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**Vacuolar convolution: possible mechanisms and role of PtdIns(3,5)P<sub>2</sub>**

**Running title:** Tentative vacuolar convolution mechanisms

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vacuolar convolution, vacuolar fragmentation, phosphatidylinositol 3,5-bisphosphate, hyperosmotic shock, guard cells, yeast

**Abbreviations:**

**PtdIns(3,5)P<sub>2</sub>**—phosphatidylinositol 3,5-bisphosphate; **PtdIns(3)P**—phosphatidylinositol 3-phosphate; **FAB1**—Forms Aploid and Binucleate cells, phosphatidylinositol 3-phosphate 5-kinase family; **NHX**—cation-proton exchangers family; **CLC**—chloride channels; **TPK**—two pore potassium channels; **TPC**—two pore calcium channels; **TIP**—tonoplast intrinsic protein, aquaporin subfamily; **mTORC1**—mammalian Target Of Rapamycin Complex 1; **SAC**—family of (Suppressor of Actin) domain phosphoinositide phosphatases; **ABA**—abscisic acid; **PM** — plasma membrane

**Abstract**

The central or lytic vacuole is the largest intracellular organelle in plant cells, but we know unacceptable little about the mechanisms regulating its function *in vivo*. The underlying reasons are related to difficulties in accessing this organelle without disrupting the cellular integrity, and to the dynamic morphology of the vacuole, which lacks a defined structure. Among such morphological changes, vacuolar convolution is probably the most commonly observed event, reflected in the (reversible) transformation of a large central vacuole into a structure consisting of interconnected bubbles of a smaller size. Such behavior is observed in plant cells subjected to

hyperosmotic stress, but also takes place in physiological conditions, for example during stomata closure. Although vacuolar convolution is a relatively common phenomenon in plants, works aimed at elucidating its execution mechanisms are rather scarce. In the present review we analyze available evidences of the participation of the cellular cytoskeleton and ion transporters in the vacuolar morphology dynamics, making special emphasis on available evidences on the role played by PtdIns(3,5)P<sub>2</sub> in this process.

## Introduction

Studies on the plant central or lytic vacuole dynamics are advancing very slowly due to the irregular shape and largely non-specific behavior of this amorphous organelle within vegetative cells, if compared to other intracellular organelles, or the cell itself. In few studies attempts were made to classify vacuolar structures by their morphology (e.g., Oda et al., 2009; Wiltshire and Collings, 2009; Segami et al., 2014), however, their functional importance for the vegetative cell physiology remain obscure. In this review we analyze the principal factors that are reported to influence vacuolar convolution, and provide possible mechanisms for the execution of this process, emphasizing those aspects which will require further analysis.

### Vacuolar morphology dynamics, underlying processes and possible executors.

One of the best known examples of vacuolar dynamics is convolution, which takes place in guard cells during the stomata closure (Gao et al., 2005; Tanaka et al., 2007; Oda et al., 2009). Bulb-like structures, which appear in this condition, have been proposed to play the role of reservoirs of membrane that allow a rapid cell expansion during water absorption, not only in guard cells, but also in young vegetative cells (Saito et al., 2002; Oda et al., 2009). Vacuolar convolution has been reported to occur also in cells subjected to hyperosmotic stress (Uemura et al., 2002; Kutsuna and Hasezawa, 2005; Reisen et al., 2005; Oda et al., 2009), and during the immune response of plants (Hatsugai et al., 2012). The ability to shrink the vacuole has been proposed as an adaptation of plants to drought (see in Magalhães Alvarez et al., 2008 and references therein) and appears to play an important role in thigmonastic movements (Trevisan Scorza and Carnier Dornelas, 2011). A decrease in size and apparent multiplication of vacuoles from a single large central vacuole occurs also during pollen development in *Arabidopsis*

(Yamamoto et al., 2003). However, it is not clear whether in this case the small vacuoles remain connected, as when convolution takes place, or not.

A complete fission of a single central vacuole into many independent vacuoles of a smaller size —usually referred to as *fragmentation*— is more commonly observed in fungi (yeast) under stimuli similar to those inducing convolution of the plant vacuole (see in Michailat and Mayer, 2013). For plants this process has not been reported, with a possible exception of the vacuolar multiplication taking place in developing pollen grains, mentioned above. We have found in the plant literature examples, when the term *fragmentation* is used to describe *convolution*, but in the present work we will use these terms in the meanings presented above. Nevertheless, vacuolar dynamics in plants and fungi seems to involve similar components (see in Zhang et al., 2014), which are also important in the endolysosomal system functioning of mammalian cells (Li and Kane, 2009; Armstrong, 2010). Thus, the similarity existing between all these phenomena allows obtaining insights on the physiology of a particular type of these acidic  $\text{Ca}^{2+}$  stores by analyzing similar aspects of their functioning in the other organisms.

To date, the mechanisms responsible for the dynamic shaping of these compartments, and particularly — for the vacuolar convolution, are unknown. Two possible processes, or a combination of both, could produce this effect: 1) the vacuole could be passively deformed by some extravacuolar factor (e.g., by the cell cytoskeleton; Verbelen and Tao, 1998); or 2) the vacuole deformation pattern could result from the presence in the tonoplast of domains with different physical properties, which could affect the way the vacuolar membrane bend in response to ion/water movement across the tonoplast. Below we analyze each of these possibilities.

It should be noted that vacuolar dynamics could additionally be influenced by the intravacuolar milieu, as structural changes within the vacuole have been reported to occur in association with thigmonastic movements of plants (e.g., Toriyama and Satô, 1968). In yeast, the vacuolar fragmentation efficiency apparently depends on the vacuolar polyphosphates content (references in Zieger and Mayer, 2012), and in *Arabidopsis*, the regulation of the tonoplast cation-proton exchanger NHX1 (see below) by an intravacuolar calmodulin-like protein has been reported (Yamaguchi et al., 2005). All-in-all, our knowledge on the role the vacuolar content plays in the vacuolar morphology dynamics is very limited due to difficulties in controlling it experimentally.

1 *Possibility 1: Actin-induced vacuole shaping*

2 Depolymerization of the actin cytoskeleton has been reported to induce shrinking and splitting of  
3 the plant vacuole (Kim et al., 1995; Higaki et al., 2006; Kutsuna et al., 2003), though this effect,  
4 at least in guard cells, seems to be a result of the vacuolar dynamics coupling to the  $K^+$  flux  
5 through plasma membrane-located channels, activated by the cytoskeleton disruption (Hwang et  
6 al., 1997, also see below). Other studies report no effect of the actin cytoskeleton  
7 depolymerization on the vacuolar morphology, but rather on its dynamics (Verbelen and Tao,  
8 1998; Uemura et al., 2002). The role of microtubules in the movement of guard cells has been  
9 debated, but microtubules are seemingly important in events upstream of those directly involved  
10 in the execution of vacuolar shaping, as application of fusicoccin, an activator of the PM  $H^+$ -  
11 ATPase, and, apparently, also of PM  $K^+$  channels, restores the stomata aperture, abolished by  
12 microtubule inhibitors treatment (Marcus et al., 2001). A compelling proof for the lack of a  
13 direct role of the actin cytoskeleton in the execution of vacuolar convolution would be the  
14 observation of this process in the presence of actin-stabilizing drugs (e.g., phalloidin), or its  
15 reproduction *in vitro* in the absence of actin. For isolated yeast vacuoles such actin-independence  
16 of fragmentation on the cytoskeleton has been demonstrated (Michaillat et al., 2012). Yet, it  
17 should be noted that actin may be directly involved in the vacuolar shaping in some cases, e.g. in  
18 case of morphological changes produced by transvacuolar strands dynamics (Ruthardt et al.,  
19 2005; Hoffmann and Nebenführ, 2004).

20 *Possibility 2: Vacuole shaping induced by ionic fluxes across the tonoplast*

21 Convolution can alternatively be pictured as a simple “deflation” of the vacuole, provoked by the  
22 efflux of water and ions when hyperosmotic stress is applied. This hypothetical mechanism is at  
23 a first glance supported by cellular plasmolysis, which can be observed when intact cells are  
24 subjected to osmotic shock (Shavrukov, 2013). We have not found a direct mention of this  
25 phenomenon in the literature, but application of solutes at plasmolysis-inducing concentrations  
26 (e.g. 300-500 mM KCl or NaCl; Niu et al., 1996; Pottosin et al., 2001; Pottosin and Martínez  
27 Estévez, 2003) to *isolated vacuoles* tend only to slightly reduce their volume *without* producing  
28 notable changes in their shape (I.I. Pottosin, Universidad de Colima, Mexico and Sergey Shabala,  
29 University of Tasmania, pers. comm.). It suggests that plasmolysis requires a coordinated

1 function of vacuolar and plasma membranes, but the osmotic shock *per se* is not sufficient to  
2 induce such an ion efflux from the vacuole that could mediate its wrinkling.

