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PROGRESS REPORT
REGULATION OF THE SYNTHESIS AND ASSEMBLY OF THE PLANT VACUOLAR H^+-ATPASE

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OVERVIEW

During the past three years we have focused on four main areas: the characterization of the 5'-upstream sequence of the gene for the V-ATPase 70 kDa (A) subunit gene, the generation of V-ATPase-deficient mutants using antisense constructs of the A subunit cDNA, analysis of V-ATPase ultrastructure by negative staining and the charactization of organelle-specific isoforms of the A subunit of carrot. In addition we have extended our studies on the cellular distribution of the V-ATPase and we have continued our investigation of the evolution of the V-ATPases by characterizing the A and B subunits of two species of the archaebacterium, Methanococcus. The major findings are listed below.

1. We have sequenced the 5' upstream region of one of the genes for the carrot A subunit and have characterized its promoter activity using GUS gene fusions (**Struve et al. 1990**).

2. We have demonstrated by negative staining of purified carrot vacuoles and mitochondria that the plant V-ATPase has an F_1-like "ball and stalk" structure similar to, but distinct from, the mitochondrial ATPase (**Taiz and Taiz, 1991**).

3. We have shown that plant coated vesicles, like animal coated vesicles, contain a V-ATPase (**Fichmann, et al. 1989**).

4. We have generated carrot V-ATPase-deficient mutants using antisense DNA to the coding and noncoding regions of the A subunit cDNA. The antisense mutant cell lines have been characterized biochemically and in transport experiments and the phenotypes of regenerated plants have been described. The V-ATPase-deficient mutants are characterized by a slower rate of leaf and tap root expansion, particularly during the early stages of development. In addition, evidence for separate tonoplast and Golgi isoforms of the A subunit has been obtained (**Gogarten et al., 1991**, submitted).

5. We have sequenced a cDNA clone of the gene for an isoform of the B subunit of human brain (**Bernasconi et al., 1990**), and we have obtained partial sequences for three isoforms of the A subunit of carrot. Based on the cDNA sequence, we have tentatively identified one of the genomic clones as the tonoplast isoform, and the other two as Golgi and possibly coated vesicle isoforms (Braun and Taiz, unpublished).

6. We have used used universal PCR primers, which recognize either V- or F-ATPase sequences, to amplify and clone sequences from the genomic DNA of two species of the methanogenic archae-

bacterium, Methanococcus; the sequences are homologous to the eukaryotic V-ATPase A and B subunits, consistent with the previously established close relationship between the V-ATPases of eukaryotes and the plasma membrane ATPase of another archaebacterium, Sulfolobus (Gogarten, et al. 1989; Bernasconi et al. 1989).

7. In collaboration with Richard Cross, a model for the evolution of the F_oF_1 superfamily (including both F-type and V-type) H^+-ATPases was proposed in which gene duplications followed by partial loss of function altered the ratio of proton channels to catalytic sites, thereby altering the H^+/ATP ratio of the enzyme (Cross and Taiz, 1990).

RESEARCH DESCRIPTION

1. Characterization of the 5'-Upstream Regulatory Sequence of the Gene for the Carrot A Subunit: Struve et al., 1990.

We isolated a genomic clone from a carrot sublibrary containing the first two exons and about 3 kb of the 5'-upstream sequence of the A subunit gene (Struve et al. 1990). Three TATA boxes were present in the 240 bp region upstream of the translation start site. Ribonuclease protection and primer extension analysis indicated that the TATA boxes corresponded to one minor (long transcript) and two major (long transcripts) transcription start sites. The flanking sequences of the two major TATA boxes were nearly identical. Additional sequences with putative regulatory roles, including an Sp1 transcription factor-binding sequence (GC box) and an ABA-box, have also been identified. The putative ABA response element (CGAGAAG) occurs at position -190 to -198 relative to the translational start site, similar to the Em2 ABA box (CGAGCAG) identified in wheat by Marcotte et al. (The Plant Cell 1: 969-976 (1989)). Three extended $(TATA)_n$ sequences were also present within 800 bp of the 5'-upstream region.

