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# *ZMVHA-B1*, the Gene for Subunit B of Vacuolar $H^+$-ATPase from the Eelgrass *Zostera marina* L. Is Able to Replace *vma2* in a Yeast Null Mutant

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**A vacuolar $H^+$-ATPase (VHA) gene (*ZMVHA-B1*) was isolated from an eelgrass (*Zostera marina*) leaf cDNA library and was characterized to be approximately 1.4 kbp in length and to encode the B subunit protein of VHA comprising 488 amino acids. *ZMVHA-B1* was highly expressed in all organs of eelgrass; the expression level was highest in the leaves. On transformation of a yeast *vma2* null mutant with *ZMVHA-B1*, yeast cells became able to grow at pH 7.5, accompanied by the vesicular accumulation of LysoSensor green DND-189. Thus, *ZMVHA-B1* expressed in yeast cells produced a functional B subunit that was efficiently incorporated into the VHA complex and eventually restored vacuolar morphology and activity. This success expedites the application of heterologous expression in yeast mutant cells to the screening of eelgrass genes involved in salt-resistance mechanisms, which are to be utilized in improving important crops.**

[**Key words:** *Zostera marina*, *ZMVHA-B1*, yeast complementation, salt stress, vacuolar $H^+$-ATPase]

The $H^+$-ATPase pump is a major protein of the tonoplast in plant species, comprising 6.5–35% of total tonoplast proteins (1). It uses the energy released during the cleavage of the γ-phosphate group of ATP to pump protons into the vacuolar lumen for the primary active transport (1, 2). The proton motive force provides a driving force for the secondary transport of ions and metabolites across the tonoplast through antiporters, symporters and channels (1). Vacuolar $H^+$-ATPase (VHA) has also been localized to endomembranes, such as the Golgi bodies, small vesicles and endoplasmic reticulum (3–6), as well as to the plasma membrane (3). In contrast to plasma membrane $H^+$-ATPase, VHA is a multimeric protein with two sectors, the integral membrane $V_0$, which provides the pathway for proton conductance, and peripheral $V_1$, which binds and hydrolyses ATP. Subunits A through H constitute the peripheral $V_1$ sector, and subunits a, c, c″, d and e constitute the integral membrane $V_0$ sector (1, 4–7). Most of the $V_1$ subunits (subunits A, C, D, F and H) are encoded by single-copy genes; subunits B, E and G are the exceptions. Unlike the $V_1$ sector, all of the $V_0$ subunits are encoded by at least two genes (5).

There have been several reports indicating that salt stress induces the accumulation of VHA proteins and mRNAs in different plants (8, 9), suggesting an important role for these proteins in salt tolerance or resistance. Subunit B may have an important role in this salt tolerance because ATP hydrolysis is catalyzed in the $V_1$ sector by subunit A (catalytic ATP binding), whereas subunit B (non catalytic ATP binding) is believed to play a regulatory role in VHA (5, 10); that is, it may regulate the net activity of VHA under high-salt conditions. In what way do the regulatory mechanisms of this protein differ between halophytes and other plants? How salt stress affects and regulates the expression of the proton ATPase pump may be clarified by examining such differences between halophytic and ordinary plants.

The majority of plants are sensitive to high-salt environments, but halophytic plants are able to tolerate salt stress and thrive in this environment. Eelgrass, *Zostera marina*, is a monocotyledonous angiosperm that can thrive in seawater with a salinity range of 0.5–3.3% (11, 12). There have been several studies on salt resistance of eelgrass, but little is known about the mechanism underlying this resistance (13). We are interested in the isolation and characterization of various genes involved in the salt resistance of this plant.

