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Expression of Two Related Vacuolar H^+-ATPase 16-Kilodalton Proteolipid Genes Is Differentially Regulated in a Tissue-Specific Manner[1]

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The 16-kD proteolipid subunit is the principal integral membrane protein of the vacuolar H^+-ATPase (V-ATPase) complex that forms the proton channel responsible for translocating protons across lipid bilayers. Two degenerate synthetic oligonucleotides, COT11 and COT12, corresponding to highly conserved transmembrane domains in all 16-kD subunits sequenced so far, were used to amplify a partial cDNA of the V-ATPase proteolipid subunit from cotton (*Gossypium hirsutum* L.) by polymerase chain reaction (PCR). These PCR products were used to isolate two full-length cDNAs from a −3 d postanthesis cotton ovule library. Both clones, CVA16.2 and CVA16.4, consisting of 816 and 895 bp, respectively, encode the 16-kD proteolipid subunit of the V-ATPase. At the nucleotide level, the complete sequences of the two clones show 73.5% identity, but share about 95% identity within the coding region, although the two polypeptides differ by only one amino acid. Comparison of deduced amino acid sequences of the proteolipid subunits revealed that the four transmembrane domains and the two cytosolic extramembrane domains are highly conserved in all eukaryotes. Southern blot analysis of cotton genomic DNA showed that these clones belong to small gene families in related diploid and allotetraploid species. Northern blot analysis suggested that the three major V-ATPase subunits (69, 60, and 16 kD) are coordinately regulated, in part, at the transcriptional level. RNA analysis and reverse-transcription PCR established that 16-kD proteolipid transcripts differentially accumulate in different tissues and increase dramatically in tissues undergoing rapid expansion, particularly in anthers, ovules, and petals. The CVA16.4 proteolipid transcript is the most prevalent of the two proteolipid messages in expanding ovules harvested 10 d postanthesis. In contrast, the two proteolipid mRNAs accumulate to similar levels in developing petals.

The generation of a negative osmotic potential in the vacuole is a key contributing factor in regulating cell turgor, the driving force of cell expansion. The transport of water and osmoregulatory solutes into the vacuole is mediated by two electrogenic proton pumps, a proton-translocating inorganic pyrophosphatase (Maeshima and Yoshida, 1989; Rea and Poole, 1993) and a H^+-ATPase (Sze, 1985; Nelson and Taiz, 1989), located in the vacuolar membrane (tonoplast). In developing cotton (*Gossypium hirsutum* L.) seed trichomes (fibers), the histochemical localization of significant ATPase activity only on the tonoplast of elongating fibers (Joshi et al., 1988) suggests that one or both of the proton pumps are regulated during rapid cell expansion.

The structure and function of the V-ATPase in plants, animals, and fungi have received increasing scrutiny in recent years. The V-ATPase holoenzyme is a large complex of 450 to 750 kD comprising 8 to 10 different subunits (Parry et al., 1989; Matsuura-Endo et al., 1990; Dupont and Morrissey, 1992; Ward and Sze, 1992) that reside in most of the endomembrane compartments of a cell (reviewed by Gluck, 1993). Analogous to the structure of F_1F_0-ATPases, the V-ATPase consists of two sectors, a hydrophilic, V_1 peripheral subunit complex located on the cytosolic face of the membrane and a hydrophobic, V_0 integral membrane complex. The principal subunit of the V_0 sector is the 16-kD proteolipid subunit, an integral membrane protein that is the primary constituent of a proton channel (Kaestner et al., 1988; Sze et al., 1992).

The structure and stoichiometry of the proteolipid in the proton pore is a characteristic feature that distinguishes the V-ATPase and related F-type ATPases of eubacteria, mitochondria, and chloroplasts. The proton channel consists of 16-kD proteolipids present in a stoichiometry of six subunits per V-ATPase holoenzyme, whereas each F-ATPase contains twelve 8-kD proteolipids (Forgac, 1989). Gene duplication and fusion of the 8-kD F-ATPase proteolipid genes is believed to have given rise to the V-ATPase proteolipid, during the early evolution of eukaryotes (Mandel et al., 1988). Cross and Taiz (1990) proposed that this gene duplication event produced an altered H^+:ATP ratio that is related to the number of proteolipid subunits/catalytic subunits and a switch in function from an ATPase synthase to a proton-pumping ATPase.

The primary amino acid sequence of the 16-kD proteolipid subunit of eukaryotes contains four membrane-spanning domains (Mandel et al., 1988). A glutamate residue in transmembrane domain IV is believed to function in H^+-translocation across the lipid bilayer (Mandel et al., 1988; Noumi et al., 1991). Inhibition of ATPase activity by the H^+ pore blocker DCCD presumably occurs via specific binding

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Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; dpa, days postanthesis; F-ATPase, coupling factor-ATPase; RT-PCR, reverse transcription-polymerase chain reaction; V-ATPase, vacuolar H^+-ATPase.

of DCCD to the glutamate residue of transmembrane domain IV (Manolson et al., 1985; Rea et al., 1987; Arai et al., 1988; Kaestner et al., 1988; Finbow et al., 1992). Disruption of yeast genes encoding the V-ATPase proteolipid (subunit c) or major peripheral subunits (subunits A and B) results in the loss of vacuole acidification (Nelson and Nelson, 1990; Yamashiro et al., 1990; Umemoto et al., 1991). Soluble vacuolar proteins, and to a lesser extent membrane-associated proteins, are missorted as a consequence of the increase in vacuolar pH associated with V-ATPase (*vat*) mutants (Nelson and Nelson, 1990; Yamashiro et al., 1990; Klionsky et al., 1992). In plants, inhibition of expression of a tonoplast-associated subunit A gene by antisense mRNA in transgenic carrots produced altered leaf morphologies as a result of defective cell expansion (Gogarten et al., 1992).

