Arabidopsis Vacuolar H\(^+\)-ATPase (V-ATPase) B Subunits Are Involved in Actin Cytoskeleton Remodeling via Binding to, Bundling, and Stabilizing F-actin*

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Vacular H\(^+\)-ATPase (V-ATPase) is a membrane-bound multishubunit enzyme complex composed of at least 14 different subunits. The complex regulates the physiological processes of a cell by controlling the acidic environment, which is necessary for certain activities and the interaction with the actin cytoskeleton through its B and C subunits in both humans and yeast. Arabidopsis V-ATPase has three B subunits (AtVAB1, AtVAB2, and AtVAB3), which share 97.27% sequence identity and have two potential actin-binding sites, indicating that these AtVABs may have crucial functions in actin cytoskeleton remodeling and plant cell development. However, their biochemical functions are poorly understood. In this study, we demonstrated that AtVABs bind to and co-localize with F-actin, bundle F-actin to form higher order structures, and stabilize actin filaments \textit{in vitro}. In addition, the AtVABs also show different degrees of activities in capping the barbed ends but no nucleating activities, and these activities were not regulated by calcium. The functional similarity and differences of the AtVABs implied that they may play cooperative and distinct roles in \textit{Arabidopsis} cells.

Vacular H\(^+\)-ATPase (V-ATPase)\(^4\) is a highly conserved, ATP-driven proton pump that is functionally and structurally related to the mitochondrial and chloroplast F-ATPase (1–3). V-ATPase is widely distributed in eukaryotic and prokaryotic cells and is responsible for proton transport across the plasma membrane and for the acidification of different intracellular organelles, including phagosomes, early endosomes, lysosomes, synaptosomes, the Golgi apparatus, clathrin-coated vesicles, dense core secretory granules, and plant vacuoles (4–6). In these organelles, V-ATPase is a multisubunit complex consisting of a water-soluble V1 subcomplex located in the cytosol, which catalyzes ATP binding and hydrolyzing, and a membrane-embedded V0 subcomplex containing the proton translocation pore that transports protons (6, 7). The peripheral V1 contains at least eight different subunits of molecular masses ranging between 13 and 70 kDa (subunits A, B, C, D, E, F, G, and H), whereas the membrane-integral V0 contains at least six different subunits of molecular masses ranging between 17 and 100 kDa (subunits a, c, c', c'', d, and e) (6–9).

Recent studies have indicated that V-ATPase is directly involved in interactions with the actin cytoskeleton through actin-binding sites located in the N-terminal domain of subunit B (10–12) and in two domains of subunit C (13, 14). The B subunit N-terminal domains of plants, mammals, and yeast have a profilin-like motif that includes several conserved Gly, Leu, Val, Asp, and Phe residues (6, 10, 11) and conserved L(K/R)XXE(S/T)-like motifs. In the latter motifs, the conserved Leu, Arg, and Ser residues are identical to the L(K/R)XXE(S/T) motifs of several actin-binding proteins, including Dp71, PKCε, α-actinin, actobindin, and tropomyosin, in which Leu and Arg (or Lys) are crucial residues that can directly interact with the N terminus of actin (6). Thus, the V-ATPase B subunit may be involved in the dynamics of the actin cytoskeleton.

The actin cytoskeleton is a highly organized and dynamic structure present in all eukaryotic cells, where it plays a central role and is involved in numerous cellular processes, including intracellular transport, cell growth, cell division, cytoplasmic streaming, organelle positioning, and cell-to-cell communication (15–19). For such processes, a regulatory system is required that contains actin filaments and actin-binding proteins (ABPs) that directly interact with G-actin and/or F-actin to promote the nucleation, polymerization, depolymerization, stabilization, severing, capping, bundling, and cross-linking of actin filaments (20–23). Recently, some ABPs were proved to be involved in actin cytoskeleton remodeling, including \textit{Arabidopsis} FIM5 (24), \textit{Arabidopsis} VLN5 (25), tobacco WLIM1 (26), \textit{Arabidopsis} LIM proteins (27), \textit{Arabidopsis} VLN4 (28), and rice formin (29). These proteins have different activities in dynamic actin cytoskeleton remodeling and are involved in cell growth and development. In \textit{Arabidopsis} \textit{thaliana}, three V-ATPase B subunits (AtVAB1, AtVAB2, and AtVAB3) (30–34) are expressed in different tissues and may play various functions in growth, differentiation, development, adaptation to changing environmental conditions, and maintenance of cell morphology (32, 35, 36). Nevertheless, how the B subunits of V-ATPase interact with actin filaments and regulate dynamic actin cytoskeleton remains poorly understood.