Two different promoter constructs fused to the beta-glucuronidase (GUS) reporter gene were tested in transformation experiments using the Ti plasmid of Agrobacterium tumefaciens. The first 240 bp sequence containing all three TATA boxes stimulated GUS activity about 4X above background in carrot callus. However, the 800 bp construct containing the $(TATA)_n$ region led to a 20-fold stimulation of GUS activity. Further studies are planned to further characterize the specific sequences involved in the constitutive, hormonal and environmental regulation of this reporter gene construct (see proposal).

2. Ultrastructural Comparison of the Vacuolar and Mitochondrial H^+-ATPases of Carrot: Taiz and Taiz, 1991.

Highly purified carrot root vacuoles were negatively stained with phosphotungstic acid and viewed in the electron microscope. F_1-like "ball and stalk" structures were observed coating the tonoplast surface which were similar to, but distinct from, F_1-particles observed on similarly prepared carrot mitochondrial membranes. The vacuolar "V_1" particles were about 9.4 nm in diameter and about 13.6 nm. The head group had a characteristic cleft, and smaller structures were frequently observed emerging from the base. In contrast, the mitochondrial F_1 averaged 8.7 nm

in diameter and 11.7 nm in height. The F_1 headgroup was spherical and contained no smaller basal structures. The ultrastructural observations confirmed those of Bowman et al. (18) in Neurospora and Klink and Luttge (36) in Mesembryanthemum.

3. Identification of the V-ATPase on Purified Plant Coated Vesicles: Fichmann et al., 1989.

In animal cells, V-ATPases have been identified on lysosomes, endosomes, Golgi bodies, chromaffin granules, neurosecretory vesicles and coated vesicles. In plants, only vacuoles and Golgi bodies had been shown to contain a V-type H^+-ATPase. We obtained highly purified zucchini coated vesicle (CV) preparations from David Robinson (University of Gottingen). Western blots of SDS!PAGE gels of intact CVs, stripped CVs and reassembled coats were probed with polyclonal antibodies raised against the A and B subunits of Zea mays (Fichmann et al., 1989). Both antibodies cross-reacted with the appropriate antigen of the intact and stripped CVs, but, as expected, failed to cross-react with the reassembled coats. Since the yields of plant CVs are still too low for direct enzyme assays, these immunological results are the only evidence thus far that plant plant coated vesicles, like those of animals, contain the V-ATPase.

4. Characterization of Carrot V-ATPase Mutants Generated by Antisense DNA: Gogarten et al.[submitted].

Carrot root tissue was transformed with two different antisense constructs of the A subunit cDNA: the entire coding region and the 5' upstream untranslated region (150 bp). These inserts were excised from the original clones in pBluescript and religated in the antisense orientation into disarmed Ti plasmids, pGA643, behind the cauliflower mosaic virus 35S RNA promoter. Transformed calli were selected on kanamycin and either maintained as cell suspensions in liquid culture or were regenerated into whole plants (T1 generation). Since the plants were not regenerated from single cells, the cells making up the T1 plants may be quite variable with respect to the dosage and genomic location of the antisense gene. Nevertheless, T1 plants exhibited a consistent phenotype (see below). Seed obtained from the T1 plants have also been grown in the greenhouse (T2 generation). All biochemical studies have been performed either on T1 callus or cell suspensions (see enclosed manuscript).

a. Biochemical Studies

The presence of the antisense transcript in the transformed cells was confirmed using a combination of reverse transcriptase and the polymerase chain reaction (PCR). Microsomal membranes were isolated and fractionated into tonoplast-enriched and Golgi-enriched fractions on sucrose step gradients. Western blots of these fractions were probed with a monospecific antibody to the A subunit of carrot. As expected, very little A subunit antigen was present in the tonoplast fractions of the antisense transformants, compared with wild-type cellsn Unexpectedly, the Golgi fractions of the antisense cells contained normal levels of the A subunit. Similar results were obtained using an antibody to the B subunit. Thus, both antisense constructs appeared to specifically block the tonoplast V-ATPase without affecting the

Golgi V-ATPase.

The western blot results were confirmed in assays of enzyme and transport activity. ATPase and proton-pumping activities by the tonoplast fractions of both antisense mutants were strongly inhibited (70-80%), and ATP-dependent ^{14}C-OMG uptake was inhibited by ~95%. No inhibition was observed in the Golgi fractions, in agreement with the western blotting results.

