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FINAL TECHNICAL REPORT
Department of Energy Grant
"Regulation of Vacuolar pH in *Citrus limon*"
1998-2003

Introduction

The primary objective of this grant was to characterize the vacuolar V-ATPase of lemon fruits. Lemon fruit vacuoles have an internal pH of about 2.5. Since a typical plant vacuole has a luminal pH of around 5.5, the lemon fruit V-ATPase must have special properties which allow it to acidify the lumen to such a low pH:

1. It might have a different structure.
2. It might have a different H⁺/ATP stoichiometry.
3. It might be regulated differently.

During the course of our investigations (which began in 1996) we characterized these aspects of the V-ATPases of both lemon fruits and lime fruits. We examined lime fruits because of the availability of both acidic limes with a low vacuolar pH and sweet limes, which have a much higher vacuolar pH. The existence of two types of lime fruits allowed a comparison of the V-ATPases of the two varieties. In this report I am including two publications from 1996 and 1997 as background for the later publications. A review article with Heven Sze on V-ATPase nomenclature was also generated during the funding period.

In addition to our studies on citrus fruit vacuoles, we also initiated studies in two new areas: polar auxin transport and the regulation of stomatal opening by UV-B irradiation. These studies were intended to serve as a basis of future separate grants, but the proposals we submitted on these topics were not funded.

Publications and Results

1. Müller, M., Irkins-Kiesinger, U, Rubinstein, B. and Taiz, L. 1996. On the mechanism of hyperacidification in lemon- Comparison of the vacuolar H⁺-ATPase activities of fruits and epicotyls. *J. Biol. Chem.* 271: 1916-1924.

Lemon fruit vacuoles acidify their lumens to pH 2.5, 3 pH units lower than typical plant vacuoles. To study the mechanism of hyperacidification, the kinetics of ATP-driven proton pumping by tonoplast vesicles from lemon fruits and epicotyls were compared. Fruit vacuolar membranes were less permeable to protons than epicotyl membranes. H⁺ pumping by epicotyl membranes was chloride-dependent, stimulated by sulfate, and inhibited by the classical vacuolar ATPase (V-ATPase) inhibitors nitrate, bafilomycin, N-ethylmaleimide, and N,N'-dicyclohexylcarbodiimide. In addition, the epicotyl H⁺ pumping activity was inactivated by oxidation at room temperature, and oxidation was reversed by dithiothreitol. Cold inactivation of the epicotyl V-ATPase by nitrate (geq100

mM) was correlated with the release of V(1) complexes from the membrane. In contrast, H⁺ pumping by the fruit tonoplast-enriched membranes was chloride-independent, largely insensitive to the V-ATPase inhibitors, and resistant to oxidation. Unlike the epicotyl H-ATPase, the fruit H-ATPase activity was partially inhibited by 200 μ M vanadate. Cold inactivation treatment failed to inhibit H⁺ pumping activity of the fruit membranes, even though immunoblots showed that V(1) complexes were released from the membrane. However, cold inactivation doubled the percent inhibition by 200 μ M vanadate from 30% to 60%. These results suggest the presence of two H-ATPases in the fruit preparation: a V-ATPase and an unidentified vanadate-sensitive H-ATPase. Attempts to separate the two activities in their native membranes on linear sucrose density gradients were unsuccessful. However, following detergent-solubilization and centrifugation on a glycerol density gradient, the two ATPase activities were resolved: a nitrate-sensitive V-type ATPase that is also partially inhibited by 200 μ M vanadate, and an apparently novel vanadate-sensitive ATPase that is also partially inhibited by nitrate. Purification and Reconstitution of the Vacuolar H⁺-ATPases from Lemon Fruits and Epicotyls

2. Müller, M., Irkens-Kiesecker, U., Kramer, D. and Taiz, L. (1997)

Purification and reconstitution of the vacuolar H⁺-ATPases from lemon fruits and epicotyls. *J. Biol. Chem.* 272: 12762-12770.