To understand the biochemical functions of AtVABs \textit{in vitro}, we first analyzed their structures based on homology molecular

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\* The abbreviations used are: V-ATPase, vacuolar H\(^+\)-ATPase; AtVAB, \textit{A. thaliana} V-ATPase B subunit; ANVHA-B\(_{5}\), \textit{A. thaliana} vacuolar H\(^+\)-ATPase subunit genes, including AtVHBA-B\(_1\) (At1g76030), -B\(_2\) (At4g38510), and -B\(_3\) (At1g20620); ABPs, actin-binding proteins; F-actin, filamental actin; G-actin, global actin; LatB, latrunculin B; TRITC, tetramethylrhodamine isothiocyanate.

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modeling. The results indicated that polypeptides shared highly similar structures; however, an unconserved region and a novel residue among these subunits were found, suggesting that AtVABs may have overlapping and distinct in vitro biochemical functions and in vivo physiological activities. The recombinant proteins were expressed in bacteria for the biochemical and microscopic analysis of their functions. Our findings strongly suggested that AtVABs may act as actin-binding proteins and that they shared similar functional features with other reported ABPs.

**EXPERIMENTAL PROCEDURES**

**Materials—Arabidopsis** Columbia wild-type plants were used for total RNA extraction and cloning of the AtVAB genes. Actin was isolated from an acetone extract from rabbit skeletal muscle according to previous methods (37). Monomeric actin was dialyzed in Buffer G (5.0 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.5 mM DT, 0.2 mM CaCl₂, and 0.01% sodium azide). Pyrene-actin was prepared by labeling actin at Cys⁷⁷⁴ with pyrene iodacetamide as described previously for kinetic analyses (38). Human recombinant profilin I was purified as described previously (39). These proteins were frozen in liquid nitrogen and stored at −80 °C.

**Preparation of Recombinant AtVABs and Specific Anti-AtVAB Antibody—Total Arabidopsis RNA was extracted from** flowers using the Total RNA Isolation kit (TianGen). The cDNA coding sequences of AtVAB1, AtVAB2, and AtVAB3 were amplified by RT-PCR using the specific primer pairs, B1F/B1R, B2F/B2R, and B3F/B3R (Table 1). The amplified fragments were cloned into the pMD19-T vector (TaKaRa), and the recombinant proteins were expressed in **Escherichia coli** BL21 (DE3) by induction with 0.5 mM isopropyl 1-thio-b-D-galactopyranoside overnight at 25 °C. Recombinant AtVABs were affinity-purified using glutathione-Sepharose 4B resin (GE Healthcare) (the wash buffer was PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄·12H₂O, 1.8 mM KH₂PO₄, pH 7.4) and, with the proteins, were eluted with 50 mM Tris-HCl (pH 8.0) and 10 mM reduced glutathione. The purified proteins were concentrated in a centrifugal filter (Millipore), buffer-exchanged (10 mM Tris-HCl, 0.2 mM CaCl₂, 0.5 mM DT, and 0.2 mM ATP, pH 7.4) using a 14 kDa molecular mass cut-off dialysis cassette (Pierce), and stored on ice. Prior to any experiments, the proteins were preclarified further by centrifugation at 100,000 × g for 1 h.

The purified recombinant AtVAB2 was used as an antigen to raise anti-AtVAB polyclonal antibodies in rabbit serum, as described by Huang et al. (40). The anti-AtVAB polyclonal antibody was purified using an octanoic acid-saturated ammonium sulfate method. Rabbit antisera (2 ml) was diluted 4-fold with 0.06 M HAc-NaAc buffer (pH 4.8), and octanoic acid was added dropwise into the diluted antisera, whose final concentration reached 75 μl/ml; the mixture was stirred for 30 min. The mixture was centrifuged at 12,000 rpm for 20 min at room temperature; 0.01 M PBS (pH 7.4) was added to the supernatant, and then isopycnic saturated ammonium sulfate (pH 7.4) was gently added with stirring. The mixture was centrifuged at 12,000 rpm for 30 min at 4 °C and allowed to stand for 2 h. The pellet was dissolved and dialyzed in 0.01 M PBS (pH 7.4) overnight at 4 °C. The purified anti-AtVAB2 antibody was used for Western blot analysis.