Our working hypothesis from the above biochemical studies was that the antisense DNA construct specifically inhibits a tonoplast-specific isoform of the A subunit. We investigated the possibility of multiple isoforms of the A subunit by 2-D gel electrophoresis and western blotting. Western blots of 2-D gels of wild type and control transformant microsomal membranes revealed the presence of two major pI classes of cross-reacting spots. Similar results had been obtained previously by Dupont et al. (96). Membranes prepared from both antisense mutants contained only the more acidic pI spot, suggesting that the alkaline spot was inhibited. Highly purified carrot vacuoles contained only the more alkaline spot. We therefore concluded that the alkaline spot corresponds to a specific tonoplast isoform of the A subunit, while the alkaline spot corresponds to the Golgi isoform. The existence of separate tonoplast and Golgi isoforms is consistent with an early report from the animal literature of a mutation in CHO cells which specifically inactivated the endosomal proton pump but had no effect on the lysosomal proton pump (86).

b. Morphology and Anatomy

The leaves of T1 and T2 plants were morphologically distinct from those of wild type plants which had been regenerated from callus under the identical conditions. The petioles were about 50% shorter and the leaflets were smaller and narrower, giving the blade as a whole a more dissected, fern-like appearance. In addition, the storage root developed more slowly in the transformants than in the wild type.

Light microscopy of the leaves of T2 plants at various stages of development was carried out. At the early stages of development, the cells of the T2 leaves were smaller than those of wild type (about 50%), although central vacuoles were present. The outer epidermal walls of the antisense leaves were thinner than those of wild type and lacked a thickened cuticle. At the later stages of development, the antisense leaves expanded more rapidly, approaching the size of the wild type leaves, although the outer epidermal wall remained thin by comparison. These results suggest that the vacuolar ATPase plays an important role in cell expansion during early leaf development. The fact that the antisense leaves appear to resume expansion later in development could either mean that the H^+-pyrophosphatase is playing a greater role, or that the antisense construct is less effective in the more mature tissues, perhaps due to the weaker expression of the antisense gene. The major effect of the antisense gene on the root system was to cause a marked delay in the expansion of the storage tap root. The antisense plants developed an abundant fibrous root system, but the tap root remained unexpanded long after the wild type tap roots had achieved a large size. As in the case of the leaves, however, the tap roots of the antisense

the case of the leaves, however, the tap roots of the antisense plants eventually reached nearly full size.

5. Isoforms of Vacuolar ATPase Genes: Bernasconi et al.1990; Braun and Taiz, [unpublished data].

Based on results with microorganisms (yeast and Neurospora) it was initially assumed that the V-ATPase subunits were encoded by single genes. However, we recently reported that human brain contains an mRNA encoding a second isoform of the vacuolar ATPase B subunit (Bernasconi et al., 1990). The first isoform, obtained by Dennis Stone's lab, was from a human kidney cDNA library. The coding regions of the two cDNAs are about 77% identical at the nucleotide acid level and about 90% identical at the amino acid level. The 3'-noncoding regions differ greatly in length and are completely unrelated. This raised the possibility that multiple isoforms of V-ATPases subunits might oe found in plants as well.

Recently we used PCR to amplify and partially sequence three dkfferent genes for the A subunit of carrot from genomic DNA. The PCR primers used were for the 3'-end of the coding region, and were expected to generate a fragment size based on cDNA of 300 bp. When the PCR products were separated on an agarose gel, however, two bands were observed at 1.3 kb and 1.0 kb, suggesting the presence of introns within this region of the genomic sequence, falling into two size classes. Subcloning and sequencing the PCR products revealed that there were actually three different sequences: two of ~1.0 kb (A and B) and one of ~1.3 kb (C) (Fig. 1). All three fragments contain a 150 bp exon from the carboxy terminus flanked by a large intron on the 5'end and 3'-untranslated sequence on the 3'end. Two features strongly indicate that these sequences are isoforms. First, sequence C has a longer intron than A or B, as indicated by the length of the blank (still unsequenced) region in line 3. Second, a large region of the introns of A and B are completely unrelated (lines 2 and 3). Such radical differences would be highly unlikely in the case of alleles. Analysis of the nucleotide sequences of the exon regions indicates that A and C are more closely related to each other than they are to B. B appears to be identical to the cDNA we previously cloned from carrot (Zimniac et al., 1988). If the cDNA represents the tonoplast subunit as initially assumed, isoform B encodes the tonoplast subunit; isoforms A and C might encode the Golgi and coated vesicle isoforms. Once all the exons have been sequenced, we may may be able to correlate the calculated pIs with the acidic and alkaline spots on the 2-D

