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Connecting vacuolar and plasma membrane transport networks

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Summary

The coordinated control of ion transport across the two major membranes of differentiated plant cells, the plasma and the vacuolar membranes, is fundamental in cell physiology. The stomata responses to the fluctuating environmental conditions are an illustrative example. Indeed, they rely on the coordination of ion fluxes between the different cell compartments. The cytosolic environment, that is an interface between intracellular compartments, and the activity of the ion transporters localized in the different membranes influence one each other. Here we analyse the molecular mechanisms connecting and modulating the transport processes at both the plasma and the vacuolar membranes of guard cells.

Key words: flux coordination, intracellular network, cytosolic conditions, guard cells, ion channels, stomata, transporters, vacuole.

Boxes

Box 1 Driving forces in transport reactions

The electrochemical potential difference ($\Delta\mu_i$) for an ion i between two sides of a membrane defines the direction and the limits of ion transport reactions across membranes. $\Delta\mu_i$ combines the chemical and the electrical potentials of ion i and is defined by:

$$\Delta\mu_i = R \cdot T \cdot \ln \frac{[i]_{cyt}}{[i]_{out}} + z_i \cdot F \cdot (V_{cyt} - V_{out}) \quad \text{Eqn 1}$$

In the following equations we outline the driving force of the major types of transport reactions in cells: passive transport (ion channels), primary active transport (pumps) and secondary active transport (antiporters and symporters).

For ion channels transporting ion i , the electrochemical potential $\Delta\mu_i$ coincides with the driving force of the transport reaction ($\Delta\mu_{channel}$):

$$i_{out} \xrightleftharpoons{channel} i_{cyt}$$

$$= \Delta\mu_i = R \cdot T \cdot \ln \frac{[i]_{cyt}}{[i]_{out}} + z_i \cdot F \cdot (V_{cyt} - V_{out}) \quad \text{Eqn 2}$$

For pumps that catalyse the transport of n ion i against $\Delta\mu_i$ coupling it to ATP hydrolysis, the driving force of the transport reaction ($\Delta\mu_{pump}$) is:

$$n i_{cyt} + ATP \xrightleftharpoons{pump} n i_{out} + ADP + P_i$$

$$\Delta\mu_{pump} = n\Delta\mu_i + \Delta\mu_{ATP} =$$

$$= R \cdot T \cdot \ln \left[\frac{ADP \cdot P_i}{ATP} \cdot \frac{[i]_{out}^n}{[i]_{cyt}^n} \right] - n \cdot z_i \cdot F \cdot (V_{cyt} - V_{out}) \quad \text{Eqn 3}$$

For an antiporter that catalyse the exchange of n ions i and m ions j , the driving force ($\Delta\mu_{antiporter}$) of the transport reaction is defined as:

$$n \cdot i_{out} + m \cdot j_{cyt} \xrightleftharpoons{antiporter} n \cdot i_{cyt} + m \cdot j_{out}$$

$$\Delta\mu_{antiporter} = n\Delta\mu_i - m\Delta\mu_j =$$

$$= R \cdot T \cdot \ln \left[\frac{[i]_{cyt}^n \cdot [j]_{out}^m}{[i]_{out}^n \cdot [j]_{cyt}^m} \right] + (n \cdot z_i - m \cdot z_j) \cdot F \cdot (V_{cyt} - V_{out}) \quad \text{Eqn 4}$$

For a symporters mediating the co-transport of n ions i and m ions j , the driving force of the transport reaction ($\Delta\mu_{\text{symporter}}$) is defined as:

$$n \cdot i_{\text{out}} + m \cdot j_{\text{out}} \xrightleftharpoons{\text{symporter}} n \cdot i_{\text{cyt}} + m \cdot j_{\text{cyt}}$$

$$\Delta\mu_{\text{symporter}} = n\Delta\mu_i + m\Delta\mu_j =$$

$$= R \cdot T \cdot \ln \left[\frac{[i]_{\text{cyt}}^n \cdot [j]_{\text{cyt}}^m}{[i]_{\text{out}}^n \cdot [j]_{\text{out}}^m} \right] + (n \cdot z_i + m \cdot z_j) \cdot F \cdot (V_{\text{cyt}} - V_{\text{out}}) \quad \text{Eqn 5}$$

