New Phytologist Supporting Information

Article title: High V-PPase activity is beneficial under high salt loads, but detrimental without salinity

Authors: Dorothea Graus, Kai R. Konrad, Felix Bemm, Meliha Görkem Patir Nebioglu, Christian Lorey, Kerstin Duscha, Tilman Güthoff, Johannes Herrmann, Ali Ferjani, Tracey Ann Cuin, M. Rob G. Roelfsema, Karin Schumacher, H. Ekkehard Neuhaus, Irene Marten, Rainer Hedrich

Article acceptance date: 15 May 2018

The following Supporting Information is available for this article:

Fig. S1 Vectors generated for transient overexpression of pyrophosphatases together with free GFP in *N. benthamiana* using the agroinfiltration method.

Fig. S2 Function of AtVHP1 in *N. benthamiana* mesophyll cells.

Fig. S3 Comparison of V-PPase amino acid sequences.

Fig. S4 Subcellular localization of NbVHP1.

Fig. S5 Enzyme activity of IPP1.

Fig. S6 Sodium content, osmolality of apoplastic fluid and NbVHP expression of salt-treated tobacco leaves.

Fig. S7 Proton pump activity of V-PPases overexpressed in *N. benthamiana* mesophyll cells.

Fig. S8 pH calibration.

Method S1 Generation of mGFP-NbVHP1 construct.

Method S2 Protein extraction and enzyme activity measurements.

Method S3 Quantification of leaf sodium content

Method S4 Apoplast washing

Method S5 Determination of endogenous NbVHP transcripts under salt treatment

Method S6 pH calibration with BCECF

Fig. S1 Vectors generated for transient overexpression of pyrophosphatases together with free GFP in *N. benthamiana* using the agroinfiltration method.

(a) Structure of the T-DNA with the gene of interest (NbVHP1, NbVHP2 or IPP1) under the control of the ubiquitin (UBQ) promoter and the coding sequence for the enhanced GFP (eGFP) under the control of the double CaMV35S promoter inserted within the left (LB) and right border (RB). (b) Structure of the T-DNA with NbVHP1 inserted by mGFP within cytosolic loop I (between bp 156 and 160). The NbVHP1 coding sequence was under the control of the ubiquitin (UBQ) promoter. RBC-Term = RBC terminator, CaMV35S-Term = CaMV35S terminator.



Fig. S2 Function of AtVHP1 in *N. benthamiana* mesophyll cells.

(a) Confocal fluorescence images of a protoplast (left) and a vacuole (right) released from different mesophyll cells overexpressing AVP1 together with free GFP. Red fluorescence is due to chloroplast autofluorescence. Bar scale = 20 μ m. (b) Representative pyrophosphate-induced current responses of vacuoles released from mesophyll cells overexpressing GFP alone (control) or free GFP together with AtVHP1 after agroinfiltration. Duration of pyrophosphate treatment $(150 \,\mu\text{M})$ is indicated by the superimposed grey bars. (c) Maximal pyrophosphate-induced changes in current density of vacuoles released from mesophyll cells overexpressing free GFP alone (control, n = 10) or free GFP together with AtVHP1 (n = 20). Pyrophosphate was applied at a concentration of 150 μ M. Data represent means ± SE. (d) Ca²⁺ dependence of the V-PPase pump activity. Maximal pyrophosphate-induced changes in current density of vacuoles released from mesophyll cells overexpressing free GFP alone (control, n = 3) or free GFP together with AtVHP1 (n = 4). Pyrophosphate (150 μ M) was applied to the same vacuole in the presence of either 1 mM CaCl₂ or 10 mM EGTA (instead of CaCl₂) in the application pipette solution. Otherwise, the composition of the application pipette solution was identical to the bath medium, containing 100 mM KCl, 1 mM CaCl₂, 5 mM MgCl₂, 10 mM Hepes/Tris pH 7.5. Data represent means \pm SE. (e) Images from leaves overexpressing free GFP alone (control) or together with AtVHP1 three days after agroinfiltration. (f) Maximal pyrophosphate-induced changes in current density of vacuoles released from AtVHP1/GFP-overexpressing mesophyll cells, plotted against the pyrophosphate concentration. Current responses were normalized to those recorded from the same vacuole during application of 150 μ M pyrophosphate. Experiments were performed at luminal pH 7.5 (triangle, n = 3-17) or pH 5.5 (circles, n = 3 - 9). Data points (means \pm SE) were globally fitted with a Michaelis-Menten equation (solid line).

