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Ancient origin of the vacuolar H$^+$-ATPase 69-kilodalton catalytic subunit superfamily

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Abstract Recently, two distinct cDNA clones encoding the catalytic subunit of the vacuolar H$^+$-ATPase (V-ATPase) were isolated from the allotetraploid cotton species *Gossypium hirsutum* L. cv 'Acala SJ-2' (Wilkins 1992, 1993). Differences in the nucleotide sequence of these clones were used as molecular markers to explore the organization and structure of the V-ATPase catalytic subunit genes in the A and D genomes of diploid and allotetraploid cotton species. Nucleotide sequencing of polymerase chain reaction (PCR) products amplified from *G. arboreum* (A_2, $2n$=26), *G. raimondii* (D_5, $2n$=26), and *G. hirsutum* cv 'Acala SJ-2' [$(AD)_1$, $2n=4x$=52] revealed a V-ATPase catalytic subunit organization more complex than indicated hitherto in any species, including higher plants. In the genus *Gossypium*, the V-ATPase catalytic subunit genes are organized as a superfamily comprising two diverse but closely related multigene families, designated as *vat*69A and *vat*69B, present in both diploid and allotetraploid species. As expected, each *vat*69 subfamily is correspondingly more complex in the allotetraploid species due to the presence of both A and D alloalleles. Because of this, about one-half of the complex organization of V-ATPase catalytic subunit genes predates polyploidization and speciation of New World tetraploid species. Comparison of plant and fungal V-ATPase catalytic subunit gene structure indicates that introns accrued in the plant homologs following the bifurcation of plant and fungi but prior to the gene duplication event that gave rise to the *vat*69A and *vat*69B genes approximately 45 million years ago. The structural complexity of plant V-ATPase catalytic subunit genes is highly conserved, indicating the presence of at least ten introns dispersed throughout the coding region.

Key words Cotton · *Gossypium* · Allopolyploid Vacuolar H$^+$-ATPase · Catalytic subunit · Subunit A

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Introduction

Emerging studies clearly demonstrate the utility of vacuolar or V-type H$^+$-ATPase (V-ATPase) genes as molecular markers for studying phylogenetic relationships in an evolutionary context (Gogarten and Taiz 1992; Gogarten et al. 1992b; Iwabe et al. 1989; Kibak et al. 1992; Starke and Gogarten 1993). The recent discovery that both V- and F-type H$^+$-ATPases have evolved from common ancestral genes (Gogarten et al. 1989, 1992b) is reflected in the remarkable structural and functional conservation of both H$^+$-ATPases throughout evolution. Despite apparent similarities, the F-type and V-type are differentially distributed in eukaryotic cells. The F-ATPase, which is also present in eubacteria, is found in mitochondria and chloroplasts, whereas the V-ATPase occurs in the various organelles of the cell's endomembrane compartment. The proton electrochemical gradient generated by the V-ATPases facilitates ion and metabolite transport essential for many fundamental cellular processes in growth and development. The V-ATPase holoenzyme is a large complex comprised of eight to ten subunits arranged into two subcomplexes; the V_1 peripheral subunit complex located on the cytosolic face of the membrane and the V_0 or integral membrane complex. The major components of the V_1 peripheral complex include 69-kDa catalytic and 60-kDa regulatory polypeptides. Interestingly, these V_1 nucleotide-binding subunits represent paralogous subunits that have evolved from a common ancestral gene following an ancient gene duplication event (Gogarten and Taiz 1992; Starke and Gogarten 1993).

The V-ATPase catalytic subunits of plants, mammals, and fungi exhibit a high degree of homology at the amino acid level (Gogarten et al. 1989; Zimniak et al. 1988; Bowman et al. 1988). The presence of a unique 90 amino acid region, termed the nonhomologous region (Zimniak et al. 1988), in the catalytic subunit of V-type ATPases (subunit A) is a striking feature that distinguishes the V-ATPase homolog from the paralogous catalytic B subunits of the F-ATPases, which lack this region. Although the nonho-

Table 1 The genome and geographical distribution of *Gossypium* species surveyed for the presence of two V-ATPase subunit A gene families

Species	Genomic group	Accession	Distribution
Diploids ($2n$ = 26)			
G. herbaceum L.	A_1	19	Old World cultigen
G. herbaceum L.	A_1	51	Old World cultigen
G. arboreum L.	A_2	106	Old World cultigen
G. arboreum L.	A_2	113	Old World cultigen
G. arboreum L.	A_2	163	Old World cultigen
G. harknessii Brandg.	D_{2-2}	–	Mexico
G. klotzschianum Anderss.	D_{3-k-54}	–	Galapagos Islands
G. aridum (Rose & Standl) Skov	D_4	–	Mexico
G. raimondii Ulbr.	D_5	–	Peru
G. laxum Phillips	D_8	–	Mexico
Tetraploids ($2n$= $4x$=52)			
G. hirsutum L.	$(AD)_1$	–	Central America
G. barbadense L.	$(AD)_2$	–	South America
G. tomentosum Nutt. ex Seem.	$(AD)_3$	–	Hawaii
G. mustelinum Miers ex Watt	$(AD)_4$	–	Brazil

mologous region serves as a fingerprint for subunit A of the V-ATPases, this region of unknown function is generally less conserved between different organisms (Gogarten et al. 1989). In bovids (Puopolo et al. 1991) and fungi (Bowman et al. 1988; Bowman et al. 1992; Ghislain and Bowman 1992; Hirata et al. 1990; Shih et al. 1988), the V-ATPase catalytic subunit is encoded by a single gene. In contrast, a number of higher plant species have been reported to contain multiple V-ATPase isoforms (Dupont et al. 1988; Gogarten et al. 1992a) or multigene families encoding the 69-kDa catalytic subunit (Narasimhan et al. 1991; Starke and Gogarten 1993; Wilkins 1992, 1993). Clearly, the extent of these gene families in plants and their contribution to functionally distinct isoforms remains to be resolved.