In all equations R is the gas constant, T is the absolute temperature, z_{ij} is the charge of the ion i or j , F is the Faraday's constant, $V_{\text{cyt}} - V_{\text{out}}$ is the membrane potential difference between the cytosol (cyt) and outside (out), $[i, j]_{\text{cyt}}$ is the cytosolic (cyt) concentration of the ion i or j , $[i, j]_{\text{out}}$ is the concentration of the ion i or j outside (out), and n and m are the stoichiometric coefficients of ions i and j , respectively. The outside (out) corresponds to the apoplast, to the vacuolar lumen or to the lumen of other compartments.

Box 2 Abbreviations

AKT1	Arabidopsis K ⁺ Transporter 1
ALMT	ALuminum Activated Malate Transporter
CBL	Calcineurin-B-Like protein
CIPK	CBL-Interacting Protein Kinase
GC	Guard Cell
GORK	Guard cell Outward Rectifying K ⁺ channel
HAK5	High Affinity K ⁺ Transporter 5
KAT1	K ⁺ channel in Arabidopsis Thaliana 1
KIN7	Kinase 7
MAPK	Mitogen-Activated Protein Kinases
NHX	Na ⁺ /H ⁺ eXchanger
NRT1.1	NitRate Transporter 1.1
OSCA	Reduced hyperosmolality-induced [Ca ²⁺] _i increase
OST1/SnRK2.6	Open STomata 1/ Snf1 Related protein Kinase type-2.6

PIP2;1	Plasma membrane Intrinsic Protein 2;1
PM	Plasma Membrane
PP2C	Protein Phosphatase type 2C
PYR/PYL/RCAR	PYrabactin Resistance/ PYR1-Like/ Regulatory Component of ABA Receptors
SLAC1	SLOW Anion Channel-associated 1
SLAH3	SLAC1-Homologue 3
TPC1	Two Pore Channel 1
TPK1	Two-Pore K ⁺ channel 1
VM	Vacuolar Membrane

I. Introduction

The vacuole occupies the major part of the cellular volume, up to 90% in differentiated plant cells (Krüger & Schumacher, 2018). It is an acidic compartment presenting dynamic morphology, composition and volume. The characteristics of the vacuole change during plant cell development and cellular responses to the environment (Martinoia *et al.*, 2012), making the plasticity of the vacuole an essential property of this organelle. Given its size, in differentiated cells the vacuole is a key player in the building of the turgor pressure and is part of the intracellular signalling network (Martinoia *et al.*, 2012; Peiter, 2011; Roelfsema & Hedrich, 2005). Vacuolar functions are intimately linked to the pool of transport systems residing in the vacuolar membrane (VM) and transporting a large diversity of molecules (nutrients, metals, metabolites, sugars, peptides) (Martinoia *et al.*, 2012).

The vacuolar functions are important for the adaptation of plants to their environment. For example, during exposure to high salt or heavy metals several plants accumulate toxic elements in the vacuole to preserve metabolic active compartments (Martinoia, 2018). Interestingly, the VM is also involved in the translocation mechanisms of toxic species between plant organs (Baetz *et al.*, 2016; Cosio *et al.*, 2004; Ma *et al.*, 2005; Thomine *et al.*, 2003). In petunia flowers, specific proton pumps in the VM of petal cells generate extremely acidic vacuolar pHs influencing the colour of flowers (Faraco *et al.*, 2014; Verweij *et al.*, 2008). The VM is also fundamental for the activity of the specialized motor cells generating seismonastic leaf movements of *Mimosa pudica* pulvini (Fleurat-Lessard *et al.*, 1997a,b; Hagihara & Toyota, 2020). Finally, the VM transporters

participate in the control of stomata aperture in guard cells (Martinoia, 2018). In these cells, the morphological changes of the vacuole during stomata movements are linked to the transport activity of the VM (Andrés *et al.*, 2014; Tanaka *et al.*, 2007) (Fig. 1b).

