodium pump for conferring salinity tolerance in rice plants and hopefully other important crops.

Acknowledgements

We thank Dr. Begoña Benito (Departamento de Biotecnología, Universidad Politécnica de Madrid, Madrid, Spain) for the kind gift of a plasmid carrying a full-length *PyKPA1* cDNA, and Drs. Tsuyoshi Nakagawa and Koji Nishimura (Center for Integrated Research in Science, Shimane University, Japan) for pGWB2 and pGWB5 vectors and technical assistance with confocal laser-scanning microscopy (Leica TCS SP5), respectively. Also, we thank Dr. Yasuo Niwa (Department of Food and Nutritional Sciences, University of Shizuoka, Japan) for a kind gift of the pTH-2 plasmid DNA. This work was partly supported by the Hokusui Society Foundation to K.M.

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