To enhance our understanding of the compartmentalization and regulation of the V-ATPase in different cell types and tissues, two discrete cDNA clones encoding the integral membrane, 16-kD V-ATPase subunit have been isolated from developing cotton ovules. The two cDNA clones represent members of a small, multigene family encoding the 16-kD subunit in both diploid and allotetraploid cotton species. The increased complexity of the gene family in allotetraploid cotton species is a consequence of the presence of two distinct subgenomes. The expression of the two gene family members represented by the cDNA clones is induced in rapidly expanding tissues and is differentially regulated in a tissue-specific fashion. The results suggest that there is an increase in the accumulation of V-ATPase transcripts to provide a corresponding increase in proton-pumping activity during rapid cell expansion.

MATERIALS AND METHODS

Plant Material

Upland cotton (*Gossypium hirsutum* L. cv Acala SJ-2) was grown in the greenhouse under a 27/21°C day/night temperature regime. The phyllotactic arrangement of cotton flowering nodes facilitates the recovery of staged developing ovules at 3-d intervals preceding anthesis based on the position of opened flowers at anthesis (0 dpa). Developing fruits (bolls) were collected at specific stages after anthesis from flowers tagged at anthesis. Ovules collected from developing flowers at −3, −1, 0, and +10 dpa, and petals from −9, −6, −3, −1, 0, and +1 dpa flowers, as well as other tissues, were frozen in liquid nitrogen and stored at −80°C for RNA isolation. Floral tissues (petals, anthers, and bracts) collected from −1 dpa flowers and leaves were harvested from greenhouse-grown plants. Mature embryos were isolated from seeds that imbibed overnight at room temperature on saturated filter paper. Cotton roots approximately 3 to 4 cm in length were collected from seeds germinated at 28°C for 5 d.

RNA Isolation and Construction of Cotton Ovule λgt10 cDNA Libraries

Total RNA was isolated from cotton tissues using a modified, hot borate procedure as described by Wan and Wilkins (1994a), to minimize interference by endogenous phenolics. Poly(A)$^+$ RNA was purified from −3 dpa ovule RNA by oligo(dT)-Sepharose affinity chromatography (Silflow et al., 1979) at room temperature. A cotton ovule λgt10 cDNA library was constructed from −3 dpa developing ovule poly(A)$^+$ RNA using cDNA synthesis and cloning kits (BRL) and Stratagene's Gigapack Gold in vitro packaging extract according to the manufacturer's instructions. Approximately 2.6×10^7 recombinant phage in the −3 dpa cDNA library were amplified in *Escherichia coli* strain C600*hfl* and stored in phage dilvent (0.1 M NaCl, 0.1 M $MgSO_4$, 0.01% [w/v] gelatin) and 7% (v/v) DMSO at −80°C in 1-mL aliquots.

General Molecular Biology Methods

All oligonucleotide primers were synthesized by Operon Technologies, Inc. (Alameda, CA). Unincorporated nucleotides were separated from random-primer ^{32}P-labeled DNA hybridization probes (Feinberg and Vogelstein, 1983) by centrifugation in Sephadex spun columns (Sambrook et al., 1989).

DNA Amplification of a Cotton Partial cDNA Encoding a V-ATPase Proteolipid Subunit by PCR

Two degenerate oligonucleotide primers, designated COT11 [sense: 5′-GC(CT)TA(CT)GG(CGT)AC(GT)GC(CT)AAG-3′] and COT12 [antisense: 5′-C(GA)AA(GA)AT(GC)A(GA)(GA)AT(GC)AG(GA)ATC-3′], were designed to PCR-amplify the four conserved transmembrane domains of the V-ATPase proteolipid (Fig. 1). PCR amplification (Saiki et al., 1985) was performed in a 125-μL reaction volume containing 3.75×10^8 recombinant phage from the amplified −3 dpa ovule λgt10 library, 200 μM deoxynucleotides, 1.5 mM $MgCl_2$, 25 pmol of each COT primer, and 2.5 units of *Taq* polymerase (Promega) in the buffer supplied with the enzyme. Reactions were conducted in an Ericomp (San Diego, CA) temperature cycler for 30 s at 94°C, followed by 30 cycles of 92°C for 2 min, 42°C for 2 min, and 72°C for 2 min and a final extension step for 10 min at 72°C. A 300-bp PCR product was excised from 1% (w/v) low-melting-point agarose (SeaPlaque, FMC, Rockland, ME) after gel electrophoresis and cloned directly into Novagen's pT7Blue T-vector. The recovery of a partial V-ATPase 16-kD proteolipid cDNA was confirmed by dideoxy chain-termination sequencing (Sanger et al., 1977) of single-stranded DNA (Vieira and Messing, 1987) prepared from four independent transformants.

Isolation and Characterization of Cotton V-ATPase Proteolipid cDNA Clones

Nitrocellulose membranes containing 1.5×10^5 plaque-forming units of the amplified −3 dpa ovule λgt10 cDNA library were screened at high stringency in 50% (v/v) formamide at 42°C (Sambrook et al., 1989) using radiolabeled gel-purified PCR products as hybridization probes. After purification of positive plaques to homogeneity, cDNAs were recovered from λ DNA as *Eco*RI restriction fragments in 1% (w/v) low-melting-point agarose and cloned into pUC119 (Struhl, 1985; King and Blakesley,