3 To our knowledge, there is only one study in which changes in the tonoplast electrical  
4 conductance has been estimated *in vivo* under hyperosmotic stress: application of 50 mM sorbitol  
5 + 50 mM mannitol induces an increase of vacuolar membrane conductance in root cells (Lew,  
6 2004). Yet, the identities of the ionic and transporter species involved in this phenomenon were  
7 not addressed. On its hand, stomata closure relies on large efflux of ions from the vacuole  
8 (MacRobbie, 1998; MacRobbie, 2006, Pandey et al., 2007), which can be visually estimated  
9 using the stoma aperture size as a measure. Stomata closure could be partially mediated by  
10 vacuolar K<sup>+</sup>-selective channels from the TPK family (Gobert et al., 2007; Isayenkov et al., 2011),  
11 as *Arabidopsis* plants knockout by one (TPK1) of the four TPK isoforms display a slower  
12 decrease in leaves conductivity than wild-type plants under ABA-induced stomata closure,  
13 remaining their opening unaffected (Gobert et al., 2007). In addition, TPK1 channels are  
14 activated by cytosolic Ca<sup>2+</sup> increase (Gobert et al., 2007) and mechanical stimulation (Maathuis,  
15 2011), in line with the observed properties of vacuolar tracer fluxes generated during stomata  
16 closure (MacRobbie, 2006). Stomata closure is altered also by other ion transport systems, which  
17 alteration arrests the movement of these cells. For example, the lack of function of Cl<sup>-</sup>-permeable  
18 CLCc-s from *Arabidopsis* renders stomata insensitive to ABA stimulation (Jossier et al., 2010),  
19 but does not produce other visible features (e.g., altered shape of vacuoles). A similar  
20 insensitivity of stomatal movement to ABA is also characteristic for plants lacking the vacuolar  
21 Na<sup>+</sup>- and K<sup>+</sup>-permeable NHX1 and NHX2 cation-proton exchangers, which are responsible for  
22 the vacuolar K<sup>+</sup> transport both during stomata opening and closing, and for respective  
23 vacuolar/cytosolic pH changes (Andrés et al., 2014). Notably, double *nhx1 nhx2* knockout plants  
24 display a convoluted vacuole and a smaller stomata aperture compared to wild type, suggesting  
25 that functional NHXs are required to maintain the coalescent state of the central vacuole.

26 A compelling demonstration of the role different factors play in the vacuolar convolution would  
27 be the reconstitution of this process *in vitro*. For plants, to our knowledge, such experiment has  
28 not yet been conducted. For yeast, *in vitro* fragmentation of isolated vacuoles was shown to  
29 require, among other components, ATP for fueling the vacuolar proton ATPase, an optimal  
30 saline solution at the cytosolic side, and yeast cytosolic extract. Fragmentation was optimal in the

1 presence of 100-150 mM K<sup>-</sup> or Na-acetate, but was not induced by KCl, NaCl or sorbitol, and  
2 was inhibited at higher K-acetate concentrations, whereas the omission of the cytosolic extract  
3 led to more than 80% reduction of the fragmentation (Michaillat et al., 2012). Importantly, bath  
4 application of the K<sup>+</sup>-ionophore valinomycin reduced fragmentation by about 70%. Two possible  
5 explanations for this last effect can be proposed: 1) valinomycin either served as a shunt,  
6 mediating a K<sup>+</sup> *influx* into the vacuole, countering the K<sup>+</sup> wrinkling-inducing efflux, or 2) if  
7 convulsion depends on a patterned distribution of vacuolar ion transporters and of the fluxes  
8 they generate, valinomycin application could disrupt this membrane heterogeneity, making ion  
9 release homogeneous throughout the entire tonoplast surface. It is worth noting that in the  
10 experiments conducted by Michaillat and coworkers (2012) vacuolar convulsion took place in  
11 isolated vacuoles, and thus, adhesion of some vacuolar sites to other intracellular structures is  
12 apparently not indispensable for this process to occur.

13 Testing the “shunt” hypothesis of the valinomycin effect requires knowing the trans-tonoplast  
14 electrical potential difference and K<sup>+</sup> concentrations on both sides of the vacuolar membrane. As  
15 K<sup>+</sup> cytosolic/vacuolar concentrations can vary, depending on the K<sup>+</sup>-supply conditions, the  
16 physiological state and the cell type (in the case of plants), an estimation of the K<sup>+</sup> trans-  
17 tonoplast gradient contribution to the vacuolar morphology dynamics is difficult, and seemingly  
18 can be resolved only in experiments with application of controlled K<sup>+</sup> concentrations on both  
19 sides of the tonoplast. Such experiments could also allow discerning scenarios where  
20 convulsion/fragmentation depends on relative or absolute changes in ion concentrations inside  
21 and outside of the vacuole.

22 The second explanation of the effect of valinomycin implies that the tonoplast has an  
23 heterogeneous structure, i.e., that in this membrane adjacent zones with different permeability  
24 properties can exist, resulting from a differential distribution of transporters, lipids, or both. In  
25 the case of lipids, such a “structuring” role could belong to phosphatidylinositol phosphates,  
26 particularly PtdIns(3,5)P<sub>2</sub>, which importance in the physiology of acidic Ca<sup>2+</sup> stores has drawn  
27 the attention of many recent studies.

## **PtdIns(3,5)P<sub>2</sub> and the vacuolar morphology dynamics**

Phosphatidylinositol 3,5-bisphosphate, PtdIns(3,5)P<sub>2</sub>, is a minor lipid species that can be found in the vacuolar membrane of yeast and in the late endosomes and lysosomes of mammalian cells (Takatori et al., 2015; Shen et al., 2011). Since the identification of this compound in 1997 (Dove et al., 1997; Whiteford et al., 1997) its role as second messenger has been proposed, and numerous evidences have already been obtained on the linkage between its abnormal metabolism and diseases in mammalian systems (Shen et al., 2011; Takasuga and Sasaki, 2013). In plants, PtdIns(3,5)P<sub>2</sub> is required for endosomes maturation and for the interaction of these organelles with microtubules (Hirano et al., 2015), as also for the generation and remodeling of the cortical actin array (van Gisbergen et al., 2012).

Using the PtdIns(3,5)P<sub>2</sub>-sensitive genetically encoded tagRFP-ML1N\*2 probe (Li et al., 2013), PtdIns(3,5)P<sub>2</sub> was shown to predominantly localize on late endosomes of cortical cells of the root differentiation zone (Hirano et al., 2017b). Of the 4 *Arabidopsis* FAB1 kinases that synthesize PtdIns(3,5)P<sub>2</sub>, two (FAB1C and FAB1D) are seemingly not targeted to membranes containing PtdIns(3)P — the PtdIns(3,5)P<sub>2</sub>'s precursor and dephosphorylation product, while the other two (FAB1A and FAB1B) have been shown to localize to (late) endosomes (Hirano et al., 2011; Hirano et al., 2015; Hirano et al., 2017b; Table 1). This last localization is slightly narrower than that of FAB1A/B's substrate, PtdIns(3)P, which can be found in late endosomes/prevacuolar compartments (Zhang et al., 2015 and references therein). Nevertheless, the most evident result of manipulations of the expression level of FAB1-family kinases is an aberrant vacuolar morphology (Whitley et al., 2009; Hirano et al., 2011), indicating that their product, PtdIns(3,5)P<sub>2</sub>, is functionally important at the tonoplast. Intriguingly, no signs of PtdIns(3,5)P<sub>2</sub> presence in the tonoplast are detected with the tagRFP-ML1N\*2 probe, however, it maybe due to insufficient sensitivity of this probe toward the available levels of this phospholipid in the examined conditions (Hirano et al., 2017b). So, to our knowledge, the question of how do spatially isolated PtdIns(3,5)P<sub>2</sub>-synthesizing enzymes alter the tonoplast dynamics through their product, has not yet been addressed.

PtdIns(3,5)P<sub>2</sub> level increase have been well documented under stimuli similar to those inducing vacuolar convolution/fragmentation. In yeast, hyperosmotic stress induces a 20-30-min transient PtdIns(3,5)P<sub>2</sub> increase (Dove et al., 1997; Bonangelino et al., 2002; Dove et al., 2004)



1 accompanied by a parallel decrease in the vacuolar volume, being this last restored when  
2 PtdIns(3,5)P<sub>2</sub> falls to its original level (Duex et al., 2006). In plants, available evidences of the  
3 signaling role of PtdIns(3,5)P<sub>2</sub> in hyperosmotic or salt stress are less consistent. There are reports  
4 that PtdIns(3,5)P<sub>2</sub> level transiently increases within minutes after the application of NaCl to  
5 *Chlamydomonas*, cultured cells of tomato and alfalfa, pea plants, or tobacco pollen tubes (Meijer  
6 et al., 1999; Munnik and Vermeer, 2010; Zonia and Munnik, 2004). However, only subtle  
7 changes in the PtdIns(3,5)P<sub>2</sub> content were reported for *Arabidopsis* plants grown in liquid  
8 medium (DeWald et al., 2001) or cultured cells of the same plant (Pical et al., 1999), subjected to  
9 hyperosmotic or salt stress. The question of whether is the transient increase in the PtdIns(3,5)P<sub>2</sub>  
10 level (and also — the ability to convolute the vacuole) somehow related to salt tolerance in  
11 plants, to our knowledge, has not yet been addressed, though, such a relationship was proposed  
12 for drought-resistance (see in Magalhães Alvarez et al., 2008). For salt-tolerant species an  
13 increase of the vacuolar volume and of the luminal Na<sup>+</sup> concentration under salt stress has been  
14 reported (Mimura et al., 2003), however, the time scale of these measurements (hours to days)  
15 exceeded by far the duration of the processes we focus on (thirty minutes at most), and whether  
16 convolution preceded the vacuole enlargement was not specified.

