Differential Expression of Vacuolar H^+-ATPase Subunit c Genes in Tissues Active in Membrane Trafficking and Their Roles in Plant Growth as Revealed by RNAi[1]

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Acidification of intracellular compartments by the vacuolar-type H^+-ATPases (VHA) is known to energize ion and metabolite transport, though cellular processes influenced by this activity are poorly understood. At least 26 *VHA* genes encode 12 subunits of the V_1V_o-ATPase complex in Arabidopsis, and how the expression, assembly, and activity of the pump are integrated into signaling networks that govern growth and adaptation are largely unknown. The role of multiple *VHA*-c genes encoding the 16-kD subunit of the membrane V_o sector was investigated. Expression of *VHA-c1,* monitored by promoter-driven β-glucuronidase (GUS) activity was responsive to light or dark in an organ-specific manner. *VHA-c1* expression in expanding cotyledons, hypocotyls of etiolated seedlings, and elongation zone of roots supported a role for V-ATPase in cell enlargement. Mutants reduced in *VHA-c1* transcript using dsRNA-mediated interference showed reduction in root growth relative to wild-type seedlings. In contrast, *VHA-c3* promoter::*GUS* expression was undetectable in most organs of seedlings, but strong in the root cap. Interestingly, dsRNA-mediated mutants of *vha-c3* also showed reduced root length and decreased tolerance to moderate salt stress. The results suggest that V-ATPase functions in the root cap influenced root growth. Expression of *VHA-c1* and *VHA-c3* in tissues with active membrane flow, including root cap, vascular strands, and floral style would support a model for participation of the V_o sector and V_1V_o-ATPase in membrane trafficking and fusion. Two *VHA-c* genes are thus differentially expressed to support growth in expanding cells and to supply increased demand for V-ATPase in cells with active exocytosis.

From the perspective of physiologists and biochemists, it is well established that primary proton pumps are crucial for plant growth and survival. Among three distinct proton pumps, the most complex is the vacuolar-type H^+-ATPase. Physiological and biochemical studies have demonstrated that acidification of the vacuolar compartment by this pump energizes the uptake and release of ions and metabolites (Sze et al., 1992; Lüttge and Ratajczak, 1997). However, due to its structural complexity, we know very little about how the expression, assembly, and activity of this pump are integrated into the signaling networks that govern the life cycle of plants. In addition to its association with vacuolar membranes in plant cells, the pump has been localized to diverse subcellular membranes, including the Golgi (Ali and Akazawa, 1986; Matsuoka et al., 1997), endoplasmic reticulum (ER; Herman et al., 1994), intracellular vesicles, and the plasma membrane (Sze et al., 1999; Schumacher et al., 1999; Dietz et al., 2001). Yet the specific subcellular functions of V-ATPases in distinct cell types and their consequences on a developing multicellular plant are largely unknown. Thus, from the perspectives of molecular, cell, and developmental biologists, many questions remain.

Based on extensive biochemical and structural studies from animal, yeast and plant V-ATPases, and the complete set of V-ATPase genes in Arabidopsis (Sze et al., 2002), a eukaryote V-ATPase consists of at least 12 distinct subunits organized in two large subcomplexes: the cytosolic V_1 and membrane V_o (Arata et al., 2002b; Domgall et al., 2002). The large cytosolic V_1 complex of subunits A through H catalyzes the hydrolysis of ATP that is coupled to the pumping of protons into a compartment via the membrane-bound V_o complex. The V_o complex includes three integral proteins, named subunits a, c, c″, and one hydrophilic subunit d. Models based on electron micrographs of a purified plant V-ATPase (Domgall et al., 2002), or of brain coated vesicles (Wilkens et al., 1999) have been proposed, though the arrangement of the stalk subunits (C–G) connecting the V_1 and V_o sector as being central or peripheral is still unresolved. Subunit A and B, present in three copies each per pump, form a hexamer to catalyze ATP. The putative central stalk, of subunits D and F, links the V_1 with V_o, and is thought to function in coupling V_1 activity to V_o. Subunit E has been reported to be part of the central stalk or the peripheral stalk based on its association with subunits G, C, and H (Arata et al., 2002a; Grüber et al., 2000). The cytosolic end of the integral a subunit

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associates with subunit A and with the peripheral stalk. Subunit c is thought to carry the protons and the rotation of a ring of 6 subunit c is essential in driving proton transport (Hirata et al., 2003).

The biochemical and transport properties of V-ATPases led to several working ideas about the cellular and physiological consequences of vacuolar acidification in plants (Sze et al., 1992). One idea was that acidification would facilitate cell expansion by generating turgor pressure through solute accumulation in the vacuole energized by the pump. Increases in transcript of V-ATPase after anthesis in rapidly elongating cotton fiber cells (Smart et al., 1998) would support this hypothesis. Another idea was the pump would promote the sequestration of excess toxic ions in vacuoles and so aid in adaptation to salt stress. Many studies support this idea. For instance, the transcript or activity of the pump (Barkla et al., 1999) was enhanced by salt stress in a number of plants. Transcript levels of subunit A, B, E, and c are increased by salt in the common ice plant (*Mesembryanthemum crystallinum*; Low et al., 1996; Tsiantis et al., 1996; Lüttge and Ratajczak, 1997; Dietz et al., 2001 and references therein). These correlative observations strongly suggest the V-pump has a role in osmoregulation and salt tolerance, though the molecular and cellular basis for adaptation is still unclear.

Genetic evidence that V-ATPase influenced plant development and signaling came from the first V-ATPase mutant, *det3*. The T→A mutation lies within the first intron of *VHA-C* (a single gene) in Arabidopsis, and presumably destroys a consensus sequence for splicing resulting in reduced subunit C transcript and V-ATPase activity (Schumacher et al., 1999). The mutant is deetiolated when germinated in the dark and the mature plant is dwarfed compared to the wild-type plants. Curiously, the defect is conditional and inverted seedlings overcome the reduction in growth. Furthermore, mutants were defective in guard cell signaling or movement. External Ca^{2+} induced Ca^{2+} oscillations in guard cells of wild-type plants; however similar oscillations were not observed in the mutant. Stomatal closure induced by external Ca^{2+} was abolished in the mutant (Allen et al., 2000), though the mechanism of this is not understood. The picture emerging from the mutant studies is that the endomembrane proton pump may be integrated with hormone signaling, and its roles are much more complex and diverse than previously assumed.

With the completed genome of Arabidopsis (Arabidopsis Genome Initiative, 2000), major questions about the roles and regulation of V-ATPases in plants can now be fully explored. In Arabidopsis, V_1 subunits are mostly encoded by single copy genes except for B, E, and G; however, all V_o subunits are encoded by two or more genes. Among 26 Vacuolar H^+-ATPase genes, now named as *VHA* for consistency (Sze et al., 2002), what specific functions do multiple genes of selected subunits serve in a flowering plant? Working ideas that V-ATPase isotypes differ in their subunit composition, pump properties, membrane localization, and tissue distribution (Sze et al., 1992; 1999) have been supported in part by biochemical studies. For instance, two enzymes purified from pea (*Pisum sativum*) epicotyl differed in the size of subunit E1 and E2, with E1 nearly undetectable in the leaf and cotyledons (Kawamura et al., 2000).

Much less is known about the roles of subunits from the membrane V_o sector in plant biology. The 16-kD integral protein or subunit c is encoded by the largest multigene family with five members in Arabidopsis (Sze et al., 2002), though the biological significance of this is unknown. Subunit c was the first multigene family reported in eukaryote V-ATPases (Sze et al., 1992; Perera et al., 1995), and this feature is conserved in other plants, including cotton (*Gossypium hirsutum*; Hasenfratz et al., 1995), rice (*Oryza sativa*; Rice Genome Databases), and *Acetabularia acetabulum* (Ikeda et al., 2001), and in worms (Oka et al., 1998). In contrast, there are no multiple isoforms of subunit c in human or mouse, as only one out of several genes encodes a functional subunit (Hasebe et al., 1992; Hayami et al., 2001). Thus, additional isoforms seen in plants may shed light on the diversity and dynamism of V-ATPase function in the life cycle of flowering plants. Because the VHA-c proteins within each species are extremely conserved (Perera et al., 1995), they are unlikely to result in activity changes or to contain specific sorting information. Thus we hypothesized that multiple subunit c genes may be expressed differentially in space, developmental stage, or in response to stress, and that in vivo roles might depend on the specific cell type.