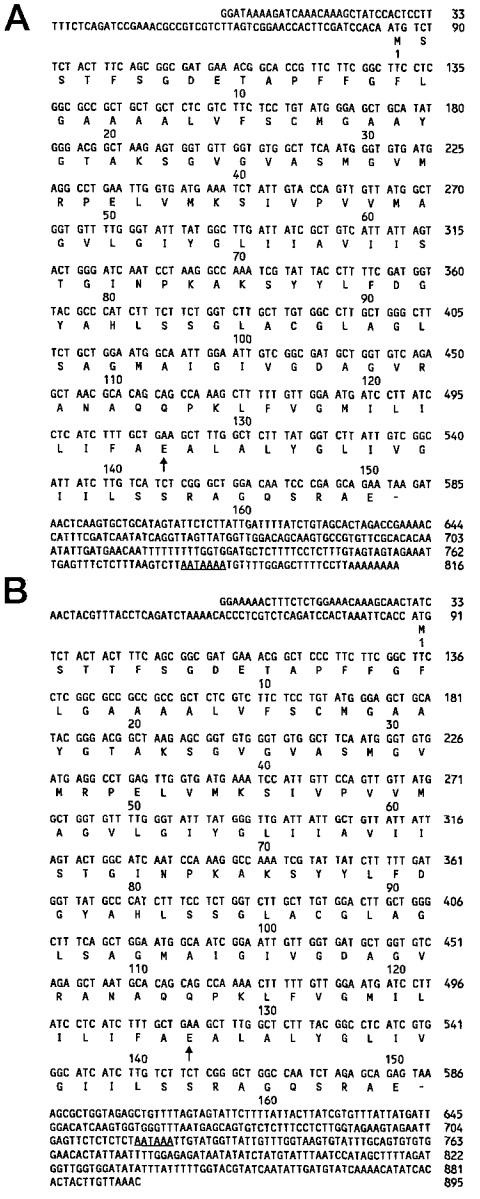

Figure 1. Nucleotide and deduced amino acid sequences of two cotton 16-kD V-ATPase proteolipid cDNA clones. A, CVA16.2. B, CVA16.4. Conserved polyadenylation signals are underlined. The arrow denotes glutamate-142 (E), the putative binding site of the ATPase inhibitor DCCD.

1986). The complete nucleotide sequence of cDNA clones CVA16.2 and CVA16.4 was determined by dideoxy chain-termination sequencing of a series of overlapping single-stranded DNA deletions (Dale and Arrow, 1987).

DNA and RNA Gel Blot Analysis

Southern blots of genomic DNA from *Gossypium arboreum* L. (A_2 genome), *Gossypium raimondii* Ulbr. (D_5 genome), and *G. hirsutum* L. cv Acala SJ-2 ($[AD]_1$ genome) were prepared and hybridized as described previously (Wilkins et al., 1994). Total RNA was electrophoresed in 1.2% (w/v) agarose:3% (v/v) formaldehyde gels and transferred to HyBond-N nylon membrane (Amersham) by capillary blotting (Ausubel et al., 1987). All membranes were stained in an aqueous solution of 0.04% (w/v) methylene blue (Sambrook et al., 1989) to ensure that equal amounts of RNA were loaded in each lane. Prehybridization and hybridization of RNA blots were performed at 65°C (Sambrook et al., 1989), except that the final concentration of SDS was decreased to 0.1% (w/v). Posthybridization washes of all membranes were conducted at high stringency in 0.1 or 0.2× SSC and 0.1% (w/v) SDS at 60 to 65°C, and the membranes were exposed to x-ray film at −80°C, with two intensifying screens (DuPont).

RT-PCR

Reverse transcription of total RNA (300 ng) from cotton tissues for first-strand cDNA synthesis was performed in separate reactions using the 5′ RACE System from BRL. Optimal RT-PCR conditions using 300 ng of total RNA amplified for 25 cycles were determined after reactions performed with varying RNA concentrations ranging from 100 ng to 1 μg and PCR-amplified for 25, 30, or 35 cycles. Antisense oligonucleotide primers COT99 (5′-ATTAAGACTTAAAGAGAAACTC-3′) and COT98 (5′-GTAGTGTGATATGTTTTGATAC-3′), specific to unique sites within the 3′ untranslated regions of CVA16.2 and CVA16.4, respectively, were used to synthesize first-strand cDNAs. COT43 [5′-C(CT)(CA)GAGCAGA(AG)TAAG-3′], a degenerate oligonucleotide primer designed against the sense strand encoding the terminal four amino acids and stop codon in both cDNA clones, was used in subsequent PCR amplification reactions using primer pairs [COT43 × COT98] or [COT43 × COT99]. Two microliters of the newly synthesized cDNAs were used directly as templates for PCR amplification under the following conditions: 1× buffer, 100 μM deoxynucleotides, 5 pmol of each COT primer, and 1 unit of *Taq* polymerase. The reactions were supplemented with 1 mM spermidine (Wan and Wilkins, 1993) to optimize amplification of specific products at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min and a final polymerization step at 72°C for 10 min.

Computer Analysis of DNA and Protein Sequences

Protein analysis, sequence analysis, and nucleotide sequence comparisons to genes recovered from the GenBank were conducted using PCGENE software (Intelligenetics, Mountain View, CA).

RESULTS

Isolation of V-ATPase 16-kD Proteolipid cDNA Clones from Cotton

The four highly conserved transmembrane domains of the V-ATPase 16-kD proteolipid subunits from oat (Lai et al., 1991), yeast (Nelson and Nelson, 1989), and *Drosophila* (Meagher et al., 1990) were used as the basis for PCR amplification of a cotton homolog. Degenerate oligonucleotide primers, designated COT11 and COT12, were de-

signed against membrane-spanning domains I and IV from the deduced amino acid sequence of the aforementioned species. The expected [COT11 × COT12] PCR fragment of 300 bp spanning domains I through IV of the proteolipid was amplified from a cotton ovule λgt10 cDNA library. DNA sequencing of the PCR product confirmed that the partial cDNA clone encoded the proteolipid, based on amino acid similarity to the oat proteolipid. The 300-bp PCR fragment was used as a homologous hybridization probe to retrieve full-length cotton proteolipid cDNA clones from the ovule library. Eleven of 19 phage clones purified to homogeneity produced the 300-bp [COT11 × COT12] product after PCR. Two of these clones, designated CVA16.2 and CVA16.4, exhibited different restriction enzyme patterns and were selected for further characterization.