17 In addition, PtdIns(3,5)P<sub>2</sub> was recently shown to be required for abscisic acid-induced vacuole  
18 convolution and stomata closure (Bak et al., 2013).

19 The functional importance of the vacuolar PtdIns(3,5)P<sub>2</sub> increase, and the role this compound  
20 plays during hyperosmotic stress is currently unknown. In our experiments, using atomic force  
21 microscopy and patch-clamp, we observed that bulk PtdIns(3,5)P<sub>2</sub> application to isolated plant  
22 vacuoles increases the vacuolar membrane stiffness (well in agreement with the prediction by  
23 Slochower et al., 2015), reducing at the same time the current mediated by the largest passive  
24 vacuolar conductance. These observations allowed us to propose a role for PtdIns(3,5)P<sub>2</sub> as a  
25 rapidly acting “emergency brake” preventing massive ion-water leak from vacuoles of cells,  
26 subjected to hyperosmotic stress, giving the plant time to activate the mechanisms of resistance,  
27 based on inducible expression (V.P.K., Erasmo Ovalle García, Armando Antillón, Iván Ortega  
28 Blake and Omar Pantoja, unpublished data).

29 This explanation, however, contrasts with the apparent requirement of PtdIns(3,5)P<sub>2</sub> increase for  
30 vacuolar convolution during stomata closure (Bak et al., 2013) or vacuolar fragmentation (Zieger

1 and Mayer, 2012) — processes which rely on large ion/water efflux from the vacuole  
2 (MacRobbie, 1998; MacRobbie, 2006, Pandey et al., 2007), and with the overall minute amounts  
3 of PtdIns(3,5)P<sub>2</sub> in cells, even when elevated in response to stresses (Meijer et al., 1999; Meijer  
4 and Munnik, 2003). For this review, we tried to find published results on the effect of mutations  
5 altering PtdIns(3,5)P<sub>2</sub> turnover in plants on the water permeability of the vacuolar membrane, but  
6 seems this issue has not been explicitly analyzed. Thus, we can only speculate on it using  
7 available indirect evidences. For example, Bak et al. (2013) observed that loss-of-function plants,  
8 defective in the expression of FAB1B and FAB1C, display a faster water loss than wild-type  
9 plants, though this effect could be alternatively explained by a slower stomata closure in these  
10 plants (Bak et al., 2013). Similarly, *Arabidopsis* plants with a reduced activity of the At5PTase9,  
11 a phosphatidylinositol 5-phosphatase, show a higher resistance to osmotic stress, yet they are  
12 sensitive to salt stress (Golani et al., 2013). The substrate of this enzyme is currently unknown,  
13 but a possible inhibition of PtdIns(3,5)P<sub>2</sub> dephosphorylation could be responsible for the  
14 hyperosmotic stress resistance. Excessive water loss seems also to underlie the curly-leaves  
15 phenotype observed in plants knockout by either *FAB1A* or *FAB1B*, and the pollen non-viability  
16 during the preparation for dehydration in the double knockout mutant (Whitley et al., 2009).

17 Alterations of the PtdIns(3,5)P<sub>2</sub> turnover results in vacuoles with abnormal morphology  
18 (McCartney et al., 2014). For example, plants overexpressing SAC2-SAC5 phosphatases display  
19 a reduced PtdIns(3,5)P<sub>2</sub> level and their vacuoles are larger and more coalescent than those of WT  
20 (Col-0, background) plants (Nováková et al, 2014). On the other hand, plants knockout by these  
21 phosphatases show a larger number and more shrunk vacuoles (50-60% of vacuoles in roots of  
22 the quadruple *sac2 sac 3 sac4 sac5* mutant), possibly associated with the slightly increased level  
23 of PtdIns(3,5)P<sub>2</sub> (Nováková et al, 2014). Contrary to these results, prolonged (24 hrs) chemical  
24 inhibition of PtdIns(3,5)P<sub>2</sub> biosynthesis was reported to produce shrunk, compact central  
25 vacuoles (Hirano et al., 2017a). Possible alterations of tonoplast ionic fluxes or stomata function  
26 in all these cases were not addressed. It is interesting to note here that mammalian cells, on their  
27 hand, also respond with a massive vacuolization to the inhibition of PIKfyve — the enzyme  
28 responsible for the synthesis of PtdIns(3,5)P<sub>2</sub> in these cells (Balla, 2013 and references therein).  
29 In yeast, PtdIns(3,5)P<sub>2</sub> was shown to be required for fragmentation of the vacuole under  
30 hyperosmotic shock. The  $\Delta fab1$  strain (PtdIns(3,5)P<sub>2</sub>-deficient) form initial vacuolar  
31 invaginations, enriched in PtdIns(3)P, which remain over long time and ultimately detach from

1 the vacuole as intravacuolar vesicles, no vacuolar fragmentation being observed in this condition  
2 (Zieger and Mayer, 2012). Application of a similar treatment to a PtdIns(3)P-deficient strain,  
3  $\Delta Vps34$ , also does not result in vacuole fragmentation, but pronounced invaginations are formed,  
4 which disappear with time (Zieger and Mayer, 2012). Nevertheless, in yeast the PtdIns(3,5)P<sub>2</sub>  
5 level may not be the only factor responsible for the vacuolar dynamics. It was shown that the  
6 strain  $\Delta atg18$  and the *fab1-5* mutant, both characterized by increased PtdIns(3,5)P<sub>2</sub> content,  
7 display opposite vacuolar morphologies: the first strain has enlarged vacuoles, characterized by a  
8 delayed fragmentation, while the second one has hyperfragmented vacuoles (Zieger and Mayer,  
9 2012 and references therein).

10 Within this section we did not mention it explicitly, but a homogenous PtdIns(3,5)P<sub>2</sub> distribution  
11 in the tonoplast is implied, which is not necessarily the case. Any clustering of PtdIns(3,5)P<sub>2</sub> (a  
12 behavior already shown for PtdIns(4,5)P<sub>2</sub> upon Ca<sup>2+</sup> application at concentrations mimicking  
13 physiological elevation, Sarmiento et al., 2014) would contribute to significant local changes in  
14 the physical properties of the vacuolar membrane, not requiring an extraordinary global increase  
15 of this minor lipid component. In mammalian cells, local concentration increase of PIKfyve-  
16 enriched microdomains can be observed, reaching concentrations up to 10  $\mu$ M, similar to the  
17 PtdIns(4,5)P<sub>2</sub> levels that can be found in the plasma membrane (Shen et al., 2011 and references  
18 therein). In yeast subjected to salt stress, apart from a detected global increase of the  
19 PtdIns(3,5)P<sub>2</sub> level, the presence of domains enriched in PtdIns(3,5)P<sub>2</sub> (but apparently deficient  
20 in transmembrane proteins) was directly shown using the quick-freezing and freeze-fracture  
21 replica labeling (QF-FRL) technique (Takatori et al., 2015). This result raises the question on  
22 whether do convoluted plant vacuoles also present such PtdIns(3,5)P<sub>2</sub>-enriched domains, and, in  
23 a case of a positive answer — how could the transient, and apparently tonoplast-stiffening  
24 PtdIns(3,5)P<sub>2</sub> increase be related to the vacuolar ion/water efflux and vacuolar convolution. In  
25 mammalian cells PtdIns(3,5)P<sub>2</sub> has been reported to be an activator of lysosomal mucolipin  
26 transient receptor potential (TRPML1) channels (Dong et al., 2010) and TPC channels (Wang et  
27 al., 2012), though the lysoNa<sub>ATP</sub> current mediated by the latter in association with mTORC1 was  
28 shown to be insensitive to PtdIns(3,5)P<sub>2</sub> (Cang et al., 2013). Nevertheless, plant TPC1 channels,  
29 which mediate the largest and ubiquitous vacuolar conductance, showed no response to the  
30 application of this lipid (Boccaccio et al., 2014). To our knowledge, possible effects of  
31 PtdIns(3,5)P<sub>2</sub> on the TPK channels, which are plausible mediators of vacuolar convolution

during stomata closure, have not been tested yet. Similarly, it would be interesting to test the effect of PtdIns(3,5)P<sub>2</sub> on the activity of vacuolar NHX exchangers, which are enriched in vacuolar invaginations of closed stomata (Andrés et al., 2015), where, according to the data obtained in yeast (Takatori et al., 2015), higher PtdIns(3,5)P<sub>2</sub> levels could be expected.