The genus *Gossypium* is divided into seven genomic groups (Endrizzi et al. 1985) distributed throughout tropical and subtropical regions of the world. The four cultivated species of the genus include the Old World Asiatic-African diploids *Gossypium arboreum* L. and *G. herbaceum* L. ($2n$=26) and the New World tetraploids *G. hirsutum* L. and *G. barbadense* L. ($2n$=52). Widely varying theories describing the origin of allotetraploid cotton species abound in the literature (Endrizzi et al. 1985; Skovsted 1934; Stebbins 1947; Hutchinson et al. 1947; Johnson 1975; Fryxell 1979; Phillips 1963). On the basis of cytogenetic (Phillips 1963), ecological (Fryxell 1979), and molecular analysis of nuclear (Galau et al. 1988) and chloroplast (Wendel 1989) genomes, it is apparent that the allopolyploids emerged within the last 1–2 million years. Cytogenetically, the New World tetraploid cotton species are allopolyploids containing an A genome derived from the Old World diploids and a D genome from an ancestral taxon of the New World diploids closely related to *G. raimondii* (Hutchinson et al. 1947; Phillips 1963; Stephens 1944a,b, 1950). Restriction analysis of the maternally inherited chloroplast genome (cpDNA) has unambiguously established that an A-genome species contributed the cytoplasm to the tetraploids, and thus served as the maternal parent during polyploidization (Galau and Wilkins 1989; Wendel 1989).

The recent isolation of two cDNA clones from cotton (*Gossypium hirsutum*) that encode the V-ATPase catalytic subunit indicates the presence of at least two genes in this allotetraploid species. We were interested, therefore, in exploring the relationship between the two cDNAs as potential homologs from the A and D subgenomes, as well as the structure and organization of V-ATPase catalytic genes in related diploid and polyploid cotton species. The increased structural complexity of plant V-ATPase catalytic subunit genes is also discussed relative to that of fungal species.

Materials and methods

Genomic DNA isolation

The cotton (*Gossypium*) species, genome designations, and corresponding ploidy levels used in this study are itemized in Table 1. Genomic DNA was isolated from young expanding leaves harvested from greenhouse-grown plants of A_2, D_5, and $(AD)_1$ species following the procedure of Galau et al. (1988) except that dithiothreitol replaced β-mercaptoethanol in the extraction buffer. Genomic DNA from other A, D, and AD species used in the survey (Table 1) was isolated according to procedures described in Wan and Wilkins (1993).

Amplification of cotton genomic DNA by polymerase chain reaction

Oligonucleotide "COT" primers (Fig. 1), designed from the cotton CVA69.24 cDNA clone encoding the vacuolar H^+-ATPase subunit A (Wilkins 1993), were synthesized by Operon Technologies. The nucleotide sequence of key COT primers are: COT2, 5′-CATGATGTTGCATCAAACTG-3′; COT8, 5′-CTTGACAAAGATGCTCTTTGG-3′; and COT22, 5′-GCCATGTATGAATTAGTCC-3′. The sequence of COT25 is complementary to COT8. Polymerase chain reactions (Saiki et al. 1985) were conducted in an Ericomp temperature cycler using 20 ng of genomic DNA with an initial denaturation step of 94 °C for 30 s followed by 25 cycles at 94 °C for 1 min, 48 °–60 °C (T_m–5 °C) for 1 min, and 72 °C for 1 min. After the last cycle, PCRs were extended for 10 min at 72 °C. Un-

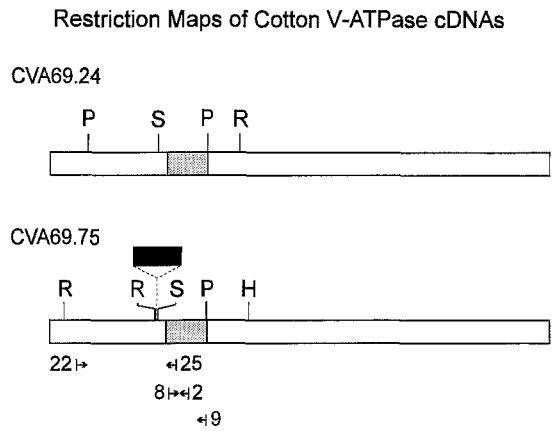

Fig. 1 Restriction maps of cotton vacuolar H^+-ATPase subunit A cDNA clones CVA69.24 and CVA69.75. Schematic diagram of the coding region highlighting key restriction sites that distinguish the two cDNA clones in the 5′ region of the clones. The *shaded box* represents the "nonhomologous" region encoding a stretch of 98 amino acids unique to subunit A of V-type H^+-ATPases (Zimniak et al. 1988). The *solid box* indicates the site of a 161-bp intron discovered in an independent isolate of CVA69.75. The *numbers* and *arrows* located *below* the restriction map indicate the identity and direction of oligonucleotide primers synthesized for PCR amplification. *Scale:* 1 mm=15 bp. *H Hin*dIII, *P Pst*I, *R Eco*RI, *S Stu*I, *X Xba*I

less otherwise specified, the PCR products were analyzed by agarose gel electrophoresis. PCR amplification of genomic DNA from A, D, and AD species by COT2 × COT8 in the survey (Table 1) was optimized by the inclusion of 1 m*M* spermidine-trihydrochloride in the reactions (Wan and Wilkins 1993).

Restriction enzyme digestion of cotton genomic DNA PCR-amplified products

PCR products were purified using Promega's Magic PCR System and separated by gel electrophoresis in 4% NuSieve GTG agarose (FMC). Individual bands were excised from gels and subjected to a second round of PCR amplification. The reamplified PCR products were purified on Magic PCR columns, digested by the indicated restriction endonucleases, and fractionated by gel electrophoresis in 1% agarose.