Based on sequence similarity to deduced amino acids of published clones from oat, yeast, and *Drosophila*, the two cotton cDNA clones encode the 16-kD proteolipid V-ATPase subunit. The 816-bp CVA16.2 (Fig. 1A) and the 895-bp CVA16.4 (Fig. 1B) cotton cDNA clones share 94.7% nucleotide identity within the coding region, although the sequences of the extramembrane loop between transmembrane domains II and III (L2) and the carboxyl terminus are discernibly more divergent, sharing 89.6 and 87.8% nucleotide identity, respectively. The untranslated regions, however, are less well conserved, exhibiting a nucleotide identity of 38.1% in the 5′ region and 46.2% in the 3′ region. A putative polyadenylation signal (AATAAA) is located 20 bp upstream from the poly(A)$^+$ tail in CVA16.2 (Fig. 1A) but is positioned greater than 175 bp distal to the terminus of CVA16.4 (Fig. 1B). However, the CVA16.4 clone (Fig. 1B) does not include a poly(A)$^+$ tail. Despite the nucleotide differences indicating that CVA16.2 and CVA16.4 represent two distinct 16-kD V-ATPase genes, the subunits are nearly perfectly conserved at the amino acid level (Figs. 1 and 2). The 165-amino acid polypeptides encoded by CVA16.2 and CVA16.4 have a calculated molecular weight of 16,747 and 16,661, respectively. The sole difference between the deduced cotton polypeptides is a conserved amino acid change between Ser^3 in CVA16.2 (Fig. 1A) and Thr^3 in CVA16.4 (Fig. 1B).

Protein Sequences and Comparisons

Comparison of the deduced amino acid sequence of the cotton and oat (Lai et al., 1991) 16-kD proteolipid revealed 96.7% amino acid identity (Fig. 2). Compared to the polypeptide from fungal, insect, and mammalian species, the cotton 16-kD subunit exhibits 52 to 57% amino acid identity to the homologs of yeast (Nelson and Nelson, 1989), *Manduca sexta* (Dow et al., 1992), and mouse (Hanada et al., 1991). It is clear, however, that some domains of the proteolipid are more highly conserved than others in these species (Fig. 2). For purposes of comparison, the proteolipid domains depicted in Figures 2 and 3 and Table I were defined on the basis of amino acid similarity, hydropathy profiles, and secondary structure algorithms. The amino acid similarity of each domain in the proteolipid from pairwise comparisons between species is presented in Table I. In general, the transmembrane domains (I–IV) and extramembrane domains or loops (L1 and L3) are the most highly conserved regions among the diverse proteolipid subunits. Transmembrane domain IV is the most conserved domain among the species, which is consistent with the role of this domain in proton translocation (Mandel et al., 1988; Noumi et al., 1991). With the exception of transmembrane domain III, the conserved domains of the plant proteolipids are more closely related to *M. sexta* and mouse sequences than to yeast sequences. The degree of divergence apparent in the amino and carboxyl termini and in the extramembrane domain L2 amino acid sequences indicates that these regions are more subject to evolutionary change. Even among the highly homologous plant proteolipids, four of the five amino acids that differ between the cotton and oat subunits reside within the less-conserved domains.

The most striking difference between the proteolipids from plants, fungi, and animals resides within the carboxyl-terminal domain. The proteolipid carboxyl terminus in animal species is characterized by a short stretch of three to four hydrophilic amino acids. However, this hydrophilic region is extended by an additional seven amino acids in plants (Fig. 2). This heptapeptide terminates in a glutamate residue (E) in both cotton proteolipids and Asp (D) in oat (Fig. 2). Yeast also contains an extended carboxyl terminus comprising seven amino acids (Fig. 2). However, the primary amino acid sequence of this peptide is not strongly conserved, and in contrast to plant proteolipids, the yeast

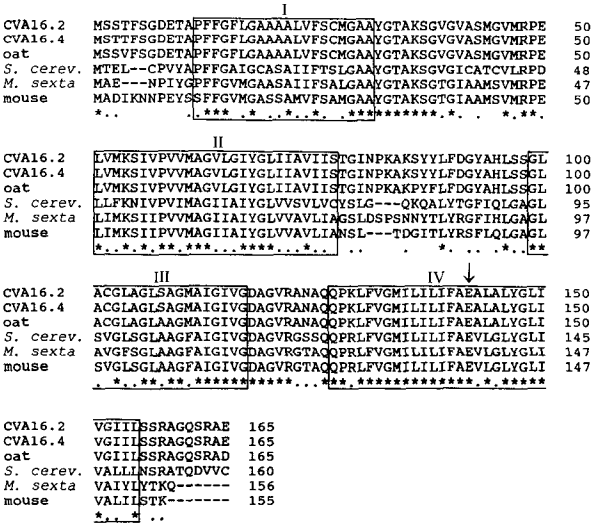

Figure 2. Alignment of 16-kD V-ATPase polypeptides from plants, animals, and yeast. The deduced amino acid sequence from mouse (Hanada et al., 1991), *M. sexta* (Dow et al., 1992), *Saccharomyces cerevisiae* (Nelson and Nelson, 1989), oat (Lai et al., 1991), and cotton were aligned for maximal homology. Asterisks denote amino acid residues conserved in all species shown in the figure, whereas conserved amino acid replacements are indicated by dots. The boxed regions correspond to the putative transmembrane domains as defined by amino acid similarity and secondary structure predictions. The arrow identifies the glutamate residue (E) in transmembrane domain IV that is the site of DCCD inhibition. Numbers on the right refer to the last amino acid in the row.

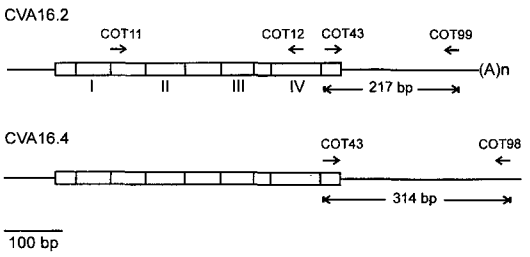

Figure 3. Schematic diagram of the cotton 16-kD V-ATPase cDNA clones CVA16.2 and CVA16.4. The shaded boxes represent transmembrane domains I through IV in the coding region. The arrows denote the position and orientation of the corresponding oligonucleotide COT primers used for PCR amplification. The 217- and 314-bp clone-specific fragments were PCR-amplified by primer pairs [COT43 × COT99] from CVA16.2 and [COT43 × COT98] from CVA16.4. For purposes of comparison, the amino acids representing the transmembrane domains were defined on the basis of amino acid similarities, hydropathy profiles, and secondary structure algorithms (Garnier et al., 1978; Chou and Fasman, 1979) as follows: domain I (12–31), II (51–77), III (99–117), and IV (127–155).

heptapeptide is predominantly hydrophobic. The Ala (A) and Gln (Q) at positions 1 and 3 of the heptapeptide are conserved in both plant and yeast species.

