2014

Developmental Changes and H+-ATPase Co-localization in Nepenthes alata Peristomal Nectary Glands

Alison Carini
Connecticut College, carini.alison@gmail.com

Developmental Changes and H^+-ATPase Co-localization in *Nepenthes alata* Peristomal Nectary Glands

A Thesis submitted in partial satisfaction of the

Requirements for the degree of

Bachelor of Arts

With Honors in Biology

By

Alison Carini

May 1, 2014

Thesis Committee:

Professor T. Page Owen, Jr., Advisor
Department of Botany, Connecticut College

Professor Joseph Schroeder
Department of Psychology, Connecticut College

Table of Contents

List of Figures

Acknowledgements

The author wishes to thank Dr. T. Page Owen, Jr. for advising me on all aspects of this project, Dr. Joseph Schroeder for assisting with project development and for second-reading this thesis, Dr. Deborah Eastman for providing and assisting with *Arabidopsis thaliana* seed tissue cultures for experiments that were ultimately not included in this thesis, Dr. Rachel Spicer for providing L.R. White resin, Ms. Kathy Gehring for providing much appreciated assistance with the technical preparation of this written thesis, Dr. Stephen Smith for his support and encouragement of all of my academic pursuits, and finally my family and friends for their constant support and encouragement. Without their support, this thesis would never have been realized. In addition, the TEM was supported by an NSF grant to T.P.O.

Abstract

Nepenthes alata is an east-Asian species of tropical carnivorous plant more commonly known as a pitcher plant. The pitcher is a modified epiascidiate leaf used to passively capture insects as nutritious supplements to its diet. In this study, the ultrastructure of different age stages of peristomal extrafloral nectary glands were surveyed, selected ages were immunolabeled for H^+-ATPases. All nectary cells had numerous mitochondria, endoplasmic reticulum, Golgi bodies, plastids, and vacuoles in a dense cytoplasm consistent with an active secretory system. The larger class of immature pitchers (approx. 4 cm in diameter, lid closed) had budding structures affecting the plasma membrane of inner nectary gland cells, suggesting that secondary cell wall protrusions begin their development at this age. Older mature pitchers (approx. 3 cm diameter with an open lid) have interior nectary gland cells with full-sized cell wall protrusions, as well as numerous plasmodesmata, suggestive of a possible combined apoplastic and sympastic method of transportation for pre-nectar and nectar in the gland. In addition, although H^+-ATPase immunogold labeled sections demonstrated a degree of non-specific binding, there was some localization between the secondary cell wall and the plasma membrane.

Introduction

The *Nepenthes* carnivorous pitcher plants are native to the tropical climates of eastern Asia, especially the islands of the Philippines, Borneo and Sumatra (Moran and Clarke, 2010). Although some *Nepenthes* are epiphtic and have been observed in montane habits, most are terrestrial vines or subscandent scrubs that grow in extremely nutrient-poor habits (Bauer et al., 2011). They acquire supplemental mineral nutrition by ingesting insects passively in modified epiascidiate leaves in a pitcher shape, instead of actively through the snap mechanisms of the more well-known Venus fly trap (*Dionea*) or the sticky flypaper type (*Drosera*) (Juniper et al., 1989, Moran and Clarke, 2010). This pitcher structure is formed by the adaxial surface of the leaf when it curls in on itself creating the innermost wall of the pitcher.

Pitchers are divided into three visible zones. At the top of the pitcher is the "attractive zone" containing the lid as well as a ridged and double-edged lip of the pitcher called the peristome. The upper inside surface of the pitcher containing slippery epicuticular waxes is the "conductive zone" that prevents arthropod prey from leaving the pitcher, and directs the insect prey to the "digestive zone" in the base of the pitcher where digestive glands secrete digestive juices that aid in the pitcher's digestion and absorption of arthropod nutrients. (Owen et al., 1999, Pavlovic et al., 2007, Clarke, 1997).

In the attractive zone, nectar is excreted by extrafloral nectary glands located on the tendril, the outside of the pitcher, the pitcher lid, and the peristome

(Bauer et al., 2011). The peristome has the highest concentration of extrafloral nectaries, with nectaries regularly placed between prominent visible radial ridges (Moran and Clarke, 2010, Bauer et. al, 2011). Arthropod prey is attracted to the pitchers through optical cues as well as the secretion of sweet smelling nectar. (Bauer et al., 2011, Pavlovic et al., 2007, Moran et al., 1999). The pitchers of *N. rafflesiana* have a peristome that color contrasts the pitcher body in the ultraviolet (350-370 nm), blue (430-470 nm), and green (490-540 nm) wavelengths (Moran and Clarke, 2010). Surprisingly, in addition to attracting prey, the peristome also helps to trap it through "aquaplaning", forming a water film to prevent the adhesive pads of insects from making contact with the surface of the peristome, causing it to fall towards the pitcher's mouth into the conductive zone (Bauer et al., 2011, Bauer et al., 2012).

The conductive zone, in contrast, has unusual “lunate” cells, possibly a modified single stomata guard cell, which points in a downward direction and, along with the entire epidermis in this region, is coated with an exfoliating slippery surface of wax crystals (Moran and Clarke, 2010). These waxes prevent the prey from gaining traction by contaminating the insect's adhesive pads, ultimately resulting in the prey's fall into the digestive zone of the pitcher. Digestive glands then secrete enzymes and reabsorb digestive juices containing mineral nutrients from the prey (Wong et al., 2011, Moran and Clarke, 2010, Moran et al., 2010, Bauer et al., 2011). In contrast, the nectar is comprised of water and carbohydrates transported to the nectary gland from the vascular tissue, as well as trace amounts of other substances like lipids and amino acids. The

carbohydrates and amino acids play roles in attracting insects to the pitcher by providing the insects with a digestible form of energy. The role of the other components of the nectar, including the lipids and antioxidants are still not very well understood at this time (Nepi, 2007; Baker, 1977)

Nepenthes pitchers are considered mature when the lid sealed against the peristome across the pitcher opening releases (Owen and Lennon, 1999). Preceding opening, pitchers of *N. alata* develop at a uniform rate progressing through several stages of development (Owen and Lennon, 1999). The first sign of pitcher development is a swollen tip of a tendril of a leaf. Then, a terminal spur, located at the apex of the lid, develops before the pitcher elongates during a period of 6-8 weeks. During this period of time, the digestive glands secrete digestive juices partially filling the pitcher. After the lid opens, the nectary glands of the peristome secrete nectar and the pitcher is fully developed (Owen and Lennon, 1999).

