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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

Abbas Alemzadeh,[1] Makoto Fujie,[1] Shoji Usami,[1] Tsukasa Yoshizaki,[2] Keiji Oyama,[2] Toyoki Kawabata,[2] and Takashi Yamada[1]*

Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan[1] *and Energia, Economic and Technical Research Institute, 3-9-1 Kagamiyama, Higashi-Hiroshima 739-0046, Japan*[2]

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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

* Corresponding author. e-mail: tayamad@hiroshima-u.ac.jp
phone/fax: +81-(0)82-424-7752

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

ATPase inhibitor), and 2 μg of proteins. The reaction was stopped after 17 min by adding of 60 μl of TCA, 30 μl of ammonium molybdate (in 2 M H_2SO_4), 30 μl of 1-amino-2-naphthol-4-sulfonic acid and 3% $NaHSO_3$. After 17 min, the absorbance of the solution is measured using a spectrophotometer (Amersham Biosciences) at 700 nm.

RESULTS

cDNA cloning of *ZMVHA-B1* from *Z. marina* and sequence analysis A cDNA clone encoding the entire VHA subunit B was isolated from a leaf cDNA library using the P1 probe as described in Materials and Methods. The nucleotide sequence determined for this clone revealed an ORF of 1464 bp encoding 488 amino acids (DDBJ accession no. AB248117) (Fig. 1A). The multiple alignment of the predicted amino acid sequences of plant VHAs from *Arabidopsis*, barley, *Citrus*, cotton, eelgrass, *Mesembryanthemum*, *Suaeda*, rice, tobacco and *S. cerevisiae* was performed using the ClustalW program to construct a phylogenetic tree (Fig. 1B). On the basis of this dendrogram, we found that ZMVHA-B1 was most closely related to GHVHA-B1, a VHA subunit B isoform from cotton (*Gossypium hirsutum*) (96%). The amino acid sequences of VHA subunit B are highly conserved among different plant species, but there is no specific similarity between ZMVHA-B1 and VHA subunit B of yeast (Fig. 1B). Incidentally, yeast protein (VMA2) is larger (515 amino acids) and distantly separated from the plant clusters.

Number of isoforms of vacuolar H^+-ATPase subunit B in eelgrass Genomic DNA of eelgrass was digested with different restriction enzymes and Southern blot hybridization was performed using the P2 probe containing the coding region of *ZMVHA-B1* (Fig. 1A) under low- and high-stringency conditions. When hybridization was performed under low-stringency conditions, a few hybridizing bands were detected (Fig. 2B), but under high-stringency conditions, only one band appeared (Fig. 2A). The strong band that appeared under high-stringency conditions corresponded to the intrinsic *ZMVHA-B1*, and other faint bands that appeared under low-stringency conditions may be due to similar genes (isoforms) of VHA subunit B, indicating a multigene family in eelgrass.

Comparison of expression levels of *ZMVHA-B1* in leaves, rhizomes and roots of eelgrass The expression of *ZMVHA-B1* was quantitatively studied by real-time PCR. cDNAs prepared from the leaves, rhizomes and roots of eelgrass were used as templates for real-time PCR. *ZMVHA-B1* was expressed in all organs: leaves, rhizomes and roots. The expression of *ZMVHA-B1* in leaves was two fold and 1.2-fold higher than that in rhizomes and roots, respectively (Fig. 3). The expression of *ZMVHA-B1* in the leaves of *Z. marina* at different concentrations of NaCl was quantitatively investigated using real-time PCR.

Functional complementation of yeast *vma2* null mutant with ZMVHA-B1 It was investigated whether ZMVHA-B1 is able to support the growth of yeast *vma2*