Here we show that two *VHA-c* genes in plants are differentially expressed and that *VHA-c3* is particularly tissue- and cell-type specific. dsRNA-mediated reduction in *VHA-c* transcripts highlight for the first time a significant role of the root cap V-ATPase in root growth. Based on the expression patterns of *VHA-c* genes in root cap cells, vascular tissue, pollen, and style, a model is proposed that compartment acidification by the V-ATPase affects a broad range of cellular activities. In addition to energizing solute transport and maintaining ion homeostasis, the V-ATPase and the V_o sector could be involved in protein sorting and membrane fusion events that are needed to promote growth, exocytosis of wall materials in certain cell types, and tolerance to salt stress.

RESULTS

Differential Expression of *VHA-c1* and *VHA-c3*

To determine the expression patterns of *VHA-c* in wild-type plants, transcriptional fusions were made between the promoter of *VHA-c* genes and the reporter gene, *GUS*. The VHA-c1 and VHA-c3 promoter region of 1 kb and 2.1 kb, arbitrarily selected before completion of the Arabidopsis genome, included the entire intergenic region of 724 bp and 749 bp upstream

of the coding sequence for *VHA-c1* (At4g34720) and *VHA-c3* (At4g38920), respectively (Arabidopsis Genome Initiative, 2000). T2 seeds bearing the GUS gene were collected and the resulting plants were stained for GUS activity. Ten lines of *VHA-c1* promoter::*GUS* reporter gene and six lines of *VHA-c3*::*GUS* analyzed gave reproducible expression patterns.

Light-Regulated Expression of VHA-c1 in Young Seedlings

Light- and dark-grown seedlings showed dramatic differences in *VHA-c1* expression. GUS activity was observed in expanded cotyledons of light-germinated seedlings with particular strong staining in the vascular strands (Fig. 1, A and B). However, unexpanded cotyledons of dark-grown seedlings showed no detectable GUS staining after a similar reaction period (Fig. 1C). In contrast, the hypocotyl (12.25 mm in length) of etiolated seedlings showed GUS staining, including the vascular strands, while the short hypocotyl (2.87 mm) of light-grown seedlings was not stained under similar reaction conditions (Fig. 1, D and E). Under blue, as well as far red light, both hypocotyl length (3.16 or 3.9 mm) and GUS staining were almost completely inhibited similar to those grown under white light. Under red light, hypocotyl length (8 mm) and GUS staining (data not shown) were both reduced relative to seedlings grown under complete darkness. The parallel increase in *VHA-c1* expression and cell elongation would point to a role for V-ATPase activity in the cell enlargement phase of growth.

In contrast, *VHA-c1* expression in the root was not altered by light. GUS activity was strong at the root tip and in the elongation zone, as well as the differentiated regions (Fig. 1, F–H) in seedlings germinated in either the light or the dark. Thus, the VHA-c1 promoter is differentially regulated by light or dark in an organ-or tissue-specific manner that probably depends on cell-specific light receptors and signaling networks that are coupled to cell growth.

Preferential Expression of VHA-c3 in Root Cap and Pollen

In contrast to the ubiquitous expression of VHA-c1, VHA-c3 promoter activity was especially strong in the root and the shoot apices of seedlings. Expanded cotyledons or the mature root of light-grown seedlings showed very low or no VHA-c3::GUS staining (Fig. 2, A and D) under the same conditions used to detect VHA-c1::GUS. In etiolated seedlings, VHA-c3::GUS staining was also undetectable in the hypocotyl and the vascular strands (Fig. 2, C and D). However, *VHA-3* expression was detected in the shoot apex, especially in the developing stipules surrounding the leaf primordia (Fig. 2B). Intense GUS staining was also

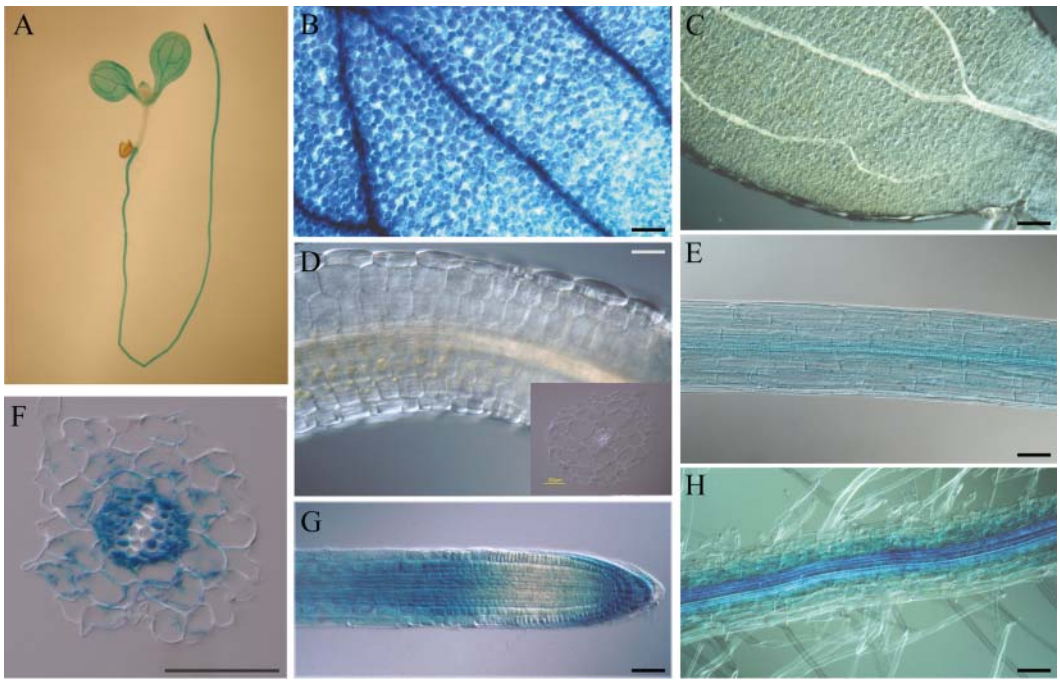

Figure 1. *VHA-c1*::*GUS* is highly expressed in expanding tissues. Seven-d-old seedlings expressing *VHA-c1*::*GUS* were incubated with 1.5 mM X-Gluc for 2 h at 37°C. Light-grown seedling is shown in A, B, D, and F–H. Dark-grown seedling is shown in C and E. Results are from one line representative of ten independent transformants. Bar = 50 μm. A, Light-grown seedling with GUS staining in cotyledon and root but not in the hypocotyl. B and C, Enlarged view of cotyledon from light- (B) or dark- (C) grown seedling. D and E, Hypocotyl of light- (D) or dark- (E) grown seedling. D inset shows hypocotyl in cross section. F, Cross-section of root shows *VHA-c1* expression all cells, and strong GUS staining in endodermis and vascular tissues. G, Root tip and elongation zone. H, Differentiated zone of root with root hairs.