DNA sequence of PCR-amplified cotton genomic DNA products

PCR products amplified from genomic DNA of *G. hirsutum*, *G. raimondii*, and *G. arboreum* (accession 113) by oligonucleotides COT2 × COT8 (Fig. 1) were cloned into Novagen's pT7Blue PCR vector and transformed into *E. coli* strain NovaBlue. Individual transformants were divided into two classes based upon molecular weight following restriction digestion of plasmid DNA. The nucleotide sequence of PCR products was determined by the dideoxy chain termination method (Sanger et al. 1977) from single-stranded DNA templates (Vieira and Messing 1987) prepared from three to seven independent transformants for each size class from each species. Sequence analysis was performed using PC-Gene computer software from Intelligenetics.

DNA blot hybridization

Genomic DNA from *G. arboreum* and *G. raimondii* (3 µg) and *G. hirsutum* (6 µg) was digested to completion with the indicated restriction enzymes and separated by gel electrophoresis in 1% agarose at 1.8 V/cm for 12 h. DNA was transferred onto Hybond N nylon membrane by capillary blotting (Sambrook et al. 1989) in 10×SSC and affixed to the membrane via UV crosslinking. Duplicate membranes were hybridized overnight at 65 °C in 5×SSPE and 7% SDS with random-primer [^{32}P]-labeled probes (Feinberg and Vogelstein 1983). Radiolabeled probes included full-length V-ATPase subunit A cDNA clones CVA69.24 (Wilkins 1993) and CVA69.75 (Wilkins 1992), the nonhomologous region PCR-amplified by COT8 × COT9 from CVA69.24, or PCR products amplified by COT2 × COT8 from *G. hirsutum* genomic DNA. Membranes washed in 0.2×SSC and 0.1% SDS at 65°C were exposed at −80 °C for 24–36 h using Kodak XAR-5 OMAT film and two intensifying screens.

Results

Characteristics of two cotton V-ATPase 69 kDa catalytic subunit cDNA clones

Two discrete cDNA clones encoding subunit A of the vacuolar H^+-ATPase, designated as CVA69.24 (Wilkins 1993) and CVA69.75 (Wilkins 1992), were isolated from allotetraploid Upland cotton (*Gossypium hirsutum* L.) (Fig. 1). The 69-kDa polypeptide encoded by the two cDNA clones shared 99.4% amino acid identity. At the nucleotide level, the cotton V-ATPase clones exhibit 93% identity within the coding region. The cotton clones were readily distinguished on the basis of unique restriction enzyme patterns clustered near the nonhomologous region located in the 5′-terminus of the coding region (Fig. 1). An independent cDNA clone (CVA69.73) identical to CVA69.75 was discovered to contain an unprocessed intron of 161 bp located immediately downstream to one of the unique *Eco*RI restriction sites in the 5′-region of CVA69.75 between amino acids 106 and 107 (Fig. 1). The structural differences evident between the two V-ATPase cDNA clones were utilized as diagnostic tools to investigate the genetic relationship between these possible alloalleles and to explore the structural organization of V-ATPase subunit A genes in diploid and allotetraploid cotton species.

PCR amplification of V-ATPase catalytic subunit genes from cotton

To examine the V-ATPase subunit A gene structure in the 5′-region proximal to the nonhomologous region, pairs of oligonucleotide primers were synthesized to PCR-amplify the region of the gene(s) spanning the intron site present in cDNA CVA69.73 (Fig. 1). The COT2 primer, anchored in the nonhomologous region, functioned to discriminate against possible amplification of F-type H^+-ATPase homologs. PCR products of 381 bp were expected to be amplified from the genome by the primer-pair [COT2 × COT22] based on the cDNA sequence and the absence of introns. However, PCR amplification of two products of 1.45 kbp and 1.32 kbp from the allotetraploid *G. hirsutum* genome by primers COT2 × COT22 (Fig. 2) indicated the presence of introns totalling 1069 bp and 939 bp in the PCR products, respectively. Moreover, the molecular weight difference of approximately 130 bp between the two PCR products presumably resulted from discrete differences in the number and/or size of introns within this amplified region,

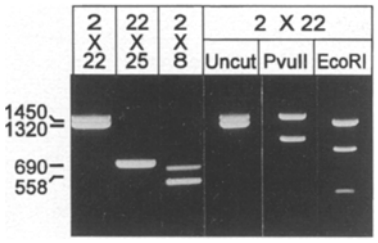

Fig. 2 PCR analysis of *G. hirsutum* vacuolar H^+-ATPase subunit A gene organization. PCR products amplified from 20 ng of genomic DNA by the indicated COT primer-pairs were fractionated by gel electrophoresis in 1% agarose. Purified [COT2 × COT22]-amplified PCR products were subjected to restriction digestion by *Pvu*II or *Eco*RI and separated by agarose gel electrophoresis. Molecular weights of PCR products (in bp) are indicated to the *left* of the figure

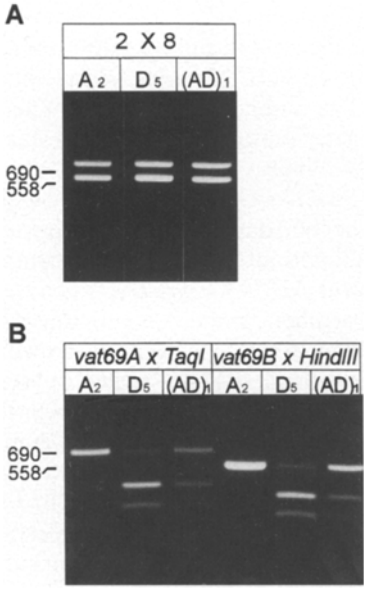

Fig. 3 A, B PCR amplification of genomic DNA from diploid and tetraploid cotton species for portions of the vacuolar H^+-ATPase 69-kDa catalytic subunit. Paired groupings at the *top* of the figure indicate COT primers used in PCR amplification. Letters correspond to genome designations of the following species: A_2 *G. herbaceum*, D_5 *G. raimondii*, $(AD)_1$ *G. hirsutum*. **A** Amplification of two [COT2 × COT8] PCR products from cotton genomic DNA. The 690-bp PCR fragment corresponds to the V-ATPase gene designated as *vat*69A and the 558-bp fragment to *vat*69B. **B** Purified [COT2 × COT8] PCR products of V-ATPase genes *vat*69A and *vat*69B subjected to restriction digestion by the enzymes *Taq*I and *Hin*dIII, respectively. Molecular weights of PCR fragments (in bp) are indicated to the *left* of the figure