**Immunofluorescence Labeling—In vitro immunofluorescence labeling was employed to test whether the AtVABs co-localized with F-actin. Prepolymerized F-actin (3.0 μM) was incubated with 2.0 μM AtVAB1, AtVAB2, and AtVAB3, respectively, for 1 h at 22 °C. Alexa-488 phalloidin-stabilized F-actin and TRITC-conjugated goat anti-rabbit IgG (H+L) (1:200)–stained AtVABs were visualized using a Leica DFC420C microscope equipped with a ×100 objective and a CCD camera. The F-actin and TRITC-conjugated goat anti-rabbit IgG alone were included as negative controls. Merged images were acquired using Image-Pro Plus 6.0 (version 6.0.0.260; Media Cybernetics).

**High and Low Speed Co-sedimentation Assays—High and low speed co-sedimentation assays were used to assess the actin-binding, -bundling, and -stabilizing activities of the AtVABs.**

The high speed co-sedimentation assays were initially employed to determine the activity of AtVAB binding to F-actin, as described by Khurana et al. (41). Preassembled F-actin (5.0 μM) was incubated with 0.5 or 2.0 μM AtVABs, GST (as a negative control) or ABP29 (as a positive control) (42) for 1 h at 22 °C, and the samples were centrifuged at 100,000 × g for 1 h at 4 °C (CS120GXII, Hitachi). After centrifugation, the supernatant and pellet fractions were resolved on SDS-polyacrylamide gels and stained with Coomassie Brilliant Blue. The intensities of the resulting bands were quantified densitometrically using Quantity One software (version 4.6.2; Bio-Rad). For the statistical analysis to characterize the ability of the AtVABs to bind to F-actin, we used Student’s t-test of SPSS Statistics 17.0.

Additionally, high speed co-sedimentation assays were also employed to assess the F-actin-stabilizing activity of the AtVABs with a 20 μM LatB treatment. Preformed F-actin (5.0 μM) was incubated with 0, 0.5, or 5.0 μM AtVABs and GST (as a negative control) at 22 °C for 1 h prior to treatment with 20 μM LatB for 6 h. As another negative control, the F-actin was incubated and treated without the AtVABs and LatB. As a positive control, the F-actin was incubated without the AtVABs but treated with 20 μM LatB. The samples were centrifuged at 100,000 × g for 1 h, and the resulting pellets and supernatants were analyzed by SDS-PAGE. After quantification, the results were expressed as the percentage of actin in the supernatant as a function of the AtVAB concentration. Statistical analysis was performed as described above.

**Low speed sedimentation experiments were used to assess the actin bundling activity of the AtVABs.** Preassembled F-actin (5.0 μM) was incubated with 0, 0.5, or 2.0 μM AtVABs and ABP29 (as a negative control) (42) in the presence of 200.0 μM calcium for 1 h at 22 °C. The samples were centrifuged at 13,500 × g for 30 min in a microcentrifuge at 4 °C to pellet the F-actin. The presence of actin in the resulting supernatants (nonbundled F-actins) and pellets (bundled F-actins) was analyzed by SDS-PAGE as described above. After quantification,
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**TABLE 1**

| Primer name | Primer sequence | Purpose and restriction sites |
|-------------|-----------------|------------------------------|
| B1p         | GGTACCGGATCCATGGGACGATGATCTCGACAT | AtVAB1 cds for. KpnI/BamHI |
| B1a         | GACTCTTAAATGGTGGACGATCTCGACAT | AtVAB1 cds rev. SacI |
| B2p         | GATACCGGATCCATGGGACGATCTCGACAT | AtVAB2 cds for. BamHI |
| B2a         | GACTCTTAAATGGTGGACGATCTCGACAT | AtVAB2 cds rev. SacI |
| B3p         | GGTACCGGATCCATGGGACGATCTCGACAT | AtVAB3 cds for. KpnI/BamHI |
| B3a         | GACTCTTAAATGGTGGACGATCTCGACAT | AtVAB3 cds rev. SacI |

The restriction enzyme sites are underlined. Start and stop codons are highlighted by boldface letters. F and for., forward primer; R and rev., reverse primer.

