Highlights

- Modulation of PM H^+-ATPase activity plays a critical role in plant physiology.
- The acid growth theory has received additional support from hormonal studies focused on regulation of PM H^+-ATPase. Thus, BRs, auxin and ABA regulate the PM H^+-ATPase activity by phosphorylation/dephosphorylation of the penultimate residue, Thr
- BRs stimulate hypocotyl elongation and induce phosphorylation of this residue through either direct interaction of PM H^+-ATPase with BRI1 or as a result of BR signalling downstream of BRI1.
- Low ABA concentrations relieve the ABI1-dependent inhibition of PM H^+-ATPase activity and stimulate root growth.
- Auxin rapidly induces the interaction of the cell surface-located TMK1 with PM H^+-ATPase and the phosphorylation of its penultimate Thr residue by TMK1.
- BRs- and auxin-induced SAUR proteins inhibit clade D PP2Cs, which prevents dephosphorylation of this Thr residue.

Tripartite hormonal regulation of plasma membrane H^+-ATPase activity

Rui Miao[a, *], Eugenia Russinova[b,c], Pedro L. Rodriguez[d, *]

[a]College of Life Sciences, Fujian Agriculture & Forestry University, Jinshan Fuzhou 350002, China

[b]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent, Belgium.

[c]Center for Plant Systems Biology, VIB, Ghent, Belgium

[d]Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Cientificas-Universidad Politecnica de Valencia, ES-46022 Valencia, Spain

[*]Correspondence: ruimiao@fafu.edu.cn, prodriguez@ibmcp.upv.es

Keywords: hypocotyl elongation, root growth and hydrotropism, acid-growth theory, clade A and D PP2Cs, phosphorylation, TMK, BRI1, ABI1, PM H^+-ATPase

Abstract

The enzyme activity of the plasma membrane (PM) proton pump, well known as Arabidopsis PM H^+-ATPase (AHA) in the model plant arabidopsis (*Arabidopsis thaliana*), is controlled by phosphorylation. Three different classes of phytohormones, brassinosteroids, abscisic acid and auxin, regulate plant growth and responses to environmental stimuli at least in part by modulating the activity of the pump through phosphorylation of the penultimate threonine residue in its carboxyl-terminus. Here, we review the current knowledge regarding this tripartite hormonal AHA regulation and highlight mechanisms of activation and deactivation as well as the significance of hormonal crosstalk. Understanding the complexity of PM H^+-ATPases regulation in plants might provide new strategies for sustainable agriculture.

The PM H^+-ATPase and its regulation in plants

Environmental cues influence plant growth and development by eliciting signalling networks together with phytohormones to balance plant growth and stress responses. Due to global climate changes, rainfed crop farming is at risk, facing challenging periods of alternating floods and severe droughts. Recent advances in plant biology have provided molecular approaches to alleviate the environmental impact on crop productivity. For example, overexpression of the PM H^+-ATPase improved overall nitrogen and carbon utilization in rice [1]

The PM H^+-ATPase, a ~100 kDa nanomachine of plants and fungi, belongs to the family of P-type ATPases [2]. AHAs play a central role in cell physiology through regulation of pH homeostasis and generation of proton motive force that drives transport across PM, which influence cell volume and expansion [3-7]. P-type ATPases receive their name because their reaction cycle involves a covalent phosphorylated intermediate, whereas F, V and ABC ATPases do not involve such intermediate [8]. The PM H^+-ATPase family in arabidopsis, which includes AHA1 to AHA11, belongs to the P3-ATPase subfamily, part of the large P1 to P5-type ATPase superfamily of cation pumps and lipid flippases that overall comprises 48 members in arabidopsis [2, 9]. In eukaryotes, transport processes are energized by electrochemical gradients across PM, either generated by the PM H^+-ATPase in plants and fungi (electrochemical gradient of protons, acidic outside) or by the Na^+, K^+-ATPase in animals (sodium-potassium pump that exports three Na^+ and imports two K^+) [10]. The PM H^+-ATPase is an electrogenic enzyme since it extrudes positive charges and forms a membrane potential that may exceed -200 mV (negative inside) in plant cells [10]. Roots absorb ions and nutrients using the membrane electrochemical gradient at the periphery and endodermal cell layers [11]. Additionally, carbohydrate translocation from the source to the sink organs is also dependent on it [12].

Posttranslational modifications of the PM H^+-ATPase, in particularly phosphorylation of several threonine (Thr) and serine (Ser) residues within the C-terminal R domain, negatively or positively affect its activity [5, 10, 13]. For example, AHA2 activity was up regulated after Thr^{881} phosphorylation, whereas the activity was down regulated after Ser^{899} or Ser^{931} phosphorylation [13]. In this review, we will focus on recent advances that link the plant hormones, brassinosteroids (BRs), abscisic acid (ABA) and auxin with the phosphorylation of

the penultimate Thr residue (Thr^{947} in the model pump AHA2). Moreover, these findings provide support to the acid growth theory (**BOX 1**), which was originally based on auxin-mediated activation of PM H^+-ATPase [14] and currently has been updated including the effects of BRs and ABA, as well as recent breakthroughs in auxin signalling [15, 16]. Thus, in response to these hormones, cell wall extensibility is increased and turgor pressure is maintained (reviewed for auxin by Du *et al*. [17]), which enable cell expansion (asymmetrically in some cell contexts) and growth. Remarkably, auxin also promotes H^+ influx through an unknown mechanism that inhibits root growth [15].