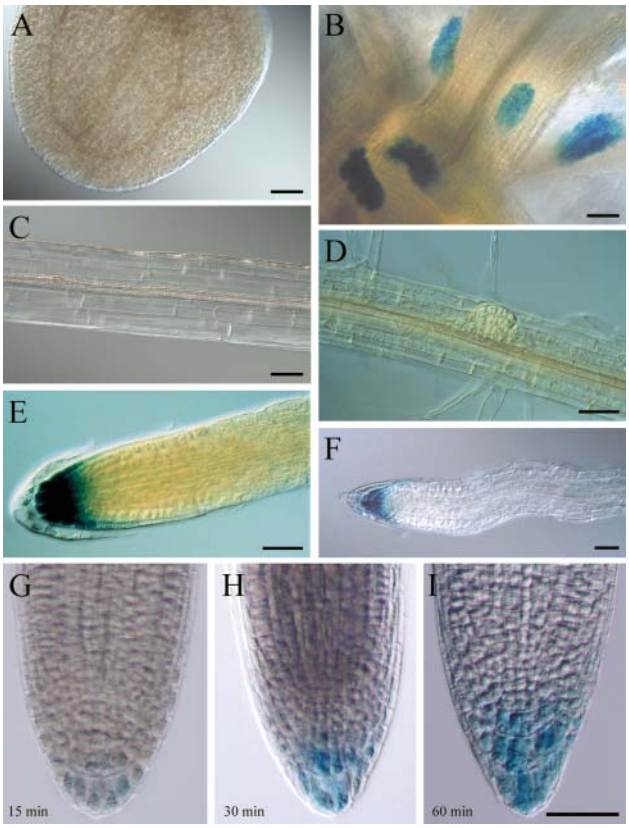

Figure 2. Expression of *VHA-c3*::*GUS* is restricted to shoot and root apices. Seven-d-old seedlings expressing *VHA-c3*::*GUS* were stained for activity with 1.5 mM X-Gluc at 37°C for 2 h (A–F). Seedlings were germinated in the light (A, B, E, G–I) or dark (C, D, and F). Results are of one line representative of six independent transformants. Bar = 50 μm. A, Cotyledon. B, Shoot apex with GUS activity in stipule primordia. C, Hypocotyl. D, Differentiated zone of root with root hairs. E and F, *GUS* expression in the root tip and cap cells. G–I, *VHA-c3* expression in lateral and columella cells of the cap. Seedlings were stained with 1.5 mM X-Gluc for 15, 30, or 60 min.

observed in the extreme root tip after 2 h incubation (Fig. 2, E and F). When the time of GUS staining was reduced, it was apparent that *VHA-c3* expression was localized to lateral and columella cells of the root cap (Fig. 2, G–I).

Interestingly, *VHA-c1*::*GUS* expression was found in all floral organs except for the petals (Fig. 3A). Histochemical staining of GUS activity was particularly high in the style and in the anther of flowers including pollen grains. In contrast, expression of VHA-c3::GUS activity was absent or very low in most floral organs, including the sepals, petals, and carpels. However, *VHA-c3* expression was strong in the anther (Fig. 3B). GUS staining was localized to the pollen grains released by shearing the anther and not in the surrounding anther tissues (data not shown).

We conducted in situ RNA hybridization and RNA gel blots to verify the results of promoter::*GUS*. Flower sections were hybridized with gene specific 3′-UTR regions of *VHA-c1* and of *VHA-c3*. The antisense probe of *VHA-c1* labeled most floral parts, and *VHA-c3* labeled the anther strongly (Fig. 3, C–F). Thus the in situ results confirm the expression patterns seen with promoter-driven GUS expression, though the latter approach does not reveal regulation by elements downstream of the transcriptional unit and/or by elements for mRNA stability. VHA-c1 was also detected in the elongation zone of roots and in vascular tissues by whole mount in situ (data not shown). The high level of *VHA-c1* expression seen with histochemical GUS activity was confirmed by RNA gel blots. *VHA-c1* and *VHA-c2* transcripts were most abundant in leaf and root. *VHA-c3* transcript was low in vegetative and reproductive organs (Fig. 4), but clearly present in the root and flower. *VHA-c4* transcript was extremely low in all organs. Analyses of other *VHA*-promoter::*GUS* expressions were not pursued at this

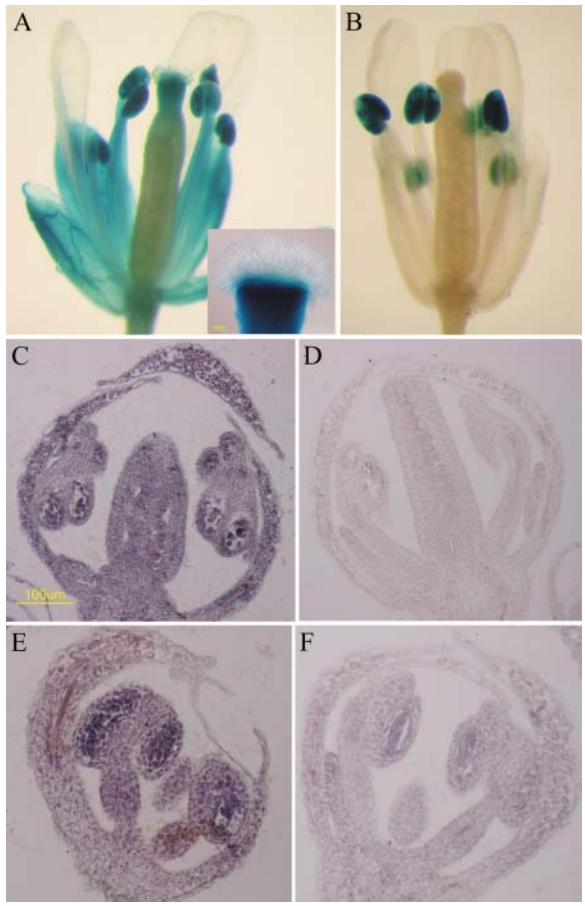

Figure 3. Differential expression of *VHA-c1* and *VHA-c3* in floral organs. A and B, Whole mount GUS staining. Flowers of 5-week-old transgenic plants were stained with 1.5 mM X-Gluc for 2 h at 37°C. A, *VHA-c1* expression is high in sepal, stamen, anther, and style (inset), but not in petal. B, *VHA-c3* expression is high in pollen grains. Results are from one line representative of several. C–F, In situ hybridization. *VHA-c1* and *VHA-c3* transcripts in wild-type plants. Flowers were paraffin-embedded, sectioned at 8 μm thickness, and hybridized with gene specific 3′-UTR probes corresponding to *VHA-c1* antisense (C) and sense (D) or to *VHA-c3* antisense (E) and sense (F) probes.

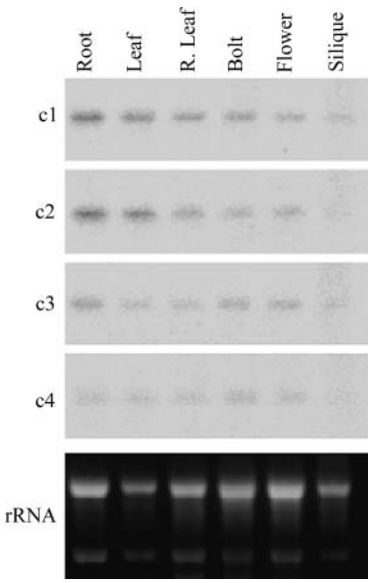

Figure 4. *VHA-c1* and *VHA-c2* are highly expressed in roots and shoots relative to other *VHA-c* genes of Arabidopsis. Five micrograms of total RNA from the organs were fractionated in formaldehyde gel and blotted to GeneScreen membrane. The blots were hybridized with random-priming-labeled probes of gene-specific regions corresponding to *VHA-c1* through *VHA-c4* (originally named *AVA-P1* to *AVA-P4*). The results were detected with a phosphorimager. Equivalent RNA loading was verified by the amount of ethidium bromide-stained 26S rRNA (bottom section).

time due to insufficient transgenic lines and weak staining patterns. Taken together, *VHA-c1* and *VHA-c3* are differentially expressed. *VHA-c3* was preferentially expressed in a few tissues. *VHA-c1* is highly expressed in many tissues, and subject to light or developmental regulation in a tissue-specific manner.