The vacuolar H⁺-ATPases (V-ATPases) of lemon fruits and epicotyls were detergent-solubilized, purified by column chromatography, and reconstituted into artificial proteoliposomes. During purification, a vanadate- and nitrate-sensitive ATPase activity, consisting of partially disassembled V-ATPase complexes, was resolved from the V-ATPase peak. ATPase and H⁺-transport activities of the purified, reconstituted V-ATPases of both fruit and epicotyl exhibited similar inhibitor profiles, except that the fruit V-ATPase retained partial vanadate sensitivity. Since the V-ATPase activity of native fruit tonoplast vesicles is insensitive to inhibitors, membrane lipids or other factors may protect the fruit V-ATPase from inactivation *in vivo*. A kinetic analysis of H⁺-pumping and H⁺-leakage indicated that the reconstituted epicotyl V-ATPase exhibited twice as much intrinsic uncoupling or slip as the reconstituted fruit V-ATPase. Comparison of their subunit compositions by SDS-polyacrylamide gel electrophoresis indicated that the reconstituted fruit V-ATPase is enriched in two polypeptides of 33/34 and 16 kDa. Moreover, the stalks of negatively stained juice sac V-ATPases appeared thicker than those of epicotyl V-ATPases in electron micrographs.

2. Müller, M.L., Jensen, M., and Taiz, L. (1999) The vacuolar H⁺-ATPase of lemon fruit is regulated by variable H⁺/ATP coupling and slip. *J. Biol. Chem.* 274: 10706-10716.

Lemon fruit tonoplasts, unlike those of seedling epicotyls, contain nitrate-insensitive H⁺-ATPase activity (Müller, M. L., Irkens-Kiesecker, U., Rubinstein, B., and Taiz, L. (1996) *J. Biol. Chem.* 271, 1916-1924). However, the degree of nitrate-insensitivity fluctuates during the course of the year with a seasonal frequency. Nitrate uncouples H⁺ pumping from ATP hydrolysis both in epicotyls and in nitrate-sensitive fruit V-ATPases. Neither bafilomycin nor oxidation cause uncoupling. The initial rate H⁺/ATP coupling ratios of epicotyl and the nitrate-sensitive fruit proton pumping activities are the same. However,

the H⁺/ATP coupling ratio of the nitrate-insensitive fruit H⁺ pumping activity is lower than that of nitrate-sensitive and epicotyl V-ATPases. Several properties of the nitrate-insensitive H⁺-ATPase of the fruit indicate that it is a modified V-ATPase rather than a P-ATPase: 1) insensitivity to low concentrations of vanadate; 2) it is initially strongly uncoupled by nitrate, but regains coupling as catalysis proceeds; 3) both the nitrate-sensitive and nitrate-insensitive fruit H⁺-pumps have identical K_m values for MgATP, and show similar pH-dependent slip and proton leakage rates. We conclude that the ability of the juice sac V-ATPase to build up steep pH gradients involves three factors: variable coupling, i.e. the ability to regain coupling under conditions that initially induce uncoupling; a low pH-dependent slip rate; the low proton permeability of the membrane.

4. Brune, A., Müller, M., Taiz, L., Gonzalez, P., and Echeverría, E. (2002) **Vacuolar acidification in citrus fruits: Comparison between acid lime (*Citrus aurantifolia*) and sweet lime (*Citrus limmetoides*) juice cells.** *Hort. Sci.* 127: 171-177. Vacuolar acidification was investigated in Palestine sweet and Persian acid lime using tonoplast vesicles isolated from juice cells. The ATPase of the tonoplast-enriched vesicles from sweet limes was strongly inhibited by bafilomycin A and nitrate, but was unaffected by vanadate. In contrast, the V-ATPase activity in acid lime membranes was only slightly inhibited by bafilomycin A and nitrate, and was strongly inhibited by high concentrations of vanadate. The vacuolar origins of the membranes was confirmed by immunoblotting. After solubilizations and partial purification of the enzymes by gel infiltration, their inhibitor profiles were largely unchanged. Based on equal ATPase activities, vesicles from sweet and acid lime vesicles were able to generate similar pH gradients. However, in tonoplast vesicles from sweet limes, the the maximum delta pH was reached four times faster than in those from acid limes. Addition of EDTA to chelate magnesium caused the rapid collapse of the pH gradient in sweet lime vesicles, but not acid lime, indicating a less permeable membrane in acid limes. The ATPase of the acid lime, but not the sweet lime, was inhibited by calcium. The data confirm that the vanadate-sensitive form of the V-ATPase found in lemon and acid limes is specific for hyperacidifying cells.