When the pitcher is immature, the nectary glands are indistinct by light microscopy from the surrounding cells in the peristome (Owen and Lennon, 1999). As the pitcher matures, the nectary glands become discernable as both a distinct collection of columnar-shaped head cells and as an expanded base of small-diametered cells. In general, both floral and extrafloral nectary glands have three different layers of cells: the nectary epidermis and nectary parenchyma, which are involved in both nectar production and secretion, and the subnectary parenchyma that contains the vascular tissue that transports pre-nectar to the gland (Nepi, 2007). The single outermost layer of cells in the nectary is called the

epidermis, and consists of polyhedric cells with an anticlinical orientation. The vacuoles of these cells are larger than in the other cells of the nectary gland, and their plastids don't normally have noticeable starch grains (Nepi, 2007, Razem and Davis, 1999).

Secretion of nectar in both floral and extrafloral nectaries occurs through these epidermal secreting cells and/or through secreting trichomes. These two methods are not always mutually exclusive and may occur together. *N. alata* possesses both a cuticle layer where nectar may collect before being secreted, and trichomes (Nepi, 2007). In addition, microchannels (Davis et al., 1998; Stpiczynska, 2003) and large pores (Kronestedt et al., 1986) have been observed in nectary glands of *Platanthera chlorantha,* and are both alternative methods of nectary secretion to the epidermal secreting cell and secreting trichome methods that may be employed in *N. alata*. Cell wall ingrowths in nectary glands have been identified before in other plants species, such as *Platanthera chlorantha, Echinacea purpurea, Brassica napus,* and *Vicia faba,* and are believed to act like transfer cells in *Senecio vulgaris* and *Lactuca sativa* by aiding the eccrine secretion of nectar, because they have been rarely seen in systems with noticeable granulocrine secretion (Nepi, 2007). Invaginations of the plasma membrane and plasmodesmata have been observed in secreting nectaries as well, but not specifically in *Nepenthes* (Durkee, 1983).

Generally, the middle layer of the extrafloral or floral nectary gland are the inner nectary gland cells, and consists of small, isodiametric cells with thin walls, dense granular cytoplasm rich with ribosomes and mitochondria, small

vacuoles, and relatively large nuclei (Nepi, 2007). The high presence of ribosomes and mitochondria is suggestive of the high amount of energy inner nectary gland cells use to produce and secrete nectar, implying that nectar is a very costly secretion for *Nepenthes alata*. Vacuole size has also been shown to vary depending on the development of the nectary gland, with small vacuoles normally present in nectary glands that are not mature and not actively secreting nectar (Nepi, 2007).

There are two main pathways that plant cells utilize in intercellular transportation: apoplastic and symplastic transport. Apoplastic transport is mediated through the cell wall and intercellular spaces, while symplastic transport occurs through the cytoplasm only using plasmodesmata. Evidence exists for both symplastic and apoplastic transport of pre-nectar, even within the same species, as with *Lonicera japonica* (Nepi, 2007). The current mechanisms of nectar transport for *Nepenthes alata* is still unknown and current literature discusses conflicting possible mechanisms. The presence of plasmodesmata between the inner nectary gland cells themselves and the subnectary inner gland cells has been reported as evidence suggestive of symplastic transport, while the presence of cell wall ingrowths has been suggestive of apoplastic transport.

In general, the last layer of nectary tissue is the subnectary inner gland cells that are under the inner nectary gland tissue. It contains cells with bigger vacuoles, less dense cytoplasm, and more plastids than the cells of the inner nectary gland. In extrafloral nectaries, the most common type of plastids are chloro-amyloplasts, with few starch grains, as extrafloral nectaries have less

evident starch formation and degradation processes (Nepi, 2007, Durkee et al., 1981, Pacini et al., 2003). In addition, its endoplasmic reticulum and Golgi bodies are less swollen and developed. It remains unclear whether the nectary gland is responsible for producing the carbohydrates used to produce the nectar, the carbohydrates are imported from other parts of the plant via the vascular tissue, or if the system is a combination of both of these scenarios. Vascular tissue is always present, with the xylem stopping in this layer of tissue, and the phloem continuing into the inner nectary gland cells. In several species including *Vicia faba*, *Eccremocarpus scaber*, *Echinacea purpurea,* and *Pisum sativum* cell wall ingrowths are common in the companion cells of nectary phloem, where the resulting increase in surface area of the plasma membrane aids the active eccrine transport across the plasma membrane of pre-nectar to the nectary gland (Nepi, 2007). The alternate method of pre-nectar transport, granulocrine, that utilizes Golgi bodies and the exocytosis of vacuoles, has been previously discarded by some authors because it contradicts current research about the function of the endoplasmic reticulum and Golgi bodies in plant secretion. (Vassilyev, 2010)

This study also examined the developmental expression of the H+-ATPase, a common proton-pump found in plant plasma membranes that is an important primary active co-transporter of solutes into and out of cells. It creates an important proton electrochemical gradient that facilitates an active proton-motive force. In addition, H^+ -ATPase is an important regulator of cell turgor and intracellular pH (Michelet, 1995). The functional unit of plant H^+ -ATPase consists of ten transmembrane α-helices and three cytoplasmic domains. The

transmembrane domain contains a large fluid-filled cavity lined with hydrophilic and charged residues that aid in proton transport against a high cell membrane potential (Pedersen, 2007).

H^+ -ATPase is associated with the eccrine and apoplastic nectar transport system from nectary cell to nectary cell (Vassilyev, 2010). It has also been identified as a key integral plasma membrane protein in floral nectar transportation in *Cucumis sativus*, where it was found to be involved in nectar secretion, specifically in the transport of nectar from secretory tissues to the intercellular space. Numerous nectary gland cells presented with invaginated plasma membranes, and the nectar was transported within the nectary gland and outside the nectary gland via an ATPase-dependent pathway (Peng et al., 2004). Additionally, the presence of H^+ -ATPase has been identified in the digestive zone of *N. alata*, where it plays an important role in regulating digestive processes (Chung-Il et al., 2000). However, to date, the expression of H^+ -ATPase in *Nepenthes* extrafloral nectary glands still remains undetermined. Therefore, the purpose of this study is to better understand the possible role of H^+ -ATPase in nectar transport in *Nepenthes alata* by determining if H^+ -ATPase localizes between the secondary cell wall ingrowths and the invaginated plasma membrane.

Materials and Methods

Nepenthes Tissue Samples

Nepenthes alata Blanco plants were grown in Connecticut College greenhouses at 22-28 ° C in sphagnum moss without fertilizer. Sample pitchers were removed at different stages of development by cutting the tendril. Tissue was collected from different ages of pitchers immature (closed lid, 1 cm diameter,) recently mature (diameter about 3 cm, < 1 day opened lid) and very mature (diameter about 3 cm, about 2-3 days opened lid) plants. In recently mature and very mature pitchers, tissue samples were taken from the peristome only, and in the immature pitchers, tissue samples were from the peristome and adjoining lid.