Structural Characteristics of the PM H^+-ATPase

The PM H^+-ATPase consists of five domains, i.e. a membrane-embedded region comprised of 10 membrane-spanning α-helices (M1–M10) and four major cytoplasmic domains named A, P, N and R domains (described below) (Figure 1). Briefly, the A domain encompasses the amino (N)-terminal segment; the P domain contains the invariant Asp residue, transiently phosphorylated as the hallmark of P-type ATPases and located within the conserved DKTGTLT sequence motif; the N domain for binding of ATP and the C-terminal R domain, so-called C-terminal autoinhibitory domain, consisting of approximately 100 amino acid residues. Phosphorylation of the penultimate Thr residue generates a high-affinity binding site for 14-3-3 proteins, whose binding abolishes the inhibitory interaction of the R-domain with its receptor site in the rest of the pump [18, 19]. The addition of glucose to yeast or stimulation by blue-light in plant guard cells, leads to phosphorylation of the R-domain and activation of the PM H^+-ATPase [10].

A crystal structure has been reported for a P-type plant proton pump, the arabidopsis AHA2 [20]. This structure represents an active form of the proton pump without its auto-inhibitory domain because no electronic density was observed for this domain, indicating that the R domain lacks defined structure in the active form of the PM H^+-ATPase [20]. The R domain is likely to interact with some regions of the pump and to inhibit its enzyme activity. For example, the R domain might potentially block the entry of protons to the transmembrane segments and restrict A domain function [10, 20].

Fusicoccin (FC) is a fungal metabolite known to mimic some of the physiological effects of auxin and its effect in plants can be explained by activation of the PM H^+-ATPase [21-23]. 14-3-3 proteins associate with plant PM H^+-ATPase to generate an FC binding complex that results in pump activation [22, 23]. Thus, FC stabilizes the association between the PM H^+-ATPase and the 14-3-3 protein; in other words, FC can induce binding of the 14-3-3 protein to the PM H^+-ATPase in the absence of Thr^{947} phosphorylation [23]. The crystal structure of 14-3-3 protein in complex with the entire 14-3-3 binding motif of a *N. benthamiana* PM H^+-ATPase (PMA2) and FC was determined and revealed that FC treatment converted the PMA2/14-3-3 complex into a stable hexameric structure [24]. RAPID ALKALINIZATION FACTOR (RALF) peptides and the PLANT PEPTIDE CONTAINING SULFATED TYROSINE 1(PSY1) glycopeptide, perceived in the PM by different receptor kinases, are also key regulators of PM H^+-ATPase activity [25], likely establishing cross-talk with the hormonal signalling pathways that we describe next [26, 27].

Regulation of PM H^+-ATPase activity by BRs

The plant steroidal hormones BRs regulate plant growth and development by governing the essential cellular processes of division and expansion [28]. In arabidopsis, a canonical BR signalling pathway has been established from the membrane receptors to the nuclear transcription factors [29] (Figure 2). **BRI1** (see Glossary) and its three homologues, BRI1-LIKE1 (BRL1), BRL2 and BRL3 are identified as transmembrane leucine-rich repeat (LRR) type of receptor kinases [30, 31] (Figure 1). BRI1, BRL1 and BRL3 strongly bind to brassinolide (BL), the most active endogenous BR, whereas BRL2 is likely a non-functional BR receptor [31-34]. BRI1 is ubiquitously expressed, whereas BRL1 and BRL3 are expressed in non-overlapping subsets of vascular cells, suggesting that they might play key roles in different cell types [31, 35].

In the presence of BRs, each BRI1, BRL1 and BRL3 interacts with a smaller LRR receptor kinase **BAK1/SERK3** [36, 37]. BAK1 belongs to the **SERK** subfamily that includes five members, three of which, i.e. SERK1, BAK1/SERK3 and SERK4, function redundantly in BR signalling [38, 39]. Consequently, *serk1 bak1 serk4* triple mutant resembles the phenotype of

bri1 mutant, confirming the essential role for SERKs in BRs signal transduction pathway [39]. BR binding to BRI1 and BAK1/SERK3 fully activates them through autophosphorylation and transphosphorylation and initiates a well-established downstream signalling cascade [29]. The active BRI1 kinase phosphorylates conserved serine residue in several **RLCKs** including BRASSINOSTEROID-SIGNALLING KINASE1 (BSK1) (Ser230), BSK2 [40], BSK3 [41] and the **CDG1** (Ser230) [42], to subsequently enhance their interactions with a **BSU1** phosphatase [43]. Then the phosphorylated and activated BSU1 dephosphorylates the conserved tyrosine residue in the negative regulator **BIN2** (Tyr200) and inactivates it [44]. The inactive BIN2 is degraded via either the proteasome [45] or by the F-box E3 ubiquitin ligase **KIB1** that mediates BIN2 ubiquitination and subsequent degradation while also blocking BIN2-substrate interactions [46]. BIN2 degradation along with the activation of the **PP2A** [47] unable the phosphorylation of two homologous transcription factors, **BZR1** and **BZR2/BES1** [48]. Consequently, dephosphorylated BZR1 and BZR2/BES1 are translocated into the nucleus where they activate or repress BR-regulated genes [48, 49].

One of the primary outputs of BR signalling is the promotion of elongation growth [31]. BRs induce cell wall relaxation via altering the expression of cell-wall-related target genes [50, 51] and in part via the acid-growth process (**BOX 1)** as a result of post-translational control of the PM H^+-ATPase activity [52]. A recent study reported that BRs induce phosphorylation of the penultimate amino acid (threonine) of the PM H^+-ATPase, as well as binding of a 14-3-3 protein to PM H^+-ATPase, which subsequently leads to the elongation of etiolated hypocotyls in seedlings [52]. The activation of the PM H^+-ATPase required functional BR signalling as treatment with bikinin, a plant specific GSK3 inhibitor known to activate the BR signalling pathway downstream of BRI1 by inhibiting the negative regulator BIN2, enhanced the phosphorylation level of the PM H^+-ATPase penultimate residue in the *bri1* mutant [52]. A model was proposed where BRs upregulate the expressions of ***SAUR9*** and *SAUR19* via the BRI1-BIN2 signalling pathway. The SAUR proteins suppress the activity of several **PP2C-D**, which dephosphorylate the phosphorylated penultimate residue in the C-terminus of PM H^+-ATPases [53] (Figure 2).