In general, the study of a specific gene in plants is difficult because each protein is encoded by a large family of genes, and several isoforms simultaneously exist within a single organ (14–17). On the other hand, heterologous expression in the yeast *Saccharomyces cerevisiae* has allowed the study of individual plant gene functions. Various types of yeast mutant including salt-sensitive mutants lacking the $Na^+$ efflux pump gene *Ena1*(18), the plasma membrane $H^+$-ATPase genes *PMA1* and *PMA2* (19), the gene for plasma membrane $Na^+/H^+$ antiporter *NHA1* (20), and the vacuolar $Na^+/H^+$ antiporter gene *NHX1* (21) are currently available

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and several plant $H^+$-ATPase genes have been actually expressed and studied in yeast (20, 22, 23). In addition, some subunits of VHA have been introduced and studied in suitable yeast mutants (24, 25). However, there has been no report of successful expression and complementation of plant VHA subunit B. Using a yeast expression system with these salt-sensitive mutants, we intend to screen the cDNA libraries of *Z. marina* for genes involved in salt-resistance mechanisms. To date, a gene of plasma membrane $H^+$-ATPase (*ZHA2*) was successfully isolated and characterized using this strategy (26).

In this paper, we report the isolation, sequencing and expression of *ZMVHA-B1* from *Z. marina*, which encodes the subunit B of VHA. For the first time, we show that a VHA subunit B from a plant successfully complemented yeast *vma2* mutant cells and supported their growth at pH 7.5. These observations increase the possibility of replacing a yeast protein in a large complex with a plant protein.

## MATERIALS AND METHODS

**Plant materials** Naturally growing *Z. marina* plants were collected in April at Yasuura Bay in Hiroshima, Japan and washed immediately with tap water, frozen in liquid nitrogen and stored at −80°C until use.

**Cloning of cDNA and sequencing** A 1800 bp fragment was amplified by PCR from eelgrass genomic DNA and labeled using a Gene Images labeling kit (P1) (Amersham Biosciences, Piscataway, NJ, USA). A cDNA library was constructed (27) and screened with a P1 probe.

One of the positive clones was subcloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) and both strands of the cDNA clone were sequenced using a Thermo Sequenase Primer Cycle Sequencing kit (Amersham Biosciences) and an ALF red automated DNA sequencer (Amersham Biosciences).

Total RNA was isolated and purified from the leaves of *Z. marina* by a modified LiCl method (27), and treated with RNase-free DNaseI (Roche Applied Science, Mannheim, Germany). cDNAs were reverse transcribed from the total RNA (1 µg/µl) using a ReverTra Ace kit (Toyobo, Osaka). To obtain a *ZMVHA-B1* construct to be introduced into and expressed in yeast cells, cDNA was amplified by PCR using specific oligonucleotide primers (forward, 5′-ATACA**GGTACC**TAATCTGAG*ATG*GGTGTGC-3′; reverse, 5′-AGTGCACTTTCTGAAT**CAGCTG**TTAGTGG-3′) and the PCR product was digested with restriction endonucleases *Kpn*I and *Pvu*II. The 1480 bp *Kpn*I–*Pvu*II fragment was connected to *Kpn*I–*Pvu*II sites of the shuttle vector pKT10 (28). The resulting plasmid [pKT10*ZMVHA-B1*] was introduced and amplified in *Escherichia coli* JM109.

**Quantitative real-time PCR** Real-time PCR was performed using a Line Gene Fluorescence quantitative detection system (BioFlux, Tokyo) with total RNA-based cDNAs prepared from the leaves, rhizomes and roots of *Z. marina* (27). For the quantitative determination of the transcripts of *ZMVHA-B1*, a PCR mixture containing SYBR-green (SYBR premix *ExTaq*; Takara Shuzo, Kyoto) was used. PCR in a final volume of 10 µl, containing 5 µl of the PCR mixture, 1 µl of diluted cDNA and 0.5 µM each of the oligonucleotide primer was carried out under the following conditions: 1 min at 95°C, 45 cycles (at 94°C for 15 s; 60°C for 15 s; 72°C for 30 s). The following primers were used: forward *ZMVHA-B1*, 5′-TGTCCTGCCATCTCTATCCC-3′; reverse *ZMVHA-B1*, 5′-AACAACAGCCTTCATTGCTTG-3′; forward actin, 5′-AGGTTC TCTTCCAGCCTTC-3′; and reverse actin, 5′-CCTTGCTCATCC TATCTGC-3′. At the end of the program, the specificity of the primer set was confirmed by melting curve analysis (65–95°C at a heating rate of 0.5°C/min). The copy number of *ZMVHA-B1* mRNA was estimated by referring to the results of real-time PCR carried out using several dilutions of *ZMVHA-B1*-containing plasmid as a template. The mRNA of actin was used to normalize the expression ratio of each gene in cDNAs obtained from different organs. To obtain more reliable results, all reactions were performed in triplicate.