and thereby indicated the existence of at least two V-ATPase catalytic subunit genes. To further localize the region accounting for the 130 bp difference between the two [COT2 × COT22] PCR products, the same region was amplified by two separate primer-pairs, [COT22 × COT25] and [COT2 × COT8], in which the latter pair served to amplify a portion of only the nonhomologous region. Generation of a single 760-bp product by COT22 × COT25 and two products of 690 bp and 558 bp, differing by approximately 130 bp, amplified by COT2 × COT8 (Fig. 2) pinpoints the molecular weight difference to the portion of the nonhomologous region delimited by COT2 X COT8.

To further explore the relationship between the two [COT2 × COT22] *G. hirsutum* genome-amplified PCR products (Fig. 2) and the cotton V-ATPase cDNA clones (Fig. 1), [COT2 × COT22] PCR products were subjected to digestion by *Eco*RI and *Pvu*II, restriction enzymes that discriminate between the cDNA clones CVA69.75 and CVA69.24, respectively (Fig. 1). The results of the restriction digests established that the [COT2 × COT22] PCR products could also be differentially digested by *Pvu*II and *Eco*RI in a manner similar to the cDNA clones (Fig. 2). This correspondence of the 1.32-kbp and 1.45-kbp PCR products to the cDNA clones, CVA69.24 and CVA69.75, respectively, permits further characterization of the structure and organization of the V-ATPase subunit A genes in cotton. The V-ATPase subunit A gene corresponding to CVA69.75 was designated *vat*69A, and that corresponding to CVA69.24 as *vat*69B.

Since *G. hirsutum* $[(AD)_1]$ is an allotetraploid species, the two V-ATPase PCR-amplified products generated in Fig. 1 may, in fact, represent alloalleles from the A- and D-genomes. To test this hypothesis, the region of the V-ATPase gene bracketed by the primer-pair [COT2 × COT8] was amplified from *G. arboreum* (A_2) and *G. raimondii* (D_5), two diploid species closely related to progenitor species of allotetraploid cotton. The [COT2 × COT8] primer-pair was selected for analysis because this region spans the structural differences, enabling the V-ATPase genes corresponding to the cDNA clones to be resolved into two discrete PCR products. If this hypothesis was correct, then each diploid species would presumably produce a single PCR product. Surprisingly, however, the results were more complex than anticipated. The two [COT2 × COT8] PCR products of the same molecular weight of 690 bp and 558 bp observed in the allotetraploid were also detected in both the A and D diploid species (Fig. 3A). Thus, the occurrence of at least two V-ATPase genes in both the diploids and tetraploid clearly indicates that the origin of these genes predates the polyploidization and speciation of present-day tetraploid cotton.

The results from Fig. 3A suggest that the two V-ATPase genes identified thus far in tetraploid cotton, *vat*69A and *vat*69B, each presumably contains homologous A and D genes donated by the parental diploid species. To address this question, two restriction enzymes, *Taq*I and *Hin*dIII, were identified that would selectively discriminate between the D genes belonging to *vat*69A or *vat*69B, respectively (Fig. 3B). An enzyme capable of differentiating the A genic variants within or between families could not be identified from among a battery of 20 enzymes. Digestion of the [COT2 × COT8] 690-bp PCR fragments of *vat*69A by *Taq*I or the 558-bp fragment of *vat*69B by *Hin*dIII revealed the existence of at least two D genes belonging to each V-ATPase gene family; one gene that is readily digested by a restriction enzyme and one minor genic variant that remains uncut (Fig. 3B). Digestion of the

tetraploid PCR products by the same restriction enzymes produced a pattern identical to that observed in the D diploid for *vat*69A and *vat*69B, thereby demonstrating the definitive presence of D genes in both *vat* gene families in the allotetraploid (Fig. 3B). However, the presence of A and/or D homologs could not be discriminated in the uncut allotetraploid PCR fragment within each family. Thus, it is not possible to ascertain if each V-ATPase gene in the allotetraploid species [$(AD)_1$] is comprised of (1A+1D) or (1A+2 D) homologs or two D genes solely on the basis of the restriction patterns. Regardless, these results suggest that *vat*69A and *vat*69B are not simply homeologs, but represent two distinct gene families containing members derived from both the A and D subgenomes.

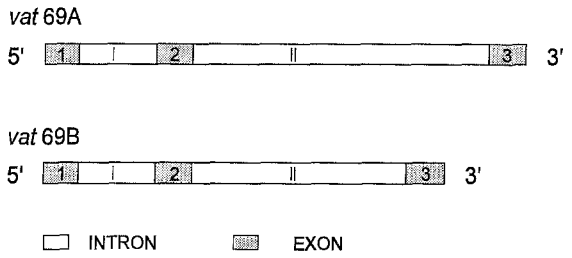

Fig. 4 Schematic diagram of a portion of two vacuolar H^+-ATPase genes, *vat*69A and *vat*69B, amplified by the PCR primer-pair COT2 X COT8 from cotton (*Gossypium*) species *G. arboreum* (A_2), *G. raimondii* (D_5), and *G. hirsutum* [$(AD)_1$]. Exons are represented by *shaded* regions and introns by *open* regions. Intron I resides within the glycine-144 codon and intron II is located between alanine-156 and threonine-157 of the coding region. The nucleotide length of each region is listed in Table 2

Nucelotide sequencing of PCR-amplified V-ATPase A and D homologous genes from diploid and allotetraploid cotton species