The Cotton V-ATPase 16-kD Subunit Gene Family

Since the cotton V-ATPase 16-kD proteolipid cDNA clones were isolated from cultivated cotton (*G. hirsutum* L. cv Acala SJ-2), an allotetraploid species ($[AD]_1$) a genomic Southern blot was prepared from the allotetraploid as well as from related A_2 (*G. arboreum* L.) and D_5 (*G. raimondii* Ulbr.) diploid species to determine the extent of the proteolipid gene family in these species. The complexity of restriction patterns detected by CVA16.4 suggests that genes encoding the 16-kD proteolipid are organized as small gene families in all three species (Fig. 4A). As might be predicted, the complexity of the allotetraploid $(AD)_1$ restriction pattern correspondingly increases due to the presence of genes derived from the A and D subgenomes and the evolution of polymorphic sites, since polyploidization occurred 1 to 2 million years ago (Wendel, 1989). The presence of six high-molecular-weight *Bam*HI restriction fragments ranging between 7 and 20 kb indicates that the 16-kD gene family in *G. hirsutum* consists of up to six family members. To determine the relationship between the cotton proteolipid cDNAs and the subgenomes of the allotetraploid, genomic DNA blots (Fig. 4B) were hybridized with clone-specific probes from the 3′ untranslated region of CVA16.2 and CVA16.4 (Fig. 3). To obtain clone-specific probes that differentiate between the two cotton cDNAs, oligonucleotide primers were synthesized to enable recovery of the 3′ untranslated regions by PCR (Fig. 3). When used in conjunction with a common primer (COT43) anchored in the carboxyl terminus of the coding region, clone-specific primers COT98 and COT99 produce PCR fragments of 217 or 314 bp from CVA16.2 and CVA16.4, respectively, that do not cross-hybridize on DNA blots. The clone-specific probes hybridize to unique restriction fragments on DNA blots in A and D diploid species (Fig. 4B), indicating that the clones do not simply represent homologs from the A and D subgenomes. Members of the proteolipid gene family recognized by the [COT43 × COT98] clone-specific probe of CVA16.4 exhibit a higher degree of polymorphism than CVA16.2-related members in both A and D diploid species.

Expression of V-ATPase 16-kD Proteolipid Subunits in Cotton

To determine if the expression of the V-ATPase subunits is coordinately regulated at the RNA level, the expression of the V_0 16-kD subunit and V_1 69-kD catalytic and 60-kD regulatory peripheral subunits of the V-ATPase was examined in +10 dpa developing ovules. Although the cDNA clones were isolated from −3 dpa ovules, all subsequent analysis was performed using +10 dpa ovules, since expression of the V-ATPase genes is higher in this developmental stage. RNA blots (Fig. 5A) containing 15 μg of +10 dpa ovule RNA were hybridized with the same level of radiolabeled cotton clones encoding the three major V-ATPase subunits: the 69-kD cDNA CVA69.24 (Wilkins, 1993), the 60-kD cDNA CVA55 (Wan and Wilkins, 1994b), and the 16-kD proteolipid cDNA CVA16.4. Each of the V-ATPase subunit RNA transcripts accumulates to discrete levels in developing ovules (Fig. 5A). Proteolipid mRNAs are the most abundant V-ATPase transcripts in this tissue relative to the 69- and 60-kD mRNAs. The marked increase of proteolipid mRNA relative to the other V-ATPase subunits may reflect the fact that there are twice as many

Table I. *Percent amino acid identity for pairwise comparisons to cotton 16 kD V-ATPase deduced amino acid sequences by domain*

The amino acids within each domain are numbered based on the cotton sequence. Transmembrane domains (I–IV) were defined on the basis of amino acid similarity and secondary structure algorithms (Garnier et al., 1978; Chou and Fasman, 1979). N, N terminus; C, carboxyl terminus; L, extramembrane loops.

Species	Amino Acid Domain								
	N	I	L1	II	L2	III	L3	IV	C
	1–11	12–31	32–50	51–77	78–98	99–117	118–126	127–155	156–165
Oat[a]	90.9	100	100	100	90.5	94.7	100	100	90
Yeast[b]	18.2	60	63.2	55	14.3	73.7	66.6	79.3	40
M. sexta[c]	9.1	65	79	66.7	23.8	73.7	77.7	82.7	0
Mouse[d]	18.2	65	79	66.7	9.5	73.7	77.7	82.7	0

[a] Lai et al., 1991. [b] Nelson and Nelson, 1989. [c] Dow et al., 1992. [d] Hanada et al., 1991.