TEM Tissue Fixation

Nepenthes pitcher tissue samples were cut into approximately 1mm^2 pieces. These tissue pieces were then fixed in 2.5% glutaraldehyde with 1% p-formaldehyde in 0.05M sodium phosphate buffer (pH 7.2) for 4 hours. TEM ultrastructure samples were then washed twice with buffer, post-fixed in buffered 1% osmium tetroxide and then dehydrated using a graded acetone series. Dehydrated samples were infiltrated and embedded with Spurr's epoxy resin and polymerized overnight at 60°C. For immunogold labeling, sections were fixed in glutaraldehyde-formaldehyde as above, washed twice with buffer, dehydrated using a graded ethanol series, infiltrated with L.R. White's resin, and polymerized overnight at 60ºC.

Thick sections (0.5-1μm) of the polymerized samples were cut using a glass or a diamond (Diatome) knife and an RMC PT-XL ultramicrotome, and stained for light microscopy using Toluidine Blue. After visual identification of nectary gland and the medial plane, thin sections (70-80 nm) were cut using the diamond knife on the ultramicrotome. For TEM ultrastructure viewing, sections were placed on copper grids, or for immunogold labeling, sections were placed on nickel grids, each coated with Formvar. Only TEM ultrastructure copper grids were post-stained in a 2% aqueous solution of uranyl acetate for 20-30 minutes, and then Reynolds' Formula lead citrate for 1-2 min (Reynolds, 1963). Grids were examined in a FEI Morgagni 268(D) transmission electron microscope (TEM) and images were taken using an AMT XR-60 CCD camera system. Images were adjusted for brightness and contrast in Adobe Photoshop.

Immunogold Labeling

Sections placed on nickel grids were blocked with Tris Buffered Saline-Triton 100x (TBS-T) with 5% Normal Goat Serum (NGS) for 15 min at room temperature, incubated with 1:50 dilution of primary *Arabidopsis thaliana* ATPase rabbit antibody (Agrisera, Vännas, Sweden) overnight at 4°C, washed in TBS-T, incubated in 1:100 dilution of secondary gold-bead conjugated Protein A antibody (Sigma-Aldrich) for 2 hours at room temperature, and washed in TBS-T followed by distilled water. Control grids were incubated with secondary antibody only.

Results

Nectary glands were taken from three different pitcher maturation stages (Figure 1). The nectary glands in all three stages of pitcher development, viewed via light microscopy, had similar sized glands, with cells that were equally visible, distinct, and identifiable (Figures 2,3,5, and 7). Previous SEM studies also revealed the nectary gland's location in between distinct peristomal ridges (Figure 2). Nectary glands, regardless of age, were still easily identifiable because of their dense cytoplasm. Transmission electron microscopy showed all ages of nectary glands had numerous organelles present consistent with a high level of molecular activity. These included numerous Golgi bodies, elliptical mitochondria, and solitary membranes identified as endoplasmic reticulum, as well as various microbodies, and underdeveloped plastids with amyloplasts occasionally present. Large nuclei were visible, with a large nucleoli present in most gland cells. The cytoplasm was electron-dense and grainy (Figures 3, 5, and 7).

Vacuoles were also frequently present, with tannins of varying sizes. However, the vacuoles in the nectary gland cells were small compared to the vacuoles of the surrounding plant tissue. In addition, no vacuoles were seen making contact with the cell membrane. The cell membrane was irregularly shaped, but remained close to the cell wall even when small protrusions were observed in some size stages (Figures 3 and 5). The cell wall was relatively thick, and there was little space between cells.

In mature pitchers (Figure 1C), most peristomal nectary glands viewed via light microscopy appeared as single recessed distinctive clusters of isodiametric,

small, darkly-staining cells (Figure 3a). The glands were always located near extensive vascular bundles and appeared to be round or oval in shape. Oval shaped secondary cell wall protrusions, causing the invagination of the cell membrane, were common and noticeable throughout all three layers of the nectary gland (Figure 3b). These cell wall protrusions were irregularly shaped, and sometimes presented as though they were not attached to the cell wall. The protrusions appeared to line up with each other, even in cell walls of adjacent cells (Figure 3c). Plasmodesmata and protrusions were found in close proximity together within the same cell (Figure 3d). Although there was non-specific binding both in between the primary and secondary cell wall and in the cytoplasm, more localization was seen in between the secondary cell wall and the plasma membrane (Figure 4).

Light microscopy revealed mature, recessed nectary glands present in the peristome of larger immature pitchers with closed lids (Figure 1B), surrounded by abundant vascular tissue. The nectary glands themselves were comprised of small, isometric, and darkly-staining cells, which made them distinctive from the surrounding tissues (Figure 5a). Nectary glands had irregularly shaped secondary cell wall structures protruding into the plasma membrane in some of the inner nectary gland cells examined, as well as numerous plasmodesmata (Figure 5b). These structures were not as dense upon examination as the cell well protrusions seen in other cells of similarly aged inner nectary gland cells. However, they did have a similar appearance to known cell wall protrusions and were located in the same location where the protrusions are visible: along the inside of the cell wall.

There were also identifiable cell wall protrusions that appeared in regular intervals along the cell wall (Figure 5c). These cell wall protrusions were less frequent, and also were smaller in diameter. Definite cell wall protrusions were visible and common among the outer epidermal layer of nectary gland cells, especially in the exposed epithelial cells bordering the cuticle of the gland (Figure 5d). In addition, some localization of H^+-ATPase between the secondary cell wall and the plasma membrane of inner nectary gland cells were present, but there also was a high amount of non-specific binding to other regions in the cell (Figure 6). There also was some localization to vacuole membranes (Figure 6b).

Light microscopy of an immature younger pitchers (Figure 1A) revealed nectary glands that appeared as a recessed cluster of isodiametric small cells, noticeably different from the surrounding tissues. The cells of the nectary were visibly darker, with the exposed head of the gland containing the smallest, darkest cells of the gland (Figure 7a). Cell wall protrusions were not found in any of the smallest immature pitchers, including the exposed epithelial cells bordering the nectary cuticle (Figure 7b), the epithelial cells bordering the surrounding vascular tissue, (Figure 7c) and the interior parenchyma cells (Figure 7d). Although the immunogold labeled H^+-ATPase was better localized between the secondary cell wall and the plasma membrane, there was till substantial non-specific binding, including near cell wall protrusions (Figure 8).

Discussion

The nectary gland cells contained the organelles involved with protein synthesis including endoplasmic reticulum and the Golgi bodies, as well as the numerous mitochondria required for the energy of the metabolic pathways utilized in nectar production, transport and secretion. The dense grainy appearance of the cytoplasm that made the nectary gland cells easy to identify is most likely due to the high concentration of proteins found in these cells. The absence of cell wall protrusions in the 1-6 cm diameter younger immature pitchers, the appearance of smaller, less common cell wall protrusions in the nectary gland cells of the larger immature pitcher, and the frequent appearance of these protrusions in the nectary gland cells of the mature pitcher suggest that protrusions are one of the later steps of *Nepenthes alata* nectary gland maturation.