The promoter::GUS expression patterns would support two ideas about the roles of V-ATPase. One is that the pump takes part in processes leading to cell expansion, as expression of subunit c, a major V_o subunit, is high in cells undergoing cell enlargement. Second, the V-ATPase may have a role in vesicular trafficking, based on the intriguing observation that both *VHA-c1* and *VHA-c3* expression is abundant in tissues with active endomembrane flow. These include the floral style, pollen, and root cap. As a first step to test these ideas, we generated mutants with reduced *VHA-c1* or *VHA-c3*.

dsRNA-Transformants Showed Reduction in *VHA-c* Expression and V-ATPase Activity

Because acidification of intracellular compartments is fundamental to eukaryote cells, we tested the role of multiple *VHA-c* genes by reducing their expression. To date, no T-DNA insertional mutants in *VHA-c* genes have been identified, suggesting that knockout mutants may be lethal (Schumacher et al., 1999). Four members of the family *VHA-c1* to *VHA-c4*, previously named *AVA-P1* to *AVA-P4* (Perera et al., 1995), showed high nucleotide sequence identity (82%–91%) in their coding region, so the 3′-UTR region was used to construct gene-specific sense and antisense sequences separated by a spacer (Fig. 5, A and B). Plants transformed with the constructs were verified for integration of the dsRNA construct in their genome by PCR amplification (Fig. 5C) and by expression of the dsRNA sequence using reverse transcription (RT)-PCR (data not shown). T2 seeds homozygous for the transgene as shown by complete resistance of their progeny to glufosinate ammonium (BASTA) were then selected. The reduction in mRNA levels targeted by dsRNAs varied in different transgenic lines. Six out of ten independent lines transformed with a dsRNA VHA-c1 contruct were classified as strong mutants based on the 80% to 96% reduction in mRNA as determined by semiquantitative RT-PCR (Fig. 6A). One line, *vha-c1-5-1*, showing about 63% reduction in mRNA, was classified as an intermediate mutant, and three transformants were considered weak mutants as they showed only 3% to 21% decrease in mRNA levels. Seven out of 10 lines transformed with dsRNA constructs corresponding to the *VHA-c3*, were strong mutants with 79% to 97% reduction in mRNA, while three other lines were classified as intermediate mutants (Fig. 6B). The reduction in transcript level mediated by dsRNA was gene-specific, as the level of transcripts of nontargeted members were unchanged in a strong mutant *vha-c1-1-5* or *vha-c3-1-1* (Fig. 6C) Thus dsRNA containing gene-specific sequences is an effective method in genetic silencing or suppression as shown previously in animal and plant systems (Montgomery and Fire, 1998; Chuang and Meyerowitz, 2000).

V-ATPase activities were determined using membranes isolated from 7-d seedlings of two separate lines of mutant and of wild-type plants. Nitrate-sensitive ATP hydrolysis activity from dsRNA-c1 mutants was consistently reduced by 20% to 27% relative to that isolated from control wild-type plants or wild-type plants transformed with an empty pMLBart vector (Table I). In contrast, the vacuolar K^+-stimulated-PPase activity was unchanged (94%–98%) in the mutants relative to the wild type indicating that the decrease in V-ATPase activity observed was not due to any difference in membrane proteins isolated from wild-type and mutant. Strong dsRNAi mutants of *vha-c3* showed a smaller (11%) decrease in specific V-ATPase activity than those of *vha-c1* mutants. These results would be consistent with the low transcript level and limited tissue distribution of *VHA-c3* expression relative to *VHA-c1* (Figs. 1, 2, and 4).

The decrease in activity was not accompanied by any visible changes in V-ATPase subunits in the mutants as determined by SDS-PAGE of membranes followed by blotting and immunostaining with monoclonal antibodies against the major subunits (data not shown).

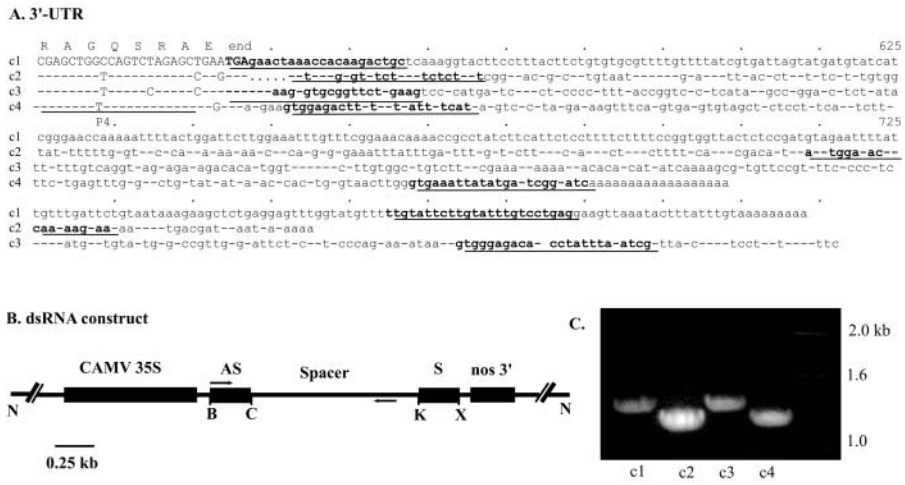

Figure 5. Constructs used to analyze dsRNA effects. A, Gene-specific sequences of *VHA-c1* to *VHA-c4* genes were amplified from the 3′-UTR. The 3′-UTR of c1 to c4 cDNAs (GenBank accession no. L44581–L44584) are shown in lower case after the stop codon TGA. Primer sequences used to amplify the antisense and sense fragments are underlined in bold. The reverse complement sequence is not shown. Appropriate restriction enzyme sites were introduced in the primers to make antisense and sense strands (see B). For in situ hybridization (Fig. 3), the forward primer underlined is labeled as P4. Reverse primers correspond to T7/T3 or SP6 promoter sequences in the plasmids. B, Map of dsRNA construct with antisense (AS), spacer, and sense (S) placed under the control of the CaMV 35S promoter and the nos 3′ terminator. *Bam*HI, *Cla*I, *Kpn*I, *Xba*I and *Not*I enzyme sites are marked as B, C, K, X, and N, respectively. The antisense (AS) or sense (S) regions ranged from 148 to 280 bp. Arrows refer to primer positions for experiment in C. C, Integration of dsRNA construct in the genome. Nuclear DNA of transformed plants was isolated from T2 homozygous plants. Gene-specific products corresponding to *VHA-c1* to *VHA-c4* were amplified using primers (arrows shown in B) from the antisense sequence and from the spacer. The PCR products are from one representative mutant line of each gene.

Decrease in Root Length of *vha-c1* and *vha-c3* Mutants

If V-ATPase is important in cell expansion, then mutants might be stunted in their growth or deetiolated in the dark. Interestingly, T1 plants expressing the transgene of either *vha-c1* or *vha-c3* were visibly smaller, had slightly reduced leaf size and delayed bolting (5–7 d) relative to wild-type plants. Plants of the T2 generation, however, were not noticeably reduced in leaf size, and bolting was delayed 0 to 3 d relative to the wild-type plants. The number of siliques per mutant was about 30% reduced compared to the wild type. In this study, all the growth measurements were conducted using T2 seedlings.

Surprisingly, hypocotyl lengths in several lines of strong *vha-c1* mutants were decreased on average by only 9% relative to the wild type (Fig. 7A). Hypocotyls of strong *vha-c3* mutants were decreased by 8%. However, strong dsRNA-c1 mutants showed a more significant (38%) decrease in root length compared to the wild type (Fig. 7A). Interestingly, roots of strong *vha-c3* mutants also showed reduced length, though on average the decrease of 25% (Fig. 7B) was less than that seen in mutants with dsRNA-c1. The reduction in root growth was not a result of impaired germination of mutants, as root growth from day 5 to day 9 in strong dsRNA-c1 mutants was also reduced (7.0 mm) relative to wild-type controls (8.7 mm). Similarly, root growth of *vha-c3* mutants from day 5 to day 9 was 14% shorter (7.35 mm) than the control (8.68 mm).

A reduction in root growth of strong *vha-c1* mutants would support the idea that V-ATPase activity is needed for cell division and cell expansion at the growing tip, perhaps for cell wall development during differentiation, and for regulating ion homeostasis. However, the substantial reduction in root growth of *vha-c3* mutants was surprising. The overall expression of *VHA-c3* is low in roots (Fig. 4), but it is concentrated in the root tip, particularly in cap cells (Fig. 2E). The results would strongly hint at a role for V-ATPase in the normal functions of the root cap.