To ascertain the number, size, and location of introns within the amplified region of the V-ATPase genes, as well as examine the relationship between the two *vat*69 genes, the nucleotide sequence of [COT2 × COT8] PCR products of the A_2, D_5, and $(AD)_1$ species were determined. Cloned PCR products recovered from the allotetraploid as individual transformants were classified as A or D homologs on the basis of restriction digests for each *vat*69 gene. The D variants that remained undigested by *Taq*I or *Hin*dIII from the D_5 and $(AD)_1$ species were cloned independently following restriction digestion of the PCR products. Subsequent analysis revealed the presence of two introns, designated I and II, located in the same position within the 115 bp of the coding region delimited by COT2 × COT8 in both the *vat*69A and *vat*69B genes (Fig. 4). Comparison of the sequence to the cDNA clones indicated that all intron/exon junctions follow the GT/AG rule. Intron I is a small intron of 79 bp that is positioned within the codon of glycine-144 (G/GT). Intron II, situated between codons encoding alanine-156 and threonine-157, is 364 bp in *vat*69B but varies in length from 487 to 493 bp in *vat*69A (Fig. 4). Thus, intron II harbors the characteristic 132-bp size difference that distinguishes the [COT2 × COT8] PCR products of the two V-ATPase genes. In addition to the diagnostic length of intron II, *vat*69A and *vat*69B can be distinguished by the nucleotide composition of the introns as well as nucleotide substitutions dispersed among the three exons.

Nucleotide sequencing of [COT2 × COT8] PCR products from *G. arboreum* (A_2), *G. raimondii* (D_5), and *G. hirsutum* [$(AD)_1$] (data not shown) confirmed the classification of the two PCR products observed in Fig. 2 as distinct genes (Fig. 4). Moreover, since the nucleotide sequence of the A and D genes in the diploid species were near identical to the genes in the allotetraploid, individual PCR products in *G. hirsutum* could be readily identified as A or D homologs within each gene family. The identification of additional genic variants not resolved by gel electrophoresis indicated that each gene actually comprised a small multigene family in the diploid and allotetraploid species. Both *vat*69A and *vat*69B in *G. hirsutum* include homologous A and D genes. The number and identity of sequence variants for each gene family in the species examined were as follows: A_2: *vat*69A (2)+*vat*69B (1)=3; D_5: *vat*69A (3)+*vat*69B (3)=6; $(AD)_1$: *vat*69A (2A+1D)+*vat*69B (2A+2D)=7. The total number of V-ATPase variants recovered from the allotetraploid *G. hirsutum* comprised a total of seven different AD (4A+3D=7AD) genes. If the number of family members in the AD genome is presumed to be the sum of the A and D homologs contributed by the parental species, the V-ATPase gene superfamily is expected to contain a minimum of nine family members (3A+6D=9AD). The possible differential amplification of A genes from the tetraploid may account for a lower than expected recovery of D genic variants. The identification of more A variants in the allotetraploid relative to the A species and the higher recovery of D genes in the D species relative to the tetraploid suggest that the number of members in the cotton V-ATPase superfamily was underestimated and may actually be higher. This is especially true since no attempt was made to conduct exhaustive sequencing of all available transformants recovered from cloning the PCR products. On the other hand, differences in the composition of the allotetraploid gene families relative to the diploids may more closely reflect the organization of progenitor species or represent changes in the genome following polyploidization, and thus account for distinct variants in the allotetraploid.

Comparison of the nucleotide sequence of *vat*69A and *vat*69B [COT2 × COT8] PCR products demonstrated a consistent degree of nucleotide divergence, whether the A and D genes from the two gene families were analyzed separately or as a pool (Table 2). Within the exons, exon 2 in both *vat*69 gene families showed the greatest divergence, with only 87.5% nucleotide identity. Not surprisingly, intron sequences were less conserved than exons, showing less than 80% or 70% nucleotide identity in introns I and II, respectively.

Nucleotide sequence comparisons between A and D genes within the same gene family, *vat*69A or *vat*69B, re-

Table 2 Nucleotide sequence comparison of V-type H^+-ATPase catalytic subunit genes from diploid and allotetraploid cotton. Percentage of identical nucleotides were derived from the alignment of 15 subunit A V-ATPase sequences PCR-amplified by primers [COT2 × COT8]. Sequences are deposited under GenBank accession numbers U06243, U06245-U06257, and U06464

		Identity between two gene families in same genome		Identity between two genomes in same gene family	
		*vat*69A *vs.* *vat*69B		A genome *vs.* D genome	
	Size (bp)	A genome	D genome	*vat*69A	*vat*69B
Exon 1	43	93.2%	89.0%	94.7%	100%
Exon 2	38	92.5%	87.6%	94.3%	100%
Exon 3	34	96.5%	94.6%	100%	100%
Intron I	79–80	80.4%	81.1%	97.3%	97.5%
Intron II[a]		69.2–71.2%	69.2–70.5%	94.0–99.8%	95.9–99.7%
*vat*69A	493–498				
*vat*69B	364				

[a] Percent comparison with A/D homologs of *vat*69A that vary in length by 5 bp

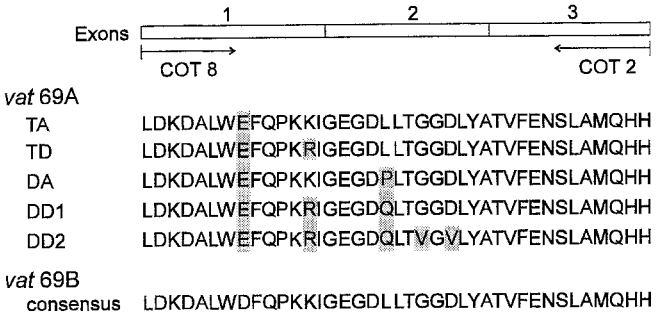

Fig. 5 Deduced amino acid sequences of V-ATPase catalytic subunit genes PCR-amplified from diploid and allotetraploid cotton by primer-pair COT2 × COT8. Amino acids corresponding to the three exons depicted in Fig. 4 are indicated. The identical peptide encoded by all eight *vat*69B gene family members is represented by a consensus sequence. Six of the seven *vat*69A genes identified exhibit amino acid substitutions in exons 1 and 2. Amino acid substitutions in *vat*69A variants are denoted by shaded residues relative to the *vat*69B consensus sequence. *T* tetraploid, *D* diploid or D genome, *A* A genome