5. Müller, M.L. and Taiz, L. (2002) **Regulation of the lemon-fruit V-ATPase by variable stoichiometry and organic acids.** *J. Membrane Biology* 185: 209-220.

The lemon-fruit V-ATPase can exist in two forms: nitrate-sensitive and nitrate-insensitive. Here we report the results of measurements of H⁺ /ATP stoichiometries using two kinetic methods: one based on steady-state ΔpH and one based on initial rates of H⁺-pumping. Our findings indicate that the nitrate-insensitive fruit V-ATPase has an H⁺ /ATP stoichiometry of ~1, while both the nitrate-sensitive fruit V-ATPase and the epicotyl V-ATPase have stoichiometries of 2, under zero-load conditions. As ΔpH increases, the stoichiometry of the nitrate-sensitive fruit V-ATPase decreases to 1. Under similar conditions, the stoichiometry of the epicotyl enzyme remains 2. Thus, the pH-dependent variable stoichiometry of the lemon-fruit V-ATPase may represent a key factor in juice sac vacuolar hyperacidification. On the other hand, the H⁺ /ATP

stoichiometry of the epicotyl V-ATPase can decrease from 2 to 1 in the presence of a membrane potential. The low pH of the fruit vacuole is not due solely to the lower H⁺/ATP stoichiometry of its pump. We show that luminal citrate and malate improve the coupling of both the epicotyl and fruit V-ATPases and enhance their ability to generate a pH gradient. Since citrate accumulation is restricted to fruit vacuoles, it may be another important determinant of vacuolar pH.

6. Sze, H., Schumacher, K., Müller, M., Padmanaban, S., and Taiz, L. (2002) A simple nomenclature for a complex proton pump: VHA genes encode the Vacuolar H⁺-ATPase. *Trends in Plant Science* 7: 157-161.

The vacuolar-type H⁺-ATPase acidifies intracellular compartments and is essential for many processes, including cotransport, guard cell movement, development, and tolerance to environmental stress. We have identified at least 26 genes encoding subunits of the vacuolar-type H⁺-ATPase in the *Arabidopsis thaliana* genome, although inconsistent nomenclature of these genes is confusing. The pump consists of subunits A through H of the peripheral V1 complex, and subunits a, c, c' and d of the Vo membrane sector. Most V1 subunits are encoded by a single gene, whereas Vo subunits are encoded by multiple genes found in duplicated segments of the genome. We propose to name these genes VHA-x, where x represents the letter code for each subunit. Applying a consistent nomenclature will help us to understand how the expression, assembly and activity of this pump are integrated with plant growth, signaling, development and adaptation.

7. Murphy, A. and Taiz, L. (1999) Localization and characterization of soluble and plasma membrane aminopeptidase activities in *Arabidopsis* seedlings. *Plant Physiology and Biochemistry* 37: 431-443.

During prolonged storage, solid naphthylphthalamic acid (NPA) turns purple due to the formation of the dye, 1,1'-azonaphthylene (ANA). ANA forms oxidatively from two molecules of the NPA degradation product, small alpha, α -naphthylamine. At concentrations $\geq \mu\text{M}$, solutions of 'purple NPA' stained *Arabidopsis thaliana* seedlings specifically at the hypocotyl-root transition zone and other regions. Staining was caused by the aggregation of ANA and could be reconstituted using undergraded NPA and either αNA or ANA. Studies with [3H]-NPA confirmed that NPA is localized at sites of staining. Genestein, curcumin and quercetin inhibited the staining reaction. Liquid chromatography-mass spectral (LC-MS) analysis of the ANA in the stained tissue indicated that ANA is synthesized at sites of staining. It was postulated that the amide bond of NPA is enzymatically cleaved, producing $\alpha\text{-NA}$. The $\alpha\text{-NA}$ combines to form ANA, which aggregates to yield an insoluble precipitate. Consistent with this hypothesis, NPA amidase activity was detected in purified plasma membranes. The NPA amidase activity was activated by 500 μM MnCl₂ and inhibited by phloretin, genestein, quercetin, bestatin and EDTA.