The examples listed above demonstrate the importance of the specialised transport capacities of the vacuole in plant cell physiology. However, for proper cellular responses the VM needs to work in concert with the other cell membranes delimiting intracellular compartments. Thus, the processes occurring at the VM and other cellular membranes are interconnected and coordinated. Given its size, the VM is a central element of the intracellular transport network, and the vacuolar transport processes must be considered integrated in a network of fluxes mutually influencing each other.

II. Vacuolar and plasma membrane transport in guard cells, a team work

Guard cells (GC) perfectly illustrate the physiological interconnection of the vacuole with the other cellular membranes. Indeed, in guard cells massive fluxes of ions and sugars across the VM and the plasma membrane (PM) control intracellular turgor pressure in response to environmental stimuli (*i.e.* light, drought, CO₂, pathogens). The rapid modification of the turgor pressure relies on the movement of ions from the apoplast to the cytosol and then to the vacuole (stomata opening), and from the vacuole to the cytosol and then to the apoplast (stomata closure; Fig. 1) (Roelfsema & Hedrich, 2005). These movements of ions induce the swelling and shrinking of guard cells for opening and closing stomata, respectively (Fig. 1). But how many molecules of solutes cross the PM and VM during stomata opening/closure? To estimate this, we used data from *Vicia faba*. To drive stomata opening a net raise of 4-5 pmol of ionic solutes per guard cell, mainly K⁺, Cl⁻, NO₃⁻ or malate²⁻, is necessary (Allaway & Hsiao, 1973). This raise of solutes induces an increase of the total intracellular concentration between 0.8 and 1 M in open stomata. Considering an opening time of 60 minutes and a *V. faba* guard cell surface of 1899 μm² (Meckel *et al.*, 2007), a net entry of up to $\sim 7 \times 10^8$ molecules·s⁻¹/GC across the PM and then across the VM can be calculated (Fig. 1a). When stomata close, ionic solutes leave the cell in about 20 minutes generating a net flux of $\sim 2 \times 10^9$ molecules·s⁻¹/GC firstly across the VM and then across the PM (Fig. 1a). If the VM and the PM were not coordinated and molecules could not enter the vacuole, the cytosolic concentration would increase of up to 150 mM of solutes·minute⁻¹. The latter is an extreme and hypothetical situation but it illustrates the importance of flux coordination between membranes.

Overall, these estimations provide the order of magnitude of the ion fluxes occurring in a guard cell.

Therefore, to avoid aberrant ionic concentrations in the cytosol and to generate coherent responses, the transport systems residing in both the VM and the PM of guard cells are co-regulated (Fig. 1). The coordination of the ion fluxes through the PM and the VM was suggested in seminal work in the 90s (summarized in MacRobbie, 2000). But which are the mechanisms allowing coordination? How fluxes of ions are coordinated between the PM and the VM? The transport properties of cellular membranes can be modified by changing the activity of transporters or by acting on the pool of transporters residing in a membrane. To coordinate the fluxes, a first level is the regulation of the solute concentrations in the cytosol; a second level is through signalling pathways targeting the transport systems residing in the different cellular membranes. These two levels will be discussed in the following sections with a special focus on the model plant *Arabidopsis thaliana*.

