Supplemental Material

Systems dynamic modelling of the stomatal guard cell predicts emergent behaviours in transport, signalling and volume control

Zhong-Hua Chen[1,3,5], Adrian Hills[1,3], Ulrike Bätz[1,6], Anna Amtmann[1], Virgilio L. Lew[2] and Michael R. Blatt[1,4]

[1]Laboratory of Plant Physiology and Biophysics, University of Glasgow, Bower Building, Glasgow G12 8QQ
[2]The Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG UK

[3]These authors contributed equally to this publication
[4]Corresponding author (Michael.Blatt@glasgow.ac.uk)
[5]Current address: School of Science and Health, University of Western Sydney, Hawkesbury Campus, Richmond, NSW 2753 Australia
[6]Current address: Plant Biology, University of Zurich, Zollikerstrasse 107, CH8008 Zurich, Switzerland

MATERIAL AND METHODS

Plant growth

Seeds of *Arabidopsis thaliana* Col0 were surface sterilized with ethanol and 5% bleach and stratified for 48 h at 4 °C. Plants were grown under a 16h/8h (light/dark) photoperiod with 150 μmol $m^{-2}s^{-1}$ of white light, a temperature of 22°C/18°C and, and relative humidity of 60%/70%. Sedlings were germinated on sterile 0.7% agar plates containing 1 mM KNO_3, 0.5 mM $Ca(NO_3)_2$, 0.04 mM FeNaEDTA, 0.5 mM $MgSO_4$, 0.625 mM KH_2PO_4, 2 mM NaCl, 1 mM micronutrients and 1 % sucrose. After 2 weeks, seedlings were transferred to hydroponic culture composed of the same media minus sucrose. Four-week-old leaf tissue (pooled from 3 plants) was harvested at 4-h intervals over a 24 h cycle.

Quantitative PCR of VH^+-ATPase transcripts

Total RNA extraction from shoots was performed by grinding the material, previously frozen in liquid nitrogen, and using the TRIZOL reagent (Sigma, Poole, UK). To determine the concentration and purity of the isolated RNA the absorbance was measured spectrophotometrically at 260nm, 230nm and 280nm. 5 μg of total RNA was used as a template for cDNA synthesis with a QuantiTect® Reverese Transcription Kit (Qiagen, Poole, UK) and oligo(dT)-priming. The samples were used to perform quantitative real-time RT-PCR with a DyNAmo™ SYBR®Green qPCR kit (Finnzymes, Thermo-Fischer, Finland). Sets of primers directed against an approximately 300 bp long fragment were designed for the VH^+-ATPase subunits α2, α3, A and C [cf. Dettmer, et al. (2006)]. The *ACT2* actin gene served as an internal control of the cDNA concentration, with the primer pair *ACT2* (At3g18780) designed according to Gutierrez et al. (2008). *UBQ10* (At4g05320) and *TUB9* (At4g20890) were used as secondary reference genes. The expression levels of the *Arabidopsis* VH^+-ATPase subunits were evaluated

in 3 independent experiments and samples were duplicated in each experiment. Primers used were (forward, reverse) for the *ACT2* gene, 5'AATTACCCGATGGGCA3' and 5'TCATACTCGGCCTTGGA3'; for the *UBQ10* gene, 5'ATCACCCTTGAAGTGGA3' and 5'GAAACCACCACGAAGAC3'; for the *TUB9* gene, 5'AGTGTCCCTGAGCTAAC3' and 5'AGTGGGAGCTATATCGC3'. Primers used for the VH^+-ATPase subunits were (forward, reverse) for the α2 subunit, 5'GCTTCAGCCCATAGAAGTGC3' and 5'GCCCCTAGTTGCACGAAATA3'; for the α3 subunit, 5'TTCCAACACCGCTTCTTACC3' and 5'GAAGGAACGCGCTTAGTGTC3'; for the A subunit, 5'TCAGGTCCTGTTGTTGTTGC3' and 5'GGCCTCTGAATTCCATCAAA3'; and for the C subunit, 5'GCTTTGTGGAAGGAGTTTCG3' and 5'CAGCAACAGAACCTTGAGA3'.

Analysis of VH^+-ATPase activity

Pooled leaf material was ground homogenization buffer (2x tissue volume) containing 0.35 M sucrose, 70 mM Trizma (pH 8), 10 % (v/v) glycerol, 3 mM Na_2EDTA, 0.15 % (w/v) BSA, 1.5 % (w/v) PVP-40, 4 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4 and 1 mM EDTA-free Protease Inhibitor Cocktail (Complete, Roche, UK). The homogenate was chilled on ice for 15 min and subsequently passed through three layers of Miracloth. After centrifugation (15 min, 15,000 g, 4 °C) the supernatant was filtered through 50 µm-diameter nylon mesh and centrifuged again at 4 °C for 1 hour at 92,000 g. The microsomal pellet was resuspended in resuspension buffer containing 0.35 M sucrose, 10 mM Tris-MES (pH 7), 2 mM DTT, 1 mM PMSF. Total microsomal protein concentration was determined using Bio-Rad Protein assay (BioRad, UK). VH^+-ATPase activity was measured as Concanamycin A-sensitive ATP hydrolysis (Palmgren 1990; Brux et al. 2008). The hydrolysis was coupled enzymatically to the oxidation of NADH which was monitored as a decrease in the absorbance at 340 nm. Each reaction contained 5 µg of the microsomal protein extract, 37.5 mM MOPS, 50 mM KCl, 4 mM $MgSO_4$, 3 mM Na_3VO_4, 1 mM phosphoenolpyruvate, 10 µg/ml pyruvate kinase, 10 µg/ml lactate dehydrogenase, 0.3 mM NADH and 2 mM ATP. As a control, 5 µg BSA was included in place of microsomal protein. Activity of the VH^+-ATPase was determined as the difference in signal without and with the addition of 100 nM of Concanamycin A, a specific inhibitor of the VH^+-ATPase. The absorption at 340 nm was followed for 20 minutes in a Biotek ELx808 plate reader. Measurements were carried out in 3 independent experiments, each with 6 replicates, and data was averaged.