Asterisks in (c, d) indicate significant differences (*P < 0.05 and ***P < 0.001, Student's t-test) between the given values.



Fig. S3 Comparison of V-PPase amino acid sequences.

(a) Amino acid sequence alignment of V-PPases from *N. benthamiana* (NbVHP1, NbVHP2), *Arabidopsis thaliana* (AtVHP1) and *Vigna radiata* (VrVHP). Percentage agreement of the residues with the consensus sequences is highlighted with a colored background (>80% mid blue, >60% light blue, >40% light grey, <40% white). Conserved amino acids with a proposed role in substrate binding (K⁺, Mg²⁺, PP_i), proton translocation, hydrophobic gate and salt-bridge interactions are highlighted by a green, bright blue, orange and purple background, respectively (Lin *et al.*, 2012). Sequences were aligned using Clustal Omega (CLUSTAL O(1.2.0) multiple sequence alignment; <u>http://www.ebi.ac.uk/Tools/msa/clustalo/</u>) (b) Percentage identical in the amino acid sequences of the V-PPases.

(a	a)																													
	NbVHP1 NbVHP2 AtVHP1 VrVHP	1 1 1 1	- <mark>mg</mark> a -mga nvap -mga	qils allp allp ailp	digt digt elwt digt	eili eivi eilv eili	p v c a p v c a p i c a p v c a	avvg avig avig avig	i a fs i v fs i a fs i a fa	l fqv l vqv l fqv l fqv	vflv vyiv vyvv vllv	skvt snvk srvk skvk	Isae Itpe Itsd Isav	ks ss Igas rd	- s g a s p s s s g g - a s p	aad- osnn gann onaa	dkng gkng gkng akng	yaae y-gd y-gd y-nd	slie ylie ylie ylie		gindh gindq gvndq gindh	nsvvq nvvv svva nvvv	kcae kcae kcae kcae	iqna iqna iqta iqna	i sega i sega i sega i sega	tsfl tsfl tsfl tsfl	fteyd fteyd fteyk fteyk	iyvgv iyvgi yvgv yvgv yvgi	fmva fa fmiafa fmiffa fmvafa	i 105 i 104 a 109 i 105
	NbVHP1 NbVHP2 AtVHP1 VrVHP	106 105 110 106	lifl lifl vifv lifl	flgs flgs flgs flgs	vegf: vegf: vegf: vegf:	stkn stks stdn stsp	qsc qpc kpc qacs	tydt tynk tydt sydk	tktc eklc trtc tktc	kpa kpa kpa kpa	lata lata lata lata	v fst i fst a fst i fst	vsfl vsfl iafv vsfl	lgav lgai lgav lggv	vtsvi tsvi vtsvi vtslv	vsgf vsgf Isgf vsgf	l gmk l gmk l gmk l gmk	iaty vaty iaty iaty	anar anar anar anar	ttle ttle ttle ttle	earkg earkg earkg earkg	vgka vgka vgka vgka	afiva afiva afiva afita	ifrsga ifrsga ifrsga ifrsga	a vmg f a vmg f a vmg f a vmg f a vmg f	a a a a a a a a	ngllv ngllv sgllv ngllv	/lyit /lyia /lyit /lyia	illfkl inlfkl invfki inlfki	y 215 y 214 y 219 y 215
	NbVHP1 NbVHP2 AtVHP1 VrVHP	216 215 220 216	ygdd ygdd ygdd ygdd	wegl wegl wegl wggl	feai feai feai feai	t g y g t g y g t g y g t g y g t g y g	g g s g g s g g s g g s	s sma s sma s sma s sma s sma	lfgr lfgr lfgr lfgr	vagg vggg vggg vggg	giyt giyt giyt giyt	k <mark>aa</mark> d kaad kaad k <mark>aa</mark> d	vgad vgad vgad vgad	vg vg vg vg	verr verr ierr verr	nipe nipe nipe nipe	ddpr ddpr ddpr ddpr	npav npav npav npav	iadr iadr iadr iadr	n vg <mark>d</mark> r n vgdr n vgdr n vg <mark>d</mark> r	n vg <mark>d</mark> i n vgdi n vgdi n vgdi	a gmg a gmg a gmg a gmg]s <mark>d</mark> ∣f]sd∣f]sd∣f]s <mark>d</mark> ∣f	gsya gsya gsya gsya	essca easca easca essca	alvv; alvv; alvv; alvv;	asiss asiss asiss asiss	fgvn fgin fgin fgln	heftam heftam hdftam heltam	11 325 11 324 1c 329 11 325
