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PROGRESS REPORT for Grant No. DE- FG03-84ER13245
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1. The Use of Antisense mRNA to Inhibit the Tonoplast H+-ATPase in Carrot.

During the first year of this proposal we completed and published a paper bearing the above title in The Plant Cell. (enclosed). In this paper we:

a) showed that the tonoplast V-ATPase could be specifically inhibited by antisense DNA to the catalytic (A) subunit;

b) showed that cell expansion was inhibited in carrot transformants deficient in the enzyme:

c) provided evidence for at least two different isoforms of the A subunit which are Golgi- and tonoplast-specific.

These findings prompted a search for sequences of the isoforms of the A subunit in carrot.

2. Cloning and Sequencing of ATPase Isoform Genes of Carrot.

We have cloned and sequenced 1.0-1.5 kb fragments of three different genes for the catalytic subunit of carrot. The fragments differ greatly in their introns, but have nearly identical exons.

We are continuing our efforts to obtain complete cDNA sequences as well as promoter sequences, for the three isoforms:

a) We are using PCR to amplify and subclone carrot seedling cDNA. Thus far two bands have been amplified and are currently being subcloned for sequencing.

b) We are preparing a carrot cDNA library with which we will screen for isoforms of the A subunit.

c) We are using PCR to amplify additional genomic fragments of the A subunit genes. Our goal is to clone the promoters of the three genes. This will allow us to study the expression of the three genes using GUS fusion constructs.

3. Screening an Arabidopsis Cosmid Library in Agrobacterium for A Subunit Genes.

Because of the large genome size of carrot, finding and sequencing all the isoforms may be difficult. In collaboration with Dr. Robert Ludwig at Santa Cruz, we are screening an Arabidopsis genomic library for A and B subunit genes. This library was prepared from a cosmid vector and contains 20-25 kb inserts. We anticipate that the same classes of isoforms will be found in Arabidopsis as have been found in carrot and cotton. A rotation graduate student, Michele Haynes, is currently working on this project.

Since the library can be used directly to transform plants, we hope to be able to test the ability of the clone(s) we isolate to rescue the antisense carrot mutant discussed in #1 above. This may lead to the identification of the tonoplast-specific isoform.

4. Characterization of the V-ATPase of Lemon Fruit

Our interest in isoforms has led us to consider the specialized functions of vacuolar ATPases, either in different orga-

nelles or different tissues. One extreme example of vacuolar specialization is the lemon fruit. Lemon fruit cells have a vacuolar pH of 2.5 due to the accumulation of citric acid. In theory, the vacuolar ATPase is capable of maintaining a pH gradient of only about 3 pH units, assuming an H+/ATP stoichiometry of 2. How does the lemon vacuole become so acidic? We can think of three possibilities:

a) The lemon fruit V-ATPase is a specialized isoform with a different H+/ATP stoichiometry.

b) Lemon fruit contains an activator protein, similar to that reported for mammalian cells, which activates the proton pump.

c) The H+-pyrophosphatase, rather than the V-ATPase, is responsible for acidifying the lemon fruit vacuole down to pH 2.5. The H+/ATP stoichiometry of the PPase is 1, which is low enough to acidify down to pH 2.5.

Preliminary results: We have worked out a procedure for isolating vacuolar membranes from lemon fruits in good yields. Our preliminary results indicate that the H+-pyrophosphatase specific activity is nearly 3X higher than that of the V-ATPase in lemon fruit vacuoles, suggesting that the PPase may acidify lemon fruit tonoplasts. This may indicate that the PPase is chiefly responsible for lowering the pH of lemon fruit vacuoles to pH 2.5. However, we have not ruled out the possibility that the V-ATPase is being inactivated during isolation.

5. Structure/Function Analysis of the Catalytic Subunit.

A. The Role of Cysteines. We recently completed a series of experiments to determine the functions of conserved cysteine residues in the catalytic subunit of the vacuolar-type ATPase in yeast. Using site-directed mutagenesis, we altered Cys 154, Cys 161 and Cys 184 to Ser. Cys 161 is highly conserved among eukaryotes and is situated adjacent to the phosphoanhydride-binding site. Cys 184 is universally conserved among both eukaryotes and archaebacteria.