III. Ion flux coordination under the control of ionic conditions in the cytosol

1. Cytosolic ion concentration, a straightforward way to coordinate fluxes between membranes

The subcellular organisation of guard cells makes the cytosol a thin layer between two large membranes, the PM and VM. Since in open stomata the vacuole occupies nearly 90% of the whole cellular volume (Fig. 1b) the majority of the solutes crossing the PM will also cross the VM during opening. Therefore, the cytosolic compartment, which accounts for only a fraction of the cell, undergoes this flux of solutes (Fig. 1b). Thus, during stomatal movements ionic concentrations in the cytosol are likely to vary, and *in silico* modelling of guard cells shows such changes (Blatt *et al.*, 2014; Chen *et al.*, 2012; Wang *et al.*, 2012). Recent *in vivo* data demonstrate that intracellular transport systems residing in the VM act on cytosolic ion homeostasis in guard cells, influencing pH and $[\text{NO}_3^-]$ (Demes *et al.*, 2020). Changes of the cytosolic concentrations of each ionic species i modifies its electrochemical potential difference ($\Delta\mu_i$) between the two sides of the cellular membranes facing the cytosol. Such modifications can influence the different types of transport reactions across the different membranes involving ion i (Box.1). Thus, $\Delta\mu_i$, together with the kinetic properties of the ion transporters, impacts on ion fluxes across cellular membranes, and changes of cytosolic ion concentrations modify the movement of ions across the membranes facing the cytosol (Horaruang *et al.*, 2020; Wang *et al.*, 2017). Therefore, changes of the ionic

conditions in the cytosol participate in the coordination of ion fluxes between cellular membranes (Fig. 1b).

2. Simultaneous regulation of VM and PM transport systems by cytosolic ions

Some ions and metabolites emerge as elements co-regulating solute transport across intracellular membranes (Fig. 2; Box 2). A first ‘classic’ candidate is Ca^{2+} . Although we still miss the genetic identity of Ca^{2+} transport systems in plants, the occurrence of cytosolic Ca^{2+} variations induced by environmental stimuli is well established (Jezek & Blatt, 2017; Konrad *et al.*, 2018). Recently, a family of channels, the OSCA, was found to be PM ion channels involved in Ca^{2+} signalling (Thor *et al.*, 2020; Yuan *et al.*, 2014). Interestingly, the OSCA channels are permeable to Ca^{2+} but also to a similar extent to Na^+ (Thor *et al.*, 2020). The VM harbours different ion transport systems sensitive to cytosolic Ca^{2+} . TPC1 is a vacuolar channel permeable to K^+ , and to a lower extent to Ca^{2+} , which is activated by cytosolic Ca^{2+} (Fig. 2) (Hedrich & Marten, 2011). One function of TPC1 seems to be linked to Ca^{2+} signalling (Vincent *et al.*, 2017), and recent data show that TPC1 regulates the VM excitability and in this way it could modulate Ca^{2+} signalling (Jašlan *et al.*, 2019). Additionally, a vacuolar anion channel permeable to malate²⁻, ALMT6, was identified to be activated by Ca^{2+} (Fig. 2) (Meyer *et al.*, 2011). In the PM, ion channels like SLAC1, SLAH3, GORK and AKT1, the K^+/H^+ symporter HAK5, and the NRT1.1 transporter are regulated by Ca^{2+} through interaction with the Ca^{2+} -dependent CBL-CIPK kinase complexes (Fig. 2) (reviewed in Kim *et al.*, 2010; Saito & Uozumi, 2019; Tang *et al.*, 2020a). Regarding the VM, CIPK9/7 with CBL2/3 were proposed to regulate the activity of the VM K^+ transporters NHX1 and NHX2 (Fig. 2) (Song *et al.*, 2018). Recently, it has been shown that modules involving CBL2/3 and CIPK3/9/23/26 activate the VM K^+ channel TPK1, leading to K^+ efflux to the cytosol (Fig. 2) (Tang *et al.*, 2020b). In the future a critical step to decipher the role of Ca^{2+} will be to identify the molecular actors mediating its fluxes in plant cells.

Nucleotides, like ATP, also modify the activity of ion transporters in both the PM and the VM (Fig. 2). ATP is the source of energy of the H^+ -ATPase pumps as P- and V-type H^+ pumps in the PM and the VM, respectively. Additionally, ATP negatively regulates the activity of the VM anion/ H^+ exchanger CLCa (De Angeli *et al.*, 2009) and of the VM anion channel ALMT9 (Fig. 2) (Zhang *et al.*, 2014). In the PM, ATP blocks Rapid-type anion currents (Frachisse *et al.*, 1999) that, in guard cells, they are mediated by ALMT12 (Fig. 2) (Meyer *et al.*, 2010). A relevant aspect

of the role of nucleotides like ATP, ADP or AMP is their potential to coordinate ion fluxes with the energetic status of the cell.