Sensitivity of *vha-c* Mutants to Salt Stress

One important function of the V-ATPase is to provide the driving force for H^+-coupled Na^+ antiporters, such as Nhx1, which sequesters sodium into the vacuole (Apse et al., 1999). We and others have shown before that salt stress enhances transcript levels of subunit c, although the specific contribution of separate *VHA-c* genes was not known (Sze et al., 1999; Dietz et al., 2001). Salt-treated seedlings showed an increase in *VHA-c1* and *VHA-c2* transcripts (Perera et al., 1995), so we tested whether the dsRNA mutants were more sensitive to salt. Strong mutants of dsRNA-c1 were germinated for 5 d in 0.5× Murashige and Skoog medium (MS, 1962), and then transferred to the 0.125× MS medium containing either 50 mM NaCl or 100 mM mannitol. The change in root length after

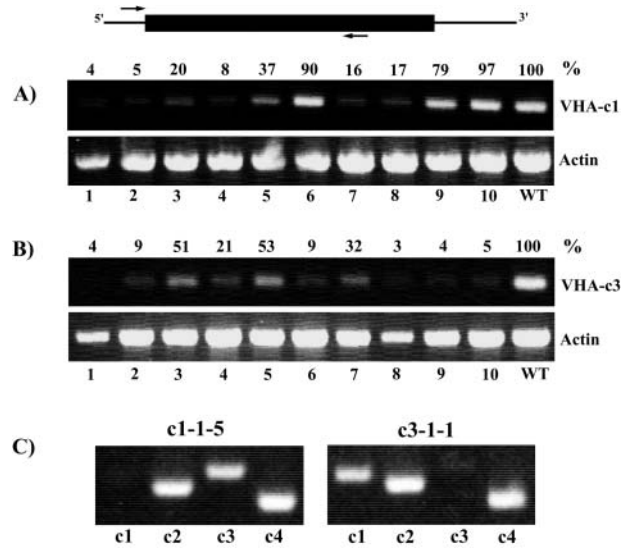

Figure 6. dsRNA reduced transcript accumulation of *VHA-c1* or *VHA-c3* specifically. RT-PCR was performed with total RNA isolated from plants expressing the dsRNA construct. After RT, cDNA was amplified for 25 cycles (94°C 30 s, 50°C 30 s, and 72°C 90 s), which was determined to be within the linear range of amplification. Forward and reverse primers (indicated as arrows in top section) correspond to the 5′-UTR upstream of ATG and a region within the coding region, respectively. RT-PCR of actin 11 (At3g12110) showed equivalent RNA was used in each reaction. A, *VHA-c1* transcript level in *vha-c1* mutants (dsRNAi). Lanes 1 to 10 correspond to mutant lines *c1-1-5, c1-2-7, c1-3-6, c1-4-4, c1-5-1, c1-6-4, c1-11-2, c1-16-2, c1-17-2,* and *c1-18-2*, respectively. WT is wild type. Six strong, one intermediate, and three weak mutants were apparent. B, *VHA-c3* mRNA in *vha-c3* mutants (dsRNAi). Lanes 1 to 10 correspond to mutant lines *c3-1-1, c3-2-2, c3-5-7, c3-6-4, c3-8-2, c3-10-4, c3-13-2, c3-14-1, c3-15-3,* and *c3-20-2*, respectively. WT is wild type. Strong and intermediate mutants are apparent. In (A) and (B), PCR-amplified product was quantitated using Scion Image analyses. Transcript level of VHA-c's in mutants was compared with that of WT which was set to 100%. C, Reduction in transcript by dsRNA was gene-specific. Reverse transcribed cDNA was amplified for 35 cycles. Relative transcript of *VHA-c1* to *VHA-c4* is shown from mutant line *c1-1-5* or in *c3-1-1*.

4 d was then determined. Root growth of wild-type plants was reduced 40% after transfer to 50 mM salt relative to those in the control medium. However, root length of two separate mutant lines, *c1-1-5* and *c1-11-2*, was reduced 57% and 57%, respectively, after 4 d in 50 mM NaCl-containing medium. Interestingly, the mutants and wild type were only slightly affected by osmotic stress from 100 mM mannitol (Fig. 8A). Thus, *vha-c1* mutants was slightly more sensitive than wild-type plants to salt stress.

Surprisingly, strong dsRNA-c3 mutants were also sensitive to mild salt stress similar to *vha-c1* mutants. Root length was reduced 56% and 50% in *c3-1-1* and *c3-14-1*, respectively, compared to a 41% reduction in wild-type plants (Fig. 8B). Together, these results point to an important role of the V-ATPase in the functions of the root cap.

DISCUSSION

Very little is known about the expression and biological roles of the membrane V_o sector in plant V-ATPases. Each V_o subcomplex consists of one copy of subunit a, c″, and d, plus up to six copies of subunit c, and the synthesis and assembly of V_o at the ER is independent of the synthesis of the V_1 complex (Sze et al., 1999 and references therein). As the most abundant component of the V_o complex, subunit c expression could conceivably alter the level and turnover of the V_o subcomplex in the membrane, and thus modulate the activity and density of fully-assembled V_1V_o-ATPase pumps (Sze et al., 1999). Of 12 subunits that make up the V_1V_o-ATPase complex, subunit c is the only one encoded by a multi-gene family with up to five members. This raises the intriguing possibility that modulation of V-ATPase activity and density during growth and development could reside in part on the transcriptional regulation of subunit c genes in a cell-specific manner. We had shown before that *VHA-c1* and *VHA-c2* were highly expressed by RNA gel blot, but *VHA-c3* expression was low and sometimes undetectable (Perera et al., 1995). To gain insights of their biological functions, we tested the expression patterns of two members of the *VHA-c* gene family and silenced the genes through dsRNA-mediated interference.

Spatial and Developmental Expression of Two *VHA-c* Genes

We provide, to our knowledge, the first evidence that multiple genes encoding a plant V-ATPase subunit are under different transcriptional regulation. First, *VHA-c1* and *VHA-c3* genes are differentially expressed in space. *VHA-c1*::*GUS* is highly expressed in nearly all organs and cell types examined consistent with the RNA gel blot analyses. In contrast, *VHA-c3* was preferentially expressed in a few tissues, such as the root cap, stipules, and pollen grains. Interestingly, *VHA-c1* expression also extends to these tissues, though the quantitative contribution of each gene cannot be assessed at this time. Second, multiple genes of subunit c are differentially influenced by light. Etiolated seedlings demonstrated strong VHA-c1::GUS staining in the hypocotyls but not in the unexpanded cotyledons. The *VHA-c1* was also highly expressed in cells of expanded cotyledons but not in the short hypocotyls of light-grown seedlings. In contrast, *VHA-c3* expression in seedling was not altered by light or by dark. Thus the promoter of *VHA-c1* is responsive to light, though it is unclear if this is a direct or indirect consequence of light/dark signaling. Light-responsive elements were identified in the upstream regulatory region of *VHA-c1*, suggesting it is a potential downstream target of light-regulated signaling networks.

Multiple subunit c genes are differentially regulated in cell-types probably depending on the functional demands and developmental stage of the cells. It is

Table I. *Reduction in V-ATPase activity but not PPase activity in dsRNAi mutants of vha-c1*

Membranes were isolated from 7-d-old seedlings of wild-type plants (WT) or wild type harboring an empty vector (WT + V) and two lines each of dsRNAi mutants of *vha-c1* or *vha-c3*. Vacuolar ATPase activity was determined as nitrate-sensitive ATP hydrolysis. PPase activity was determined as K^+-stimulated PPi hydrolysis. Number of experiments shown in parentheses using 2 to 3 independent membrane isolations.

Plant Line	$\Delta NO3^-$ ATPase Activity		ΔK^+-PPase Activity	
	μmol/mg.h ± SE	Avg %	μmol/mg.h	Avg %
WT	3.30 ± 0.65 (7)	100	5.20 ± 1.13 (5)	100
WT + vector	3.44 ± 0.70 (7)		5.16 ± 1.25 (3)	
vha-c1 mutant line				
c1-1-5	2.47 ± 0.45 (7)	76	4.95 ± 1.18 (5)	98
c1-2-7	2.71 ± 0.54 (7)		5.25 ± 1.25 (5)	
WT	2.16 ± 0.13 (3)	100	7.27 (2)	100
WT + vector	2.26 ± 0.09 (3)		7.12 (2)	
vha-c3 mutant line				
c3-1-1	2.13 ± 0.05 (3)	89	6.93 (2)	93
c3-14-2	1.81 ± 0.10 (3)		6.51 (2)	

interesting that even a single gene, *VHA-c1*, can be developmentally regulated in an organ-specific manner. For instance, *VHA-c1* expression in the root, unlike that in the cotyledon or hypocotyls, was not responsive to either light or dark. Expression of two subunit c genes were analyzed before in cotton ovules (Hasenfratz et al., 1995), though RNA gel blotting was not able to provide the spatial resolution shown here by promoter::GUS analyses. Interestingly, one of three subunit c genes in *Caenorhabditis elegans* was preferentially expressed in gastrointestinal and hypodermal cells, whereas the expression of two other genes was similar at all stages of the life cycle (Oka et al., 1998).