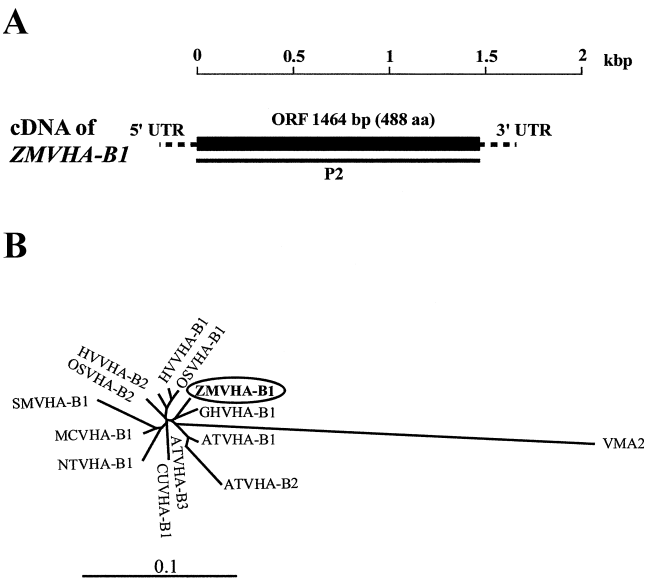

FIG. 1. (A) Schematic representation of *ZMVHA-B1* cDNA. The size and position of the probe that was used for Southern blot hybridization is shown (P2). (B) A phylogenetic dendrogram deduced from predicted amino acid sequences of 13 VHAs from the following plants: *Arabidopsis thaliana* (ATVHA-B1: AAC36485; ATVHA-B2: AAF88162; ATVHA-B3: BAD44171), *Hordeum vulgare* (HVVHA-B1: Q40078; HVVHA-B2: Q40079), *Citrus unshiu* (CUVHA-B1: BAA75517), *Gossypium hirsutum* (GHVHA-B1: Q43432), *Mesembryanthemum crystallinum* (MCVHA-B1: CAD27443), *Nicotiana tabacum* (NTVHA-B1: AAF26445), *Oryza sativa* (OSVHA-B1: AAK54617; OSVHA-B2: BAB89101), *Suaeda maritima* (SMVHA-B1: AAO73463), *Z. marina* (ZMVHA-B1: this study), *S. cerevisiae* (VMA2, AAA66890).

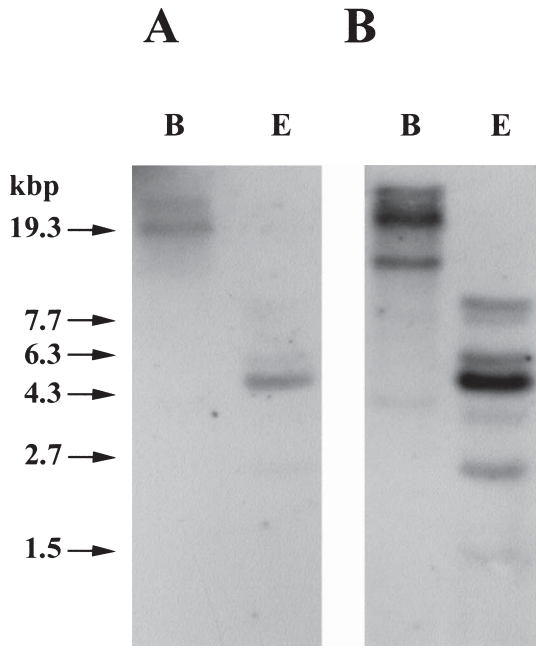

FIG. 2. Southern blot analysis of eelgrass (*Z. marina*) genomic DNA using probe of *ZMVHA-B1*. Genomic DNA (5 μg) digested with *Bam*HI (lane B) or *Eco*RI (lane E) was separated by 0.8% agarose gel electrophoresis, and separated products were transferred to a nylon membrane and hybridized with the P2 probe (Fig. 1A). Hibridization and washing were performed at 65°C (A) and 55°C (B). A few faint bands are noted under low-stringency conditions (B), indicating the presence of a small gene family.

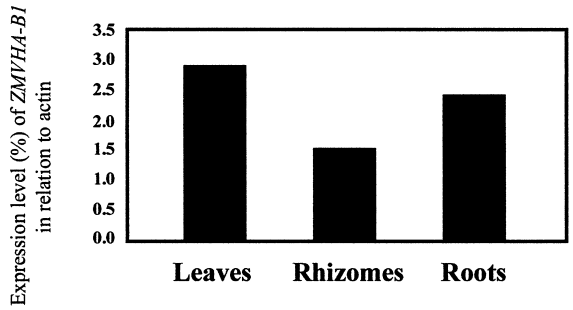

FIG. 3. Organ-specific expression of *ZMVHA-B1*. cDNAs prepared from leaves, rhizomes and roots were used as templates for real-time PCR.

null mutant cells at pHs values higher than 6.5. For the expression and functional complementation of *ZMVHA-B1*, the haploid *S. cerevisiae vma2* null mutant, lacking genomic copies of VHA subunit B, was used as a host. The mutant grows optimally in a rich medium at pH 5.5, grows much slower at lower pH, and cannot grow in a medium having a pH higher than 6.5. It was suggested that within only such a narrow pH range, the mutant can counteract the lack of vacuolar acidification by VHA by utilizing the external low pH, perhaps a mechanism of fluid-phase endocytosis (31).