vealed a striking degree of greater than 95% nucleotide identity, even within intron regions (Table 2), although the homologs were donated by an Old World and New World species, respectively. In addition to nucleotide substitutions in exon 1 and introns I and II of the *vat*69A gene family that enable differentiation between A- and D-subgenome homologs, the A genes contain a short stretch of 6 bp in intron II that is not present in the D homologs. The discrete nucleotide differences between A and D genes residing in exon 1 permitted assignment of the cDNA clone CVA69.75 as an A gene homolog. However, unlike the situation in the *vatA* gene family, differences in the nucleotide sequence of [COT2 × COT8] PCR products from *vatB* gene family members were confined solely to intron regions. Consequently, the lack of nucleotide divergence within the coding region of A and D family members of *vat*69B did not enable assignment of CVA69.24 to a particular subgenome. Results of pairwise comparisons of genic variants within and between the *vat69A* and *vat69B* gene families (Table 2) revealed that both A and D homologs within the same gene family were more closely related than A and D genes between families at the nucleotide level. The family relationships were further corroborated by phylogenetic analysis, which divided the two *vat*69 gene families into two clades (data not shown). As a measure of evolutionary distance between the families, a mean Jukes-Cantor estimate of 0.53 nucleotide substitutions per site was determined based upon pairwise comparisons of intron sequences (J. Wendel, personal communication).

Deduced amino acid sequences of the V-ATPase catalytic subunit superfamily

Within the coding region of the V-ATPase genes PCR-amplified by COT2 X COT8, members of the two *vat*69 gene families can be readily distinguished by a single conserved amino acid substitution in exon 1 (Fig. 5). All eight members of the *vat*69B gene family encode a consensus sequence of identical peptides spanning all three exons. Within the 26 amino acids delimited by COT 2 and COT8 primers, the amino acid immediately following the 3'-terminus of the COT8 primer in position 8 of exon 1 is an aspartic acid (D) in all *vat*69B family members, whereas members of the *vat*69A family are characterized by a glutamic acid (E) residue in the same position. Unlike the *vat*69B family with a conserved peptide sequence, five of the seven members of the *vat*69A family also exhibit one or more amino acid substitutions in exons 1 and 2 (Fig. 5). Interestingly, the degree of substitutions is most striking in variants belonging to the D genome, especially in the D_5 diploid species.

Structural analysis of cotton V-ATPase catalytic subunit genes

DNA blot analysis was performed to investigate the complexity of the V-ATPase gene superfamily in the genomes of the A_2, D_5, and $(AD)_1$ cotton species (Fig. 6). Genomic DNA of both diploid and tetraploid species produced complex restriction enzyme patterns when hybridized by V-ATPase cDNA clones CVA69.24 and CVA69.75 (data

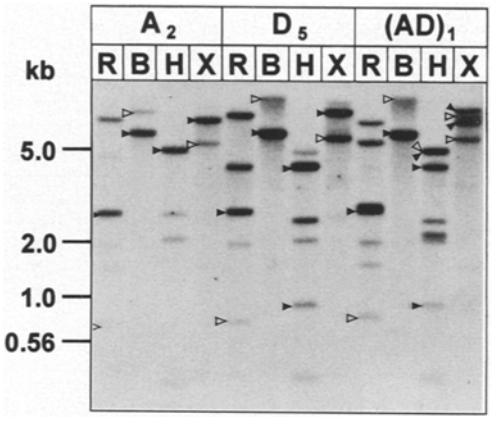

Fig. 6 Organization of the vacuolar H^+-ATPase gene superfamily in genomes of *G. arboreum* (A_2), *G. raimondii* (D_5), and *G. hirsutum* [$(AD)_1$]. DNA blot of genomic DNA was digested with indicated restriction enzymes and hybridized with the [^{32}P]-labeled cDNA CVA69.24 nonhomologous region. Restriction fragments hybridized to radiolabeled *vat*69A or *vat*69B gel-purified [COT2 × COT8] PCR products from *G. hirsutum* on duplicate DNA blots are highlighted by *open* and *closed triangles*, respectively. *R Eco*RI, *B Bam*HI, *H Hin*dIII, *X Xba*I

not shown). The complexity of the hybridization pattern was minimized when only the portion of the cDNA clones spanning the nonhomologous region was employed as a hybridization probe (Fig. 6). In many instances, the number, pattern, and intensity of the DNA bands detected in the $(AD)_1$ allotetraploid were correlated to the sum of the fragments detected in the A_2 and D_5 diploid species. Probes derived from [COT2 × COT8] PCR products representing the *vat*69A and *vat*69B gene families of the allotetraploid differentially hybridized to discrete restriction fragments on duplicate blots as indicated in Fig. 6. It was evident that the restriction fragments spanning the nonhomologous region are highly conserved within each gene family since the probes hybridize to single bands containing multiple gene family members.

Additional A, D, and AD species (Table 1) were surveyed by PCR analysis to determine the distribution of the two V-ATPase catalytic subunit gene families in the A and D genomes. PCR amplification of genomic DNA prepared from the species listed in Table 1 produced two PCR products of 690 bp and 558 bp (data not shown) that were identical to the amplified products observed in Fig. 2. These results indicated that the general organization of the V-ATPase gene(s) into two distinct groups was consistent within the genomic groups, regardless of ploidy level. The number of individual members within each subgroup remains to be determined.