Besides the importance of the canonical BR signalling pathway in ATPase activation [52], a faster PM H^+-ATPase-dependent response to BRs leading to cell wall expansion and membrane hyperpolarization was observed [54]. A direct regulation of PM H^+-ATPase activity by BRI1 through phosphorylation was suggested to generate an output of BRI1 activity independent of downstream BR signalling. Although the interaction between BRI1 and AHAs *in vivo* was demonstrated [54-56] the direct phosphorylation of the penultimate residue of AHA1 or AHA2 by BRI1 has not been established. In the arabidopsis root, BR biosynthesis is enhanced in the elongation zone [57], where it overlaps with BR signalling maxima [58]. Thus, low BR concentrations in the meristem and high in the root elongation zone contribute to the optimal root growth [57]. Interestingly, during root development the *AHA2* transcripts are also increased in the transition and elongation zone, resulting in AHA2 protein accumulation and acidic apoplastic pH in the epidermal cells in this part of the root [59]. As BRI1 interacts directly with AHA2 and AHA7 [53-56], it was proposed that AHA2-containing-BRI1-BAK1 nanocluster at least in part regulates arabidopsis root growth along the root tip axis [59].

The significance of the BR-associated H^+ efflux via regulating the activity of PM H^+-ATPases was also revealed when investigating the mechanisms underlying root hydrotropism in arabidopsis [55]. The H^+ fluxes during the hydrotropic response were decreased especially in *bri1-5* root elongation zone. Another study supported these observations by showing that triple or quadruple mutants in BRs receptor or co-receptors, including *bri1*, *bak1*, *bri1brl1brl3*, *brl1brl3bak1* and *bri1brl1brl3bak1*, displayed reduced root growth and root curvature angles in the hydrotropism assay, while the BRL3 overexpression transgenic line demonstrated an increased root hydrotropic bending compared to wild type roots [60].

ABA-mediated modulation of PM H^+-ATPase activity

Different environmental challenges (drought, salinity, freezing) lead to water deficit, which generates osmotic stress and induces ABA biosynthesis in the vascular plant tissue as well in guard cells [61, 62]. ABA elicits numerous adaptive processes to generate plant stress resistance, which involve stomatal closure, promotion of root growth and dehydration avoidance [63]. More than a decade ago, the 14-members of the ABA receptor family (**PYR/PYL/RCARs**)

were identified in arabidopsis as soluble intracellular receptors [64-67]. The structure of ABA receptors displays the classical α/β helix-grip fold of the START/Bet v proteins, including a large central hydrophobic pocket that serves to accommodate ABA [66-69]. PYR/PYL/RCARs alone can bind ABA, but only in the presence of the PP2C co-receptor can bind the ligand with nanomolar affinity [64, 66]. ABA signalling starts with ABA perception through PYR/PYL/RCARs, which leads to their interaction with and inactivation of **PP2C-A** (**BOX2**), such as **ABI1** (Figure 1), **ABI2**, **HAB1**, **HAB2** and **PP2CA/AHG3**, thereby relieving their inhibition on three **SnRK2s** termed subclass III SnRK2s. Additionally, RAF-like kinases are required to activate subclass III SnRK2s that have been previously dephosphorylated by PP2C-A [70-72].

Downregulation of PM H^+-ATPase activity is a key determinant for ABA-mediated stomatal closure as revealed by the ABA-insensitive phenotype of the constitutively active AHA1 in the ***ost2-1D and ost2-2D*** mutants [73]. In contrast, blue light-induced stimulation of PM H^+-ATPase activity promotes stomatal aperture [74, 75]. Other physiological processes, such as hypocotyl elongation (HE) in etiolated seedlings and promotion of root growth, are also strongly dependent on PM H^+-ATPase activity and molecular studies have investigated their regulation by ABA. Thus, Hayashi *et al*. [76] studied (in etiolated seedlings) HE, a crucial step to reach the seedling establishment stage, and found that ABA suppresses it through dephosphorylation of the PM H^+-ATPase. Different genetic and pharmacological studies had established that PM H^+-ATPase activation determines HE [76-78]. Thus, application of PM H^+-ATPase inhibitors, vanadate and erythrosine B, decreased HE, and an AHA2 knockout mutant, *aha2-5*, displayed a noticeably reduced hypocotyl length. In contrast, the PM H^+-ATPase activator FC enhanced HE and induced phosphorylation of the penultimate Thr residue of PM H^+-ATPase. Furthermore, the application of high ABA concentrations to etiolated seedlings suppressed HE and attenuated PM H^+-ATPase activity through Thr^{947} dephosphorylation. This was abolished in the ABA-insensitive mutant *abi1-1D*, and the authors concluded that ABI1 was involved in ABA-dependent PM H^+-ATPase inhibition [76]. Given that *abi1-1D* is a dominant allele, it was not possible to unequivocally conclude that ABI1 is directly involved in the dephosphorylation of the Thr residue [79, 80]. The *abi1-1D* allele leads to replacement of

ABI1^{Gly180} by the bulkier Asp residue and structural studies of the ABA receptors in complex with ABA and different PP2C-A have illuminated the nature of this singular mutation, which can act as hypermorphic or hypomorphic depending on the substrate assayed [79-81].