In the inner nectary gland cells of the larger immature pitchers, recognizable cell wall protrusions were visible in epidermal outer layer of cells, and irregular protrusion-like structures were seen along the inside of the cell wall. These could be demonstrative of the development of protrusions from the outer layers of the gland to the interior of the gland. Since secondary cell wall ingrowths cause invagination of the plasma membrane, increasing the surface area of the membrane, secondary cell wall ingrowths are believed to aid in eccrine transport of nectar and nectary components within the nectary gland. In addition, there is no evidence of granulocrine vesicle transport, as the observed vacuoles were smaller in comparison to the cells outside of the nectary gland, and no vacuoles were observed in direct contact with the plasma membrane.

Previous literature on active nectar sugar transport in nectary secretory cells of *Barbarea vulgaris*, *Helleborus foetidus*, and *Artrostema ciliatum*, identified its role in apoplastic transport between secretory tissue and the intercellular space and also during the secretion of nectar to the outside surfaces of the nectary gland. Since nectar production and secretion is an energetically costly process for *N. alata* and all other nectar-producing plant species, plasma membrane H^+-ATPase assays in nectary glands have been identified as a marker of nectar transportation in *Cucumis sativus*. These assays along with TEM ultrastructure studies in *Cucumis sativus* revealed electron-dense material, a high abundance of mitochondria, a low abundance of plasmodesmata, and invaginated inner nectary gland cell plasma membranes, which led the authors to conclude that an apoplastic nectar transport mechanism was being utilized. (Peng et al., 2004) Because of the high rate of non-specific binding seen in all of the immunolabeled sections, the immunolabeled data set remains inconclusive. However, because some localization was seen between the secondary cell wall and the plasma membrane, the possibility still remains that the presence and activation of H^+-ATPase is still related to the development of the secondary cell wall ingrowths. Unlike the *Cucumis sativus* study, cell wall protrusions and plasma membrane invagination in *N. alata* were often seen in combination with plasmodesmata in the same cell in the mature and immature pitchers, which suggests that both apoplastic and symplastic transportation methods are used in a combined system for pre-nectar and nectar transport.

Nepenthes alata nectary glands provide us with a model system for further studying the advanced nectar production and transport systems, found in most plants. Further research into the method of secretion of *N. alata* through comparing the ultrastructure of the extrafloral nectary glands before and after known secretion of nectar, specifically looking at the vacuoles and other organelles, is required to confirm the method of secretion outlined in this study.

References

Baker, H. 1977. Non-sugar chemical constituents of nectar. *Apidologie* **8:** 349-356.

Bauer U, Di Giusto B, Skepper J, Grafe TU, Federle W. 2012. With a flick of the lid: A novel trapping mechanism in *Nepenthes gracilis* pitcher plants. *PLoS ONE* DOI: 10.1371/journal.pone.0038951

Bauer U, Grafe TU, and Federle W. 2011. Evidence for alternative trapping strategies in two forms of the pitcher plant, *Nepenthes rafflesia*. *Journal of Experimental Botany* **62**: 3683-3692.

Clarke CM. 1997. The effects of pitcher dimorphism on the metazoan community of the carnivorous pitcher plant *Nepenthes bicalcarata*. *Malayan Nature Journal* **50**: 149–157

Chung-Il A. 2012. Plasma membrane H+ -ATPases are expressed in pitchers of the carnivorous plant *Nepenthes alata* Blanco. *Planta* **212**: 547-555.

Davis AR, Pylatuik JD, Paradis JC, and Low NH. 1998. Nectary-carbohydrate production and composition vary in relation to nectary anatomy and location within individual flowers of several species of *Brassicaceae.* *Planta* **205**: 305-318.

Dress WJ, Newell SJ, Nastase AJ, Ford JC. 1997. Analysis of amino acids in nectar from pitchers of *Sarracenia purpurea* (Sarraceniaceae). *American Journal of Botany* **84**:1701-1706.

Durkee LT, Gaal DJ, WH Reisner. 1981. The floral and extrafloral nectarines of *Passiflora.* I. The Floral Nectary. *American Journal of Botany* **68**:453-462.

Elias TS. 1983. Extrafloral nectaries: their structure and distribution. In: Bently, B; and T. Elias. *The Biology of Nectaries.* Columbia University Press. New York: 174-203.

Hager A. 2003. Role of the plasma membrane H+ -ATPase in auxin-induced elongation growth: Historical and new aspects. *Journal of Plant Research* **116**: 483-505.

Juniper BE, Robins RJ, Joel DM. 1989. *The Carnivorous Plants.* Academic Press; London: 78-81, 314-315.

Kronestedt – Robards EC, Robards AW, Strak M, Olsen P. 1986. Development of trichomes in the *Abutilon* nectary gland. *Nordic Journal of Botany* **6**: 627-639.

Lalonde S, Boles E, Hellmann H, et al. 1999. The dual function of sugar carriers: transport and sugar sensing. *Plant Cell* **11**: 707-726.

Michelet B, and Boutry, M. 1995. The plasma membrane H+ -ATPase: Highly-regulated enzyme with multiple physiological functions" *Plant Physiology*. **108**: 1-6.

Moran JA, and Clarke CM. 2010. The Carnivorous Syndrome in *Nepenthes* pitcher plants: Current state of knowledge and potential future directions. *Plant Signaling & Behavior* **5**: 644-648.

Moran JA, Hawkins BJ, Gowen BE, and Robbins SL. 2010. Ion fluxes across the pitcher of three Bornean *Nepenthes* pitcher plant species: flux rates and gland distribution patterns nitrogen sequestration strategies. *Journal of Experimental Botany* **61**: 1385-1374.

Nepi M. 2007. Nectary Structure and Ultrastructure. In: Nicolson, SW, M Nepi, E Pacini (Eds.). *Nectaries and Nectar.* Springer. Dordrecht:129-166.

Owen TP, and Lennon KA. 1999. Structure and development of the pitchers from the carnivorous plant *Nepethes alata* (Nepenthaceae). *American Journal of Botany* **86**: 1382-390.

Owen TP, Lennon KA, Santo MJ, and Anderson AN. 1999. Pathways for nutrient transport in the pitchers of the carnivorous plant *Nepenthes alata*. *Annals of Botany* **84**: 459-66.

Pacini E, Nepi M, and Vesprini JL. 2003. Nectar biodiversity: A short review. *Plant Systematics and Evolution.* **238**:7-21.

Paez-Valencia J, Patron-Soberano A, Rodriguez-Leviz A, et. al. 2011. Plasma membrane localization of the type I H+ -PPase AVP1 in sieve element–companion cell complexes from Arabidopsis thaliana. *Plant Science* **181**: 23-30.