	NbVHP1 NbVHP2 AtVHP1 VrVHP	326 325 330 326	yp yp yp yp	vssv issm issm vssv	gilvo gvlio gilvo gilvo	clit clit clit clit	t fa t fa t fa t fa	atdf atdf atdf atdf	fevk feik feik feik	avke avke Ivke avke	eiep eiep eiep eiep	alkq alkn alkn alkk	qlvi qlii qlii qlvi	stal stal stvi stvi	mtvç mtvç mtvç mtiç	giaa giai giai gvav	vtwi vtwt vswv vsfv	alps clps glpt alpt	tfti sfti sfti sfti	ifnfg ifnfg ifnfg ifnfg	jaqke jaqkv jtqkv jvqkd	evksw vknw vknw vksw	vqlfl vqlfl vqlfl vqlfl	cvgvg cvavg cvcvg cvcvg	g Iwag g Iwag g Iwag g Iwag g Iwag	liig liig liig liig	fvtey fvtey fvtey fvtey	ytsn ytsn ytsn ytsn	ayspvq tyspvq ayspvq ayspvq	d 435 d 434 d 439 d 435
	NbVHP1 NbVHP2 AtVHP1 VrVHP	436 435 440 436	vads vads vads vads	crtg: crtg: crtg: crtg: crtg:	aatn aatn aatn aatn	vifg vifg vifg vifg	lalg lalg lalg lalg	gyks gyks gyks gyks	viip viip viip viip	ifa ifa ifa ifa	iavs iaia iais iais	ifvs ifvs ifvs ifvs	fsfa fsfa fsfa ftfa	am yg am yg am yg am yg	jiava jiava jvava jiava	aalgı aalgı aalgı aalgı aalgı	m Ist m Ist m Ist m Ist	iatg iatg iatg iatg	laic laic laic laic	laygp laygp laygp laygp laygp	ois <mark>d</mark> n oisdn oisdn oisdn	naggi naggi naggi naggi	a ema a ema a ema a ema	igmsq igmsh igmsh igmsh	ri <mark>r</mark> er ri <mark>r</mark> er ri <mark>r</mark> er ri <mark>r</mark> er	tdal tdal tdal tdal tdal	daagr daagr daagr daagr	ttaa ttaa ttaa ttaa	igkgfa igkgfa igkgfa igkgfa	i 545 i 544 i 549 i 545
	NbVHP1 NbVHP2 AtVHP1 VrVHP	546 545 550 546	gsaa gsaa gsaa gsaa	vs ; vs ; vs ; vs ;	alfga alfga alfga alfga	afvs afvs afvs afvs afvs	raa raa rag ras	istv ittv ihtv ittv	dvlt dvlt dvlt dvlt	pkv pqv pkv pkv	figl figl igl figl	lvgan ivgan lvgan ivgan	nlpy nlpy nlpy nlpy nlpy	w fsa w fsa w fsa w fsa	am tmk am tmk am tmk am tmk	ksvg ksvg ksvg ksvg	saal saal saal saal	km ve km ve km ve km ve	evrr evrr evrr evrr	qfnt qfnt qfnt qfnt qfnt	ipg ipg ipg ipg	meg t meg t meg t meg t	akpd akpd akpd akpd akpd	yatc yatc yatc yatc	vkist vkist vkist vkist	dasil dasil dasil dasil dasil	kemia kemip kemip kemip	ipgal opgal opgcl opgal	vmltpl vmltpl vmltpl vmltpl	i 655 i 654 i 659 v 655
	NbVHP1 NbVHP2 AtVHP1 VrVHP	656 655 660 656	vgil vgif vgff vgil	fgve fgve fgve fgve	t I sg t I sg t I sg t I sg	vlag vlag vlag vlag	s I vs a I vs s I vs s I vs	5 g v q 5 g v q 5 g v q 5 g v q 5 g v q	iais iais iais iais	asn asn asn asn	tggan tggan tggan tggan	w <mark>d</mark> na wdna wdna wdna	k <mark>k</mark> yi k k yi k k yi k k yi	eag eaga eaga eaga	/seha aseha /seha aseha	art art aks ars	g p k g g p k g g p k g g p k g	sdah seph seph sdch	kaav kaav kaav kaav	vig <mark>d</mark> t vigdt vigdt vigdt	vgdp igdp igdp igdp	olkd tolkd tolkd tolkd tolkd	sgps sgps sgps sgps	Inil Inil Inil	i <mark>k</mark> Ima ik Ima ik Ima ik Ima	vesl vesl vesl vesl	vfapt vfapt vfapt vfapt	fath fath fath fath	ggllfk ggllfk ggilfk ggilfk	l f 766 i f 765 y f 770 i f 766