**Southern blot hybridization** Genomic DNA (5 µg) of *Z. marina* was digested with two restriction endonucleases (*Eco*RI and *Bam*HI) and separated by 0.8% agarose gel electrophoresis, and separated products were capillary transferred to a Biodyne A nylon membrane (Pall, Ann Arbor, MI, USA). The procedure for nonradioactive blotting analysis was described previously (27). Hybridization was performed using a fluorescein-labeled probe directed against the coding region of *ZMVHA-B1* prepared using a Gene Images labeling kit (P2) (Amersham Biosciences) in a mixture containing 5× SSC, 0.1% SDS, and 5% blocking reagent (Amersham Biosciences), 5% dextran sulfate (D6001; Sigma, St. Louis, MO, USA) at 65°C (high-stringency conditions) or 55°C (low-stringency conditions) for 18 h. The hybridized membrane was finally washed with 0.2× SSC containing 0.1% SDS at 65°C (high stringency) or 55°C (low stringency) for 2×15 min. The signals were detected with a Gene Images Detection Kit (Amersham Biosciences) using an X-ray film (RX-U; Fuji Film, Tokyo).

**Yeast complementation** *S. cerevisiae* W303 (*MAT*α, *ade2*, *leu2*, *his3*, *met15*, *ura3*) was used as a control strain. W303-1B Δ*vma2* (*VMA2::URA3*), kindly provided by Dr. N. Nelson (Tel Aviv University, Israel), was used as the VHA subunit B null mutant. The Δ*vma2* strain was separately transformed with the pKT10 and pKT10*ZMVHA-B1* plasmids (*vma2*pKT10 or *vma2ZMVHA-B1*) by electroporation with a Gene Pulser Xcell (Bio-Rad, Hercules, CA, USA) according to Becker and Guarente (29). The transformant and the wild-type yeast cells were stained with LysoSensor green DND-189 (30) according to Nelson and Nelson (31), and observed under an Olympus BX60 fluorescence microscope. The yeast cells were grown at 28°C in YPAD (1% yeast extract, 2% peptone, 0.0075% l-adenine, and 2% glucose), YAGlc (2% yeast extract, 0.0075% l-adenine, and 2% glucose) and SD (synthetic medium containing 2% glucose) media. The solid media contained 2% agar. The pH of each medium was adjusted with 1 M HCl or 1 M NaOH.

To measure fluorescence intensity, the yeast mutant, transformant and wild-type cells were grown at 28°C in YPAD medium, harvested at the exponential phase, washed with SD medium and stained with LysoSensor green DND-189. Fluorescence intensity was measured using a FP-6500 spectrofluorometer (Jasco, Essex, UK) at an excitation of 443 nm and an emission of 505 nm.

**Membrane preparation** Membrane preparation was obtained according to the method of Goffeau and Dufour (32). Yeast cells were grown in 800 ml of YAGlc medium and harvested by centrifugation at the late exponential phase, washed three times with ice cold water and resuspended in a mixture containing 250 mM sorbitol, 1 mM $MgCl_2$, 50 mM imidazole (pH 7.5), 5 mM DTT and 1 mM PMSF. The yeast cells were disrupted in a French press under 15,000 psi. After disruption, subcellular fractionation, and purification, the membrane was resuspended in a solution containing 10 mM imidazol (pH 7.5) and 0.1 mM sodium orthovanadate, frozen in liquid nitrogen, and stored at −80°C until use. Protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA).

**ATPase assays** ATPase activity was assayed at 30°C in 100 µl of a reaction mixture containing 5 mM $ATPNa_2$ (Wako, Osaka), 5 mM $MgCl_2$, 25 mM Mes–KOH (pH 7.0), 10 mM sodium azide (a mitochondrial ATPase inhibitor), 0.2 mM sodium molybdate (a phosphatase inhibitor), 25 µM vanadate (a plasma membrane

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