Palmieri M, and Kiss JZ. 2005. A novel technique for flat-embedding cryofixed plant specimens in LR white resin. *Microscopy Research and Technique* **68**: 80-84

Pavlovic A , Masarovicova E, and Hudak J. 2007. Carnivorous Syndrome in Asian Pitcher Plants of the Genus Nepenthes." *Annals of Botany* **100**: 527-536.

Pedersen BP, Buch-Pedersen, MJ, Morth JP, Palmgren MG, and Nissen P. 2007. Crystal structure of the plasma membrane proton pump. *Nature* **450**: 1111-1114.

Peng YB, Li YQ, Hao YJ, Xu ZH, Bai SN. 2004. Nectar production and transportation in the nectaries of the female *Cucumis sativus* L. flower during anthesis. *Protoplasma* **224**: 71-78.

Razem FA and Davis AR. 1999. Anatomical and ultrastructural changes of the floral nectary of *Pisum sativum* L. during flower development. *Protoplama* **206**: 57-72.

Reynolds ES. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *Journal of Cell Biology* **17**: 208-213.

Spurr AR. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* **26**: 31-34.

Stpiczynska M. 2003. Nectar resorption in the spur of *Platanthera chlorantha*. Custer (Rchb.) Orchidaceae-structural and microautoradiographic study. *Plant Systematics and Evolution*. **238**:119-126

Vassilyev AE. 2010. On the mechanisms of nectar secretion: revisited. *Annals of Botany*. **105**: 349-354.

Wong TS, Kang SH, Tang KY, Smythe EJ, Hatton BD, Grinthal A, and Aizenberg J. 2011. Bioinspired self-repairing slippery surfaces with pressure-stable omniphobicity. *Nature* **477**: 443-47.

Figure 1: Images of different maturation stages of whole *Nepenthes alata* pitchers before peristomal dissection and tissue preparation. Arrow indicates peristome. A, immature (closed lid, 1 cm diameter); bar = 0.75 cm. B, recently mature (diameter 3 cm, < 1 day opened lid), bar = 2 cm. C, very mature (diameter 3 cm, ~2-3 days opened lid), bar = 2.7 cm.

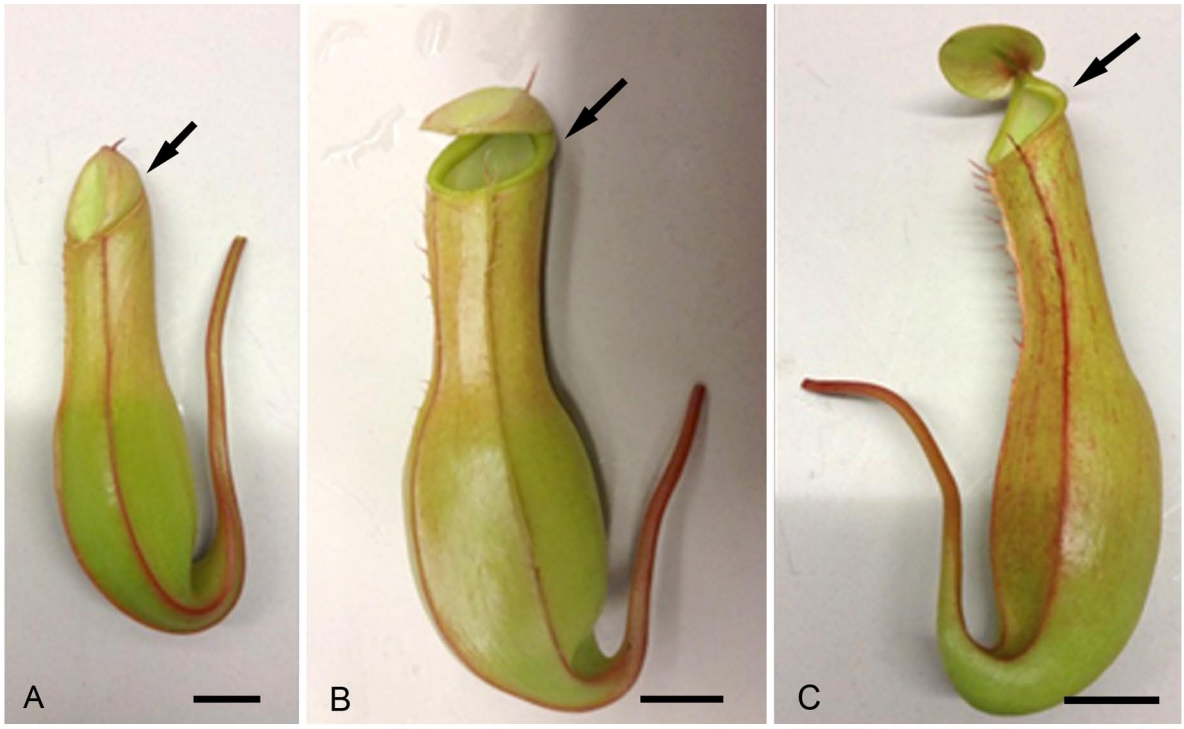

A
B
C

Figure 2. *Nepenthes alata* peristome with nectary glands. A. LM image of longitudinal section through medial nectary within the peristome. B. SEM image of similar view. Note nectary pore location in between peristomal ridges. (Images courtesy of Cara Hass ’12).

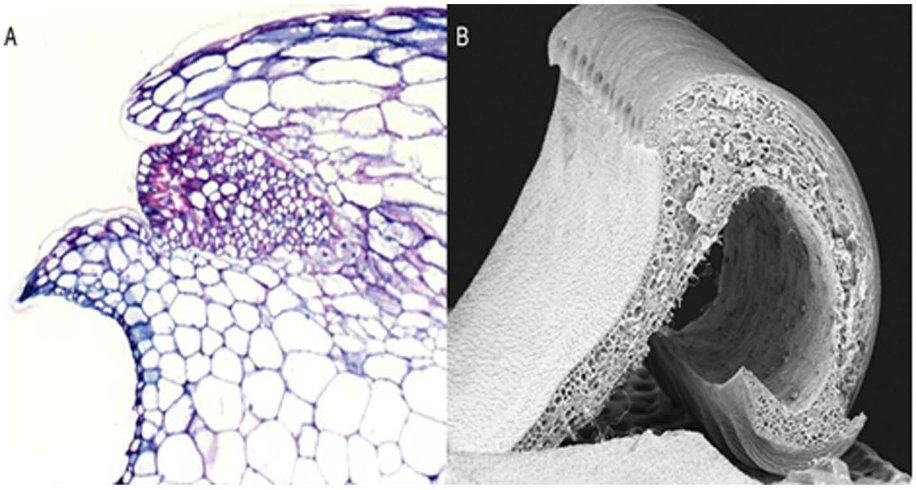

A
B

Figure 3: Mature pitcher sample (diameter 3 cm, ~2-3 days opened lid). A: Light microscopy of a mature nectary gland (NG) near vascular bundle (VB). B: Transmission electron microscopy (TEM) of interior nectary gland cell. Cell wall ingrowths (*) are numerous, with plasma membrane closely following the protuberances (arrow). Occasional plasmodesmata serving to symplastically connect the cells are visible (arrow head). Notice cell organelles including numerous mitochondrias (M), Golgi bodies (G), and vacuoles (V). Bar= 2µm. C: TEM detail of protrusions (*) and organelles in neighboring cell, including mitochondria (M), plastids (P), the nucleus (N), and Golgi bodies (G). Plasmodesmata are numerous (arrow heads), in regions between secondary cell wall (CW) ingrowth (*). Bar= 500µm. D: TEM detailed view of secondary cell wall protrusions. Notice plasma membrane invagination (arrows) caused by cell wall (CW) protrusions (*), as well as the nucleus (N), endoplasmic reticulum (ER), and numerous mitochondria (M). Bar= 500µm.