### **Energization of the vacuolar morphology dynamics and dynamics-associated water flow**

In the present review we centered our attention on the mechanisms of execution of the vacuolar shaping, leaving aside other related issues. Meanwhile, any large movement of ions through the vacuolar membrane is tightly associated to two processes: 1) the accumulation of these ions in the vacuolar lumen, and 2) the flow of water dragged by the ions movement, requiring routes for this transport to occur.

In fungi and plants the energization of the vacuolar transport is a task executed by the vacuolar H<sup>+</sup>-ATPase and H<sup>+</sup>-PPase. Thus, defects in their functioning should result in an altered vacuolar dynamics. Indeed, a yeast strain that lacks a catalytic subunit of the V-ATPase does not display any invaginations under hypertonic stress (Zieger and Mayer, 2012). This effect, however, could be related to affected accumulation of ion species directly involved in convolution/fragmentation execution, due altered secondary transport, which depends on the pH gradient across the tonoplast (though V-ATPase-lacking yeast of the  $\Delta vma$  strain can apparently maintain an acidic vacuolar content if grown in appropriate conditions, Plant et al., 1999).

To our knowledge, the PtdIns(3,5)P<sub>2</sub> effect on the plant H<sup>+</sup>-PPase has not yet been examined, while that on the vacuolar H<sup>+</sup>-ATPase is not clear. There are evidences for the activation of the V-ATPase by PtdIns(3,5)P<sub>2</sub> (Bak et al., 2013; Li et al., 2014), yet this compound was reported to be irrelevant for the *maintenance* of the steady-state vacuolar pH (Ho et al., 2015). Vacuoles of *Arabidopsis* plants with reduced expression of FAB1 kinases display acidification defects (Hirano et al., 2011). However, whether the reason is a lowered level of PtdIns(3,5)P<sub>2</sub> or another indirect mechanism, was not discerned. Similarly, PtdIns(3,5)P<sub>2</sub> effects on the V-ATPases in mammalian cells are not very clear (Balla et al, 2013). On the other hand, in yeast the importance of the proton translocation by the V-ATPase for salt-shock-induced vacuolar fragmentation has been proved (Baars et al., 2007). A recent work directly demonstrates that PtdIns(3,5)P<sub>2</sub> depletion perturbs the V-ATPase activity (Deranieh et al., 2015). The question here would be whether are

1 active V-ATPase-s directly required for this process, or rather, — the pH gradient they generate  
2 across the vacuolar membrane, which can be used by the proteins executing convolution.  
3 Available evidences point to the second option (Baars et al., 2007). Thus, a plausible alternative  
4 to explore the direct participation of vacuolar proton pumps in the execution of vacuolar shaping  
5 could be their pharmacological inhibition. Though, in this case an important problem to deal with  
6 will be the dissipation of the trans-tonoplast proton gradient, which is constantly maintained by  
7 these transporters, and which is required for the functioning of secondary transport.

8 Cellular water flow is mediated by aquaporins, which in plants constitute a substantial number of  
9 homologs belonging to five different subfamilies — plasma membrane intrinsic proteins (PIPs),  
10 tonoplast intrinsic proteins (TIPs), nodulin26-like intrinsic proteins (NIPs), and the recently  
11 discovered small basic intrinsic proteins (SIPs) and the uncategorized intrinsic proteins (XIPs)  
12 (Maurel et al., 2015). TIPs, mediating the water flow across the tonoplast, are one of the  
13 subfamilies with a largest number of members, but different isoforms have been shown to be  
14 specifically targeted to different vacuole subtypes (Maurel et al., 2015 and references therein). In  
15 our hypothesis of a patterned vacuolar efflux-producing convolution, we can expect that TIPs  
16 could also decorate specific membrane regions of the vacuolar membrane. Such structures,  
17 enriched in TIP isoforms fused to GFP, have indeed been reported in previous studies to localize  
18 in apposing tonoplast sheets (Beebo et al., 2009), or in vacuolar bubbles (Reisen et al., 2005).  
19 However, recently it was found that similar structures are artifacts resulting from the  
20 dimerization of the non-monomerized GFP tags of proteins located in tonoplast areas facing each  
21 other (Segami et al., 2014). So, additional information on the microscopic localization and  
22 dynamics of tonoplast aquaporins is required.

23 For the subject of our review it is also interesting the fact that, at least for isolated vacuoles, their  
24 volume is solely controlled by the water and solute flows across the tonoplast membrane, with no  
25 contribution from the elastic properties of the membrane, or from the non-osmotic volume (Vitali  
26 et al., 2016). Particularly, when aquaporins are expected to be inhibited (at low cytosolic pH),  
27 solute movement has no effect on the vacuolar volume, being the opposite effect observed when  
28 the water permeability is high (at higher cytosolic pH values).

29 **Possible mechanism of vacuolar convolution**

1 Following the observations made by Michailat and coworkers (2012) we can suppose that  
2 vacuolar convolution in plants can be a relatively autonomous process. However, at least in  
3 guard cells, its triggering requires an interaction between transporters located both at the plasma  
4 and the vacuolar membranes (Jezek and Blatt, 2017). Thus, a still unsolved question is whether a  
5 single upward mechanism exists, able to initiate convolution, or several such triggering  
6 mechanisms coexist.

7 Second, plant tissues differ in their vacuolization degrees, and in the composition of other  
8 organelles which appear as critical for the execution of vacuolar convolution—  
9 endosomes/prevacuolar compartments, which can, additionally, be subjected to fast post-  
10 translational modifications in response to external stimuli (Heilmann, 2016; Brumbarova and  
11 Ivanov, 2016). For example, in the *Arabidopsis* root, the meristematic zone is characterized by  
12 small multiple (“preconvoluted”?) vacuoles, which gradually enlarge and fuse in the elongation  
13 zone, and occupy most of the cell volume in the differentiation zone (see in Kriegel et al., 2015).  
14 These three zones differ also in the abundance of FAB1-containing late endosomes and in the  
15 level of PtdIns(3,5)P<sub>2</sub> reported by the tagRFP-ML1N\*2 probe (Hirano et al., 2017b), suggesting  
16 that the response of each of these zones to hyperosmotic treatment will also differ. Indeed,  
17 though it is not the short-term response we are focused on, hyperosmotic stress is known to  
18 induce the premature differentiation of the root apical meristem (Ji et al., 2014; Ji and Li, 2014).

19 Bearing in mind these observations, we can attempt to enlist the steps that, in our opinion, bring  
20 the plant vacuole to a convoluted state:

21 1) Both in guard cells upon ABA treatment (Irving et al., 1992), and in salt-tolerant species upon  
22 salinity stress (Kader and Lindberg, 2010), an increase in cytosolic pH is observed. No such pH  
23 increase is observed if the osmolyte has a non-ionic nature or in salt-sensitive species.

24 2) We consider the above effect to be a result of vacuolar NHXs activity inhibition by a transient  
25 increase of the tonoplast PtdIns(3,5)P<sub>2</sub> level, taking place upon the fusion of PtdIns(3,5)P<sub>2</sub>-  
26 (FAB1)-containing late endosomes/prevacuolar compartments with the lytic vacuole.

27 3) Inhibition of the vacuolar NHXs activity (and apparently—activation of the V-ATPase by the  
28 increased PtdIns(3,5)P<sub>2</sub> level) creates cytosolic zones with high pH, increasing the water-to-

1 solute permeability ratio of the tonoplast areas adjacent to these zones, and thus—the effect of  
2 solutes movement on the vacuolar volume (Vitali et al., 2016).

3 4) Large  $K^+$  efflux takes place, provoked both by the inhibition of the  $K^+$ -transporting vacuolar  
4 NHXs, and the activation of the TPK channels by the cytosolic  $Ca^{2+}$  increase (Irving et al., 1992).  
5 Activation of TPKs by the PtdIns(3,5)P<sub>2</sub> increase could also be possible.

6 5) The vacuole is “deflated” in patterns by the efflux of  $K^+$  and water, and by the structural  
7 (stiffening) role of PtdIns(3,5)P<sub>2</sub>.

8 This rough (and for sure—incomplete) scheme is depicted in Fig. 1, and although its steps are  
9 presented sequentially, it is very probable that some of them could be interdependent. In this last  
10 case convolution could be triggered by some of the conditions mentioned (either cytosolic pH  
11 rise, cytosolic  $Ca^{2+}$  or  $K^+$  increase, increase in the tonoplast PtdIns(3,5)P<sub>2</sub> level, NHX  
12 inactivation, etc.), on its hand mediated by an external stimulus.