The results were expressed as the percentage of actin in the pellet as a function of the AtVAB concentration.

**Fluorescence Microscopy Visualization of Actin Filaments and Bundles**—Fluorescence microscopy was used to visualize the bundling activity of the AtVABs. Prepolymerized actin (3.0 μM) was incubated with or without 2.0 μM AtVABs for 1 h and labeled with 200 nM Alexa-488 phalloidin for 5 min at room temperature. The actin filaments were then diluted 10-fold with 1× F buffers (10× stock: 50 mM Tris-HCl, 5 mM ATP, 10 mM DTT, 0.5 mM KCl, and 50 mM MgCl₂). A 1-μl aliquot of the diluted sample was used to visualize the actin filaments and bundles. Micrographs of the filament bundles were obtained using a Leica DFC420C fluorescence microscope equipped with a ×100 objective, a CCD camera, and Leica Application Suite software.

**Actin Polymerization Assay**—Actin nucleation assays were essentially employed according to a method described previously (42). Monomeric actin (5.0 μM; 5% pyrene-labeled) was incubated with various concentrations of AtVABs for 5 min in the presence of 0.1 μM (treated with 1 mM EGTA) (42, 43), 10.0 μM (treated with 0.2 mM EGTA) (42, 43), or 200.0 μM free Ca²⁺ at room temperature. The polymerization of actin filaments was detected for 400 s by pyrene fluorescence with a FluoroMax®-4 spectrofluorometer immediately after the addition of one-tenth volume of 10× F buffers. The experiments were repeated at least three times under the same conditions.

**Barbed End-capping Assay**—To determine whether AtVAB1, AtVAB2, and AtVAB3 cap the barbed end of actin filaments, a seeded elongation assay was performed as described previously (25). Preformed F-actin at a final concentration of 0.4 μM was incubated with various concentrations of the AtVABs in the presence of 0.1 μM (treated with 1 mM EGTA) (42, 43), 10.0 μM (treated with 0.2 mM EGTA) (42, 43), or 200.0 μM free Ca²⁺ for 5 min at room temperature. Then 1 μM G-actin (5% pyrene-labeled) saturated with 4 μM human profilin 1 was added to the actin filament mixture. One-tenth volume of 10× F buffer was added to initiate actin elongation at the barbed end of the actin filaments. The time course of actin elongation was tracked by monitoring the increase of pyrene fluorescence with a FluoroMax®-4 spectrofluorometer, with the excitation wavelength set at 365 nm and emission wavelength at 407 nm after the actin elongation was initiated.

**Actin Filament Depolymerization Assay**—To test the effect of the AtVABs on actin depolymerization, a depolymerization assay was performed, roughly according to a described method (42). Preassembled 5 μM F-actin (50% pyrene-labeled) was incubated with various concentrations of the AtVABs in the presence of 200.0 μM free Ca²⁺ for 5 min at room temperature. Depolymerization was initiated by diluting the mixtures 25-fold with low ionic strength buffer G (5.0 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM CaCl₂, and 0.01% sodium azide). The decrease in pyrene fluorescence intensity accompanying the actin depolymerization was monitored over 400 s at room temperature using a FluoroMax®-4 spectrofluorometer (HORIBA Jobin Yvon). The experiments were repeated at least three times under the same conditions.

**RESULTS**

**Binding and Co-localization between AtVABs and Actin Cytoskeleton in Vitro**—Using the primer pairs B1p/B1a, B2p/B2a, and B3p/B3a (Table 1), the full-length cDNAs of AtVAB1, AtVAB2, and AtVAB3 were generated by RT-PCR. We found that the three subunits share high sequence identity (prediction identity = 97.27%) and that there was a conserved profilin-like actin-binding motif within each subunit (Fig. 1A), which shared a high identity with previously reported motifs (6, 10, 11). Several conserved residues (Gly³⁷, Leu³⁹, Val⁴⁰, Asp⁴³, and Lys⁴⁴ in AtVAB1 and AtVAB3, Gly³⁸, Leu⁴⁰, Val⁴¹, Asp⁴⁴, and Lys⁴⁵ in AtVAB2) were also found in yeast and humans. We considered that the AtVABs can bind to F-actin through their profilin-like motifs located on the N-terminal β-barrel domain, an actin-binding region composed of 44 amino acids that are required for high affinity F-actin binding (11) (Fig. 1A). The recombinant AtVABs and rabbit skeletal muscle actin were purified for the investigation of the interaction of AtVABs with F-actin (filamentous actin) (Fig. 1B).