(b)

		NbVHP2
	NbVHP1	91%
AtVHP1	88%	90%
VrVHP	91%	91%

Fig. S4 Subcellular localization of NbVHP1.

Confocal fluorescence images of a protoplast (a) and a vacuole (b) released from different *N*. *benthamiana* mesophyll cells overexpressing mGFP-labelled NbVHP1. (a) The plasma membrane is highlighted with red fluorescence caused by staining with FM4-64. Bar scale = $10 \mu m$. Note that the GFP and FM4-64 fluorescence signals do not co-localize as marked by the arrow. (b) Red fluorescence is due to chloroplast autofluorescence. Bar scale = $20 \mu m$.



Fig. S5 Enzyme activity of IPP1.

The hydrolytic pyrophosphate activity of the soluble fraction of *N. benthamiana* leaves overexpressing free GFP alone (control) or together with IPP1 or NbVHP1. Data points (means \pm SE, **P < 0.01, Student's t-test) represent three independent experiments.



Fig. S6 Sodium content, osmolality of apoplastic fluid and NbVHP expression of salt-treated tobacco leaves.

(a) Sodium content of *N. benthamiana* wild type leaves (DW= Dry weight) without salt treatment (open markers) and at different times after treatment with 200 mM NaCl (closed markers). Plant leaves were directly injected with NaCl (triangle markers) or NaCl was applied in solution to the soil ('poured', circle and square markers). The NaCl content of the 3rd and 4th leaves from the base of the plant were measured separately. Note, that when pots with soil-grown plants were soaked with either water or NaCl, the Na⁺ content in the third and fourth leaf increased over 48 h to the same Na^+ level as observed in Na^+ infiltrated leaves, and likewise, then remained stable. (b) Sodium content of N. benthamiana leaves overexpressing free GFP alone (control) or free GFP together with the indicated pyrophosphatases two days after agroinfiltration with or without 200 mM NaCl. FW = Fresh weight. Significant differences between values at P < 0.05 are indicated by different characters (Student's t-test). (c) Osmolality of the apoplastic fluid was determined from N. benthamiana leaves injected with agromix (light grey) or agromix enriched with 200 mM NaCl (dark grey) after 3-5 h after infiltration. Note that the osmolality of infiltrated pure agromix solution is 25 mosmol kg⁻¹. ***P < 0.001, Student's t-test. (d) Transcript levels of *NbVHP1* and NbVHP2 in leaves overexpressing free GFP two days after agroinfiltration with or without 200 mM NaCl. *P < 0.05, Student's t-test. The transcript levels were normalized to the measured number of GFP transcripts.

The number of independent experiments was n = 4 in (a) and (b), n = 7 in (c) and n = 5 in (d) (means \pm SE).



Fig. S7 Proton pump activity of V-PPases overexpressed in *N. benthamiana* mesophyll cells. Maximal pyrophosphate-induced current responses of vacuoles released from mesophyll cells overexpressing free GFP alone (control, n = 10) or free GFP together with either AtVHP1 (n = 22), NbVHP1 (n = 14) or NbVHP2 (n = 4) after agroinfiltration. Pyrophosphate was applied at a concentration of 150 μ M. Data points represent means ± SE (****P* < 0.001, Student's t-test).



Fig. S8 pH calibration

BCECF calibration curve generated ex vivo by dissolving the free acid form of the dye (2.5 μ M) in a medium containing 1 mM CaCl₂, 1 mM MES adjusted to the designated pH with TRIS. Data points (n = 3) were fitted with a linear fit (solid line) to convert ratio values into pH values.