13 An unsolved question in this description is the destiny of the FAB1 enzymes: apparently they are  
14 not detected in the vacuolar membrane. Thus, they could be internalized into the vacuole, as has  
15 been shown for plasma membrane proteins (Ueda et al., 2016), or be readily recycled by the  
16 retromer complex, to stay at the endosomes (Robinson et al., 2012; Zelazny et al., 2013).  
17 Interestingly, in contrast to the lateral roots initiation-promoting effect of hyperosmotic stress,  
18 plants knockout by one or several retromer core members display severe inhibition of lateral root  
19 initiation (Zelazny et al., 2013). It is tempting to think that hyperosmotic stress acts in plants by  
20 recapitulating the developmental signals that induce lateral roots formation. **Conclusion**

21 A thorough reconstruction of the events leading to vacuolar convolution requires knowledge on  
22 details of vacuole functioning, which in many cases are not available for plants. In the previous  
23 sections we tried to complete the lacking information with data obtained on yeast. However,  
24 despite similarities of the factors producing vacuolar convolution in plants and fragmentation in  
25 yeast vacuoles, and of the compounds involved in these processes, differences between the  
26 mechanisms of vacuolar shaping in these phylogenetically-distant organisms have to be expected.  
27 It is clear that the intracellular location of the plant vacuole poses formidable technical  
28 difficulties to study its dynamics, however, it is possible that the availability of plants expressing

1 genetically encoded tools (tagged proteins and indicators, Hirano et al., 2017b) can help in  
2 shedding light on how does vacuolar convolution proceed *in vivo*.

3 Still, it is difficult to discern whether convolution/fragmentation and other types of vacuolar  
4 remodeling share a common mechanism or, alternatively, several mechanisms of vacuolar  
5 shaping coexist, each one for a different purpose. For instance, yeast cells lacking Vps1p or a  
6 function vacuolar V-ATPase, which are not able to fragment the vacuole under hyperosmotic  
7 shock, are nevertheless, not deficient for vacuolar inheritance — a process that involves vacuole  
8 fission as an essential step. On its hand, vacuole inheritance requires a functional actin  
9 cytoskeleton, which is not required for vacuolar fragmentation induced by hyperosmotic shock  
10 (Zieger and Mayer, 2012 and references therein). This observation suggests that the timing of  
11 vacuole remodeling could be important, and the rate at which vacuolar shaping occurs could give  
12 idea on the nature of the mechanisms involved in each case. For this purpose, techniques like  
13 digital image correlation (Higaki et al., 2006; Oda et al., 2009), which allows the evaluation of  
14 morphological changes occurring in the vacuole between frames of an acquired timelapse series,  
15 could be useful.

16 Finally, we should not discard the possibility that most of the components localized on the  
17 tonoplast membrane, or, more generally, on the membrane of acidic  $\text{Ca}^{2+}$  stores, are, to different  
18 extent, involved in the morphology dynamics of these organelles by regulating their  
19 electrochemical potential and ion fluxes. For example, Wang et al. (2012) proposed that  
20  $\text{PtdIns}(3,5)\text{P}_2$  could act as a regulator of lysosomal  $\text{H}^+$  homeostasis through the action on the  
21 allegedly- $\text{PtdIns}(3,5)\text{P}_2$ -activated  $\text{Na}^+$ -permeable TPC channels located in these organelles.  
22 According to the authors, large  $\text{Na}^+$  (but not  $\text{K}^+$ ) changes in these compartments by release  
23 through TPC would, in its turn, control the activity of lysosome-located cation-proton (in this  
24 case —  $\text{Na}^+/\text{H}^+$ ) exchangers, also affecting the luminal pH in these organelles. This possibility  
25 raises the question of whether does the plant vacuole within the cell act as a unified compartment,  
26 or its responses can vary locally, depending on the surging vacuole-organelles interfaces, as has  
27 been proposed for plant TPC1 activation (Pérez et al., 2008). Resolving this question could be of  
28 a great importance in light of the emerging evidences that acidic  $\text{Ca}^{2+}$  stores can serve as  
29 integrating hubs of several signaling cascades, defining in several eukaryotic lineages the  
30 developmental program to be followed (Michaillat et al., 2012; Cang et al., 2013).



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## Conflicts of Interest

The authors declare no conflicts of interest

## References

- Andrés Z., Pérez-Hormaeche J., Leidi E.O., Schlücking K., Steinhorst L., McLachlan D.H., Schumacher K., Hetherington A.M., Kudla J., Cubero B., Pardo J.M. (2014) Control of vacuolar dynamics and regulation of stomatal aperture by tonoplast potassium uptake. *Proceedings of the National Academy of Sciences of the United States of America* **111**(17), E1806-E1814.
- Armstrong J. (2010) Yeast vacuoles: more than a model lysosome. *Trends in Cell Biology* **20**(10), 580-585.
- Baars T.L., Petri S., Peters C., Mayer A. (2007) Role of the V-ATPase in regulation of the vacuolar fission-fusion equilibrium. *Molecular Biology of the Cell* **18**(10), 3873-3882.
- Bak G., Lee E.-J., Lee Y., Kato M., Segami S., Sze H., Maeshima M., Hwang J.-U., Lee Y. (2013) Rapid structural changes and acidification of guard cell vacuoles during stomatal closure require phosphatidylinositol 3,5-bisphosphate. *The Plant Cell* **25**, 2202-2216.
- Balla T. (2013). Phosphoinositides: tiny lipids with giant impact on cell regulation. *Physiological Reviews* **93**, 1019–137.

1 Beebo A., Thomas D., Der C., Sanchez L., Leborgne-Castel N., Marty F., Schoefs B., Bouhidel  
2 K. (2009) Life with and without AtTIP1;1, and Arabidopsis aquaporin preferentially localized in  
3 the apposing tonoplasts of adjacent vacuoles. *Plant Molecular Biology* **70**(1): 193-209.

4 Boccaccio A., Scholz-Starke J., Hamamoto S., Larsich N., Festa M., Gutla P.V., Costa A.,  
5 Dietrich P., Uozumi N., Carpaneto A. (2014) The phosphoinositide PI(3,5)P<sub>2</sub> mediates activation  
6 of mammalian but not plant TPC proteins: functional expression of endolysosomal channels in  
7 yeast and plant cells. *Cellular and Molecular Life Sciences* **71**(21), 4275-4283.

8 Bonangelino C.J., Nau J.J., Duex J.E., Brinkman M., Wurmser A.E., Gary J.D., Emr S.D.,  
9 Weisman L.S. (2002) Osmotic stress-induced increase of phosphatidylinositol 3,5-bisphosphate  
10 requires Vac14p, an activator of the lipid kinase Fab1p. *Journal of Cell Biology* **156**(6), 1015-  
11 1028.

12 Brumbarova T., Ivanov R. (2016) Differential gene expression and protein phosphorylation as  
13 factors regulating the state of the *Arabidopsis* SNX1 protein complexes in response to  
14 environmental stimuli. *Frontiers in Plant Science* **7**, 1456.

15 Cang C., Zhou Y., Navarro B., Seo Y.-j., Aranda K., Shi L., Battaglia-Hsu S., Nissim I.,  
16 Clapham D.E., Ren D. (2013) mTOR regulates lysosomal ATP-sensitive Two-Pore Na<sup>+</sup> channels  
17 to adapt to metabolic state. *Cell* **152**, 778-790.

18 Deranieh R.M., Shi Y., Tarsio M., Chen Y., McCaffery J.M., Kane P.M., Greenberg M.L.  
19 (2015) Perturbation of the vacuolar ATPase: a novel consequence of inositol depletion. *The*  
20 *Journal of Biological Chemistry* **290**(46), 27460-27472.

21 DeWald D., Torabinejad J., Jones C.A., Shope J.C., Cangelosi A.R., Thompson J.E., Prestwich  
22 G.D., Hama H. (2001) Rapid accumulation of phosphatidylinositol 4,5-bisphosphate and inositol  
23 1,4,5-trisphosphate correlates with calcium mobilization in salt-stressed *Arabidopsis*. *Plant*  
24 *Physiology* **126**, 759-769.

25 Dong X-p., Shen D., Wang X., Dawson T., Li X., Zhang Q., Cheng X., Zhang Y., Weisman L.S.,  
26 Delling M., Xu H. (2010) PI(3,5)P<sub>2</sub> controls membrane trafficking by direct activation of  
27 mucolipin Ca<sup>2+</sup> release channels in the endolysosome. *Nature Communications* **1**(4): 38.

1 Dove S.K., Cooke F.T., Douglas M.R., Sayers L.G., Parker P.J., Michell R.H. (1997) Osmotic  
2 stress activates phosphatidylinositol-3,5-bisphosphate synthesis. *Nature* **390**, 187-192.

3 Dove S.K., Piper R.C., McEwen R.K., Yu J.W., King M.C., Hughes D.C., Thuring J., Holmes  
4 A.B., Cooke F.T., Michell R.H., Parker P.J., Lemmon M.A. (2004) Svp1p defines a family of  
5 phosphatidylinositol 3,5-bisphosphate effectors. *The EMBO Journal* **23**, 1922-1933.