To test the hypothesis of the AtVABs binding to F-actin, a high speed co-sedimentation assay was performed in the presence of 0.5 and 2.0 μM AtVAB1, AtVAB2, and AtVAB3, respectively (Fig. 1C). The results showed that AtVAB1, AtVAB2, and AtVAB3 bound to and co-sedimented with actin filaments (5.0 μM) (when centrifuged at 100,000 g at 4°C for 1 h) in a dose-dependent manner but that their abilities to bind to F-actin were similar. ABP29, a positive control, bound to F-actin as reported previously (42) (Fig. 1D), indicating that the AtVABs can bind to F-actin with similar affinity in vitro.

To determine whether the AtVABs and actin filaments co-localize, immunofluorescence labeling experiments were employed in vitro. The co-localization between the AtVABs and actin filaments was observed when the AtVABs were incubated with Alexa-488 phalloidin-stabilized F-actin (shown in green) and then labeled with an anti-AtVAB antibody followed by TRITC-conjugated goat anti-rabbit IgG (shown in red) (Fig. 1E). No co-localization was detected when labeling was performed with the secondary antibody alone (data not shown). Taken together, these results suggested that AtVAB1, AtVAB2,
and AtVAB3 cap the barbed end of actin filaments in Ca\(^{2+}\)-insensitive manner. Many ABPs with capping activity bind to the barbed end of actin filaments with high affinity and prevent subunit addition and loss (44, 45). To determine whether AtVAB1, AtVAB2, and AtVAB3 cap the barbed end of actin filaments, a seeded actin elongation assay was performed (40, 46). Preformed F-actin (0.4 \( \mu M \)) was incubated with varying concentrations of AtVAB1, AtVAB2, and AtVAB3 for 5 min, and polymerization was initiated with the addition of 1 \( \mu M \) G-actin (5% pyrene-labeled) at a free Ca\(^{2+}\) of 0.1, 10.0, and...
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The G-actin was saturated with 4 mM human profilin I to suppress nucleation and the addition of actin to the pointed ends of the filaments, which permits the addition at the barbed ends unless they are capped (46). As shown in Fig. 4A-4C, AtVAB1, AtVAB2, and AtVAB3 decreased the initial rate of actin elongation, confirming their barbed end-capping activity, in which AtVAB1 and AtVAB2 capped the barbed ends of F-actin in a dose-dependent manner, and AtVAB3 had a marked capping activity at various concentrations. As reported previously (42), ABP29 capped the barbed ends of F-actin when present at a low concentration (0.1 mM). Compared with the assays in the presence of 200.0 μM free Ca2+, the activities of 0.5 μM AtVABs capping the barbed ends of F-actin were not regulated in the presence of 0.1 and 10.0 μM Ca2+.

FIGURE 2. AtVAB1, AtVAB2, and AtVAB3 bundle actin filaments in vitro. A, a low speed co-sedimentation assay was performed to determine the bundling activity of the AtVABs. Prepolymerized actin (5.0 μM) was incubated for 1 h with 0.5 and 2.0 μM AtVAB1, AtVAB2, AtVAB3, and ABP29 (as a negative control), respectively. Then the mixtures were centrifuged at 13,500 × g for 1 h to separate the bundled versus unbundled actin filaments. After centrifugation, equal amounts of supernatant (Sup) and pellet (Pel) were separated by SDS-PAGE. B, AtVABs bundled actin filaments in a dose-dependent manner, but ABP29 had no bundling actin filament activity. The graph shows the percentage of sedimented actin in A (n = 3). Bars, mean ± S.E. (error bars) based on Student’s t test. C-F, fluorescence microscopy was performed to determine the bundling activity of AtVAB1, AtVAB2, and AtVAB3. F-actin (3.0 μM) was incubated for 1 h with 2.0 μM AtVAB1, AtVAB2, and AtVAB3, respectively, and subsequently labeled with 1 μM Alexa-488 phalloidin for 5 min at room temperature. Micrographs of actin bundles captured using fluorescence microscopy showed filament bundles. C, micrographs of actin filaments in the absence of AtVAB1, AtVAB2, and AtVAB3. D–F, micrographs of actin filaments in the presence of 2.0 μM AtVAB1, AtVAB2, and AtVAB3, respectively.