Role in Cell Enlargement

Several observations strongly point to a major role of *VHA-c1* in cell expansion. First, high VHA-c1 promoter activity was consistently seen in the elongating zone of roots, expanding cotyledons or elongating hypocotyls, but not in nonexpanding organs, such as cotyledons of dark-grown seedlings or hypocotyls of light-grown seedlings. Second, reduction of *VHA-c1* message by dsRNA-mediated interference reduced root and hypocotyl length of mutants relative to wild type. These results are consistent with previous studies of V_1 subunits. For example, transcript levels of subunit A or E increased during cotton fiber elongation (Smart et al., 1998) or in expanding barley (*Hordeum vulgare*) leaves (Dietz et al., 1995), respectively. Reduced expression of subunit C, a product of a single copy gene, caused severe stunting in *det3* Arabidopsis mutants (Schumacher et al., 1999). Moreover, analysis of transgenic carrot (*Daucus carota*) plants showed cell size in leaves was reduced by antisense inhibition of subunit A (Gogarten et al., 1992). Collectively, the results show that several genes of the VHA are downstream targets for up-regulation in response to cues that stimulate cell expansion. One model is that the V-ATPase activity is regulated differentially by phytohormones, as subunit C is necessary for brassinolide-induced cell elongation (Schumacher et al., 1999).

However, the roles of multiple c subunits may differ depending on the organ, tissue, or cell types. The reduction in hypocotyl length of mutant expressing the ds-RNA VHA-c1 was consistent though small, suggesting that VHA-c1 function was partially compensated by other genes. The 16-kD proteins are nearly identical (Perera et al., 1995), so it is very likely that VHA-c2 in shoots (Fig. 4) could functionally replace VHA-c1. However, severe reduction (38%) in root growth of *vha-c1* mutants indicated that other subunit c's were unable to fully compensate for the loss of one isoform.

Importance of Root Cap in Root Growth

Surprisingly, V-ATPase function may be critical for root growth via a role in the root cap. First, root length of *vha-c1* mutants was significantly reduced (38%) in etiolated seedlings relative to the wild type. It is possible that reduced *VHA-c1* expression in the root tip and elongation zone would limit cell division, cell elongation, or both, and that other *VHA-c* genes could not compensate for this loss. Second, *vha-c3* mutants consistently showed reduced (14%–25%) root growth, although *VHA-c3* expression is mainly expressed at the root cap (Fig. 2). Third, salt stress reduced root growth of *vha-c3* mutants nearly as well as *vha-c1* mutants. Thus, V-ATPase in root caps is needed to support root growth under normal nutrient conditions as well as under mild salt stress. To our knowledge, this is the first report to highlight the potential importance of V-ATPase to root cap functions.

The root cap is a structure that protects the root apical meristem, facilitates penetration through the soil by the root, secretes chemicals to create a chemical microenvironment, and senses environmental signals and gravity. In Arabidopsis, the root cap consists of the central columella cells that are thought to perceive

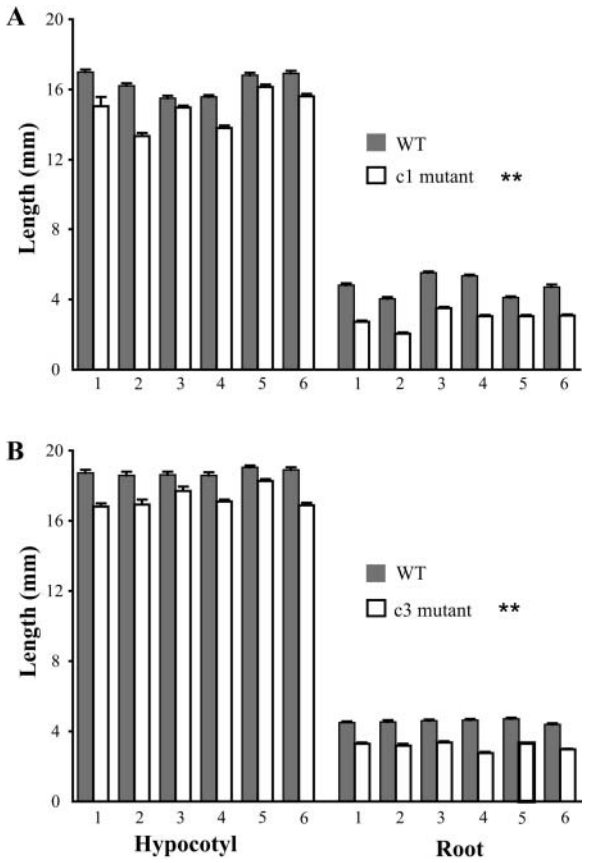

Figure 7. Reduction in root length of *vha-c* mutant (dsRNAi) seedlings. Wild-type and mutant seeds were germinated on the same plate containing 0.5× MS for 5 d in the dark. Hypocotyl and root lengths were then measured. Results are from duplicate plates each with 26 to 48 plants per line. One experiment is representative of two. ** indicates that a significant difference was observed in all mutants from values of the wild type based on a Student's *t* test (**, $P<0.01$). A, Hypocotyl and root length of wild-type and *vha-c1* mutants. Numbers 1 to 6 correspond to six mutant lines *c1-1-5, c1-2-7, c1-4-4, c1-5-1, c1-11-2,* and *c1-16-2,* respectively. On average, mutant hypocotyl was 91% and mutant roots were 62% of the wild type. Weak mutant lines, *c1-6-4* and *c1-17-2,* showed little or no (0%–7%) decreased root length compared to the wild type (not shown). B, Hypocotyl and root length of wild-type and *vha-c3* mutants. Numbers 1 to 6 refer to six mutant lines *c3-1-1, c3-2-2, c3-5-7, c3-14-1, c3-15-3,* and *c3-20-2,* respectively. Hypocotyl length was 92% and mutant roots were 75% of wild type.

gravity (Blancaflor et al., 1998), and lateral cells and epidermis that secrete mucilage, proteins, and metabolites. Root caps are highly dynamic and able to regrow and redifferentiate rapidly when the cap is removed or sloughed. A genetic study recently highlighted the essentiality of root cap to signaling systems that determine root growth and architecture. When Arabidopsis root cap cells were genetically ablated by expressing a diphtheria toxin, root growth was severely inhibited, and roots were agravitropic (Tsugeki and Fedoroff, 1999). Thus, a major disruption of root cap function alters root architecture. We speculate that reduced *VHA-c* message resulting in reduced V_o formation or V-ATPase activity in cap cells may impair hormone perception, transport, or transduction in the root. For instance, the pump or V_o subunits may influence auxin transport. Auxin from the shoot is translocated to the root tip and to the columella cells of the cap. The hormone is then moved radially to the lateral cap cells and then basipetally to the elongation zone. The direction of auxin transport and the cellular auxin level are thus determined by the location of PIN efflux carriers. Recent studies suggest that the distribution of auxin efflux carriers at a plasma membrane is regulated by endosomal vesicle cycling of PIN proteins (Friml, 2003).