The yeast mutant cells were transformed as described in Materials and Methods. Cells of mutant *vma2* and *vma2*pKT10 could not grow at pH 7.5, whereas *vma2ZMVHA-B1* cells were able to grow at a higher pH as did W303 cells (Fig. 4A), indicating that the yeast cells recovered their VHA function, namely, the subunit B of eelgrass could be successfully expressed, incorporated into the yeast V_1 sector at the right position and exert its function. The growth rate of the transformant *vma2ZMVHA-B1* cells was comparable to that of W303 in YPAD medium at pH 7.5 (data not shown). To confirm the actual recovery of VHA activity, the membrane of yeast cells was prepared and H^+-ATPase activity was measured in the presence of appropriate specific inhibitors. Net VHA activities obtained were 2.9, 1.7, and 3.1 (μmolPi/min/mg protein) for strains W303, *vma2* and *vma2ZMVHA-B1*, respectively, indicating that VHA activity was recovered at the wild-type level (even higher) in the transformant.

Cytologically or morphologically, this restoration was also confirmed: The yeast mutant cells had smaller vacuoles than the wild-type cells and did not accumulate LysoSensor green DND-189 in the vacuoles (Fig. 4B, C). The cDNA clone *ZMVHA-B1* derived from *Z. marina* and expressed in yeast cells restored vacuole morphology; in transformed cells, large vacuoles were observed with a morphology indistinguishable from that of wild-type cells (Fig. 4C). The average sizes of vacuoles were 2.4 ± 0.65 μm (n=32), 1.6 ± 0.9 μm (n=29), and 3.0 ± 1.31 μm (n=32) for strains W303, *vma2*, and *vma2ZMVHA-B1*, respectively. The vacuoles of transformant cells showed efficient accumulation of the dye, indicating a normal activity of the VHA (Fig. 4B). Fluorescence emission with LysoSensor green DND-189 measured for strains W303, *vma2*, and *vma2ZMVHA-B1* was 610.0, 316.3 and $805.7/2.3 \times 10^6$ cells, respectively.

This is the first report indicating that VHA subunit B from a plant is able to replace that of yeast.

DISCUSSION

Recently, the eelgrass *Z. marina* has attracted public attention because of its environmental and ecological importance. *Z. marina* beds are important for sediment deposition, for substrate stabilization, as substrate for epiphytic algae and microinvertebrates, and as nursery grounds for many species of economically important fish and shellfish. For increasing productivity and improving polluted environments of bay areas, the sustainable cultivation of eelgrass is a matter of concern (33). For this purpose, molecular biological and physiological information about the growth of this plant is invaluable. In addition, the salt resistance of *Z. marina*, which allows the plant to thrive in seawater with a salinity range of 0.5–3.3%, is also attractive for use in the plant breeding. Genes involved in the salt-resistance mechanisms of halophytes can be good candidates for improving important salt-sensitive crops for cultivation in salt environments. Particularly in breeding halo-tolerant rice cultivars, eelgrass genes conferring salt resistance will be very useful. Genome information of rice, which is currently available (http:/rgp.dna.affrc.go.jp/Analysis.html), may help advance molecular studies of eelgrass genes.

To enhance the isolation of eelgrass genes conferring salt resistance, we applied the heterologous expression of the genes in salt-sensitive mutant yeast cells. Many clones have been so far obtained including genes for plasma membrane H^+-ATPase ZHA1 and ZHA2 (26), putative plasma membrane Na^+/H^+ antiporter, vacuolar Na^+/H^+ antiporter and other unknown proteins. Among them, a clone, *ZMVHA-B1*, was characterized in this work to encode a functional B subunit of vacuolar H^+-ATPase. Although the amino acid of ZMVHA-B1 was not specifically similar to the yeast protein (Fig. 1B), *ZMVHA-B1* expressed in the yeast *vma2* mutant successfully replaced the yeast mutant protein and formed the functional VHA complex. The VHA activity of the transformant was even slightly higher than that of the wild-type. Because of the recovered activity of VHA, large vacuoles were also recovered in the transformant cells (Fig. 4C), which have even a stronger fluorescence intensity with LysoSensor green DND-189 than the wild-type vacuoles, indicating stable and adequate functioning of the composite VHA. In *vma2* mutant cells, vacuoles became very small and vesicles are abundant in the cytoplasm (Fig. 4C). Previously, the fluid phase endocytosis mechanism was proposed for the growth of this mutant in a medium at a narrow pH range from 5.5 to 6.5 (31). In this mechanism, the fluid with external pH conditions is taken up by endocytosis vesicles and transported to vacuoles via trans Golgi vesicles. As shown in Fig. 4C large vacuoles were obvious in the transformant, confirming the overall restoration of the yeast cellular system.