8. Murphy, A. and Taiz, L. (1999) Naphthylphthalamic acid is enzymatically hydrolyzed at the hypocotyl-root transition zone and other tissues of *Arabidopsis thaliana* seedlings. *Plant Physiology and Biochemistry* 37: 413-430

We previously demonstrated that N,1-naphthylphthalamic acid is hydrolyzed at the root-hypocotyl transition zone and other regions of *Arabidopsis thaliana* seedlings, and that this reaction, like NPA-induced growth inhibition, is strongly promoted by blue light. In addition, NPA amidase activity was detected in plasma membrane-enriched fractions obtained from *Arabidopsis* seedlings. To further investigate this phenomenon, we tested the hypothesis that the arylamidase(s) responsible for NPA hydrolysis may also have aminopeptidase activity. The responses of *Arabidopsis* seedlings to various aminopeptidase substrates were tested. The hydrolysis of Tyr-, Trp-, Pro- and Gly-Pro-small beta, Greek-naphthylamide aminopeptidase substrates was shown to be histochemically localized at the root-hypocotyl transition zone and other regions where NPA hydrolysis also occurs. Blue light stimulated the *in vivo* activity of Tyr- and Pro-aminopeptidase activities, and far-red light stimulated the activity of the Trp-aminopeptidase. These same substrates also induced NPA-like growth inhibitory effects. In parallel experiments, aminopeptidase activities were detected in the supernatant and plasma membrane fractions of seedling extracts. The soluble AP activities resemble previously described neutral aminopeptidases with specificity for aromatic residues. The plasma membrane fraction hydrolyzed Tyr-, Trp-, Ala-Pro- and Pro-AP substrates, and also exhibited activity against Phe- and Leu-substrates. Many of the properties of the aminopeptidases, such as pH optima, metal requirements and responses to inhibitors, overlap with those of the previously characterized NPA amidase, suggesting that the latter may represent the combined activities of multiple aminopeptidases.

9. Murphy, A., Peer, W., and Taiz, L. (2000) Regulation of polar auxin transport by aminopeptidases and flavonoids. *Planta* 211: 315-324

The 1-N-naphthylphthalamic acid (NPA)-binding protein is a putative negative regulator of polar auxin transport that has been shown to block auxin efflux from both whole plant tissues and microsomal membrane vesicles. We previously showed that NPA is hydrolyzed by plasma-membrane amidohydrolases that co-localize with tyrosine, proline, and tryptophan-specific aminopeptidases (APs) in the cotyledonary node, hypocotyl-root transition zone and root distal elongation zone of *Arabidopsis thaliana* (L.) Heynh. seedlings. Moreover, amino acyl- β -naphthylamide (aa-NA) conjugates resembling NPA in structure have NPA-like inhibitory activity on growth, suggesting a possible role of APs in NPA action. Here we report that the same aa-NA conjugates and the AP inhibitor bestatin also block auxin efflux from seedling tissue. Bestatin and, to a lesser extent, some aa-NA conjugates were more effective inhibitors of low-affinity specific [3 H]NPA-binding than were the flavonoids quercetin and kaempferol but had no effect on high-affinity binding. Since the APs are inhibited by flavonoids, we compared the localization of endogenous flavonoids and APs in seedling tissue. A correlation between AP and flavonoid localization was found in 5- to 6-d-old seedlings. Evidence that these flavonoids regulate auxin accumulation *in vivo* was obtained using the flavonoid-deficient mutant, *tt4*. In whole-seedling [14 C]indole-3-acetic acid transport studies, the pattern of auxin distribution in the *tt4* mutant was shown to be altered. The defect appeared to be in auxin accumulation, as a considerable amount of auxin escaped from the roots. Treatment of the *tt4* mutant with the missing intermediate naringenin restored normal auxin distribution and accumulation by the root. These results implicate APs and endogenous flavonoids in the regulation of auxin efflux.