Recently, malate emerge as a regulator of ion transport systems in both the VM and the PM. Cytosolic malate concentration depends on the starch degradation/synthesis cycle, on the metabolic consumption, on the vacuolar stocks and on its transport from the apoplast (Santelia & Lawson, 2016). In the last years it was found that the vacuolar anion channels ALMT4 (Eisenach *et al.*, 2017) and ALMT9 (De Angeli *et al.*, 2013) are directly activated by cytosolic malate (Fig. 2). In the PM SLAC1 (Wang & Blatt, 2011; Wang *et al.*, 2018) and ALMT12 (Meyer *et al.*, 2010) channels are also regulated by malate (Fig. 2). These data open to the possibility that cytosolic malate plays a role in the coordination of ion fluxes between both membranes during the opening and closure of stomata.

IV. Kinases and phosphatases targeting vacuolar and plasma membrane transporters

PM ion channels and transporters are target of kinases and phosphatases (Kim *et al.*, 2010; Saito & Uozumi, 2019). Less is known on the VM side. Nonetheless, signalling pathways targeting vacuolar ion transporters and channels are emerging (Carpaneto *et al.*, 2017; Eisenach *et al.*, 2017; Wege *et al.*, 2014). The signalling pathway responsible of ABA-induced stomata closure is an illustrative example (Fig. 2). In the last decade, the identification of the PYR/PYL/RCAR ABA receptors was a considerable advance (Ma *et al.*, 2009; Park *et al.*, 2009). PYR/PYL/RCAR interaction with ABA is the starting point of the signalling cascade inducing stomata closure (Kim *et al.*, 2010). Interestingly PYR/PYL/RCAR receptors reside in the cytosol as soluble proteins (Fig. 2) (Ma *et al.*, 2009). The cytosolic localisation of PYR/PYL/RCAR receptors is conceptually intriguing. It suggests that the PM and the VM events taking place during ABA response are not hierarchically organised in time and space. In other words, the signalling cascade starts in the cytosol with ABA binding to PYR/PYL/RCAR and is then targeting transport systems in the PM and the VM coordinating the release of ions from the vacuole to the apoplast. The ABA-PYR/PYL/RCAR complex inhibits a PP2C phosphatase, and this leads to the activation of the cytosolic kinase OST1/SnRK2.6 (Fig. 2) (Lee *et al.*, 2013; Park *et al.*, 2009). This kinase is central in ABA response targeting and activating, among others, the PM anion channels SLAC1 (Geiger *et al.*, 2009; Lee *et al.*, 2009) and ALMT12 (Imes *et al.*, 2013), the aquaporin PIP2;1 (Grondin *et al.*, 2015) as well as the VM anion/H⁺ exchanger CLCa (Fig. 2) (Wege *et al.*, 2014). The activation of these ion transport systems residing in the VM and PM is required for the release of

ions and water to close stomata (Fig. 1b). In addition, OST1 inhibits inward K⁺ currents mediated by KAT1 through protein interaction (Fig 2) (Acharya *et al.*, 2013). Besides OST1/SnRK2.6, other kinase proteins were found to regulate the activity of PM and VM ion transport systems. Recently, the vacuolar K⁺ channel TPK1 was shown to be the target of the kinase KIN7 (Isner *et al.*, 2018). This kinase phosphorylates TPK1 and seems to participate in ABA and CO₂ signalling (Fig. 2) (Gobert *et al.*, 2007; Isner *et al.*, 2018). Interestingly, KIN7 is localised in both the PM and the VM (Isner *et al.*, 2018). Although its role in the PM is unknown, the dual localisation suggests that it could regulate transport systems in both membranes. Finally, MAPK were also found to target ion channels (Fig. 2) (Lee *et al.*, 2016). A phosphorylation site targeted by MAPK was identified in the vacuolar ion channel ALMT4 (Eisenach *et al.*, 2017). It was shown that the phosphorylation of ALMT4 inhibits its ion transport activity and that it is involved in ABA-induced stomata closure (Fig. 2) (Eisenach *et al.*, 2017). Apart from SLAC1 (Fig. 2) (Prodhan *et al.*, 2018), no target of MAPKs in the PM have been identified so far.