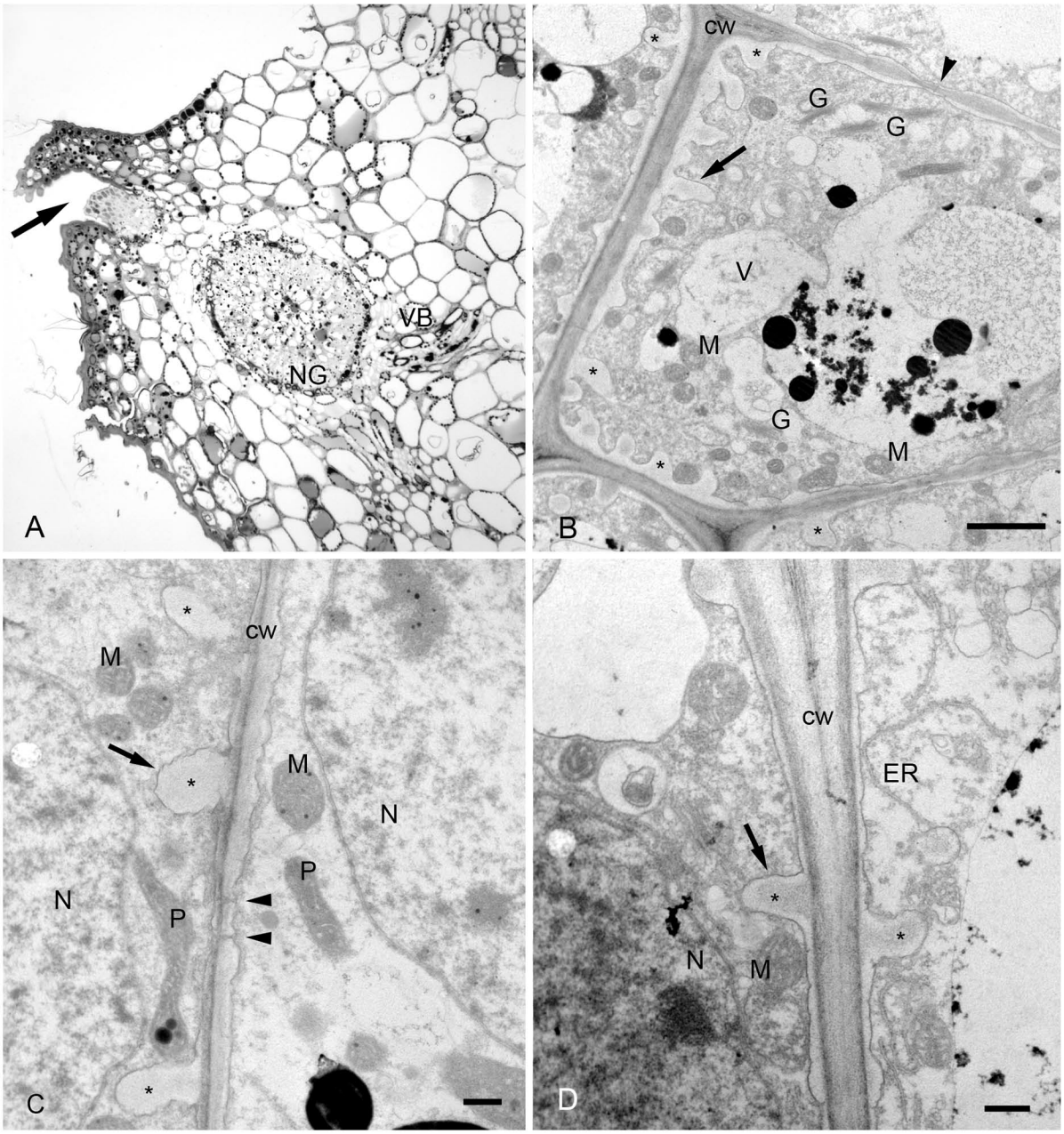

A
VB
NG
B
cw
G
G
V
M
G
M
C
cw
M
M
N
P
N
P
D
cw
ER
N
M

Figure 4: H^+-ATPase immunolabeled TEM mature (diameter about 3 cm, about 2-3 days opened lid) sections. A: Overview of outer nectary gland and nectary cuticle (bar = 10 μm). B and C: TEM detail of colloidal gold localization between secondary cell wall (CW) and plasma membrane, as well as some non-specific binding. Arrow indicates specific localization between secondary cell wall (CW) and plasma membrane, and arrow heads indicate non-specific localization between primary and secondary cell wall or in the cytoplasm. (B bar = 2 μm; C bar = 500 nm).

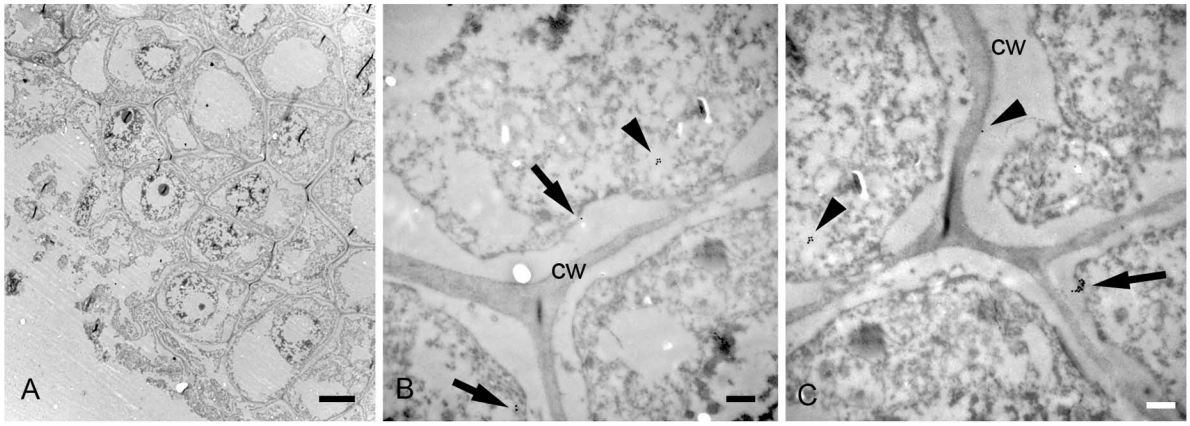

A
B
CW
C
CW

Figure 5: Older immature pitcher sample. A: Light microscopy of mature nectary gland (NG) and surrounding peristomal epidermis (PE). B: TEM of interior nectary gland cell with smaller plasma membrane invaginations (arrows), caused by cell wall (CW) protrusions as compared to mature tissue sample. Bar= 2μm. C: TEM detail of cell wall (CW) protrusions in cell (*), with Golgi bodies (G), mitochondria (M), plastids (P), and nuclei (N) present. Bar= 500μm D: TEM detail of exterior nectary epidermis and nectary cuticle (NC) of gland. Bar= 2μm.