6 Duex J.E., Nau J.J., Kaufmann E.J., Weisman L.S. (2006) Phosphoinositide 5-phosphatase Fig4p  
7 is required for both acute rise and subsequent fall in stress-induced phosphatidylinositol 3,5-  
8 bisphosphat levels. *Eukaryotic Cell* **5**(4), 723-731.

9 Gao X.Q., Li C.H., Wei P.C., Zhang X.Y., Chen J., Wang X.C. (2005) The dynamic changes of  
10 tonoplasts in guard cells are important for stomatal movement in *Vicia faba*. *Plant Physiology*  
11 **139**, 1207-1216.

12 Gobert A., Isayenkov S., Voelker C., Czempinski K., Maathuis F.J.M. (2007) The two-pore  
13 channel *TPK1* gene encodes the vacuolar K<sup>+</sup> conductance and plays a role in K<sup>+</sup> homeostasis.  
14 *Proceedures of the National Academy of Sciences of the U.S.A.* **104**(25), 10726-10731.

15 Golani Y., Kaye Y., Gilhar O., Ercetin M., Gillaspay G., Levine A. (2013) Inositol polyphosphate  
16 phosphatidylinositol 5-phosphatase9 (At5ptase9) controls plant salt tolerance by regulating  
17 endocytosis. *Molecular Plant* **6**(6), 1781-1794.

18 Hatsugai N., Pérez Koldenkova V., Imamura H., Noji H., Nagai T. (2012) Changes in cytosolic  
19 ATP levels and intracellular morphology during bacteria-induced hypersensitive cell death as  
20 revealed by real-time fluorescence microscopy imaging. *Plant and Cell Physiology* **53**(10),  
21 1768-1775.

22 Heilmann I. (2016) Plant phosphoinositide signaling — dynamics on demand. *BBA - Molecular*  
23 *and Cell Biology of Lipids* **1861**(9 Pt B), 1345-1351.

24 Higaki T., Kutsuna N., Okubo E., Sano T., Hasezawa S. (2006) Actin microfilaments regulate  
25 vacuolar structures and dynamics: dual observation of actin microfilaments and vacuolar  
26 membrane in living tobacco BY-2 cells. *Plant and Cell Physiology* **47**(7), 839-852.

1 Hirano T., Matsuzawa T., Takegawa K., Sato M.H. (2011) Loss-of-function and gain-of-function  
2 mutations in *FAB1A/B* impair endomembrane homeostasis, conferring pleiotropic developmental  
3 abnormalities in *Arabidopsis*. *Plant Physiology* **155**, 797-807.

4 Hirano T., Munnik T., Sato M.H. (2015) Phosphatidylinositol 3-phosphate 5-kinase,  
5 FAB1/PIKfyve kinase mediates endosome maturation to establish endosome-cortical  
6 microtubule interaction in *Arabidopsis*. *Plant Physiology* **169**, 1961-1974.

7 Hirano T., Munnik T., Sato M.H. (2017a) Inhibition of phosphatidylinositol 3,5-bisphosphate  
8 production has pleiotropic effects on various membrane trafficking routes in *Arabidopsis*. *Plant*  
9 *and Cell Physiology* **58**(1), 120-129.

10 Hirano T., Stecker K., Munnik T., Xu H., Sato M.H. (2017b) Visualization of  
11 phosphatidylinositol 3,5-bisphosphate by a tandem ML1N-based fluorescent protein probe in  
12 *Arabidopsis*. *Plant and Cell Physiology*, doi: 10.1093/pcp/pcx011

13 Ho C.Y., Choy C.H., Wattson C.A., Johnson D.E., Botelho R.J. (2015) The Fab1/PIKfyve  
14 phosphoinositide phosphate kinase is not necessary to maintain the pH of lysosomes and of the  
15 yeast vacuole. *The Journal of Biological Chemistry* **290**(15), 9919-9928.

16 Hoffmann A., Nebenführ A. (2004) Dynamic rearrangements of transvacuolar strands in BY-2  
17 cells imply a role of myosin in remodeling the plant actin cytoskeleton. *Protoplasma* **224**, 201-  
18 210.

19 Hwang J-U., Suh S., Yi H., Kim J., Lee Y. (1997) Actin filaments modulate both stomatal  
20 opening and inward K<sup>+</sup>-channel activities in guard cells of *Vicia faba*. *Plant Physiology* **115**,  
21 335-342.

22 Irving H.R., Gehring C.A., Parish R.W. (1992) Changes in cytosolic pH and calcium of guard  
23 cells precede stomatal movements. *Proceedings of the National Academy of Sciences of the*  
24 *United States of America* **89**(5), 1790-1794.

25 Isayenkov S., Isner J.-C., Maathuis F.J.M. (2011) Membrane localization diversity of TPK  
26 channels and their physiological role. *Plant Signaling and Behavior* **6**(8), 1201-1204.

1 Jezek M., Blatt M.R. (2017) The membrane transport system of the guard cell and its integration  
2 for stomatal dynamics. *Plant Physiology*, doi: 10.1104/pp.16.01949

3 Ji H., Liu L., Li K., Xie Q., Wang Z., Zhao X., Li X. (2014) PEG-mediated osmotic stress  
4 induces premature differentiation of the root apical meristem and outgrowth of lateral roots in  
5 wheat. *Journal of Experimental Botany* **65**, 4863-4872.

6 Ji H., Li X. (2014) ABA mediates PEG-mediated premature differentiation of root apical  
7 meristem in plants. *Plant Signaling and Behavior* **9**, e977720.

8 Jossier M., Kroniewicz L., Dalmas F., Le Thiec D., Ephritikhine G., Thomine S., Barbier-  
9 Brygoo H., Filleur S., Leonhardt N. (2010) The *Arabidopsis* vacuolar anion transporter, AtCLCc,  
10 is involved in the regulation of stomatal movements and contributes to salt tolerance. *The Plant*  
11 *Journal* **64**, 563-576.

12 Kader M.A., Lindberg S. (2010) Cytosolic calcium and pH signaling in plants under salinity  
13 stress. *Plant Signaling and Behavior* **5**(3), 233-238.

14 Kim M., Hepler P.K., Eun S.-O., Ha K.S., Lee Y. (1995) Actin filaments in mature guard cells  
15 are radially distributed and involved in stomatal movement. *Plant Physiology* **109**, 1077-1084.

16 Kriegel A., Andrés Z., Medzihradsky A., Krüger F., Scholl S., Delang S., Görkem Patir-  
17 Nebioglu M., Gute G., Yang H., Murphy A.S., Peer W.A., Pfeiffer A., Krebs M., Lohmann J.U.,  
18 Schumacher K. (2015) Job sharing in the endomembrane system: vacuolar acidification requires  
19 the combined activity of V-ATPase and V-PPase. *The Plant Cell* **27**(12), 3383-3396.

20 Kutsuna N., Hasezawa S. (2005) Dynamic organization of vacuolar and microtubule structures  
21 during cell cycle progression in synchronized tobacco BY-2 cells. *Plant and Cell Physiology* **43**,  
22 965-973.

23 Kutsuna N., Kumagai F., Sato M.H., Hasezawa S. (2003) Three-dimensional reconstruction of  
24 tubular structure of vacuolar membrane throughout mitosis in living tobacco cells. *Plant and Cell*  
25 *Physiology* **44**(10), 1045-1054.

- 1    Lew R.R. (2004) Osmotic effects on the electrical properties of *Arabidopsis* root hair vacuoles *in*  
2    *situ*. *Plant Physiology* **134**, 352-360.
- 3    Li S.C., Diakov T.T., Xu T., Tarsio M., Zhu W., Couoh-Cardel S., Weisman L.S., Kane P.M.  
4    (2014) The signaling lipid PI(3,5)P<sub>2</sub> stabilizes V<sub>1</sub>-V<sub>0</sub> sector interactions and activates the V-  
5    ATPase. *Molecular Biology of the Cell* **25**, 1251-1262.
- 6    Li S.C., Kane P.M. (2009) The yeast lysosome-like vacuole: endpoint and crossroads.  
7    *Biochimica et Biophysica Acta* **1793**(4), 650-663.
- 8    Li X., Wang X., Zhang X., Zhao M., Tsang W.L., Zhang Y., Yau R.G., Weisman L.S., Xu H.  
9    (2013) Genetically encoded fluorescent probe to visualize intracellular phosphatidylinositol 3,5-  
10    bisphosphate localization and dynamics. *Proceedings of the National Academy of Sciences of the*  
11    *United States of America* **110**(52), 21165-21170.
- 12    Maathuis F.J.M. (2011) Vacuolar two-pore K<sup>+</sup> channels act as vacuolar osmosensors. *New*  
13    *Phytologist* **191**, 84-91.
- 14    MacRobbie E.A.C. (1998) Signal transduction and ion channels in guard cells. *Philosophical*  
15    *Transactions of the Royal Society B: Biological Sciences* **353**(1374), 1475-1488.
- 16    MacRobbie E.A.C. (2006) Control of volume and turgor in stomatal guard cells. *The Journal of*  
17    *Membrane Biology* **210**, 131-142.
- 18    Magalhães Alvarez J., Francisco Rocha J., Rodriguez Machado S. (2008) Bulliform cells in  
19    *Loudetiopsis chrysothrix* (Nees) conert and *Tristachya leiostachya* Nees (*Poaceae*): structure in  
20    relation to function. *Brazilian Archives of Biology and Technology* **51**(1), 113-119.
- 21    Marcus A.I., Moore R.C., Cyr R.J. (2001) The role of microtubules in guard cell function. *Plant*  
22    *Physiology* **125**(1), 387-395.
- 23    Maurel C., Boursiac Y., Luu D.-T., Santoni V., Shahzad Z., Verdoucq L. (2015) Aquaporins in  
24    plants. *Physiological Reviews* **95**(4), 1321-1358.
- 25    McCartney A., Zhang Y., Weisman L.S. (2014) Phosphatidylinositol 3,5-bisphosphate: low  
26    abundance, high significance. *Bioessays* **36**(1), 52-64.