Role of V-ATPase in Vesicular Traffic: A Model

Our results and those of others provide valuable insights that suggest acidification of intracellular compartments is important for protein trafficking and perhaps vesicle fusion in plants. First, a significant and interesting observation is that V-ATPase message and subunits are abundant in cells active in vesicular traffic and exocytosis. For instance, style tissues secrete a mixture of glycoproteins to stimulate and perhaps guide pollen tube growth through the transmitting tissue (Wheeler et al., 2001). Expression in the vascular parenchyma (Fig. 1F) may be related to vascular differentiation that involves the synthesis and exocytosis of pectins, hemicelluloses, and wall

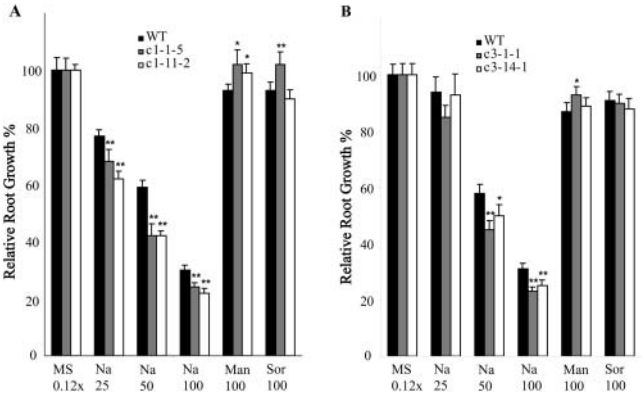

Figure 8. Mutants of *vha-c1* or *vha-c3* are sensitive to salt stress. Seeds were germinated in 0.5× MS for 5 d and then transferred to plates containing 0.125× MS supplemented with NaCl, mannitol, or sorbitol. Mutants and wild-type seedlings were placed on each half of the same plate. The apices of the roots were positioned above a horizontal line drawn across the petri dish. After 4 d growth in 16 h light and 8 h dark at 20°C, the increase in root length from day 5 to day 9 was measured. Results are from 2 to 3 experiments with a sample size of 18 to 22 per treatment. The asterisk represents a significant difference from values of the wild type based on a Student's *t* test (*, $P<0.05$; **, $P<0.01$). (A) Relative root growth of *vha-c1*mutants. Average root growth of wild-type, *c1-1-5,* and *c1-11-2* mutant seedlings on control 0.125× MS medium was 9.81 ± 0.45 (100%), 8.12 ± 0.34, and 8.45 ± 0.18 mm (100%), respectively. (B) Relative root growth of *vha-c3* mutant. Average root growth of wild-type, *c3-1-1,* and *c3-14-1* mutant plants on control 0.125× MS medium was 9.36 ± 0.37 (100%), 8.5 ± 0.35, and 8.66 ± 0.34 mm (100%), respectively.

proteins (Carpita and McCann, 2000), and their delivery from ER- or Golgi-derived vesicles to the plasma membrane (Battey and Blackbourn, 1993). Pollen tubes show active vesicular traffic including secreting wall materials during rapid growth. All these tissues show strong expression of either *VHA-c1* or *VHA-c3* or both in Arabidopsis. Moreover, subunit E is abundant in xylem parenchyma cells (Golldack and Dietz, 2001), which have been shown to synthesize and secrete wall proteins, including Gly-rich proteins (Carpita and McCann, 2000). Not only are *VHA-c* expressed highly in pollen, growing pollen tubes also showed intense fluorescence from a VHA-C protein tagged with the green fluorescent protein (Schumacher et al., 1999).

Second, the distribution of V-ATPase activity in diverse plant endomembranes indicates that their essentiality extends beyond the roles of the central vacuole (Sze et al., 1999). V-ATPase activity or protein has been demonstrated in membranes from young roots lacking large central vacuoles (Herman et al., 1994), in Golgi of suspension-cultured cells (Ali and Akazawa, 1986), and in the plasma membrane (Robinson et al., 1996). Fluorescence-labeled antibodies against subunit B or green fluorescent protein-tagged subunit C stained the perinuclear region of root tips (Schumacher et al., 1999), and immuno-electron microscopy demonstrated that V-ATPase was associated with the ER and small vacuoles or vesicles (Herman et al., 1994). Subunit E has been also localized by immunofluorescence at the exodermis of ice plant root tip (Golldack and Dietz, 2001). Vesicle acidification by the V-ATPase is thought to be a critical component in receptor-ligand endocytosis, intravesicular trafficking processes in animal cells, and in trafficking of membrane and soluble vacuolar proteins in yeast (Stevens and Forgac, 1997). Concanamycin A, a specific inhibitor of V-ATPase, caused missorting of soluble vacuolar proteins in tobacco (*Nicotiana tabacum*), supporting the idea that a Golgi V-ATPase is similarly involved in proper protein sorting in plants (Matsuoka et al., 1997).

The collection of results from this and other studies led us to propose that V-ATPase and compartment acidification are important for cell expansion, though a combination of cellular mechanisms needs to be considered. The model that V-ATPase energizes the uptake of osmotic solutes and water into large central vacuoles and so regulates ion homeostasis and generates turgor pressure for cell expansion is only a part of the story. A fresh perspective that is frequently overlooked in the plant literature is that acidification and the osmotic status of compartments are critical also to an endomembrane system active in vesicular traffic, protein sorting, and membrane fusion. These processes contribute to the formation of new vacuolar and plasma membranes in growing cells, and in the delivery of new polysaccharides and proteins to the cell wall. In differentiating and nonexpanding cells, high expression of V-ATPase, such as in the style and root cap, would support the idea for a role of V-ATPase in exocytotic activities.

What functions would a V-ATPase serve in an active endomembrane system? Several mechanisms are considered here, though other possibilities are not excluded (Stevens and Forgac, 1997). First, the maintenance of an appropriate pH in ER, Golgi, and secretory compartments is thought to be important for posttranslational modification and sorting of proteins along the secretory pathway. The balance of H^+ pumping by the V-ATPase and H^+ leak has been proposed to regulate pH homeostasis (Wu et al., 2001), so any change in pH could affect pH-sensitive enzymes that process or modify proteins and lead to significant changes in sorting and secretion. Second, an acidic intravesicular pH could promote fusion of two membranes, similar to that of the influenza viral and endosomal membranes where pH triggered refolding of a Haemagglutinin2 protein crucial for fusion (Gaudin et al., 1995). Third, free V_o subcomplexes in two opposing membranes might associate to form a proteinaceous fusion pore complex that mediate the bilayer merger, as suggested in the homotypic fusion of two vacuoles in yeast (Peters et al., 2001). These models suggest exciting possibilities for the biological roles of the V_1V_o-pump and the free V_o sector in a plant's life cycle. So far the results of our work do not allow us to distinguish whether the free V_o subcomplex is involved in functions separate from the assembled V_1V_o-H^+-ATPase pump. Considerable V_o subcomplexes are free in plant endomembranes (Li and Sze, 1999), so future investigations are needed to test whether V_o subcomplexes are involved in functions other than proton pumping, such as vesicular trafficking and fusion.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All experiments were conducted with Arabidopsis ecotype Columbia. Plants were grown in Metromix-200 soil (E.C. Geiger, Harleysville, PA). Seeds in soil were incubated at 4°C for 3 d and then placed under cool-white fluorescent light for 16 h and 8 h in dark at 20°C. Two weeks after germination, plants were given Miracle-Gro (Stern's Miracle-Gro, Port Washington, NY) plant food at 20 d intervals. To test for *promoter*::*GUS* expression, seeds were grown in the dark or under light (approximately 100 μE m^{-2} s^{-1}) at 20°C on plates containing 0.5× MS salts, 3% sucrose, and 1.0% agar pH 5.8. For light quality experiments, 13 to 14 μmol m^{-2} s^{-1} of photosynthetically active radiation was used for white, blue, or red light.

DNA Constructs

Promoter-GUS Fusion

DNA fragments containing the promoter region and the 5′ untranslated region (UTR) just upstream of the translation start codon of *VHA-c* genes were amplified by PCR from genomic clones previously isolated (Perera et al., 1995). A *Sal*I site is located 1 kb or 2.8 kb upstream of the open reading frame of *VHA-c1*/AVA-P1 (At4g34720) or *VHA-c3*/AVA-P3 (At4g38920), respectively. An XbaI site was introduced in the gene-specific primers. PCR products were then digested with *Sal*I and *Xba*I and subcloned into the *Sal*I/*Xba*I site upstream to GUS coding region in pGPTV-HPT vector, which contains a hygromycin resistance gene (Becker et al., 1992). Clones were sequenced using the GUS oligonucleotide to confirm the correct construct. The pGPTV-HPT vector without promoter insert was used as a promoterless control in this study. The plasmid pGPTV-HPT and its derivative constructs in *Escherichia coli* strain DH5α were mobilized into *Agrobacterium tumefaciens* strain C58 (pGV2260) by triparental mating with HB101 and pRK2013 as helper.

dSRNA Constructs

The gene-specific sequences of the 3′-UTR from *VHA-c1* to *VHA-c4* were amplified by PCR (Fig. 5A). Suitable restriction enzyme sites were introduced in the primer sequences. *Bam*HI and *Cla*I restriction sites were introduced to amplify the antisense (AS) strand. *Kpn*I and *Xba*I restriction sites were added to amplify the fragments of sense (S) orientation. The antisense and sense DNA fragments were linked with a 1-kb spacer coding for a partial sequence of GUS. All the cloning was carried out in pGEM 7Zf vector (Promega, Madison, WI) and sequenced to verify the constructs. Then the BamHI and *Xba*I fragment containing antisense, spacer, and sense was subcloned in pART 7 vector between the 35S cauliflower mosaic virus (CAMV) promoter and the ocs 3′ end. The entire fragment containing the 35S CAMV, antisense, spacer, Sense, and ocs 3′ end was cloned into a plant T-DNA binary vector pMLBart using *Not*I enzyme. pMLBart vector contains BASTA resistant gene as plant selection marker. *A. tumefaciens* strain GV 3101 was transformed with plasmid constructs by electroporation (Mersereau et al., 1990).