Method S1 Generation of mGFP-NbVHP1 construct

According to Segami *et al.* (2014), NbVHP1 was labelled with mGFP (monomeric Green Fluorescence Protein) which was inserted into loop 1 exactly between the amino acids Gly53 and Ala54 (Fig. S1b) via USER cloning (Nour-Eldin *et al.*, 2006). Additionally, the following linker sequence was fused to the N-terminus of the mGFP sequence: 5'-GGC GGC GGA GGT TCA GGT GGT GGC GGG TCT -3' (= [Gly₄Ser]₂). The following primers were used: mGFPfwd with partial linker-sequence (5'-AGG TTC AGG TGG UGG CGG GTC TGC TGT GAG CAA GGG CGA GG-3'), mGFPrev (5'-AGA TGC CTT GUA CAG CTC GTC CAT GCC GTG A-3'); NbVHP1-L1rev with partial linker sequence (5'-ACC ACC TGA ACC UCC GCC GCC TGA CTT TTC AGC ACT GAG CGT C-3'); NbVHP1_L1fwd (5'-ACA AGG CAT CTG GAG CAG CAG CAG ACG ATA AGA ATG G-3'). Using the USER cloning system, the NbVHP1-mGFP construct was transferred into the pCambia2200 vector equipped with UBQ promotor and RBC terminator.

For staining the plasma membrane, isolated protoplasts were briefly incubated with FMTM4-64 (10 μ M) (Thermo Fisher Scientific). The FM4-64 dye was excited with 514 nm and the emission detected at 625-690 nm.

Method S2 Protein extraction and enzyme activity measurements

Two days after agroinfiltration, tobacco leaves were harvested, immediately frozen in liquid nitrogen and stored at -80 °C until microsomal membranes and soluble protein fractions were prepared as described previously (Krebs *et al.*, 2010), with minor modifications. Tissue was homogenized with 2 ml homogenization buffer per g fresh weight (350 mM sucrose, 70 mM Tris-HCl pH 8.0, 10 % (v/v) glycerol, 3 mM Na₂EDTA, 0.15 % (w/v) BSA, 1.5 % (w/v) PVP-40, 4 mM DTT, and 1 x complete protease inhibitor mixture (Roche)). The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was filtered again through Miracloth, then centrifuged at 100,000 g for 1 h at 4 °C. To determine enzyme activity of the soluble protein fraction, 200 µl of the supernatant was retained and the remaining supernatant was carefully removed and discarded. The pellet (microsomal

membranes) was re-suspended in 350 mM sucrose, 10 mM Tris-Mes pH 7.0, 2 mM DTT and $1 \times$ complete protease inhibitor mixture.

To determine the hydrolytic PPase enzyme activity, the P_i release was determined colorimetrically as described previously (Krebs *et al.*, 2010), with minor modifications. Soluble protein fraction (10 µg) were incubated for 20 min at 28 °C. Reactions were terminated by adding 40 mM citric acid. 10 µg BSA was used for the blank value. The PPase reaction medium contained 25 mM Tris-MES pH 7.5, 2 mM MgSO₄ × 7 H₂O, 0.1 mM Na₂ MoO₄, 0.1 % Brij 58, and 0.2 mM K₄P₂O₇. PPase activity was calculated as the difference measured in the presence and absence of 50 mM KCl.

Method S3 Quantification of leaf sodium content

N. benthamiana leaves infiltrated only with 200 mM NaCl were harvested at different times after salt treatment, dried at 60 °C to constant weight, pulverized and homogenized. After the exact amount of the samples (usually 10 - 20 mg) were precisely weighed in a quartz digestion vessel, the leaf tissue was treated with 1 ml of nitric acid (65%, suprapur, Merck KGaA, Darmstadt) and digested at 180 °C for 10 hours inside a Teflon pressure vessel. After cooling, samples were diluted 1:20 with nanopure water. The sodium content was determined using a flame atomic absorption spectrometer (AAnalyst 400, PerkinElmer) three times and averaged.

For tobacco leaves infiltrated with a NaCl-free or 200 mM NaCl-containing agrobacterium suspension, leaves were collected two days after infiltration, immediately frozen in liquid nitrogen and stored at -80 °C until use. After re-suspension in double-distilled water the plant leaf material was subjected to complete hydrolysis using a temperature step gradient with a maximum temperature of 210 °C. Hydrolysis was carried in a MLS-Ethos microwave oven (http://www.mls-mikrowellen.de/) with 5 ml HNO₃ (60% v/v), 2 ml H₂O₂ (30% v/v), and the preparation was diluted to a final volume of 12 ml with double-distilled water. Quantification was performed by inductively coupled plasma/optical emission spectrometry, on an iCAP 6300 DUO apparatus (Thermo-Fischer). Sodium was detected and quantified at 589.5 nm (Krebs *et al.*, 2010; Müller *et al.*, 2014).