6. Identification of a V-type ATPase in Two Species of Methanococcus: Gogarten, et al. 1989; Bernasconi et al. 1989.

At the beginning of the current granting period in 1989, it had just been shown that the plasma membrane ATPase of Sulfolobus was a V-type, rather than an F-type, ATPase. If the archaebacteria are monophyletic as initially proposed by Carl Woese, they should all have V-type ATPases. However, Jim Lake at UCLA has recently disputed the original monophyletic classification and has suggested that the halobacteria and methanogenic bacteria are more closely related to the eubacteria than to Sulfolobus. According to Lake, the eocytes, including Sulfolobus, and euka-

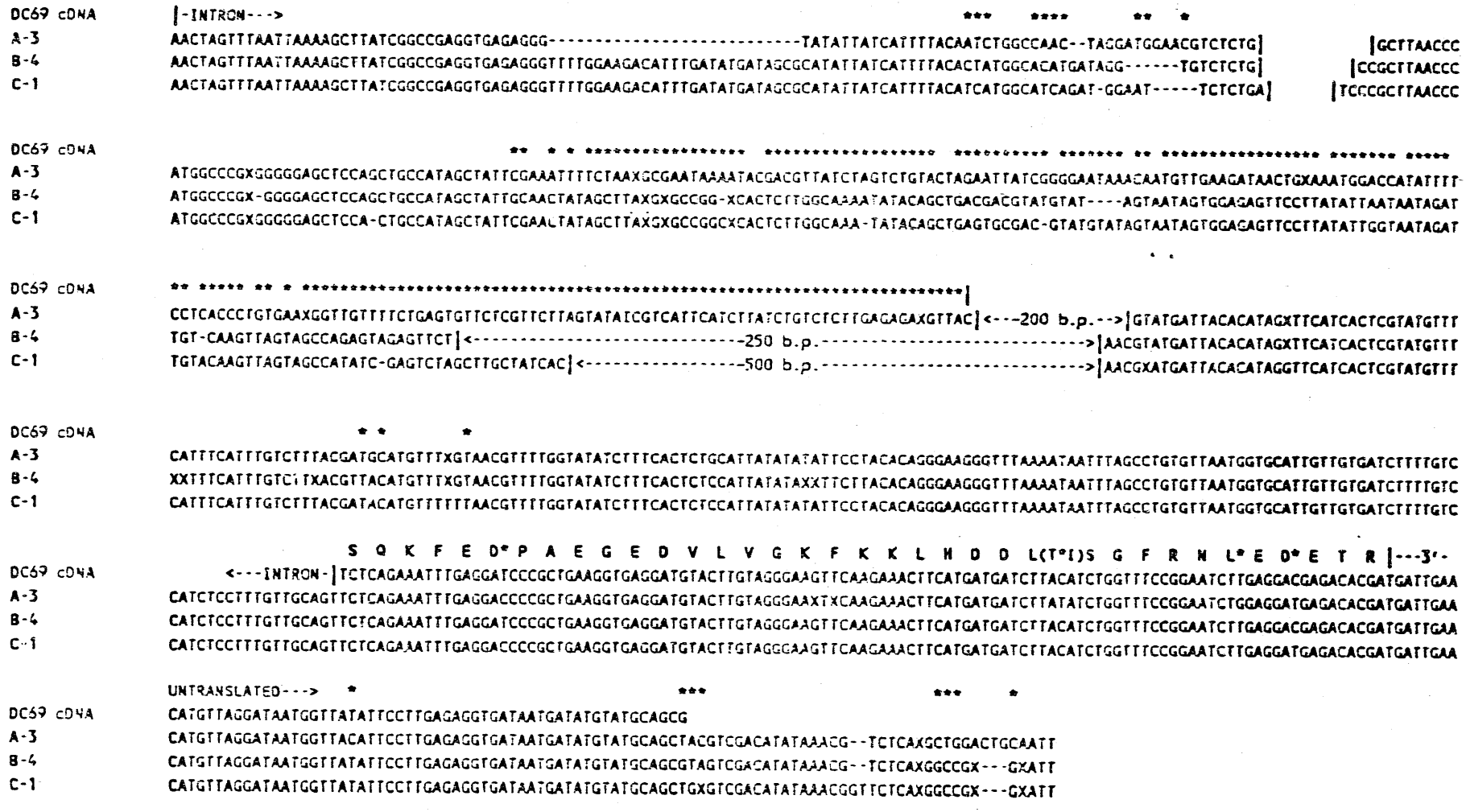

Fig. 1. Partial sequences of three potential isoforms (A-C) of the carrot A subunit of the vacuolar ATPase. The previously sequenced A subunit cDNA, believed to correspond to the tonoplast ATPase, is given on the upper line of each horizontal row. Nucleotide differences are indicated by asterisks. Incomplete sequences are indicated by the vertical breaks on the first and third rows. Note the differences in intron lengths shown on the third row, and the large difference in intron sequence on the second and third rows. Only one amino acid difference was observed in the exon: threonine vs. isoleucine. Data from Braun and Taiz, unpublished results.

ryotes share a common ancestor which branched off very early in evolution. According to the Woese model, then, halobacteria and methanogens should have V-type ATPases, while the Lake model would suggest that they have eubacterial-type F-ATPases.

We designed "universal" PCR primers that would amplify the highly conserved core sequences of the two major subunits of either F-type of V-type ATPases. We hoped to use these primers to amplify sequences from the genomic DNA of two species of Methanococcus that would correspond to either F-type, V-type, or both types of ATPases. In control experiments we demonstrated that the primers were, indeed, able to amplify both types of ATPase sequences in the case of known organisms. We then showed that the primers amplified two sequences from both of the Methanococcus species which clearly belonged to the V-ATPases . No F-type sequences