In this study, we successfully demonstrated the complementation of yeast mutation in a subunit of a large protein complex such as VHA by introducing an eelgrass gene. This success encourages us to apply the method of plant gene screening using yeast mutants, particularly genes conferring salt resistance or salt tolerance. Although the possible con-

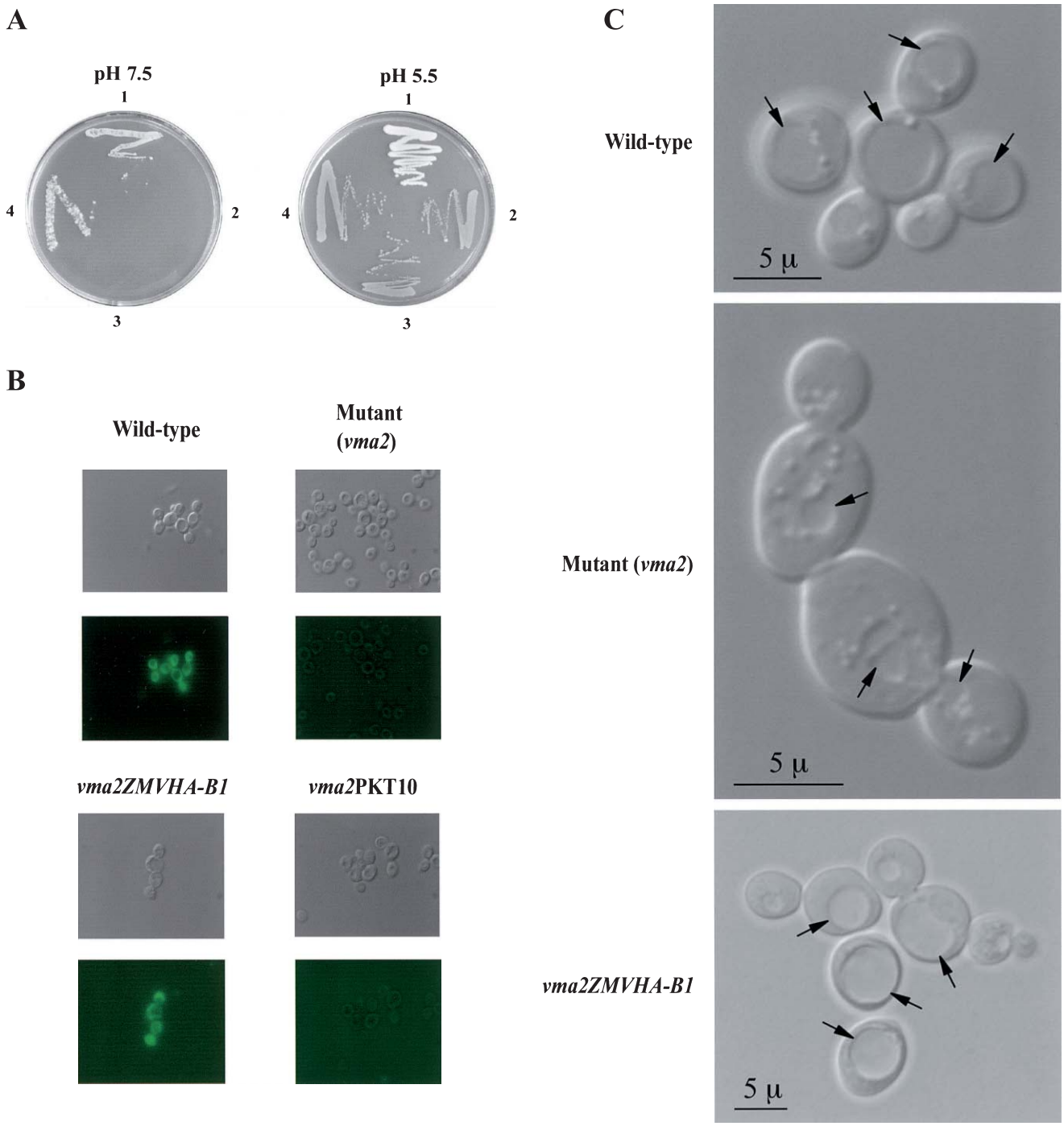

FIG. 4. (A) Functional complementation of yeast *vma2* null mutant *ZMVHA-B1*. The growths of wild-type W303, *vma2*pKT10, *vma2ZMVHA-B1*, and *vma2* null mutant at pHs 5.5 and 7.5 were compared. W303 and *vma2ZMVHA-B1* cells were able to grow in YPAD medium at pH 7.5, but yeast *vma2* null mutant and *vma2*pKT10 cells were not able to grow at this pH. (B) Nomarski images (top panels) and LysoSensor green DND-189 fluorescence images (bottom panels) of wild-type, mutant and transformant yeast cells. 1, W303; 2, *vma2* mutant; 3, *vma2*pk10; 4, *vma2ZMVHA-B1*. (C) Comparison of size and morphology of vacuoles among wild-type, mutant, and transformant yeast cells. Vacuoles are indicated by arrows.

tribution of eelgrass VHA to the salt resistance of this plant was not examined in this study, heterologous expression in the yeast will allow the efficient study of individual plant functions.

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