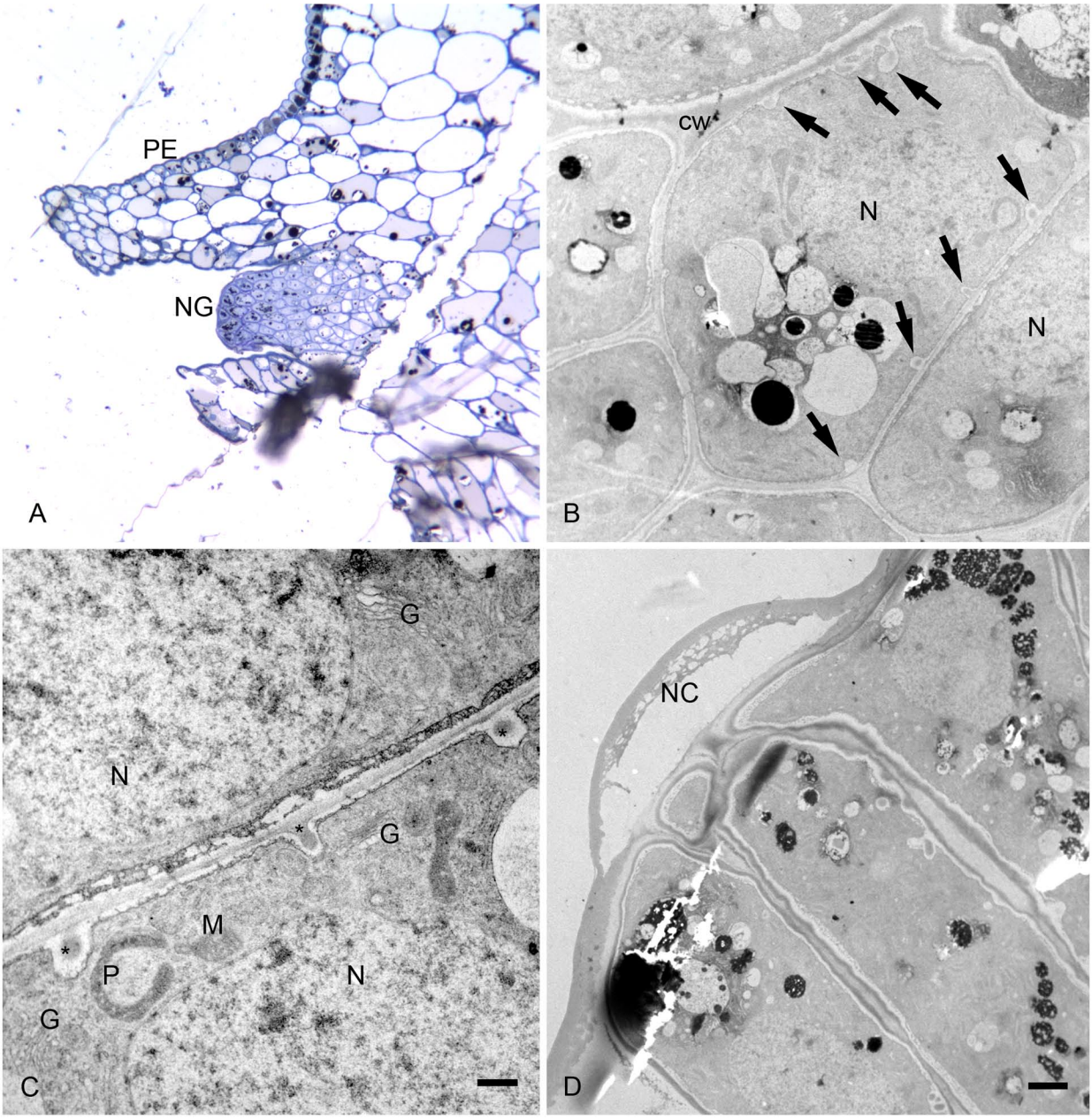

PE
NG
A
cw
N
N
B
G
N
*
*
G
M
*
P
N
G
C
NC
D

Figure 6: TEM images of recently mature (diameter 3 cm, < 1 day opened lid) immunolabeled sections. A: Nectary gland and surrounding tissue (bar = 2 μm). B: TEM detail of immunolabeling (bar = 500 nm). C: TEM detail of H^+-ATPase labeling localized in between secondary cell wall and plasma membrane (bar = 500 nm). Asterisk indicates specific localization between secondary cell wall (CW) and plasma membrane. Arrow indicates non-specific localization.

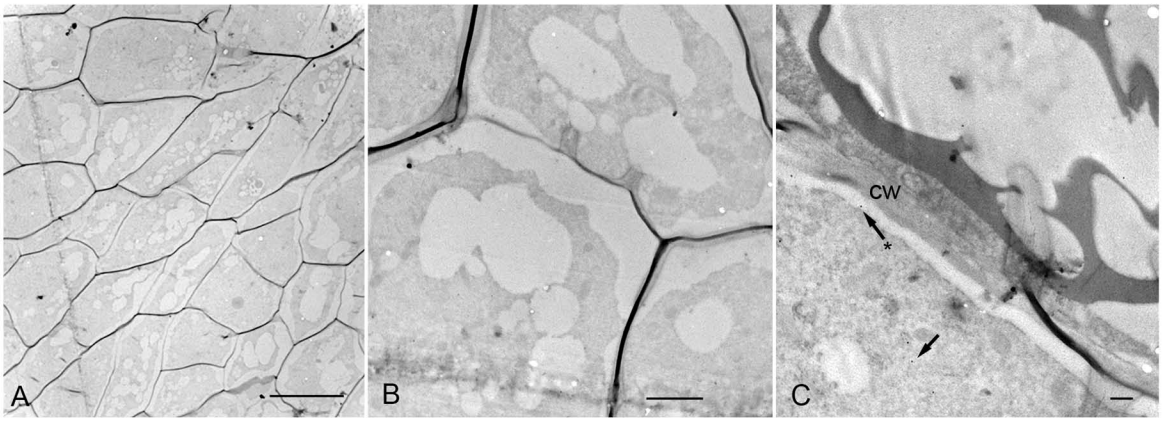

A
B
CW
C

Figure 7: Youngest immature pitcher sample (closed lid, 1 cm diameter). A: Light microscopy of youngest immature nectary gland (NG) and surrounding peristomal epidermis (PE). B: TEM image of nectary cuticle (NC), nectary cuticle space (S) and open end of nectary (bar = 400μm). C: Interior nectary gland cells as well as nectary epithelial cells (bar = 4μm). D: TEM detail of junction between interior and exterior nectary cells. Notice lack of cell wall (CW) protrusions and normal plasma membrane. In addition, a nucleus (N) is present (bar = 1μm).