we laboratories showed that the ATPases of another methanogen, Merthanosarcina, as well as Halobacterium, also group with the V-ATPases. There is now general agreement that all prokaryotes classified as archaebacteria have V-type ATPases. All eubacteria contain F-type ATPases. The only apparent exception to this rule is Thermus thermophilus, a eubacterium which M. Yoshida in Japan recently reported to contain a nitrate-sensitive V-type ATPase, based on a carboxy terminal protein sequence. If this is confirmed, it would suggest that lateral transfer of the ATPase can occur.

7. A Model for the Evolution of the F_oF_1-ATPase Superfamily: Cross and Taiz, 1990.

It is generally assumed that F_oF_1-ATPases have undergone two reversals in function, first from an H^+-pumping ATPase to an ATP synthase during the transition from anaerobic to aerobic life, then back again to an H^+-ATPase when the enzyme was internalized in eukaryotic cells (V-ATPases). Richard Cross and I proposed that both changes in function were facilitated by a change in the H^+/ATP ratio, to better adapt the enzyme to its function . High ratios favor synthesis, while low ratios favor hydrolysis. We further proposed that the H^+/ATP ratio is a function of the number of proton channel subunits versus the number of catalytic sites. For example, an F_oF_1-ATPase with 9-12 c subunits and 3 catalytic sites would have a H^+/ATP ratio of 3-4, which favors synthesis. V-ATPases with only 6 c subunits and 3 catalytic sites, would have an H^+/ATP ratio of 2, which favors hydrolysis. Both of these H^+/ATP ratios have been experimentally verified. Finally, we proposed that changes in the ratio were brought about by gene duplications with subsequent partial loss of function of one of the genes. Thus, the original enzyme consisted of 6 identical catalytic subunits and 9-12 proton channel subunits. During the first transition, gene duplication and loss of function of one of the genes first led to the reduction in the number of catalytic sites from six to three. During the second transition, gene duplication followed by gene fusion and partial loss of function reduced the number of proton channel subunits from twelve to six.

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