V. Conclusions and perspectives

The vacuole presents a high functional plasticity and is involved in a multitude of cellular processes. The transporters fluxing molecules across the VM determine the specialized functions of the vacuole in plant cells. In the last decade, the knowledge on the molecular identity of the vacuolar transport systems has considerably expanded. Currently, a consistent number of proteins forming transports systems in the VM is known, highlighting the role of the vacuolar fluxes in plants (Eisenach & De Angeli, 2017; Martinoia *et al.*, 2012; Zhang *et al.*, 2017). Based on this knowledge, a major step will be to decipher how vacuolar transport is integrated within the cell. Only some flux studies on *Commelina communis* L. using ⁸⁶Rb⁺ as a tracer investigated simultaneously ion fluxes across the PM and the VM (summarized in MacRobbie, 2000). Otherwise, the transport processes occurring in the VM and in other membranes, such as the PM, have been considered separately. Compared with the PM, only a restricted number of regulatory mechanisms targeting the VM transport systems is known. An integrative perspective on the intracellular transport reactions comes from *in silico* modelling, but only few data are available *in vivo*. Recently, *in vivo* data demonstrated the impact of ion transport on cytosolic conditions and connected it with stomatal aperture (Demes *et al.*, 2020). Therefore, a future challenge will be to visualise and decipher, in living cells, the connection between transport systems residing in different membranes during cellular responses.

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Figure Legends

Fig. 1 The coordination of the fluxes of solutes across the vacuolar and plasma membranes is required to control stomata movements. (a) Opening (blue) and closure (red) of the stomata are based on rapid changes of the intracellular osmotic pressure (Π) leading to water entry/release into/from the guard cell (GC). In *Vicia faba* GC, during stomata opening ionic solutes raise of ~ 4 pmol, increases the intracellular concentration of solutes of ~ 0.8 M. J_T is the total flux of solutes between the apoplast and the GC, that is majorly composed of anions (J_{A^-}) and of cations (J_{C^+}) fluxes. During closure, the total flux of ionic solutes (J_T) from the GC to the apoplast is $\sim 2 \times 10^9$ molecules \cdot s $^{-1}$ /GC. (b) Osmotically active solutes move from the apoplast to the vacuole (opening, blue) and from the vacuole to the apoplast (closure, red). In GCs, the major osmotically active solutes are cations (C^+) like K^+ , anions (A^-) like Cl^- , NO_3^- and malate $^{2-}$, and sugars. To reach the vacuole/apoplast, solutes need to cross both the plasma (PM) and vacuolar (VM) membranes (*insets*). The cytosol faces both the PM and the VM, thus the cytosolic concentration of ions

influences ion fluxes across both the VM and the PM. Notably, during stomata opening and closure the vacuole undergoes morphological changes and modifications of its relative volume.

Fig. 2 Identified mechanisms co-regulating ion transport systems in the vacuolar membrane (VM) and plasma membrane (PM). Cytosolic Ca^{2+} raise induces the activation of different CBL/CIPK kinase complexes that activates the PM anion channels SLAC1, SLAH3, the potassium (K^+) channels AKT1 and GORK, and the K^+/H^+ symporter HAK5. In the VM, CBL/CIPK target the K^+ exchangers NHX1 and NHX2 and the K^+ channel TPK1. Cytosolic Ca^{2+} can also directly interact and activate vacuolar channels like TPC1 and ALMT6. The ABA signalling induces phosphorylation by OST1 of the PM channels SLAC1, KAT1 and ALMT12, the PIP2;1 aquaporin, and of the VM anion/proton exchangers CLCa. ABA signalling also acts on the vacuolar exchanger CLCc by an unknown pathway, and on the K^+ channel TPK1 through KIN7 kinase. MAPK kinases activate SLAC1 in the PM and inhibit ALMT4 in the VM. Several cytosolic molecules induce the activation/inhibition of ion transporters like ALMT9, CLCa, and H^+ pumps. Malate activates the anion channels ALMT4 and ALMT9 in the VM, and ALMT12 and SLAC1 in the PM. ATP is the substrate for the pumping activity of the H^+ ATPases and is a negative regulator of anion channels in the PM and VM.