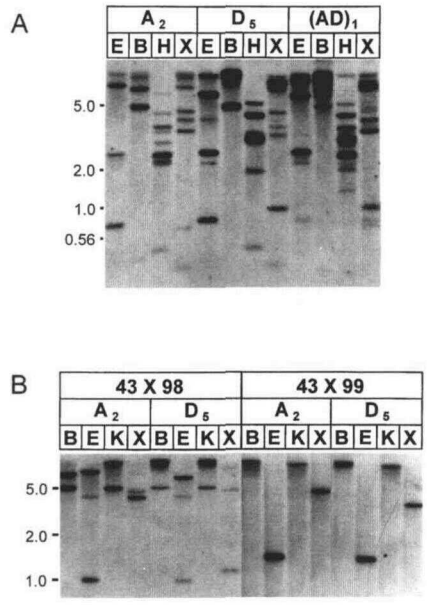

Figure 4. Southern blot of genomic DNA from diploid and allotetraploid cotton species. Genomic DNA from *G. arboreum* L. (A_2 genome, $2n = 26$) (3 μg), *G. raimondii* Ulbr. (D_5 genome, $2n = 26$) (3 μg), and *G. hirsutum* L. ($[AD]_1$ genome, $2n = 4x = 52$) (6 μg) was digested with the indicated restriction enzymes, blotted, and hybridized with radiolabeled cotton cDNA. The positions of molecular weight markers are indicated to the left of the figure, E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; X, *Xba*I. A, Blot hybridized with CVA16.4 cDNA. B, Blots hybridized with [COT43 × COT98] and [COT43 × COT99] PCR products corresponding to the 3′ untranslated regions of CVA16.4 and CVA16.2, respectively.

proteolipid subunits per holoenzyme, suggesting that the V-ATPase genes are coordinately expressed. Confirmation that the same amount of RNA was loaded in each lane was obtained by hybridization of the blot with a radiolabeled soybean 18S rDNA clone (Zimmer et al., 1988) (Fig. 5A) and a cotton cDNA (*mybA*) encoding a transcription factor (data not shown).

An RNA blot (Fig. 5B) containing a titration of ovule total RNA revealed that the 1.0-kb, 16-kD V-ATPase mRNA observed in Figure 5A is actually a doublet that can be resolved into two discrete mRNA species. The results also demonstrated that the two 16-kD mRNAs are differentially expressed in +10 dpa developing cotton ovules, such that the lower-molecular-weight mRNA species is more prevalent than the higher-molecular-weight message. Because the two ovule proteolipid mRNAs accumulate to different levels, the abundant lower-molecular-weight species tends to obscure the less-abundant message on RNA blots (Fig. 5, A and B). As the number of micrograms of RNA on the gel is decreased (Fig. 5B), the less-abundant transcript becomes barely discernible as faint, fuzzy bands that appear as a "tail" to the abundant mRNA (see lanes with 5 and 7 μg of total RNA).

The similarity in molecular weight differences between the 16-kD mRNAs and the cDNA clones suggests that the clones may, in fact, represent the two mRNA species. To determine if this is the case and to maximize resolution of the two transcripts, the clone-specific primer pairs [COT43 × COT98] and [COT43 × COT99], described in Figure 3, were used to amplify the 3′ untranslated regions from 16-kD mRNAs isolated from +10 dpa developing cotton ovules via RT-PCR. Consistent with RNA analysis, the differential recovery of the expected RT-PCR products indicates that the 314-bp RT-PCR product corresponding to cDNA CVA16.4 is more abundant than the 217-bp CVA16.2 RT-PCR product (Fig. 5C). Control experiments containing only one primer did not amplify any detectable RT-PCR products. Thus, the results established that the cotton 16-kD cDNA clones correspond to the two mRNAs observed on RNA blots and that the mRNA corresponding to CVA16.4 is the predominant proteolipid mRNA in developing ovules.

The expression of the 16-kD V-ATPase subunit in other tissues was examined by RNA blot analysis and RT-PCR to determine the relative contribution of each mRNA species. Analysis of a northern blot (Fig. 6A) established that the 16-kD proteolipid genes are differentially expressed in various tissue types, such that the 16-kD mRNAs are present at relatively low levels in leaves, bracts, and mature embryos. However, there is a striking increase of the proteolipid transcripts in roots, anthers, and petals, but it is most notable in expanding petals. The same blot was hybridized with an 18S rDNA (Zimmer et al., 1988) probe as a control. Typically, the majority of the main ribosomal RNA bands (Fig. 6A) occurs as lower-molecular-weight cleavage products in leaf and bract samples (data not shown). The sum of the signal contained within all rRNA transcripts (data not shown) indicates that each lane of the RNA blot contains an equivalent amount of total RNA. These results are reproducible with different preparations of RNA and are con-

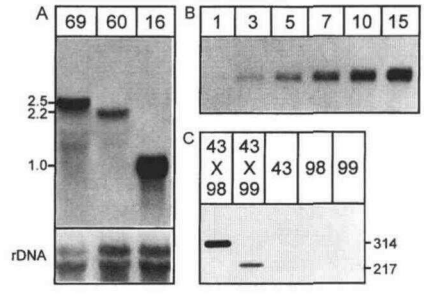

Figure 5. RNA analysis of V-ATPase expression in +10 dpa developing cotton ovules. A, RNA blot containing total ovule RNA (15 μg) hybridized with cotton cDNA clones encoding the V_1 69-kD catalytic subunit (Wilkins, 1993) and 60-kD regulatory subunit (Wan and Wilkins, 1994b) or the V_0 16-kD proteolipid (CVA16.4). Molecular mass markers are indicated to the left of the figure in kb. The same blot was hybridized with a soybean 18S rDNA clone (Zimmer et al., 1988) as a control. B, RNA titration blot of total ovule RNA probed with equal cpm of cotton 16-kD proteolipid cDNAs CVA16.2 and CVA16.4. The numbers within the boxed region indicate the amount in micrograms of RNA loaded in each lane. C, RT-PCR of ovule total RNA (300 ng) using the clone-specific primer pairs [COT43 × COT99] and [COT43 × COT98] (Fig. 3). RT-PCR products were separated by gel electrophoresis in 1.2% (w/v) agarose gels. Lanes with single COT primers indicate PCR controls. The numbers to the right of the figure indicate the size of the PCR products in bp.