The general structure of a plant V-ATPase gene encoding the catalytic subunit was investigated using available PCR primer-pairs capable of amplifying approximately 85% of the coding region (Table 3). The regions not analyzed encompass a total of 95 codons spanning between amino acids 1–40, 230–278, and 393–400. Sequence analysis conducted earlier revealed the location of three introns of 161 bp (Fig. 1), 79 bp, and 364 or 493/498 bp (Fig. 4) residing between amino acids 100 and 160 of the coding

Table 3 Gene structure of plant vacuolar H^+-ATPase subunbit A genes

COT primer-pairs	Nucleotide[a]	Amino acid[a]	cDNA size (bp)	Genomic PCR size (bp)	Known intron size (bp)	Amino acid position of intron	Estimate intron size (bp)	Estimate intron number
14 × 1	162–309	1–46	147	–	521[b]	26		1
22 × 2	291–672	41–167	287	1450, 1320	161	106		1
					79	144		1
					364 or 493/498	156		1
					>250[b]	?	~310	1
22 × 25	291–578	41–136	381	~760	161	106		1
					>250[b]	?	~310	1
8 × 2	558–672	130–167	114	690, 558	79	144		1
					364 or 493/498	156		1
8 × 9	558–856	130–229	298	1300, 1200	79	144		1
					364 or 493/498	156		1
					?	?	426–485	≥1
17 × 4	1006–1345	279–392	339	1100	80 or 130[c]	288		1
					?	?	581–781	≥1–2
18 × 5	1707–2041	513–623	334	1000, 1200	?	?	666–866	≥1–2
19 × 6	1370–1745	400–505	375	525, 850	?	?	150–475	≥1–2

[a] Nucleotide and amino acid numbering corresponds to the DNA and deduced amino acid sequence from cotton cDNA clone CVA69.24 (Wilkins 1993)
[b] Struve et al. (1990)
[c] Starke and Gogarten (1993)

region in cotton V-ATPase genes. Comparison of (1) PCR products produced from *G. hirsutum* genomic DNA by the primer-pairs [COT2 × COT22] and the primer subsets [COT22 × COT25] and [COT2 × COT8], (2) the cDNA sequence, and (3) the number and size of introns identified previously in this study indicated the presence of an additional intron of approximately 310 bp located upstream from the nonhomologous region. The 310-bp intron, in fact, probably corresponds to the partial intron (>250 bp) present between amino acids 68/69 in the carrot gene (Struve et al. 1990). If it is presumed that plant genes have a similar structural organization on the basis of work by Starke and Gogarten (1993), there are five introns ranging in length from 79 bp (this study) to 521 bp (Struve et al. 1990) residing within the first 160 amino acids of the V-ATPase coding region. Similar comparisons for the remaining PCR primer-pairs (Table 3) indicated the presence and approximate location of at least five additional introns, including a small plant intron identified in earlier studies (Starke and Gogarten 1993). Thus, plant V-ATPase catalytic subunit genes contain a minimum estimate of ten introns, in which the exact position and composition of six of the introns are known (this study; Starke et al. 1991; Struve et al. 1990). Interestingly, the three introns located upstream from the nonhomologous region are the same size in both gene subfamilies and, consequently, cannot be used to distinguish the two groups at this level. In contrast, two PCR products displaying a 100- to 200-bp difference in molecular weight, which is presumed to discriminate between *vat*69A and *vat*69B genes, were generated by all primer-pairs from the nonhomologous region to the end of the coding region, with the exception of COT4 × COT17 (Table 3).

Discussion

Evidence compiled in recent years (Narasimhan et al. 1991; Starke and Gogarten 1993; Wilkins 1992) indicates that the organization of multiple genes encoding the vacuolar H^+-ATPase (V-ATPase) catalytic subunit in higher plants is more complex than that found in any other group of organisms. The number, structure, and organization of the V-ATPase genes in the plant kingdom, however, remain unknown. Consequently, a specific region of the V-ATPase catalytic subunit was targeted for PCR amplification and nucleotide sequencing to study the structure and organization of this V-ATPase subunit in diploid and allotetraploid cotton (*Gossypium*) species.

V-ATPase catalytic subunit gene superfamily in diploid and allotetraploid cotton species

This study is the first report detailing the presence of multiple genes encoding the catalytic subunit of the V-ATPase, which are organized into a superfamiliy of related genes belonging to two distinct multigene subfamilies, designated *vat*69A and *vat*69B. Moreover, this complex organizational hierarchy is evident in both diploid and allotetraploid cotton species, although the number of family members correspondingly increases in the polyploid. Predictably, the $(AD)_1$ allotetraploid (*G. hirsutum*) contains genes derived from both the A and D subgenomes contributed by ancestral species related to the diploids used in this study.

Although the possible existence of multiple plant genes encoding the vacuolar H^+-ATPase catalytic subunit has been reported (Narasimhan et al. 1991; Starke and Gogarten 1993; Wilkins 1992), the complexity of the patterns on genomic DNA blots makes it difficult to approximate the number of genes in a given species. Starke and Gogarten (1993) contend that land plants contain two catalytic subunit V-ATPase genes, basing their conclusion on the direct sequencing of two discrete PCR products. However, additional genic variants may be obscured using this approach and, consequently, closely related genes may remain undetected. The two cDNA clones (Wilkins 1992, 1993) that are representative members of two distinct subfamilies, *vat*69A and *vat*69B, in diploid and allopolyploid cotton species are presumably analogous to the two genes identified in several other plant species (Starke and Gogarten 1993). A minimum of 2A, 3D, and 3AD (2A+1D) *vat*69A gene family members were observed in *G. arboreum* (A_2), *G. raimondii* (D_5), and *G. hirsutum* $[(AD)_1]$, respectively. Similarly, 1A, 3D, and 4AD (2A+2D) genes belonging to the *vat*69B gene family were identified in the same species. The full extent of the cotton superfamily, however, is as yet undetermined, nor has it been ascertained if this complex organization reflects a general pattern in higher plants or is, in fact, specific to the genus *Gossypium*. Clearly, the gene organization encoding the V-ATPase catalytic subunit in higher plants still remains to be resolved.