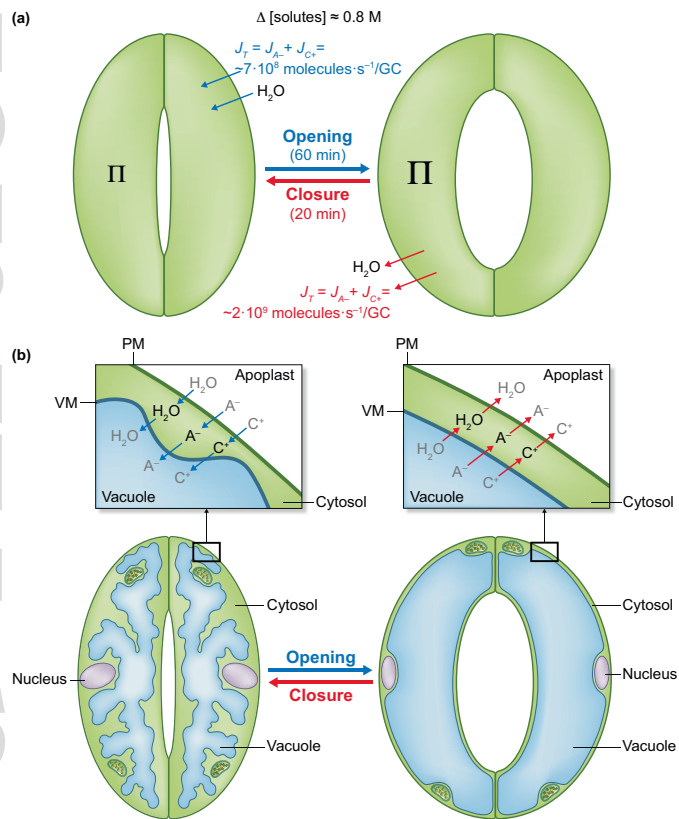


Figure 1
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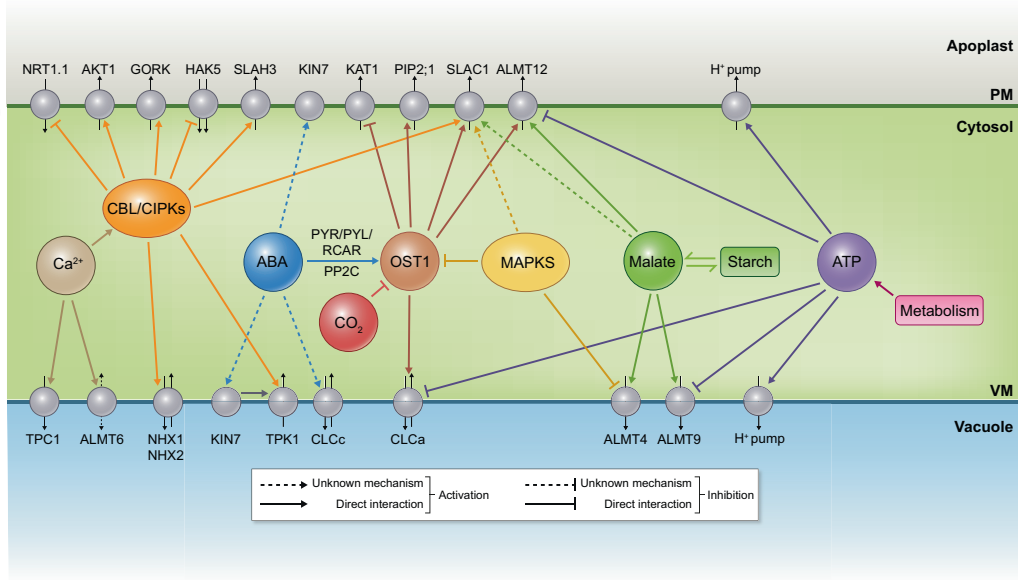


Figure 2

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