Treatment with high ABA concentrations is strongly inhibitory for root growth and suppresses 50% PM H^+-ATPase activity in arabidopsis roots, whereas different ABA-insensitive mutants as ***112458***, a line overexpressing *HAB1* and the *snrk2.2 snrk2.3* double mutant, were resistant to ABA-mediated root growth inhibition [82-85]. These results support that the ABA signal core components (ABA receptors/PP2Cs/SnRK2s) are required for the ABA inhibitory effect on root growth based on inhibition of PM H^+-ATPase in arabidopsis seedlings [85]. Certain peptide ligands also negatively regulate PM H^+-ATPase activity and root growth. For example, binding of RALF1 peptide to FERONIA receptor kinase initiates a downstream signalling cascade that represses PM H^+-ATPase activity by phosphorylation of Ser^{889}, which increases apoplastic pH, and reduces root cell elongation [86]. Moreover, FERONIA can enhance the activity of PP2C-A, as ABI1 and ABI2, which might have a cooperative effect for PM H^+-ATPase inhibition [26].

Although the inhibitory effect of ABA on PM H^+-ATPase activity had been known for a long time and attributed to ABI1/ABI2 phosphatases [76, 87], the core component directly responsible for PM H^+-ATPase inhibition had remained unknown. Prolonged treatment with high ABA concentration, in addition to promoting ABA signalling, leads to upregulation of PP2C-A [88, 89]. Therefore, to minimize ABA-induced PP2C-A increase, Miao *et al*. [90] investigated the effect of low ABA concentrations on PM H^+-ATPase activity and root growth. It was previously reported that low ABA concentrations stimulate root growth whereas high ABA concentrations inhibit it [91], which is in line with the auxin knowledge [92, 93]. Exogenous 0.1 μM ABA enhanced primary root elongation, whereas 3 μM ABA impaired primary growth, which correlated with higher and lower, respectively, apoplastic H^+ extrusion in wild type roots (elongation zone) [90]. Interestingly, the stimulatory effect on root elongation of 0.1 μM ABA phenocopied the enhanced root growth of the *pp2c* quadruple mutant ***Qabi2-2***. Without exogenous ABA treatment, the *Qabi2-2* mutant showed enhanced apoplastic H^+-extrusion, which not only contributed to root growth but also enhanced the hydrotropic bending

response [90, 94]. Therefore, these results suggested that PP2C-A might interact and directly impair PM H^+-ATPase activity, which was confirmed for ABI1 using different interaction assays [90]. Finally, using anti-pThr^{947} antibodies, the authors demonstrated that *Qabi2-2* shows enhanced phosphorylation of the Thr^{947}. This leads to higher H^+ efflux in the elongation root zone compared to the wild type in either normal or low water potential medium conditions.

The above findings suggest that PP2C-A forms a complex with AHA2 in the absence of ABA and dephosphorylate Thr^{947} of AHA2 to suppress H^+ extrusion (Figure 3). Upon rise of ABA in response to osmotic stress, ABA receptors bind to PP2C-A, thus relieving AHA2 inhibition and facilitating phosphorylation of Thr^{947}. Genetic inactivation of PP2C-A in *Qabi2-2* enables the Thr^{947} of AHA2 to be maintained in the phosphorylated state to activate apoplastic H^+ efflux, which might cause cell wall extension by activating cell wall-loosening proteins [90, 95] (Figure 2). The increase of ABA in particular cell types requires transport and uptake in target tissues. During root hydrotropic responses, ABA acts in cortical cells of the elongation zone to activate SnRK2.2 [96]. ABA transport in the context of the primary root is not well understood yet; in any case, it is complex and involves several ABA transporters and diffusion through the membrane lipid bilayer of the protonated form [97, 98]. Certain cells of the root elongation/transition zone facing the dry side (lower water potential) should accumulate more ABA than those in the higher water potential side to generate the differential growth response that occurs during hydrotropism. Indeed at 2 h after stimulation of the hydrotropic response, asymmetric H^+ efflux occurs between the dry (convex) and moist side (concave) of the root [90]. As a result, the dry side extrudes much more H^+ than the moist side, leading to root hydrotropic bending at an early stage in the hydrotropic experimental system.

Auxin-triggered H^+ fluxes and fast regulation of PM H^+-ATPase activity by auxin signalling

Recent advances in hormone signalling have provided an updated molecular framework for the acid growth theory (**BOX 1**) [17] and new insights into fast auxin-induced mechanisms for regulation of H^+ fluxes [15, 16]. Two articles focused on auxin signalling have highlighted the importance of H^+ fluxes to promote or inhibit growth [15, 16]. To promote cell elongation in

hypocotyls, auxin induces the efflux of protons, resulting in rapid apoplast acidification by activating AHA. Auxin induced proton efflux occurs within seconds and represents a fast branch of auxin signalling in the PM mediated by the TRANSMEMBRANE KINASE (TMK) pathway, different from the TRANSPORT INHIBITOR RESPONSE1 (TIR1)/ AUXIN SIGNALLING F-BOX (AFB) pathway that mediates intracellular auxin perception and signalling [16]. In arabidopsis protoplasts, the TMK proteins, namely TMK1 (Figure 1) and TMK4, show enhanced interaction with AHA within 1 min after auxin treatment [16]. This leads to phosphorylation of the penultimate Thr residue of AHA in the aerial parts of arabidopsis seedlings and a *tmk1-1 tmk4-1* double mutant lacks auxin-induced phosphorylation of this Thr residue. Activation of AHA in response to auxin is further sustained through the nuclear auxin signalling pathway mediated by TIR1/AFB auxin receptors, which induces synthesis of SAUR proteins and inactivation of PP2C-D phosphatases (described below). Interestingly, in root cells, auxin inhibits growth through rapid apoplastic alkalization [15]. This inhibitory effect involves TIR1/AFB receptors and a yet unknown non-transcriptionally based mechanism, because of the rapid increase (seconds) in apoplastic pH of root epidermal cells after auxin treatment [15]. This apoplast alkalization can be counteracted by the same auxin-dependent mechanism described above in hypocotyl, i.e. in root cells AHA are activated by TMK-based signalling in response to auxin. Therefore, in root cells two auxin-dependent mechanisms that counteract each other coexist. However, the auxin-triggered H^+ influx yet remains to be explained [15].