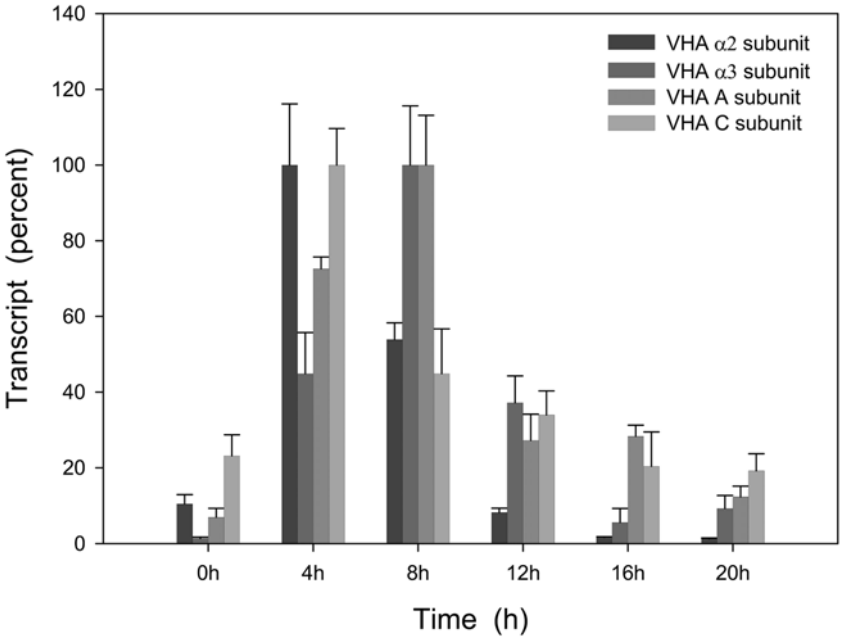

Fig. S1. Diurnal variation in VH^+-ATPase transcript levels from *Arabidopsis* leaf tissue. VH^+-ATPase subunits α2, α3, A and C were assayed by quantitative PCR. Data are means ±SEM of three independent experiments and are reported as percent of maximum expression after normalising to the *ACT2* actin gene transcript.

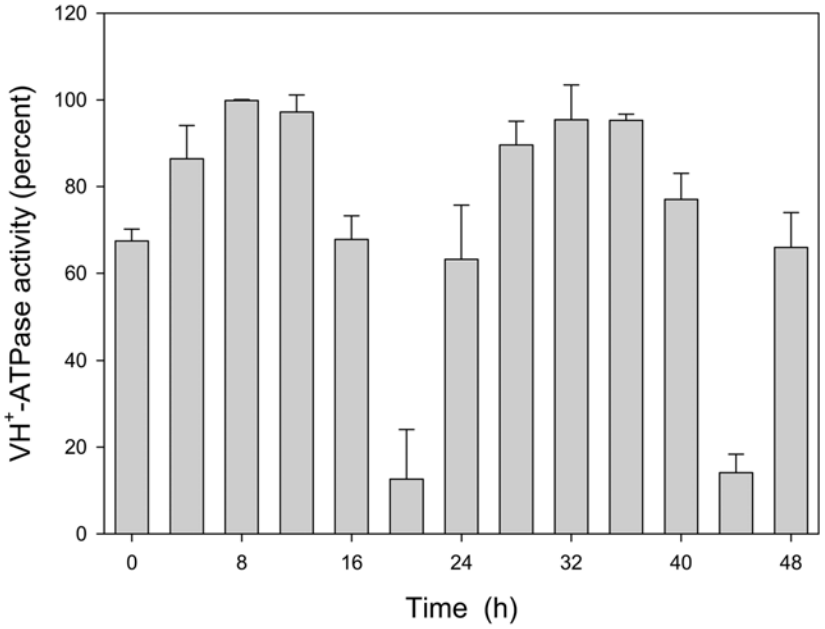

Fig. S2. Diurnal variation in VH^+-ATPase activity from *Arabidopsis* leaf tissue. VH^+-ATPase activity was determined from microsomal membrane fractions by coupling to the oxidation of NADH and was measured as a decrease in absorbance at 340 nm (Palmgren 1990) without and with the addition of 100 nM Concanamycin A. Each bar represents the means ±SEM of three independent experiments. Data were normalised to the 8-h timepoint.

References:

1. **Brux A, Liu TY, Krebs M, Stierhof YD, Lohmann JU, Miersch O, Wastermack C, Schumacher K** (2008) Reduced V-ATPase activity in the trans-Golgi network causes oxylipin-dependent hypocotyl growth inhibition in *Arabidopsis*. Plant Cell 20: 1088-1100

2. **Dettmer J, Hong-Hermesdorf A, Stierhof YD, Schumacher K** (2006) Vacuolar H^+-ATPase activity is required for endocytic and secretory trafficking in *Arabidopsis*. Plant Cell 18: 715-730

3. **Gutierrez L, Mauriat M, Guenin S, Pelloux J, Lefebvre JF, Louvet R, Rusterucci C, Moritz T, Guerineau F, Bellini C, Van Wuytswinkel O** (2008) The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants. Plant Biotechnology Journal 6: 609-618

4. **Palmgren MG** (1990) An H^+-ATPase assay - proton pumping and ATPase activity determined simultaneously in the same sample. Plant Physiol 94: 882-886