10. Brown, D.E., Rashotte, A.M., Murphy, A.S., Normanly, J., Tague, B.W., Peer, W.A., Taiz, L., and Muday, G.K. (2001) Flavonoids act as negative regulators of auxin transport in vivo in *Arabidopsis*, *Plant Physiol.* 126: 524-535

Polar transport of the plant hormone auxin controls many aspects of plant growth and development. A number of synthetic compounds have been shown to block the process of auxin transport by inhibition of the auxin efflux carrier complex. These synthetic auxin transport inhibitors may act by mimicking endogenous molecules. Flavonoids, a class of secondary plant metabolic compounds, have been suggested to be auxin transport inhibitors based on their in vitro activity. The hypothesis that flavonoids regulate auxin transport in vivo was tested in *Arabidopsis* by comparing wild-type (WT) and transparent testa (tt4) plants with a mutation in the gene encoding the first enzyme in flavonoid biosynthesis, chalcone synthase. In a comparison between tt4 and WT plants, phenotypic differences were observed, including three times as many secondary inflorescence stems, reduced plant height, decreased stem diameter, and increased secondary root development. Growth of WT *Arabidopsis* plants on naringenin, a biosynthetic precursor to those flavonoids with auxin transport inhibitor activity in vitro, leads to a reduction in root growth and gravitropism, similar to the effects of synthetic auxin transport inhibitors. Analyses of auxin transport in the inflorescence and hypocotyl of independent tt4 alleles indicate that auxin transport is elevated in plants with a tt4 mutation. In hypocotyls of tt4, this elevated transport is reversed when flavonoids are synthesized by growth of plants on the flavonoid precursor, naringenin. These results are consistent with a role for flavonoids as endogenous regulators of auxin transport.

11. Murphy, A., Hoogner, K., Peer, W., and Taiz, L. (2002) Identification, purification, and molecular cloning of N-1-naphthylphthalamic acid-binding plasma membrane-associated aminopeptidases from *Arabidopsis*, *Plant Physiol.* 128: 935-950.

Polar transport of the plant hormone auxin is regulated at the cellular level by inhibition of efflux from a plasma membrane (PM) carrier. Binding of the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) to a regulatory site associated with the carrier has been characterized, but the NPA-binding protein(s) have not been identified. Experimental disparities between levels of high-affinity NPA binding and auxin transport inhibition can be explained by the presence of a low-affinity binding site and in vivo hydrolysis of NPA. In *Arabidopsis*, colocalization of NPA amidase and aminopeptidase (AP) activities, inhibition of auxin transport by artificial beta-naphthylamide substrates, and saturable displacement of NPA by the AP inhibitor bestatin suggest that PM APs may be involved in both low-affinity NPA binding and hydrolysis. We report the purification and molecular cloning of NPA-binding PM APs and associated proteins from *Arabidopsis*. This is the first report of PM APs in plants. PM proteins were purified by gel permeation, anion exchange, and NPA affinity chromatography monitored for tyrosine-AP activity. Lower affinity fractions contained two orthologs of mammalian APs involved in signal transduction and cell surface-extracellular matrix interactions. AtAPM1 and AtAPP1 have substrate specificities and inhibitor sensitivities similar to their mammalian orthologs, and have temporal and spatial expression patterns consistent

with previous in planta histochemical data. Copurifying proteins suggest that the APs interact with secreted cell surface and cell wall proline-rich proteins. AtAPM1 and AtAPP1 are encoded by single genes. In vitro translation products of ATAPM1 and AtAPP1 have enzymatic activities similar to those of native proteins.

12. Peer, W.A., Brown, D.E., Tague, B.W., Muday, G.L., Taiz, L., and A. S. Murphy (2001) Flavonoid accumulation patterns of transparent testa mutants of *Arabidopsis*. *Plant Physiol.* 126: 536-548.

