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H+-PPase Distribution in Sieve Element-Companion Cell Complexes from *Arabidopsis thaliana* Wild Type Plants and Allelic Mutants.

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Plants have two phylogentically distinct H^+-PPases: type I and type II. Type I H^+-PPases depend on cytosolic K^+ for their activity and are moderately sensitive to inhibition by Ca^{2+}; type II are K^+-insensitive but extremely Ca^{2+} sensitive. Plant type I H^+-PPases were first isolated from vacuoles and considered to be a *bona fide* vacuolar marker [1][2]. However, later studies with immunoelectron microscopy using H^+-PPases specific antibodies and proteomic approaches showed a dual localization at the vacuole and the plasma membrane (PM) [3][4]. The over-expression of type I H^+-PPase also increased root and shoot proliferation and resulted in significantly greater leaf area (40-60%) than wild type *Arabidopsis* plants [5][6][7][8]. Previous literature has shown the presence of a plasma membrane (PM) localized type I H^+-PPase in *Ricinus communis* sieve elements-companion cell complex (SE-CC) but the physiological relevance of these findings is still obscure. We examined the spatial relationship between H^+-PPase and PIP1 (Plasma Membrane Integral Protein 1), a *bona fide* PM maker, in Arabidopsis wild types (WT) plants. In addition, we analyzed the distribution of H^+-PPase in phloem cells in two different allelic mutation of the *AVP1* gene: *avp1*-47 and *avp1-1*. *avp-1 47* harbors a T-DNA insertion at -756 bp in the *AVP1* promoter gene while *avp1-1* contains the DNA insertion in the fifth exon of the coding sequence.

Leaf tissues with minor veins were fixed with 3% glutaraldehyde in PBS buffer (pH 7.4) for 2 hours at room temperature. Fixed tissues were washed in buffer and post-fixed for 1 hour in 1% OsO_4 in the same buffer. Tissues were washed in buffer and dehydrated in a graded ethanol series at 4°C and embedded in LR-White. Ultra-thin sections (60-90nm) were mounted on nickel grids (75 mesh), coated with fomvard film. The sections were preincubated in 1% (w/v) BSA and 0.05% (v/v) Tween 20 in PBS at room temperature for 1 hour. Single and/or double immunogold was performed against PIP1 and H^+-PPase with colloidal gold of 20 and 12 nm, respectively. Single immunogold was performed only against H^+-PPase. The sections were rinsed in PBS, washed thoroughly in distilled water, and stained with 2% aqueous uranyl acetate for 15 minutes and lead citrate for 10 minutes. The sections were observed with a Transmission Electron Microscopy JEOL JEM-1200EXII (Japan).

In WT plants, H^+-PPase and PIP1 colocalize in the PM of SE-CC complex of Arabidopsis **(A, B).**

The H^+-PPase localization in *avp1-47* mutant showed a topological distribution at the plasma membrane of sieves elements and companion cells complex similarly to WT plants **(C, D).** Non signal was detected in parenchymal cells. In addition, non significant ultrastructural differences were observed when compared with the WT plants.
H^+-PPase was not detected in the *avp1-1* mutants **(E, F).** Abberrant formation of cell walls, altered morphology of chloroplasts, and non differentiations of vascular tissue were observed in the *avp1-1* mutants. The differential expression and localization pattern in the WT and allelic mutants for the *AVP1* gene suggest a potential role of this electrogenic pump in sucrose transport and phloem differentiation.

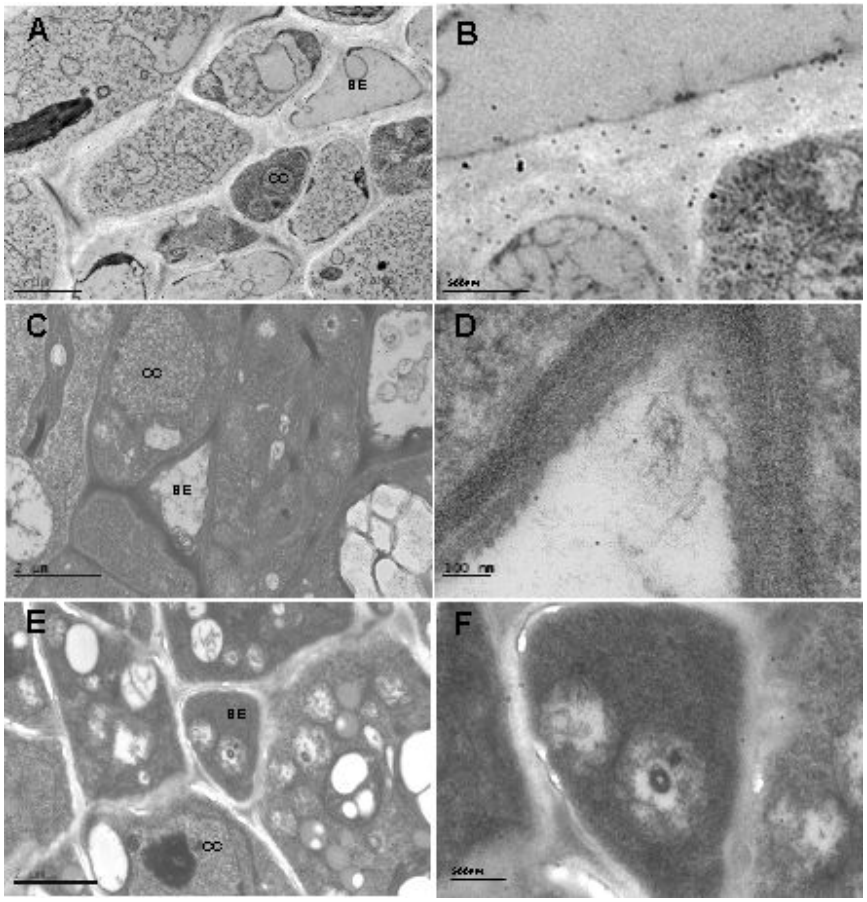

Fig. 1 Immunogold of *Arabidopsis thalinana* and *avp1-1* allelic mutants.

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