- 1 Meijer H., Divecha N., van den Ende H., Musgrave A., Munnik T. (1999) Hyperosmotic stress  
2 induces rapid synthesis of phosphatidyl-D-inositol 3,5-bisphosphate in plant cells. *Planta* **31**,  
3 294-298.
- 4 Meijer H.J., Munnik T. (2003) Phospholipid-based signaling in plants. *Annual Reviews in Plant*  
5 *Biology* **54**, 265-306.
- 6 Michailat L., Baars T.L., Mayer A. (2012) Cell-free reconstitution of vacuole membrane  
7 fragmentation reveals regulation of vacuole size and number by TORC1. *Molecular Biology of*  
8 *the Cell* **23**(5), 881-895.
- 9 Michailat L., Mayer A. (2013) Identification of genes affecting vacuole membrane  
10 fragmentation in *Saccharomyces cerevesiae*. *PLoS ONE* **8**(2), e54160.
- 11 Mimura T., Kura-Hotta M., Tsujimura T., Ohnishi M., Miura M., Okazaki Y., Mimura M.,  
12 Maeshima M., Washitani-Nemoto S. (2003) Rapid increase of vacuolar volume in response to  
13 salt stress. *Planta* **216**, 397-402.
- 14 Munnik T., Vermeer J.E.M. (2010) Osmotic stress-induced phosphoinositide and inositol  
15 phosphate signalling in plants. *Plant Cell and Environment* **33**, 655-669.
- 16 Nass R., Rao R. (1999) The yeast endosomal Na<sup>+</sup>/H<sup>+</sup> exchanger, Nhxl, confers osmotolerance  
17 following acute hypertonic shock. *Microbiology* **145**, 3221-3228.
- 18 Niu X., Damsz B., Kononowicz A.K., Bressan R.A., Hasegawa P.M. (1996) NaCl-induced  
19 alterations in both cell structure and tissue-specific plasma membrane H<sup>+</sup>-ATPase gene  
20 expression. *Plant Physiology* **111**, 679-686.
- 21 Nováková P., Hirsch S., Feraru E., Tejos R., van Wijk R., Viaene T., Heilmann M., Lerche J., de  
22 Rycke R., Feraru M., Grones P., van Montagu M., Heilmann I., Munnik T., Friml J. (2014) SAC  
23 phosphoinositide phosphatase at the tonoplast mediate vacuolar function in *Arabidopsis*.  
24 *Proceedings of the National Academy of Sciences of the United States of America* **111**(7), 2818-  
25 2823.

- 1 Oda Y., Higaki T., Hasezawa S., Kutsuna N. (2009) New insights into plant vacuolar structure  
2 and dynamics. *International Review of Cell and Molecular Biology* **277**, 103-135.
- 3 Pandey S., Zhang W., Assmann S.M. (2007) Roles of ion channels and transporters in guard cell  
4 signal transduction. *FEBS Letters* **581**(12), 2325-2336.
- 5 Pérez V., Wherrett T., Shabala S., Muñiz J., Dobrovinskaya O., Pottosin I. (2008) Homeostatic  
6 control of slow vacuolar channels by luminal cations and evaluation of the channel-mediated  
7 tonoplast  $\text{Ca}^{2+}$  fluxes *in situ*. *Journal of Experimental Botany* **59**(14), 3845-3855.
- 8 Pical C., Westergren T., Dove S.K., Larsson C., Sommarin M. (1999) Salinity and hyperosmotic  
9 stress induce rapid increases in phosphatidylinositol 4,5-bisphosphate, diacylglycerol  
10 pyrophosphate, and phosphatidylcholine in *Arabidopsis thaliana* cells. *The Journal of Biological*  
11 *Chemistry* **274**(53), 38232-38240.
- 12 Plant P.J., Manolson M.F., Grinstein S., Demareux N. (1999) Alternative mechanisms of  
13 vacuolar acidification in  $\text{H}^+$ -ATPase-deficient yeast. *The Journal of Biological Chemistry* **274**,  
14 37270-37279.
- 15 Pottosin I.I., Dobrovinskaya O.R., Muñiz J. (2001) Conduction of monovalent and divalent  
16 cations in the slow vacuolar channel. *Journal of Membrane Biology* **181**, 55-65.
- 17 Pottosin I.I., Martínez Estévez M. (2003) Regulation of the fast vacuolar channel by cytosolic  
18 and vacuolar potassium. *Biophysical Journal* **84**(2), 977-986.
- 19 Reisen D., Marty F., Leborgne-Castel N. (2005) New insights into the tonoplast architecture of  
20 plant vacuoles and vacuolar dynamics during osmotic stress. *BMC Plant Biology*, **5**, 13. doi:  
21 10.1186/1471-2229-5-13.
- 22 Robinson D.G., Pimpl P., Scheuring D., Stierhof Y.-D., Sturm S., Viotti C. (2012) Trying to  
23 make sense of retromer. *Trends in Plant Science* **17**(7), 431-439.
- 24 Ruthardt N., Gulde N., Spiegel H., Fischer R., Emans N. (2005) Four-dimensional imaging of  
25 transvacuolar strand dynamics in tobacco BY-2 cells. *Protoplasma* **225**(3-4), 205-215.



- 1 Saito C., Ueda T., Abe H., Wada Y., Kuroiwa T., Hisada A. et al. (2002) A complex and mobile  
2 structure forms a distinct subregion within the continuous vacuolar membrane in young  
3 cotyledons of *Arabidopsis*. *The Plant Journal* **29**, 245-255.
- 4 Sarmiento M.J., Coutinho A., Fedorov A., Prieto M., Fernandes F. (2014)  $\text{Ca}^{2+}$  induces PI(4,5)P<sub>2</sub>  
5 clusters on lipid bilayers at physiological PI(4,5)P<sub>2</sub> and  $\text{Ca}^{2+}$  concentrations. *Biochimica et*  
6 *Biophysica Acta* **1838**, 822-830.
- 7 Segami S., Makino S, Miyake A., Asaoka M., Maeshima M. (2014) Dynamics of vacuoles and  
8  $\text{H}^{+}$ -pyrophosphatase visualized by monomeric green fluorescent protein in *Arabidopsis*:  
9 artifactual bulbs and native intravacuolar spherical structures. *The Plant Cell* **26**(8), 3416-3434.
- 10 Serrazina S., Vaz Dias F., Malhó R. (2014) Characterization of FAB1 phosphatidylinositol  
11 kinases in *Arabidopsis* pollen tube growth and fertilization. *New Phytologist* **203**(3), 784-793.
- 12 Shavrukov Y. (2013) Salt stress or salt shock: which genes are we studying? *Journal of*  
13 *Experimental Botany* **64**(1), 119-127
- 14 Shen D., Wang X., Xu H. (2011) Pairing phosphoinositides with calcium ions in endolysosomal  
15 dynamics. *Bioessays* **33**, 448-457.
- 16 Slochower D.R., Wang Y.-H., Radhakrishnan R., Jahmey P.A. (2015) Physical chemistry and  
17 membrane properties of two phosphatidylinositol bisphosphate isomers. *Physical Chemistry*  
18 *Chemical Physics* **17**, 12608.
- 19 Takasuga S., Sasaki T. (2013) Phosphatidylinositol-3,5-bisphosphate: metabolism and  
20 physiological functions. *The Journal of Biochemistry* **154**(3), 211-218.
- 21 Takatori S., Tatematsu T., Cheng J., Matsumoto J., Akano T., Fujimoto T. (2015)  
22 Phosphatidylinositol 3,5-bisphosphate-rich membrane domains in endosomes and lysosomes.  
23 *Traffic* **17**, 154-167.
- 24 Tanaka Y., Kutsuna N., Kanazawa Y., Kondo N., Hasezawa S., Sano T. (2007) Intra-vacuolar  
25 reserves of membranes during stomatal closure: the possible role of guard cell vacuoles  
26 estimated by 3-D reconstruction. *Plant and Cell Physiology* **48**, 1159-1169.