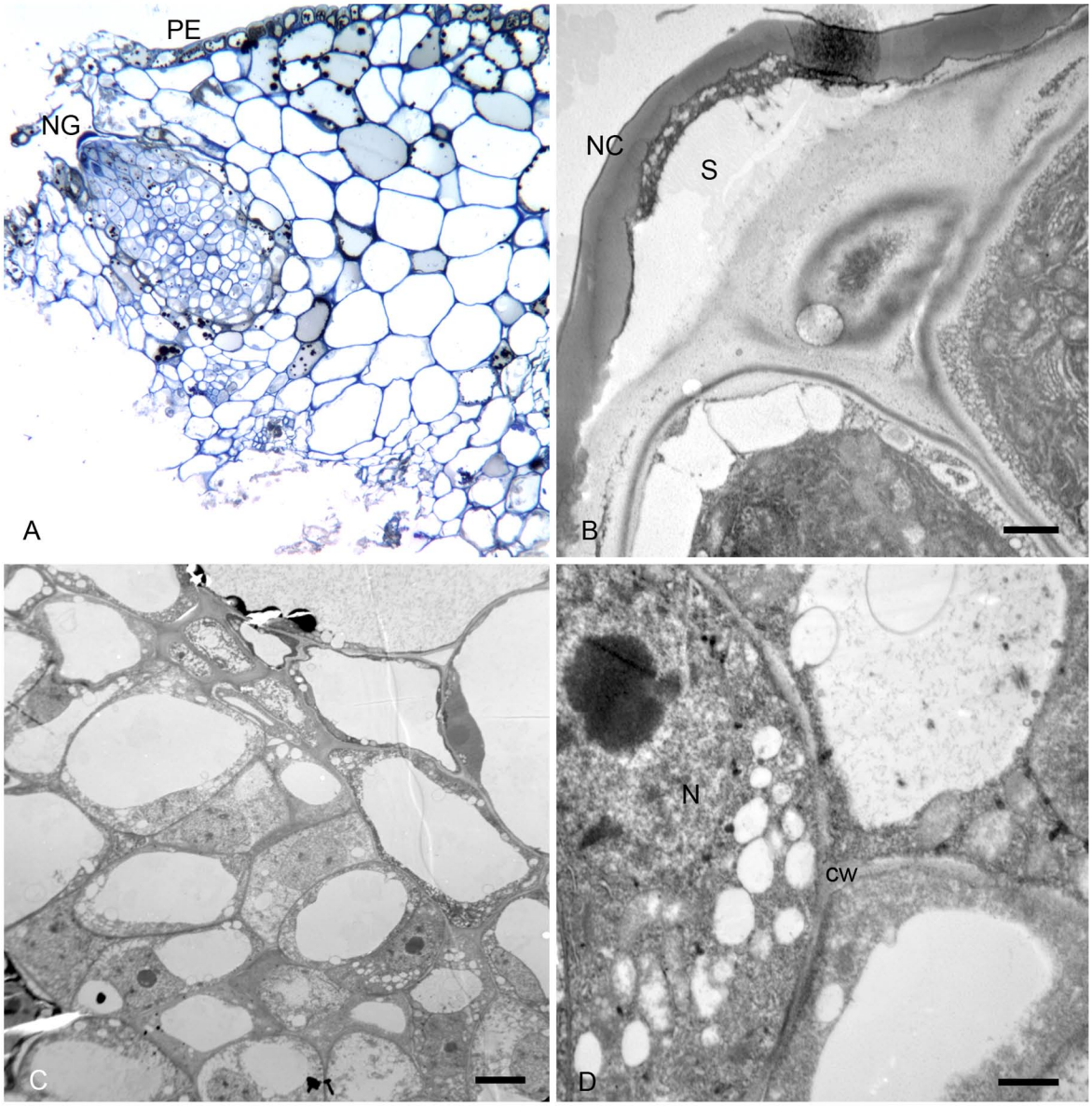
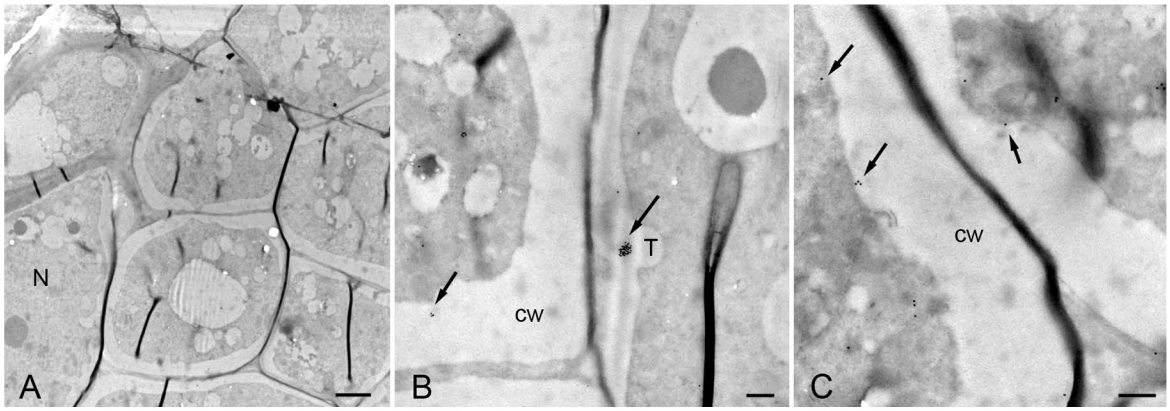

PE
NG
A
NC
S
B
C
N
CW
D

Figure 8: H^+-ATPase immunolabeled TEM immature (closed lid, 1 cm diameter) sections. A: Overview of nectary. Bar = 2μm. B: TEM detail of non-specific binding near wall secondary cell wall (CW) ingrowth (T). Arrow indicates non-specific localization (bar = 500 μm). C: TEM detail of immunolabeling localized between secondary cell wall (CW) and plasma membrane. Arrow indicates specific localization (bar = 500 μm).


N
A
cw
T
B
cw
C