Transformation of Arabidopsis

The constructs were introduced into Arabidopsis using the vacuum infiltration method (Bent et al., 1994). The transformants were selected on 0.5× MS plates containing hygromycin (25 μg mL^{-1}). Homozygous T2 transformant seeds were used for *GUS* expression studies.

Plants transformed with dsRNA constructs were selected on soil after spraying with BASTA (T1). Seeds resulting from self-pollinated transformants (T2) were scored for herbicide resistance on 0.5× MS medium containing 50 μM BASTA. Complete BASTA resistance identified homozygous lines of the T2 progeny. Using this method, 11 to 14 independent homozygous lines were obtained for each dsRNA-expressing mutant.

GUS Staining

Young seedlings or organs from transgenic plants were stained for GUS activity. Materials were incubated in a mixture containing 84 mM Na_2HPO_4 phosphate pH 7.0, 0.5 mM $K_4Fe[CN]_6$, 0.5 mM $K_4Fe[CN]_6$, 0.5% Triton X-100, and 1.5 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-Gluc) at 37°C for 2 h. Samples were then fixed in 70% ethanol overnight to clear chlorophyll. Photograph was taken under a dissecting scope or with differential interference contrast using a Nikon (Tokyo) E600W microscope.

RNA Expression

RNA Extraction and Gel Blot

Total RNA was isolated from Arabidopsis tissues by the guanidine/acid-phenol method (Chomczynski and Sacchi, 1987). RNA was fractionated on 1.5% agarose formaldehyde gel and transferred to GeneScreen (DuPont, Wilmington, DE) nylon membrane by the capillary blot method. Hybridization was conducted following the protocol of the manufacturer.

RT-PCR

To check the expression of dsRNA and native *VHA-c* RNAs in transgenic plants, total RNA was isolated from independent transformants using the method of Chomczynski and Sacchi (1987). First strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, catalog no.11904–018), and then was used to amplify actin 11 (At3g12110) or *VHA-c* genes by PCR. Sense and antisense primers for actin were -5′-ATGGCAGATGGTGAAGACATTCAG-3′, and -5′-GAAGCACTTCCTGTGGACTATTGA-3′. The forward and reverse *VHA-c* primers corresponded to the 5′-UTR and a part of the coding region, respectively, as follows:

VHAc1-F 5′-GATTTAAGATCTCAGATACAAAACTCCGAC-3′,
c1-R-5′TCCTACAATAAGCCCGTAAAGAGCAAGCGC-3′;
c2-F-5′-CACGAGCCATGGCTTCAACTTTCAGCGGCG-3′,
c2-R-5′-CGTACAGGGCAAGTGCTTCAGCAAAGATGA-3′;
c3-F-5′-CATATTTTAAGATCCAGAATCGCCTGAGAG-3′,
c3-R-5′-TTCGGCTCTGGACTGACCAGCTCGGGAGGA-3′;
c4-F-5′-CTCATCGGAGCAACAGTCATCAAAGAGCCA-3′,
c4-R-5′-TCACCGACGATTCCAATAGCCATACCAGCA-3′.

Semiquantitative PCR was started at 94°C for 5 min followed by 25 cycles of 94°C (30 s), 50°C (30 s), and 72°C (90 s). Amplified product was quantitated using Scion Image Analysis (Scion Corporation, Frederick, MD).

In-Situ Hybridization

The gene-specific 3′-UTR regions of *VHAc1* and of *VHA-c3* (Fig. 5A) were amplified using *Taq* DNA polymerase and cloned into a TA cloning vector pCR 2.1 (Invitrogen,). Clones in the antisense and sense orientation to the T7 transcription site were selected after the sequencing. Probes were synthesized using the AmpliScribe T7 transcription kit (Epicenter Technologies, Madison, WI); manufacturer's instructions were followed except that the T7 RNA polymerase reaction contained 1.4 mM each of ATP, CTP, and GTP, and 0.7 mM UTP plus 0.5 mM digoxigenin-labeled UTP. Probes were chemically degraded into 75 to 100 base fragments by treating with hydrolysis buffer (50 μL RNA, 30 μL 200 mM Na_2CO_3, 20 μL 200 mM $NaHCO_3$) at 60°C. Arabidopsis inflorescence flower samples were fixed in a solution containing 50% ethanol, 5% acetic acid, and 3.7% formaldehyde for 1 h. The fixed tissue was dehydrated with ethanol, cleared with xylene, and embedded in Paraplast Plus (Oxford Labware, St. Louis). Tissue sections (8 μm thick) were fixed on superfrost plus slides (Fisher Scientific, Pittsburgh). After removing the wax, tissues were pretreated with proteinase K (1 μg mL^{-1}) at 37°C for 30 min. Hybridization and the subsequent treatment were carried out as described by Lincoln et al. (1994). After hybridization, slides were washed twice at high stringency (0.2× SSC, 55°C), and then washed with a solution containing 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA followed by RNase A (20 μg mL^{-1}) treatment for 30 min at 37° C. After blocking with a solution of 1% bovine serum albumin, 100 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.3% Triton X-100, slides were incubated in anti-digoxigenin antisera conjugated to alkaline phosphatase for 2 h. Slides were washed four times with blocking buffer, and then incubated with the substrate (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) overnight. Sections were passed through an ethanol series and xylene before mounting. Sections were visualized under bright field using a Nikon E600 microscope.

Membrane Isolation

Arabidopsis seedlings were grown on 0.5× MS plates for 10 d. About 8 g of seedlings were ground in a cold mortar with 12 mL of homogenization buffer containing 50 mM HEPES-bis tris propane (HEPES-BTP) pH 7.2, 250 mM D-sorbitol, 6 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was filtered through four layers of cheesecloth and centrifuged at 11,000 rpm (Sorvall SS34) for 15 min at 4° C. The supernatant (approximately 12 mL) was layered on three tubes with 15% and 35% sucrose step gradient (in 10 mM HEPES-1,3-bis(tris[hydroxymethyl]methylamino) propane (BTP) pH 7.2, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 100,000*g* for 1 h (Beckman SW 28). Membrane at the interface were collected and assayed directly for ATPase or PPase activity. Protein concentration was determined with the Bio-Rad (Hercules, CA) protein assay reagent (catalog no. 500–0006).

ATPase and PPase Activity

ATPase and PPase hydrolysis in isolated membranes were determined using the Ames method (Briskin et al., 1987). Fifty μL of membrane sample in a test tube was mixed with 200 μL of an ATPase or a PPase reaction mixture and incubated in a 30°C water bath for 30 min. The ATPase mixture contained 3 mM ATP, 3mM $MgSO_4$, 1 mM $NaMoO_4$, 50 mM NH_4Cl, 25 mM HEPES-BTP pH 7.2, 5 mM NaN_3, and 0.2 mM Na orthovanadate, with or without 200 mM KNO_3. The PPase mix included 1.5 mM PPi-Na_4, 25 mM HEPES-BTP pH 7.2, 1.5 mM $MgSO_4$, and 1 mM $NaMoO_4$, with or without 50 mM KCl. The reaction was terminated by adding 0.7 mL of a stop solution containing one part of 10% ascorbic acid mixed with six parts of 0.42% ammonium molybdate made in 1N H_2SO_4. After 20 min at room temperature, the absorbance was read at 700 nm. NH_4Cl was added to dissipate any pH gradient as Triton X-100 interfered with the Ames assay. Specific V-ATPase or PPase activity was determined as nitrate-inhibited or K^+-stimulated hydrolysis, respectively, as described before (Churchill and Sze, 1984; Wang et al., 1986).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers L44581–L44584.

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