Our results showed that neither Cys 154 nor Cys 161 are required for enzyme activity. However, a double mutant was considerably less active than wild-type in vivo. Hence at least one of these cysteines is required for maximal activity in vivo. Mutating Cys 184 to Ser completely inactivated the enzyme. Cys 184 is in a highly conserved region believed to be involved in magnesium-binding. However, the homologous region in the F-type ATPases has a valine in this position. Mutating Cys 184 to Val also inactivated the enzyme. This may indicate a mechanistic difference between V- and F-ATPases at their metal-binding sites.

These results are currently being written up for submission to J.B.C. and will cite DOE support exclusively.

B. Sulfite-Induced Intrinsic Uncoupling of the Vacuolar ATPase and its Inhibition by Delta pH.

We have just completed a study on the effects of sulfite on the vacuolar ATPases of mung bean and yeast. Sulfite stimulates the hydrolytic activity of the yeast vacuolar ATPase by as much as 100% (mung bean is stimulated up to 50%). The stimulation of hydrolytic activity by sulfite is completely blocked by the presence of a delta pH. In fact, sulfite becomes inhibitory in

the presence of a delta pH. Imposition of a delta psi across the tonoplast does not block the stimulatory effect of sulfite.

What is the mechanism of sulfite-activation? We have shown that the V-ATPases of yeast and mung bean exhibit nonlinear hydrolysis kinetics: an initial rapid rate is followed by a slow steady rate. The slow steady rate is apparently caused by the tight binding of nucleotide to the catalytic site. Sulfite-activation appears to be related to an ability to cause the dissociation of tightly-bound nucleotide from the catalytic site. Surprisingly, proton transport is not stimulated during sulfite-enhanced hydrolysis. Hence, it appears that sulfite induces intrinsic uncoupling, or "slip".

Futai's group in Japan has recently provided evidence for slip in the F-type ATPase of E. coli. A mutation of the gamma subunit causes slip to occur. Our results are consistent with those of Futai's lab, and suggest that sulfite may alter the interaction between the catalytic subunit and one of the minor subunits involved in energy transduction to the channel.

REQUEST FOR CONTINUATION OF FUNDING

As noted earlier, my laboratory is now exclusively supported by DOE. Projects that were initiated using seed funds from the university with the expectation of future NSF support will probably be phased out after completion and publication. These include the site-directed mutagenesis experiments with yeast and the studies on sulfite-activation. During the transition period, I request permission to use DOE funds to complete these projects. My research goals for next year are as follows:

1. COMPLETE THE SEQUENCING OF THE THREE GENES FOR THE PUTATIVE ISOFORMS OF THE 70 KD SUBUNIT.

We will screen genomic DNA by PCR for the remaining genomic sequences of the three isoforms. We will also screen cDNA libraries. This will be the major focus of the next year, since it is crucial to our goal of characterizing the isoforms and determining their function- the main objective of our original proposal.

2. SCREEN ARABIDOPSIS GENOMIC LIBRARY FOR V-ATPase A SUBUNIT GENES

We are turning to Arabidopsis because its small genome may allow us to isolate isoform genes more rapidly. Unfortunately, most of the subunits for the plant enzyme have been identified in different plants. Our goal is to isolate all the genes in Arabidopsis, which should proceed more quickly than in plants with larger geneomes.

3. IDENTIFY THE ACIDIFICATION MECHANISM OF LEMON FRUIT VACUOLES

We wish to determine whether the lemon fruit V-ATPase is specialized to pump down to a lower pH, or whether acidification is due to the PPase. We will compare the ATPase and PPase activities and proton pumping activities of tonoplasts isolated from both fruits and hypocotyls. The role of organic acids will also be investigated. Since citrate is the major counter ion in the fruit, we predict that the PPase of lemon fruits should pump down

to a much lower delta pH in the presence of citrate than the ATPase, because of its lower H+/ATP stoichiometry. Thus the citrate carrier may play a crucial role in regulating the pH of lemon fruit vacuoles.

4. COMPLETE STRUCTURE/FUNCTION ANALYSIS OF THE ROLE OF CYSTEINES IN THE YEAST VACUOLAR ATPASE.

Because of the high degree of conservation among the eukaryotic V-ATPases, information gained about the yeast enzyme is directly applicable to plants. This work is essentially complete and is in the process of being written up for publication. However, we may have to tie up some loose ends in the process.

5. COMPLETE KINETIC STUDIES ON THE EFFECTS OF SULFITE

Like the cysteine mutant work, this study is essentially complete and is being written up for publication.

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