FIGURE 3. Effect of AtVABs on actin filament polymerization in a Ca2+-insensitive manner. Actin polymerization was performed with 5.0 μM actin (5% pyrene-labeled actin) in the presence of various concentrations of AtVABs (0, 0.5, 1.0, and 2.0 μM), and various concentrations of free Ca2+ (0.1, 10.0, and 200.0 μM). ABP29 (0.5 μM) was used as a positive control. The time course of actin polymerization was recorded by measuring the pyrene fluorescence (arbitrary units (a.u.)), which was plotted versus time after the addition of polymerization salts to initiate the polymerization. A–C, various concentrations of AtVABs and ABP29 were incubated for 5 min with 5.0 μM actin (5% pyrene-labeled actin) in the presence of 200.0 μM free Ca2+. D–F, AtVABs (2.0 μM) were incubated with 5.0 μM F-actin (5% pyrene-labeled actin) for 5 min in the presence of 0.1, 10.0, and 200.0 μM free Ca2+.
In this assay, actin depolymerization assay using pyrene-labeled F-actin (26). The samples were centrifuged at 100,000 \(20^\text{g}\), whereas 40% of the actin was detected in the supernatant with the supernatant of the control sample without LatB treatment, shown in Fig. 5A and expressed as a percentage of the total actin (Fig. 5B). The amounts of actin in the resulting supernatants were quantified and subsequently submitted to LatB treatment for 6 h (Fig. 5A). As shown in Fig. 5B, less than 10% of the actin was detected in the supernatant of the control sample without LatB treatment, whereas 40% of the actin was detected in the supernatant with 20 \(\mu M\) LatB treatment in the absence of AtVABs. The percentage of actin in the supernatant was significantly decreased proportionally in the presence of 0.5 and 5.0 \(\mu M\) AtVAB1, AtVAB2, and AtVAB3 after 6 h of LatB treatment. Interestingly, AtVAB1 and AtVAB3 inhibited the depolymerization of the actin filaments induced by the LatB treatment in a dose-dependent manner, but AtVAB2 did not. As a negative control, GST did not inhibit the depolymerization of the actin filaments induced by LatB (Fig. 5, A and B). These results indicated that AtVAB1, AtVAB2, and AtVAB3 have distinct stabilizing activities on actin filaments in vitro.

The effect of AtVABs on the actin filament depolymerization kinetics was also investigated by a dilution-mediated actin depolymerization assay using pyrene-labeled F-actin (26). In this assay, AtVAB1 and AtVAB3 inhibited depolymerization significantly (Fig. 5, C and E), whereas AtVAB2 weakly inhibited actin depolymerization (Fig. 5D). In all of these assays, 0.5 \(\mu M\) ABP29 consistently markedly inhibited the actin depolymerization, as reported previously (42). These results further indicated that AtVAB1, AtVAB2, and AtVAB3 have different activities for stabilizing actin filaments in vitro.

Taken together, these data suggested that actin polymerization/depolymerization may be regulated by AtVAB1, AtVAB2, and AtVAB3 to varying degrees in vivo.

**DISCUSSION**

V-ATPases are ubiquitous proton pumps in eukaryotic cells that are responsible for the acidification of compartments of the endocytic and exocytic pathways (7). Previous reports indicated that V-ATPase may interact with microfilaments via both the B (10, 11) and C subunits (13, 14). The V-ATPases bind to F-actin (microfilaments) through actin-binding domains on the C subunit B in mammalian cells (10), tobacco hornworm cells (13), and yeast cells (47). In mammalian and yeast cells, the B subunit was inhibited when these 11 amino acids were deleted (10, 48). To date, it is not known whether the Arabidopsis V-ATPase B subunits also interact with F-actin.