The existence of both physiologically and biochemically distinct isoforms of the V-ATPase in plants is well documented in the literature (Chanson and Taiz 1985; Depta et al. 1991; Dupont et al. 1988; Randall and Sze 1986; Wang and Sze 1985). Certainly the presence of similar or related V-ATPases in the functionally diverse compartments of the endomembrane system bespeaks a requirement for organelle-specific isoforms. The significant reduction of V-ATPase activity specifically associated with the tonoplast of vacuoles in carrots using antisense constructs, while the activity of a Golgi-associated V-ATPase is relatively unaffected, provides compelling evidence in support of organelle-specific V-ATPase isozymes (Gogarten et al. 1992a). On the basis of this evidence, one can propose that the two distinct genes identified in *Gossypium* potentially represent organelle-specific isoforms, in which individual family members are subject to tissue-specific regulation. The number of V-ATPase catalytic genes actually expressed, especially in the allotetraploid species, is as yet unknown. In the functionally amphidiploid allotetraploid species, gene silencing may play a role in the regulation of V-ATPase gene expression since diploidization and loss of duplicate gene expression has been observed for several isozymes (Suiter 1988) and many morphological traits (Endrizzi et al. 1985).

V-ATPase catalytic subunit gene structure in higher plants

The organization of plant catalytic subunit V-ATPase gene(s) into exons and introns is more complex than the situation in fungal species, but is apparently highly conserved within the plant kingdom. The number and position of introns are identical among family members of the two *vat*69 subfamilies in both diploid and allotetraploid cotton species. The nucleotide composition of introns and, in some cases, the size of introns are diagnostic features that serve to distinguish between the two *vat*69 families and among the A and D alloalleles within a gene family. Similar results regarding the site of insertion and nucleotide composition of an intron located elsewhere in the V-ATPase gene have been reported recently in other plant species (Starke and Gogarten 1993; Starke et al. 1991). Although the entire organization of plant V-ATPase catalytic subunit genes is as yet unknown, at least six introns (Starke and Gogarten 1993; Struve et al. 1990) have been identified within the first 300 amino acids of the polypeptide, including the three new introns discovered in this study. PCR amplification of the carboxyl-terminal 323 amino acids of the cotton V-ATPase catalytic subunit genes indicates the presence of four or more introns within this region. Thus, a minimum of ten introns, dispersed throughout the coding region, are estimated to occur in plant V-ATPase catalytic subunit genes. In marked contrast to plant genes, the number and position of relatively few introns vary significantly among several fungi, and in all these cases there is no structural similarity to plant V-ATPase catalytic subunit genes. The dissimilarities in structural complexity between fungal and plant V-ATPase genes indicate that introns accrued in the catalytic subunit followed the evolutionary separation of these two kingdoms. These findings concur with evidence indicating that many introns were acquired in plant and animal lineages following divergence from a common ancestor (Palmer and Logsdon 1991). Moreover, the incorporation of introns must have preceded the gene duplication event giving rise to the two *vat*69 genes because all plant V-ATPase catalytic subunit genes appear to contain the same number of introns located in the same position. If we use a corrected mean of 0.53 nucleotide substitutions per site in intron sequences between *vat*69 families and the synonomous nucleotide substitution rate of 6×10^{-9} substitutions/site/year (Wolfe et al. 1989), the duplication event that gave rise to the two primary families is estimated to have occurred approximately 45 million years ago. This evolutionary estimate is consistent with the presence of both *vat*69 gene families in diploid *Gossypium* and suggests that the families will likely occur throughout the Malvaceae, which is at most 50 million years old.

Origin of multiple V-ATPase catalytic subunit genes in the phylogeny of A and D diploid cotton species

The number of V-ATPase catalytic subunit genes identified in the A_2, D_5, and $(AD)_1$ species of *Gossypium* has interesting implications for the phylogeny of the genus. Following the bifurcation of plants and fungi, a gene duplication event must have occurred to give rise to the two *vat*69A and *vat*69B genes observed in cotton and other plant species (Starke et al. 1991; Starke and Gogarten 1993). Thus, the amplification of the *vat*69 genes into two multigene families in ancient diploid taxa prior to polyploidization of New World allotetraploid species lends credence to the long-held belief that the genus *Gossypium* has undergone two ploidy amplification events during evolution (Endrizzi et al. 1985). The allozyme variation observed by Suiter (1988) in accessions of *G. arboreum* (A_2) and *G. herbaceum* (A_1) is also consistent with an ancient polyploidization, followed by a loss of duplicate gene expression at some loci. Although gene amplification can occur via several possible mechanisms, including dysploidy and auto- or allopolyploidization, early cytological data (Abraham 1940; Beasley 1942; Davie 1933; Lawrence 1931; Skovsted 1933) suggest that present-day diploids *G. arboreum* and *G. herbaceum* are paleopolyploids that evolved from ancestral allopolyploid species. Evidence in primitive angiosperms (Soltis and Soltis 1990) supports the hypothesis that a high chromosome number near x=11 or 12 demarcates species derived via ancient polyploidy (Ehrendorfer et al. 1968; Grant 1981; Raven 1975; Stebbins 1971). This is certainly in agreement with cytogeneticists who have argued for decades that the basic chromosome number (x=13) of *Gossypium* is polyploid, having been initially derived from an ancestral base number of 6 or 7. This contention is entirely feasible, given the range in the basic chromosome number from 5 to 13 in the Malvaceae (Darlington and Wylie 1955). Clearly, the evolutionary conservation of distinctive genic variants of the V-ATPase catalytic subunit and the ability to distinguish A and D alloalleles at the nucleotide level provide a means to study the phylogeny of the seven genomic groups within the genus *Gossypium* as well as trace the interrelationships of V-ATPase genes among the genera of the Malvaceae.

Recent estimates, predicated upon a basic chromosome number greater than 11, propose that as many as 70–80% of both monocot and dicot plant species have experienced polyploidy at some time in their evolutionary history (Goldblatt 1980; Lewis 1980). Given the extent of polyploidy in angiosperms, the complex organization and structure of the V-ATPase catalytic subunit genes identified in *Gossypium* species are likely, therefore, to be more common among higher plant species than is currently believed.

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