Auxin, BR and ABA signalling converge in the regulation of PM H^+-ATPase

Phosphorylation of the penultimate Thr residue of AHA is counteracted by auxin- or ABA-regulated protein phosphatases, i.e. auxin/PP2C-D and ABA/PP2C-A [53, 90]. PP2C-D negatively regulate PM H^+-ATPase activity by dephosphorylating the Thr^{947} residue, which is also a target of the PP2C-A ABI1 when the phosphatase is not inhibited by the ABA receptors [53, 90] (Figure 3). What are the similarities and differences between these PP2Cs in terms of PM H^+-ATPase regulation?

First, while some PP2C-D contain a putative membrane-spanning domain, PP2C-A are soluble proteins that will require either auxiliary proteins to localize in PM or interaction with

PM targets. However, a recent research has revealed that PP2C-D2, D5 and D6, major regulators of cell expansion in hypocotyl growth, are associated with PM, but only PP2C-D6 contains a predicted transmembrane domain [99]. In other model systems, such as the apical hook of etiolated seedlings, PP2C-D1 is the predominant phosphatase and contains the transmembrane domain [99].

Second, SAUR proteins regulate the enzymatic activity of PP2C-D, whereas the activity of PP2C-A is regulated by ABA and ABA receptors (Figure 2 and 3) [90]. Thus, while PP2C-A are inhibited in an ABA-dependent manner in response to abiotic stress, PP2C-D are inhibited by different auxin-induced SAUR proteins and are therefore sensitive to auxin signalling (Figure 2) [53]. Auxin leads to the accumulation of many SAUR proteins that show distinct subcellular localization [99]. Particularly SAUR19 and SAUR63 are associated with the PM, where they can inhibit the phosphatase activity of membrane-associated PP2C-D. ABA can inhibit ABI1 (and other PP2C-A) at low concentration because monomeric ABA receptors, such as PYL8, perceive ABA in the nanomolar range [90]. Indeed, in root cells, exogenous treatment with low ABA concentration phenocopies the *pp2c* quadruple *Qabi2-2* mutant [90]. It is likely that activation of SnRK2 might have a positive effect for PM H^+-ATPase activity (although not elucidated at a molecular level yet) because SnRK2 activity is required for differential expansion of cortical cells in the root hydrotropic response [96]. High ABA levels have the opposite effect on PM H^+-ATPase activity in suspension cell cultures, guard cells and hypocotyls; however, in roots 10 μM ABA was not found to inhibit Thr^{947} phosphorylation [85]. Sustained high ABA levels increase the PP2C-A protein levels [88, 89] and degrade SnRK2s [72].

Expression of stabilized SAUR proteins confers increased PM H^+-ATPase activity, as the *ost2* dominant mutations in the *AHA1* gene, leading to increased phosphorylation of $pThr^{947}$ [53, 100, 101]. This SAUR-based molecular mechanism can explain how auxin sustains cell expansion via an acid growth mechanism in the hypocotyl and perhaps in the root when combined with the fast TMK-dependent phosphorylation of the penultimate Thr residue [17, 53]. *SAUR* genes are also induced by BRs, which enables integration of PM H^+-ATPase regulation by auxin and BR signalling. Many *SAUR* genes were identified as potential direct

targets of BZR1 and BES1/BZR2 transcription factors [51]. Both BZR1 and BES1/BZR2 bind to the promoter of *SAUR15* gene [51, 102], and BES1/BZR2 binds to *SAUR36* and *SAUR59* promoters [51] whose gene products inhibit PP2C-D [52]. While the precise roles of SAUR proteins in BR action remain unclear, given the well-established role of BR in promoting cell expansion, it seems likely that SAURs are downstream effectors that mediate at least some aspects of BR-mediated expansion growth. Thus, a new scenario emerges where the BRs-ABA-Auxin signalling network (Figure 2 and 3) can regulate plant growth by regulating phosphorylation of the penultimate Thr residue at the R domain of PM H^+-ATPase.

Concluding Remarks and Future Perspectives

The decades-old acid growth theory (**BOX 1**) has been further supported by different physiological and molecular studies on hormone signalling. Recent breakthroughs in auxin signalling have identified TMK1 and TMK4 as the auxin-dependent kinases that catalyse phosphorylation of Thr^{947}, although the auxin perception mechanism of the TMK pathway remains a mystery. Interestingly, in the *tmk1 tmk4* double mutant, FC treatment still increased the level of phosphorylation of the penultimate Thr residue, which suggests that auxin-independent kinases are also able to phosphorylate this residue [16]. Thus, whereas TMK1 and TMK4 catalyse auxin-induced phosphorylation of Thr^{947} within seconds, it is possible that either BR/ABA-regulated or yet unknown kinases mediate Thr^{947} phosphorylation in response to other stimuli. The BR-induced PM H^+-ATPase phosphorylation is slower, so it might involve downstream signalling to induce SAURs and perhaps basal phosphorylation by BRI1 [52, 55]. Low ABA concentration, in addition to inhibiting PP2C-A, leads to activation of subfamily III SnRK2s (such as SnRK2.2), which is also a good candidate kinase to phosphorylate directly Thr^{947} in the root hydrotropic response [85, 90]. Finally, the molecular mechanisms (downstream TIR1/AFB) for auxin-triggered H^+ influx and apoplast alkalization to inhibit root growth are yet unknown [15]. Therefore new queries emerge to fully understand the molecular mechanism of PM H^+-ATPase activation and growth regulation (see Outstanding Questions).

Acknowledgments

Work in the P.L.R. laboratory was supported by grant PID2020-113100RB funded by MCIN/AEI/ 10.13039/501100011033 and by "ERDF A way of making Europe". Figure 2 and Figure 3 are created using the software BioRender (BioRender.com).