Flavonoids have been implicated in the regulation of auxin movements in *Arabidopsis*. To understand when and where flavonoids may be acting to control auxin movement, the flavonoid accumulation pattern was examined in young seedlings and mature tissues of wild-type *Arabidopsis*. Using a variety of biochemical and visualization techniques, flavonoid accumulation in mature plants was localized in cauline leaves, pollen, stigmata, and floral primordia, and in the stems of young, actively growing inflorescences. In young Landsberg erecta seedlings, aglycone flavonoids accumulated developmentally in three regions, the cotyledonary node, the hypocotyl-root transition zone, and the root tip. Aglycone flavonoids accumulated at the hypocotyl-root transition zone in a developmental and tissue-specific manner with kaempferol in the epidermis and quercetin in the cortex. Quercetin localized subcellularly in the nuclear region, plasma membrane, and endomembrane system, whereas kaempferol localized in the nuclear region and plasma membrane. The flavonoid accumulation pattern was also examined in transparent testa mutants blocked at different steps in the flavonoid biosynthesis pathway. The transparent testa mutants were shown to have precursor accumulation patterns similar to those of end product flavonoids in wild-type Landsberg erecta, suggesting that synthesis and end product accumulation occur in the same cells.

13. Eisinger, W., Swartz, T., Bogomolni, R., and Taiz, L. (2000) The ultraviolet action spectrum for stomatal opening in broad bean. *Plant Physiology* 122: 99-105.

The ultraviolet action spectrum for stomatal opening was measured using epidermal peels from leaves of broad bean (*Vicia faba*). The spectrum was calculated from hyperbolic fluence response curves using 11 wavelengths ranging from 275 to 459 nm. The action spectrum exhibits a major peak at approximately 280 nm and a minor peak at approximately 360 nm. The response at 280 nm is about three times greater than the response at 459 nm. Under the conditions utilized (i.e. the absence of saturating red light), stomatal opening saturated at extremely low fluence rates: $<0.2 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 280 nm, and approximately $1.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ at 459 nm. The threshold for blue-light-induced stomatal opening was approximately $0.02 \mu\text{mol m}^{-2} \text{s}^{-1}$. In light-mixing experiments, the addition of 280 nm light to saturating 650 nm (red) light caused additional stomatal opening, which is indicative of separate photoreceptors. In contrast, adding 280 nm of light to saturating 459 nm (blue) light did not increase stomatal opening, suggesting that they both excite the same receptor. The results with white light were similar to those with blue light. We infer that ultraviolet light acts via the blue light photoreceptor rather than through photosynthesis. The additional absorbance peak at 360 nm suggests that the chromophore is either a flavin or a cis-carotenoid, both of which exhibit peaks in this region. It is proposed that the chromophore can be excited either

directly by blue light or by energy transferred from the protein portion of the protein-pigment complex after it absorbs 280 nm light.

14. Eisinger, W., Bogomolni, R., and Taiz, L. (2003) Interactions between a blue-green reversible photoreceptor and a separate UV-B receptor in stomatal guard cells. *Am. J. Botany* 90: 1560-1566.

Stomatal opening exhibits two main peaks of activity in the visible range—a red peak, mediated by photosynthesis, and a blue peak, mediated by one or more blue light (BL) photoreceptors. In addition, a pronounced peak in the UV-B region has been characterized, as has a smaller UV-A peak. The BL-induced stomatal opening can be reversed by green light (GL). Here we report that UV-B-induced opening is also antagonized by GL. To determine whether UV-B is being absorbed by the BL photoreceptor or by a separate UV-B receptor, the UV-B responses of two different *Arabidopsis* mutants, *npq1* and *phot1/phot2*, were tested. Both putative BL-photoreceptor mutants exhibited normal stomatal opening in response to UV-B, consistent with the existence of a separate UV-B photoreceptor. Moreover, GL failed to antagonize UV-B-induced stomatal opening in the *phot1/phot2* double mutant and only partially antagonized UV-B opening in *npq1*. Thus, both *phot1* and *phot2*, as well as zeaxanthin, are required for the normal GL inhibition of UV-B. A model for a photoreceptor network that regulates stomatal opening is presented. Unlike the situation in guard cells, the UV-B bending response of *Arabidopsis* hypocotyls during phototropism appears to be mediated by phototropins.