AtVABs Share Overlapping Activities of Binding to and Bundling Filamentous Actin and Capping Barbed Ends of Actin Filaments—We analyzed the sequences of the V-ATPase B subunits from mammals, yeast, and Arabidopsis and found that there were two potential actin-binding domains, a profilin-like motif (6, 10, 11, 14) and L(K/R)XXE(S/T)-like motifs (6), among these B subunits. Therefore, we can propose a logical hypothe-
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siss of AtVAB binding to F-actin. Our data also suggest that each of the AtVABs can bind to actin filaments and that these subunits were co-localized along the elongating direction of F-actin.

The conservation of actin-binding domains in AtVABs allowed us to predict that they may perform conserved actin-related functions, such as binding, bundling, capping, and stabilizing activities. F-actin bundling is generally mediated by proteins or protein complexes containing multiple actin-binding domains or two discrete actin-binding domains separated by longer distance (20). In fact, our low speed co-sedimentation and fluorescence microscopy assays unambiguously demonstrated that the AtVABs bundled F-actin and induced the formation of higher order actin structures.

Previous reports indicated that several classes of ABPs are known to control filament barbed ends both in vitro and in vivo (40, 49) and stabilize actin filaments by preventing subunit loss or addition at that end. These ABPs regulate the addition and loss of subunits from either the barbed (plus) or pointed (minus) ends of filaments; nucleating proteins initiate efficient actin polymerization and create new barbed ends for assembly. To determine whether AtVABs cap actin filaments, a seeded actin elongation assay was employed. The results demonstrate that the capping of the filament barbed end activities of AtVAB1, AtVAB2, and AtVAB3 were not regulated by free calcium.

In plant cells, the formation of higher order actin structures, such as bundles and cables, is crucial to stabilize the organization of transvacuolar strands and maintain the overall cellular architecture (50). As mentioned above, a subset of overlapping activities of AtVABs may be involved in stabilizing long actin filaments. This result prompted us to investigate whether AtVABs stabilize actin filaments directly. In vitro LatB treatment experiments and pyrene fluorescence assays indicated that AtVABs stabilize actin filaments/bundles against LatB with distinct activity. Our data suggest that the actin filament-stabilizing activity may be regulated by AtVAB1, AtVAB2, and AtVAB3 to varying degrees in vivo.

Molecular Bases of Distinct Activities of AtVABs—The results from our investigation on the activities of AtVAB1, AtVAB2, and AtVAB3 in the regulation of actin cytoskeleton remodeling demonstrated differences among AtVAB1, AtVAB2, and AtVAB3, despite the fact that these proteins share a high sequence identity (identity = 97.27%). We found a novel residue (Phe81 in AtVAB1 and AtVAB3 and Tyr82 in AtVAB2) in the N-terminal β-barrel domain (Fig. 6A) and an unconserved region in the central α/β domains (Fig. 6B) that may result in the distinct activities of the AtVABs.

To test this hypothesis, we reconstructed the three-dimensional structures of AtVAB1, AtVAB2, and AtVAB3 based on the putative amino acid sequences by homology comparative
modeling. To confirm structural differences of the sites and regions, the structures of AtVAB1, AtVAB2, and AtVAB3 were superimposed onto each other (Fig. 6C); the results indicated two distinct structural differences at the novel residues and the unconserved regions (Fig. 6, D–G). Compared with AtVAB1 and AtVAB3, the structure of AtVAB2 displayed a great alteration due to Tyr (Fig. 6E); in addition, the unconserved regions created distinct structures (Fig. 6G). These data suggested that the structural differences among AtVAB1, AtVAB2, and AtVAB3 might form the basis for their distinct activities in the regulation of actin cytoskeleton dynamics.

The analysis of the expression level of the AtVHA-B1, -B2, and -B3 genes using the e-FP Browser indicated that the three genes are expressed in all tissues in every developmental stage (51); however, the expression level of AtVHA-B2 is lower than the other two. Specifically, in cauline leaves, rosette leaf 2, and stem (second internode), the GeneChip operating software expression signal of AtVHA-B2 was markedly lower than AtVHA-B1 and AtVHA-B3. However, in mature pollen, the GeneChip operating software expression signal of AtVHA-B2 is significantly higher than AtVHA-B1 and AtVHA-B3.

Taken together, the functional differences of AtVAB1, AtVAB2, and AtVAB3 imply that they may be involved in different physiological processes of the same cell trafficking or different regulation pathways of different physiological processes, resulting in conserved heredity of AtVAB1, AtVAB2, and AtVAB3 over the course of evolution in A. thaliana.

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