References

1. Zhang, M. *et al*. (2021) Plasma membrane H+-ATPase overexpression increases rice yield via simultaneous enhancement of nutrient uptake and photosynthesis. *Nat Commun.* 12, 735.
2. Palmgren, M.G. and Nissen, P. (2011) P-type ATPases. *Annu. Rev. Biophys.* 40, 243–266.
3. Serrano R, *et al.* (1986) Yeast plasma membrane ATPase is essential for growth and has homology with (Na+ + K+), K+- and Ca2+-ATPases. *Nature* 319, 689–693.
4. Harper, J.F. et al. (1989) Molecular cloning and sequence of cDNA encoding the plasma membrane proton pump (H+ -ATPase) of Arabidopsis thaliana. *Proc. Natl. Acad. Sci. U. S. A.* 86, 1234–1238.
5. Pardo, J.M. and Serrano, R. (1989) Structure of a plasma membrane H+-ATPase gene from the plant Arabidopsis thaliana. *J. Biol. Chem.* 264, 8557–8562.
6. Haruta, M. *et al*. (2015) Regulation of the plasma membrane proton pump (H(+)-ATPase) by phosphorylation. *Curr. Opin. Plant Biol.* 28, 68–75.
7. Morth, J.P. *et al.* (2011) A structural overview of the plasma membrane Na+,K+-ATPase and H+-ATPase ion pumps. *Nat. Rev. Mol. Cell Biol.* 12, 60–70.
8. Pedersen, P. and Carafoli, E. (1987) Ion motive ATPases. 1. Ubiquity, properties, and significance to cell function. *Trends Biochem. Sci.* 12, 146–150.
9. Pedersen C.N., Axelsen K.B., Harper J.F., Palmgren M.G. (2012) Evolution of plant p-type ATPases. *Front. Plant Sci.* 3, 31.
10. Palmgren, M.G. (2001) Plant plasma membrane H+-ATPases: powerhouses for nutrient uptake. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 52, 817–845
11. Sondergaard, T.E. *et al.* (2004) Energization of transport processes in plants. roles of the plasma membrane H+-ATPase. *Plant Physiol.* 136, 2475–2482.
12. Zhao, R. *et al.* (2000) Cosuppression of a plasma membrane H(+)-ATPase isoform

impairs sucrose translocation, stomatal opening, plant growth, and male fertility. *Plant Cell* 12, 535–546.

13. Falhof, J. *et al.* (2016) Plasma Membrane H(+)-ATPase Regulation in the Center of Plant Physiology. *Mol. Plant* 9, 323–337.
14. Cleland, R. (1973) Auxin-induced hydrogen ion excretion from Avena coleoptiles. *Proc. Natl. Acad. Sci. U. S. A.* 70, 3092-3093.
15. Li, L.X. *et al.* (2021) Cell surface and intracellular auxin signalling for H+ fluxes in root growth. Nature. https://doi.org/10.1038/s41586-021-04037-6.
16. Lin, W.W. *et al*. (2021) TMK-based cell-surface auxin signalling activates cell-wall acidification. Nature. https://doi.org/10.1038/s41586-021-03976-4.
17. Du, M. *et al.* (2020) Rapid Auxin-Mediated Cell Expansion. *Annu. Rev. Plant Biol.* 71, 379-402.
18. Svennelid, F. *et al.* (1999) Phosphorylation of Thr-948 at the C terminus of the plasma membrane H+-ATPase creates a binding site for the regulatory 14-3-3 protein. *Plant Cell* 11, 2379–2391.
19. Maudoux, O. *et al.* (2000) A plant plasma membrane H+-ATPase expressed in yeast is activated by phosphorylation at its penultimate residue and binding of 14-3-3 regulatory proteins in the absence of fusicoccin. *J. Biol. Chem.* 275, 17762–17770.
20. Pedersen, B.P. *et al.* (2007) Crystal structure of the plasma membrane proton pump. *Nature* 450, 1111–1114.
21. Oecking, C. *et al.* (1997) Topology and target interaction of the fusicoccin-binding 14-3-3 homologs of Commelina communis. *Plant J.* 12, 441–453.
22. Piotrowski, M. *et al.* (1998) Complementation of the Saccharomyces cerevisiae plasma membrane H+-ATPase by a plant H+-ATPase generates a highly abundant fusicoccin binding site. *J. Biol. Chem.* 273, 30018–30023.
23. Baunsgaard, L. *et al.* (1998) The 14-3-3 proteins associate with the plant plasma membrane H(+)-ATPase to generate a fusicoccin binding complex and a fusicoccin responsive system. *Plant J.*13, 661-671.

24. Ottmann, C. *et al.* (2007) Structure of a 14-3-3 coordinated hexamer of the plant plasma membrane H+ -ATPase by combining X-ray crystallography and electron cryomicroscopy. *Mol Cell.* 25, 427–440.
25. Gjetting, S.K. *et al.* (2020) Evidence for multiple receptors mediating RALF-triggered Ca2+ signaling and proton pump inhibition. *Plant J.* 104, 433-446.
26. Chen, J. *et al.* (2016) FERONIA interacts with ABI2-type phosphatases to facilitate signaling cross-talk between abscisic acid and RALF peptide in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 113, E5519-E5527.
27. Ladwig, F. *et al*. (2015) Phytosulfokine Regulates Growth in Arabidopsis through a Response Module at the Plasma Membrane That Includes CYCLIC NUCLEOTIDE-GATED CHANNEL17, H+-ATPase, and BAK1. *Plant Cell* 27, 1718–29.
28. Clouse, S. and Sasse, J. (1998) Brassinosteroids: Essential regulators of plant growth and development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49, 427–451.
29. Nolan, T.M. *et al.* (2020) Brassinosteroids: Multidimensional Regulators of Plant Growth, Development, and Stress Responses. *Plant Cell* 32, 295–318.
30. Li, J. and Chory, J. (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* 90, 929–938.
31. Cano-Delgado, A. *et al.* (2004) BRL1 and BRL3 are novel brassinosteroid receptors that function in vascular differentiation in Arabidopsis. *Development* 131, 5341–5351.
32. Kinoshita, T. *et al.* (2005) Binding of brassinosteroids to the extracellular domain of plant receptor kinase BRI1. *Nature* 433, 167–171.
33. Hothorn, M. *et al.* (2011) Structural basis of steroid hormone perception by the receptor kinase BRI1. *Nature* 474, 467–471.
34. She, J. *et al.* (2011) Structural insight into brassinosteroid perception by BRI1. *Nature* 474, 472–476.
35. Friedrichsen, D.M. *et al.* (2000) Brassinosteroid-insensitive-1 is a ubiquitously expressed leucine-rich repeat receptor serine/threonine kinase. *Plant Physiol.* 123, 1247–1256.
36. Nam, K.H., and Li, J. (2002) BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell* 110, 203–212.