Method S4 Apoplast washing

Agromix solution with or without 200 mM NaCl was infiltrated into the apoplast of wild type *N*. *benthamiana* leaves through the open stomata via a syringe. After recovery from infiltration (about 3-5 h) leaves were harvested and the surface washed and infiltrated a second time with distilled water. The leaf apoplast fluid was then extracted by centrifugation as described by O'Leary *et al.* (2014), and its osmolality measured with an osmometer (Vapor Pressure Osmometer 5520, Wescor, Vapro).

Method S5 Determination of endogenous NbVHP transcripts under salt treatment

N. benthamiana leaves were harvested two days after agroinfiltration with the appropriate constructs for overexpression of GFP alone (control) in the presence or absence of 200 mM NaCl. Plant material was immediately frozen in liquid nitrogen, crushed with a pestle and mortar and stored at -80°C until use. Total RNA of the leaf was isolated using Plant RNA Kit R6827-02 (OMEGA, <u>www.omegabiotek.com</u>). Each of the collected RNAs was treated with recombinant DNase (Thermo Scientific) to remove any genomic DNA contamination. Generation of cDNA and Quantitative real-time PCR (pPCR) were done like described in the main text. Transcription data were normalized to the coexpressed GFP molecules using standard curves calculated for the individual PCR products. GFPfwd (5'-CCT GAA GTT CAT CTG CAC CA-3') and GFPrev (5'-TGC TCA GGT AGT GGT TGT CG-3') (Gaddam *et al.*, 2013).

Method S6 pH calibration with BCECF

A BCECF calibration curve was generated *ex vivo* with exactly the same imaging parameters and setup used for the *in vivo* measurements described in the main text by dissolving the free acid form of the dye (2.5 μ M) in a medium containing 1 mM CaCl₂, 1 mM MES adjusted to the designated pH with TRIS (Fig. S8). The generated calibration curve (n = 3) was used to convert the ratio values into pH-values.

References

Gaddam D, Stevens N, Hollien J. 2013. Comparison of mRNA localization and regulation during endoplasmic reticulum stress in Drosophila cells. *Molecular Biology of the Cell* **24**: 14–20.

Krebs M, Beyhl D, Görlich E, Al-Rasheid KAS, Marten I, Stierhof Y-D, Hedrich R, Schumacher K. 2010. Arabidopsis V-ATPase activity at the tonoplast is required for efficient nutrient storage but not for sodium accumulation. *Proceedings of the National Academy of Sciences of the United States of America* 107: 3251–3256.

Lin S-M, Tsai J-Y, Hsiao C-D, Huang Y-T, Chiu C-L, Liu M-H, Tung J-Y, Liu T-H, Pan R-L, Sun Y-J. 2012. Crystal structure of a membrane-embedded H⁺-translocating pyrophosphatase. *Nature* **484**: 399–403.

Müller M, Kunz H-H, Schroeder JI, Kemp G, Young HS, Neuhaus HE. 2014. Decreased capacity for sodium export out of Arabidopsis chloroplasts impairs salt tolerance, photosynthesis and plant performance. *The Plant Journal* 78: 646–658.

Nour-Eldin HH, Hansen BG, Nørholm MHH, Jensen JK, Halkier BA. 2006. Advancing uracil-excision based cloning towards an ideal technique for cloning PCR fragments. *Nucleic Acids Research* 34: e122.

O'Leary BM, Rico A, McCraw S, Fones HN, Preston GM. **2014**. The infiltration-centrifugation technique for extraction of apoplastic fluid from plant leaves using phaseolus vulgaris as an example. *Journal of Visualized Experiments : JoVE* **19**:e52113.

Segami S, Makino S, Miyake A, Asaoka M, Maeshima M. **2014**. Dynamics of vacuoles and H⁺-pyrophosphatase visualized by monomeric green fluorescent protein in Arabidopsis: artifactual bulbs and native intravacuolar spherical structures. *The Plant Cell* **26**: 3416–3434.