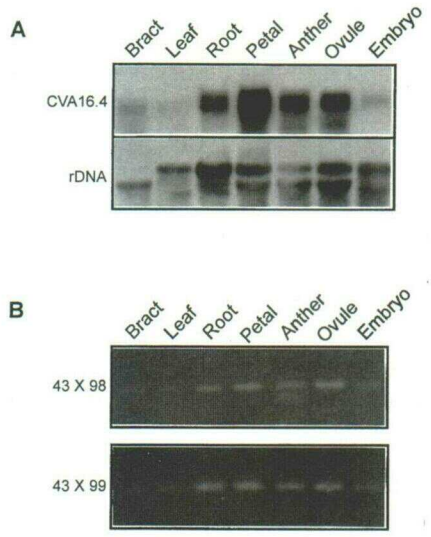

Figure 6. RNA analysis of the V-ATPase 16-kD subunit expression in different tissues. A, An RNA blot containing total RNA (15 μg) isolated from different tissues was hybridized with the radiolabeled cotton 16-kD cDNA CVA16.4. The size of the proteolipid mRNA transcript is approximately 1 kb. As a control, the blot was hybridized with an 18S rDNA clone from soybean (Zimmer et al., 1988). B, RT-PCR of total RNA from various cotton tissues using V-ATPase 16-kD clone-specific primers. RT-PCR products were fractionated by gel electrophoresis in 1.5% (w/v) agarose. CVA16.4 primer pair [COT43 × COT98] (Fig. 3) amplifies a 314-bp product. CVA16.2 primer pair [COT43 × COT99] (Fig. 3) amplifies a 217-bp product. The amount of [COT43 × COT99] RT-PCR products loaded on the gel was increased 4-fold to allow visual detection. RNA was isolated from fully expanded, mature leaves and bracts. Petals and anthers were harvested from flowers collected the day before anthesis. Ovule RNA was obtained from +10 dpa developing bolls. Mature embryos were recovered from seeds that imbibed overnight on wet filter paper.

sistent on replicated RNA blots hybridized with cDNA probes encoding two other V-ATPase subunits. Based on RT-PCR experiments (Fig. 6B) performed using clone-specific primers (Fig. 3), both of the proteolipid mRNA species are present in all tissue types examined. However, to visually detect the 217-bp CVA16.2 RT-PCR product on agarose gels, 4-fold more of the reaction product was required.

Control reactions were performed using equimolar amounts of each primer pair and the corresponding cDNA template (data not shown) and demonstrated that differential PCR amplification was not a major factor. These results would suggest that the 314-bp RT-PCR product corresponding to the CVA16.4 mRNA is the more prevalent proteolipid message in a diversity of tissues. This observation is consistent with RNA analysis demonstrating that the CVA16.4 transcript is more abundant in +10 dpa ovules (Fig. 5, B and C). However, it is unknown if the clone-specific PCR primers also amplify other members of the 16-kD V-ATPase gene family. The CVA16.4 clone-specific primer pair [COT43 × COT98] consistently amplified a 250-bp band in addition to the expected 314-bp RT-PCR product in anthers and embryos (Fig. 6B). The amplification of this unidentified extraneous product is presumably a PCR artifact and probably does not represent an additional 16-kD variant in these tissues.

To determine if the abundance of the V-ATPase proteolipid transcripts is modulated in expanding tissues in a developmental context, the expression of proteolipid genes was examined by RNA blot analysis in flower petals. The petals of the ephemeral flower type of cotton expand dramatically in the days preceding anthesis, increasing approximately 10-fold in size between −9 dpa and anthesis (Fig. 7). Shortly after pollination on the day of anthesis (0 dpa), the rapid onset of petal senescence is accompanied by the discernible accumulation of anthocyanin pigments (Fig. 7). RNA blot analysis (Fig. 8) showed that the level of two discrete 16-kD proteolipid mRNAs is modulated during petal development. The two V-ATPase proteolipid mRNAs are already quite abundant in young petals of −9 dpa flower buds relative to the low abundance of these transcripts in vegetative tissues (Fig. 6A). The most dramatic increase of the proteolipid mRNAs to peak level occurs in −1 dpa petals of flowers in the "candle stage." The proteolipid mRNAs begin to decline on anthesis (Fig. 8), reaching preanthesis levels in senescing petals collected the day after anthesis (+1 dpa). The two proteolipid transcripts accumulate to similar levels in developing petals (Fig. 8), which is in marked contrast to the relative abundance of the mRNAs in +10 dpa ovules (Fig. 5B). Hybridization of the same blot with an 18S rDNA clone (Zimmer et al., 1988) showed that the same amount of total RNA was loaded for each developmental stage.

DISCUSSION

Given the diversity of biological functions associated with the various organelles of the endomembrane system,

Figure 7. Developing cotton flower buds. Top, from left to right: cotton flower buds harvested at −9, −6, and −3 dpa; bottom, from left to right: −1 (candle stage), 0, and +1 dpa flowers. All the buds were photographed at the same magnification and are reduced to one-third of their original size.

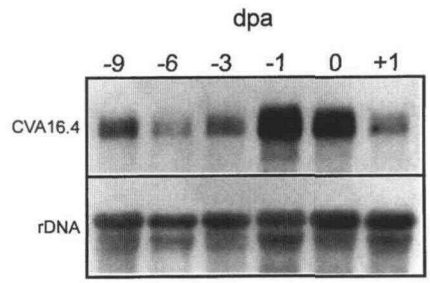

Figure 8. RNA analysis of the V-ATPase 16-kD subunit expression in developing cotton petals. An RNA blot containing total RNA (15 μg) isolated from petals at different stages of development (−9 to +1 dpa) was hybridized with the radiolabeled cotton 16-kD cDNA CVA16.4. The size of the proteolipid mRNA transcript is approximately 1 kb. The blot was hybridized with a soybean 18S rDNA clone (Zimmer et al., 1988) as a control.

the dynamics of which may vary between tissues and cell types, the occurrence of distinct V-ATPase isoforms (Gluck, 1993) is not wholly unexpected. However, very little is known regarding the expression of the various V-ATPase subunits in eukaryotes. To address this issue, two cDNA clones encoding the V-ATPase 16-kD proteolipid subunit were isolated and characterized from cotton. The polypeptides encoded by the two cDNAs, CVA16.2 and CVA16.4, differ by a single, conserved amino acid change and thus are essentially identical. The cotton clones represent two unique members of a small, multigene family, a genome organization similar to that of the proteolipid gene family reported in oat (Lai et al., 1991). This complex genome organization is not due to the polyploid nature of these two plant species, since multiple genes are also evident in related diploid cotton species. Although at least two genes of the cotton proteolipid gene family are expressed, it is as yet undetermined if other family members are subject to tissue-specific regulation, respond to environmental stimuli, or may encode pseudogenes. In humans, for instance, four proteolipid genomic clones have been characterized, although three of the clones may in fact be derived from pseudogenes (Hasabe et al., 1992). Thus, determining the potential contribution of each proteolipid gene to unique cellular functions remains an important question.