37. Li, J. *et al.* (2002). BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* 110, 213–222.
38. Hecht V. *et al.* (2001) The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant Physiol.* 127, 803–816.
39. Gou, X. *et al.* (2012). Genetic evidence for an indispensable role of somatic embryogenesis receptor kinases in brassinosteroid signaling. *PLoS Genet.* 8, e1002452.
40. Tang, W. *et al.* (2008) BSKs mediate signal transduction from the receptor kinase BRI1 in Arabidopsis. *Science* 321, 557–560.
41. Ren, H. *et al.* (2019) BRASSINOSTEROID-SIGNALING KINASE 3, a plasma membrane-associated scaffold protein involved in early brassinosteroid signaling. *PLoS Genet.* 15, e1007904.
42. Kim, T.W. *et al.* (2011) The CDG1 kinase mediates brassinosteroid signal transduction from BRI1 receptor kinase to BSU1 phosphatase and GSK3-like kinase BIN2. *Mol. Cell* 43, 561–571.
43. Mora-García S. *et al.* (2004) Nuclear protein phosphatases with Kelch-repeat domains modulate the response to brassinosteroids in Arabidopsis. *Genes Dev.* 18, 448–460.
44. Kim, T.W. *et al.* (2009) Brassinosteroid signal transduction from cell-surface receptor kinases to nuclear transcription factors. *Nat. Cell Biol.* 11, 1254–1260.
45. Peng, P. *et al.* (2008) Regulation of the Arabidopsis GSK3-like kinase BRASSINOSTEROID-INSENSITIVE 2 through proteasome-mediated protein degradation. *Mol. Plant* 1, 338–346.
46. Zhu, J.Y. et al. (2017) The F-box Protein KIB1 Mediates Brassinosteroid-Induced Inactivation and Degradation of GSK3-like Kinases in Arabidopsis. *Mol. Cell* 66, 648–657.
47. Tang, W. *et al.* (2011) PP2A activates brassinosteroid-responsive gene expression and plant growth by dephosphorylating bzr1. *Nat. Cell Boil.* 13, 124–131.
48. Wang, Z.Y. *et al.* (2002) Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. *Dev. Cell* 2, 505–513.

475 49. Yin, Y. *et al.* (2002). BES1 accumulates in the nucleus in response to brassinosteroids to
476 regulate gene expression and promote stem elongation. *Cell* 109, 181–191.
477 50. Sun, Y., et al. (2010). Integration of brassinosteroid signal transduction with the
478 transcription network for plant growth regulation in Arabidopsis. *Dev. Cell* 19, 765–777.
479 51. Yu, X. *et al.* (2011). A brassinosteroid transcriptional network revealed by genome-wide
480 identification of BES1 target genes in *Arabidopsis thaliana*. *Plant J.* 65, 634–646
481 52. Minami, A. *et al.* (2019) Brassinosteroid Induces Phosphorylation of the Plasma
482 Membrane H+-ATPase during Hypocotyl Elongation in Arabidopsis thaliana. *Plant Cell*
483 *Physiol.* 60, 935–944.
484 53. Spartz, A.K. *et al.* (2014) SAUR Inhibition of PP2C-D Phosphatases Activates Plasma
485 Membrane H+-ATPases to Promote Cell Expansion in Arabidopsis. *Plant Cell* 26, 2129–
486 2142.
487 54. Caesar, K. *et al.* (2011) A fast brassinolide-regulated response pathway in the plasma
488 membrane of Arabidopsis thaliana. *Plant J.* 66, 528–540.
489 55. Miao, R. *et al.* (2018) Comparative Analysis of Arabidopsis Ecotypes Reveals a Role for
490 Brassinosteroids in Root Hydrotropism. *Plant Physiol.* 176, 2720–2736.
491 56. Yuan, W. *et al.* (2018) BR-INSENSITIVE1 regulates hydrotropic response by interacting
492 with plasma membrane H+-ATPases in Arabidopsis. *Plant Signal. Behav.* 13, e1486147.
493 57. Vukašinović, N. *et al.* (2021) Local brassinosteroid biosynthesis enables optimal root
494 growth. *Nat. Plants* 7, 619–632.
495 58. Chaiwanon, J. and Wang, Z.Y. (2015) Spatiotemporal brassinosteroid signaling and
496 antagonism with auxin pattern stem cell dynamics in Arabidopsis roots. *Curr. Biol.* 25,
497 1031–1042.
498 59. Großeholz, R. *et al.* (2021) Integration of computational modeling and quantitative cell
499 physiology reveals central parameters for the brassinosteroid-regulated elongation
500 growth along the axis of the Arabidopsis root tip. bioRxiv 2021.04.13.439595; doi:
501 https://doi.org/10.1101/2021.04.13.439595.
502 60. Fàbregas, N. *et al.* (2018) Overexpression of the vascular brassinosteroid receptor BRL3
503 confers drought resistance without penalizing plant growth. *Nat Commun.* 9, 4680.