- 1 Toriyama H., Satô S. (1968) Electrone microscope observation of the motor cell of *Mimosa*  
2 *pudica* L. II On the content of the central vacuole of the motor cell. *Proceedings of the Japan*  
3 *Academy* **44**(9), 949-953.
- 4 Trevisan Scorza L.C., Carnier Dornelas M. (2011) Plants on the move: Towards common  
5 mechanisms governing mechanically-induced plant movements. *Plant Signaling and Behavior*  
6 **6**(2), 1979-1986.
- 7 Ueda M., Tsutsumi N., Fujimoto M. (2016) Salt stress induces internalization of plasma  
8 membrane aquaporin into the vacuole in *Arabidopsis thaliana*. *Biochemical and Biophysical*  
9 *Research Communications* **474**(4), 742-746.
- 10 Uemura T., Yoshimura S.H., Takeyasu K., Sato M.H. (2002) Vacuolar membrane dynamics  
11 revealed by GFP-AtVam3p fusion protein. *Gene to Cells* **7**, 743-753.
- 12 van Gisbergen P.A.C., Li M., Wu S.-Z., Bezanilla M. (2012) Class II formin targeting to the cell  
13 cortex by binding PI(3,5)P<sub>2</sub> is essential for polarized growth. *The Journal of Cell Biology* **198**(2),  
14 235-250.
- 15 Verbelen J.-P., Tao W. (1998) Mobile arrays of vacuole ripples are common in plant cells. *Plant*  
16 *Cell Reports* **17**, 917-920.
- 17 Vitali V., Sutka M., Amodeo G., Chara O., Ozu M. (2016) The water to solute permeability ratio  
18 governs the osmotic volume dynamics in beetroot vacuoles. *Frontiers in Plant Science* **7**, 1388.
- 19 Wang X., Zhang X., Dong X.-p., Samie M., Li X., Cheng X., Goschka A., Shen D., Zhou Y.,  
20 Harlow J., Zhu M.X., Clapham D.E., Ren D., Xu H. (2012) TPC are phosphoinositide-activated  
21 sodium-selective ion channels in endosomes and lysosomes. *Cell* **151**, 372-383.
- 22 Whiteford C.C., Brearley C.A., Ulug E.T. (1997) Phosphatidylinositol 3,5-bisphosphate defines  
23 a novel PI 3-kinase pathway in resting mouse fibroblasts. *Biochemical Journal* **323**, 597-601.
- 24 Whitley P., Hinz S., Doughty J. (2009) *Arabidopsis* FAB1/PIKfyve proteins are essential for  
25 development of viable pollen. *Plant Physiology* **151**, 1812-1822.

- 1 Wiltshire E.J., Collings D.A. (2009) New dynamics in an old friend: dynamic tubular vacuoles  
2 radiate through the cortical cytoplasm of red onion epidermal cells. *Plant and Cell Physiology*  
3 **50**(10), 1826-1839.
- 4 Yamaguchi T., Aharon G.S., Sottosanto J.B., Blumwald E. (2005) Vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter  
5 cation selectivity is regulated by calmodulin from within the vacuole in a Ca<sup>2+</sup> and pH-dependent  
6 manner. *Proceedures of the National Academy of U.S.A.* **102**(44),16107-16112.
- 7 Yamamoto Y., Nishimura M., Hara-Nishimura I., Noguchi T. (2003) Behavior of vacuoles  
8 during microspore and pollen development in *Arabidopsis thaliana*. *Plant and Cell Physiology*  
9 **44**(11), 1192-1201.
- 10 Zelazny E., Santambrogio M., Gaude T. (2013) Retromer association with membranes. Plants  
11 have their own rules! *Plant Signaling and Behavior* **8**, e25312
- 12 Zhang C., Hicks G.R., Raikhel N.V. (2014) Plant vacuolar morphology and vacuolar trafficking.  
13 *Frontiers in Plant Science* **5**, 476
- 14 Zhang C., Hicks G.R., Raikhel N.V. (2015) Molecular composition of plant vacuoles: important  
15 but less understood regulations and roles of tonoplast lipids. *Plants* **4**, 320-333.
- 16 Zieger M., Mayer A. (2012) Yeast vacuoles fragment in an asymmetrical two-phase process with  
17 distinct protein requirements. *Molecular Biology of the Cell* **23**, 3438-3449.
- 18 Zimmermann P., Hirsch-Hoffmann M., Hennig L., Gruissem W. (2004) GENEVESTIGATOR.  
19 Arabidopsis microarray database and analysis toolbox. *Plant Physiology* **136**(1), 2621-2632.
- 20 Zonia L., Munnik T. (2004) Osmotically induced cell swelling versus cell shrinking elicits  
21 specific changes in phospholipid signals in tobacco pollen tubes. *Plant Physiology* **134**, 813-823.
- 22

**Figures Legends**

**Fig. 1 Schematic representation of the proposed mechanism of vacuolar convolution**

Depicted are the transporters and organelles proposed to participate in vacuolar convolution execution. An important role in convolution is apparently played by late endosomes/prevacuolar compartments (LE/PVC) containing the PtdIns(3,5)P<sub>2</sub>-synthesizing enzymes of the FAB1 family. In this scheme a localized increase of PtdIns(3,5)P<sub>2</sub> at tonoplast invaginations is postulated. It is hypothesized that this increase inhibits the activity of vacuolar NHXs exchangers; at the same time, PtdIns(3,5)P<sub>2</sub> rise apparently activates the V-ATPase. The cytosolic pH increase provoked by the inhibition of the NHXs exchangers and the activation of the V-ATPase mediates an increase in the water-to-solute permeability, during which solutes flow largely affects the vacuolar volume. A cytosolic Ca<sup>2+</sup> increase, often preceding convolution, activates the TPK channels which mediates the large K<sup>+</sup> efflux characteristic for convolution. In dotted lines are depicted the FAB1 enzymes which can be either internalized or recycled to stay at the endosomes. See details and supporting evidences for this hypothetical mechanism in the text.

1

**Table 1. Properties of plant FAB1 family kinases**

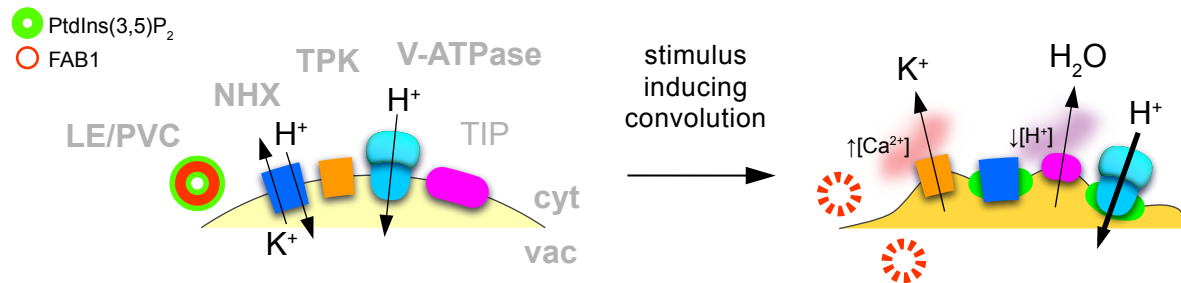
Name	Gene	Comments	Main subcellular localization*	Deficiency phenotype**	References
FAB1A	AT4G33240	Contain a FYVE domain required for binding to PtdIns(3,5)P2-containing membranes in yeasts.	Endosomal	Defects in vacuolar biogenesis Curly leaf phenotype Root growth inhibition Reduced sensitivity to exogenous auxin	Whitley et al., 2009 Hirano et al., 2011 Serrazina et al., 2014
FAB1B	AT3G14270			Impaired root gravitropism Double mutant is infertile (unviable pollen)	Hirano et al., 2017b
FAB1C	AT1G71010	Negligible expression in pollen Lack the FYVE domain	ND	Slow stomatal closure and fast water loss in double <i>fab1b-fab1c</i> mutant	Whitley et al., 2009
FAB1D	AT1G34260	Not expressed in guard cells Predominantly expressed in pollen Lack the FYVE domain	Cytosol and sperm cells in pollen	Indistinguishable from WT plants Abnormal pollen tube growth and osmoregulation in double <i>fab1b-fab1d</i> mutant	Whitley et al., 2009 Serrazina et al., 2014

2 \* Localization of FAB1 kinases in the cited references in some cases differ from that observed in microarrays,  
3 according to The Arabidopsis Information Resource (TAIR, <http://www.arabidopsis.org/>). Tissue expression  
4 levels of FAB1 kinases can be consulted in the TAIR database, and in the GENEVESTIGATOR platform  
5 (Zimmerman et al., 2004; cited in Whitley et al., 2009).

6 \*\* Some of the cited references present information only on particular aspects of FAB1 deficiency phenotypes.

7

# Fig.1



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