The major peripheral V-ATPase A and B subunits and the proteolipid (subunit c) are present in the holoenzyme in a stoichiometry of $A_3B_3c_6$. It is interesting that proteolipid RNA transcripts are more abundant than the 69-kD catalytic subunit A message, suggesting that expression of V-ATPase subunits is coordinately regulated, in part, at the transcriptional level commensurate with the number of polypeptides contributed to the holoenzyme. It was recently shown that the V-ATPase proteolipid in animals is the same protein as the ductin polypeptide of gap junctions (Finbow et al., 1994). If an analogous situation also occurs in plants, then the observed increase in proteolipid transcripts relative to the other subunits may reflect a multifunctional role of the proteolipid subunit. However, in contrast to expectations raised if the above stoichiometric relationship holds true at the RNA level, subunit A RNA transcripts are relatively more abundant than the 60-kD subunit B transcripts. This observed difference in the relative levels of the A and B V-ATPase subunit mRNAs, however, is most likely the result of a gene dosage effect, since the subunit A multigene family contains at least twice as many genes as the subunit B gene family (Wilkins et al., 1994; C.-Y. Wan and T.A. Wilkins, unpublished data). Doubtless, other levels of regulation operating at the posttranscriptional and posttranslational levels also play a role in modulating the expression and activity of the various V-ATPase subunits and holoenzyme.

Both V-ATPase proteolipid mRNA transcripts corresponding to the two cotton cDNA clones increase severalfold, specifically in tissues undergoing rapid cell expansion. The induction of the proteolipid mRNAs is particularly evident in flower petals preparing to unfurl at anthesis. Modulation of proteolipid transcript levels during petal development entails a significant increase in proteolipid mRNAs coincident with rapid petal expansion and a corresponding decline in message during senescence. The induction of V-ATPase gene expression is presumably related not only to vacuolation, but also to the mobilization of the cellular machinery necessary to support rapid cell expansion. An increase in H^+-ATPase activity on the tonoplast is expected to facilitate an increased influx of osmoregulatory solutes via secondary transport into the vacuole during turgor-driven cell expansion. In addition to osmoregulatory solutes, there is also a corresponding translocation of water into the vacuole. The observed increase in expression of the γ-tonoplast intrinsic protein water channel protein associated with cell elongation in vegetative tissues (Ludevid et al., 1992) and cell expansion stimulated by GA_3 (Phillips and Huttly, 1994) are consistent with our results.

The V-ATPase genes represented by the cDNA clones CVA16.2 and CVA16.4 are differentially expressed in mature and expanding tissues. Although both proteolipid messages are induced severalfold in expanding tissues, the majority of the total proteolipid mRNA is composed of the CVA16.4 mRNA species in ovules, whereas the two proteolipid mRNAs are equally abundant in developing petals. The differential expression of the proteolipid genes is not the result of differential gene expression of the two subgenomes in the tetraploid species, since genes corresponding to the cDNA clones were detected in both the A and D diploid species using clone-specific hybridization probes. The differentially expressed proteolipid genes may encode functionally significant isoforms present in the same cells and organelles, or even within the same holoenzyme.

Considering that the V-ATPase is not restricted to the vacuolar compartment, but is a component of the endomembrane system, the potential existence of organelle-specific isoforms is quite feasible. For instance, disruption of the *VMA3* 16-kD proteolipid subunit or the related *VMA11* 17-kD polypeptide results in the loss of vacuolar acidification and V-ATPase activity in yeast (Umemoto et al., 1991). However, since the mutants do not complement one another, *VMA3* and *VMA11* apparently encode functionally independent isoforms. In fact, the identification of

V-ATPase variants associated with vacuoles, Golgi, or coated vesicles (Chanson and Taiz, 1985; Depta et al., 1991; Gogarten et al., 1992) or that respond to different stimuli (Reuveni et al., 1990; Narasimhan et al., 1991; Sanchez-Aguayo et al., 1991) lends further credence to this premise. Thus, regulation of V-ATPase activity within a particular endomembrane compartment would depend on the control of the various subunits and isoforms via modulation of RNA and/or protein levels and enzymatic activity as determined by the function of the organelle and the physiological environment of the cell.

The correct delivery of organelle-specific components during biogenesis and ontogeny is essential for the maintenance of vacuole integrity and function. Several studies have established that different sorting mechanisms are employed to target soluble and membrane proteins to the vacuolar compartment (Klionsky and Emr, 1989; Klionsky et al., 1992; Raymond et al., 1992; Seeger and Payne, 1992; Gomez and Chrispeels, 1993). In animal cells, the two targeting motifs known to mediate sorting of membrane glycoproteins to the lysosome depend on the proteolytic processing of a cytoplasmic carboxyl-terminal sorting signal (Guarnieri et al., 1993; Ogata and Fukuda, 1994). The mechanism by which membrane proteins are sorted to the vacuole in yeast and plants is as yet undetermined. It is interesting to note, however, that plant and yeast V-ATPase proteolipid subunits contain a carboxyl-terminal extension of six to seven amino acids not found in animal or insect homologs. Whether this heptapeptide functions as a vacuolar membrane targeting signal by a mechanism similar to lysosomal membrane glycoproteins or if this peptide influences the topology of the proteolipid in the lipid bilayer is currently under investigation.

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The GenBank/EMBL accession numbers for the sequences reported in this article are U13669 (CVA16.2) and U13670 (CVA16.4).

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