61. Endo, A. *et al.* (2008) Drought induction of Arabidopsis 9-cis-epoxycarotenoid dioxygenase occurs in vascular parenchyma cells. *Plant Physiol.* 147, 1984–1993.
62. Bauer, H. *et al.* (2013) The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis. *Curr. Biol.* 23, 53-57.
63. Zhu, J.K. (2016) Abiotic Stress Signaling and Responses in Plants. *Cell* 167, 313–324.
64. Ma, Y. *et al.* (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* 324, 1064–1068.
65. Park, S.Y. *et al.* (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* 324, 1068–1071.
66. Santiago, J. *et al.* (2009) The abscisic acid receptor PYR1 in complex with abscisic acid. *Nature* 462, 665–668.
67. Nishimura, N. *et al.* (2009) Structural mechanism of abscisic acid binding and signaling by dimeric PYR1. *Science* 326, 1373–1379.
68. Melcher, K. *et al.* (2009) A Gate-Latch-Lock Mechanism for Signal Transduction by Abscisic Acid Receptors. *Nature* 462, 602–608.
69. Miyazono, K. *et al.* (2009) Structural basis of abscisic acid signalling. *Nature* 462, 609–614.
70. Takahashi, Y. *et al.* (2020) MAP3Kinase-dependent SnRK2-kinase activation is required for abscisic acid signal transduction and rapid osmotic stress response. *Nat. Commun.* 11, 12.
71. Lin, Z. *et al.* (2020). A RAF-SnRK2 kinase cascade mediates early osmotic stress signaling in higher plants. *Nat. Commun.* 11, 613.
72. Lin, Z. *et al.* (2021) Initiation and amplification of SnRK2 activation in abscisic acid signaling. *Nat. Commun.* 12, 2456.
73. Merlot, S. *et al.* (2007) Constitutive activation of a plasma membrane H(+)-ATPase prevents abscisic acid-mediated stomatal closure. *EMBO J.* 26, 3216–3226.
74. Assmann, S.M. *et al.* (1985) Blue light activates electrogenic ion pumping in guard cell protoplasts of Vicia faba. *Nature* 318, 285–287.
75. Shimazaki, K. *et al.* (1986) Blue light-dependent proton extrusion by guard-cell

protoplasts of Vicia faba. *Nature* 319, 324–326.

76. Hayashi, Y. *et al.* (2014) Abscisic acid suppresses hypocotyl elongation by dephosphorylating plasma membrane H(+)-ATPase in Arabidopsis thaliana. *Plant Cell Physiol.* 55, 845–853.

77. Hager, A. (2003) Role of the plasma membrane H+-ATPase in auxin-induced elongation growth: historical and new aspects. *J. Plant Res.* 116, 483–505.

78. Haruta, M. and Sussman,M.R. (2012). The effect of a genetically reduced plasma membrane proton motive force on vegetative growth of Arabidopsis. *Plant Physiol.* 158, 1158–1171.

79. Santiago, J. *et al.* (2012) Structural insights into PYR/PYL/RCAR ABA receptors and PP2Cs. *Plant Sci.* 182, 3–11.

80. Robert, N. *et al.* (2006) A hypermorphic mutation in the protein phosphatase 2C HAB1 strongly affects ABA signaling in Arabidopsis. *FEBS Lett.* 580, 4691–4696.

81. Umezawa, T. *et al.* (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proc. Natl. Acad. Sci. U. S. A.* 106, 17588–17593.

82. Saez, A. *et al.* (2004) Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C HAB1 reveal its role as a negative regulator of abscisic acid signalling. *Plant J.* 37, 354–369.

83. Fujii, H. *et al.* (2007) Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis. *Plant Cell* 19, 485-494.

84. Gonzalez-Guzmán, M. *et al.* (2012) Arabidopsis PYR/PYL/RCAR receptors play a major role in quantitative regulation of stomatal aperture and transcriptional response to abscisic acid. *Plant Cell* 24, 2483–2496.

85. Planes, M.D. *et al.* (2015) A mechanism of growth inhibition by abscisic acid in germinating seeds of Arabidopsis thaliana based on inhibition of plasma membrane H+-ATPase and decreased cytosolic pH, K+, and anions. *J. Exp. Bot.* 66, 813–825.

86. Haruta, M. *et al.* (2014) A peptide hormone and its receptor protein kinase regulate plant

cell expansion. *Science* 343, 408-411.

87. Roelfsema, M.R.G. *et al.* (1998) Blue light-induced apoplastic acidification of Arabidopsis thaliana guard cells is mediated through protein phosphatases. *Physiol. Plantarum* 103, 466–474.

88. Wang, X. *et al.* (2019) ABRE-BINDING FACTORS play a role in the feedback regulation of ABA signaling by mediating rapid ABA induction of ABA co-receptor genes. *New Phytol.* 221, 341–355.

89. Julian, J. *et al.* (2019) The MATH-BTB BPM3 and BPM5 subunits of Cullin3-RING E3 ubiquitin ligases target PP2CA and other clade A PP2Cs for degradation. *Proc. Natl. Acad. Sci. U. S. A.* 116, 15725–15734.

90. Miao, R. *et al.* (2021) Low ABA concentration promotes root growth and hydrotropism through relief of ABA INSENSITIVE 1-mediated inhibition of plasma membrane H(+)-ATPase 2. *Sci. Adv.* 7, eabd4113.

91. Harris, J.M. (2015) Abscisic Acid: Hidden Architect of Root System Structure. *Plants. (Basel)* 4, 548–572.

92. Fu, X. and Harberd, N.P. (2003). Auxin promotes Arabidopsis root growth by modulating gibberellin response. *Nature* 421, 740–743.

93. Barbez, E. *et al.* (2017) Auxin steers root cell expansion via apoplastic pH regulation in Arabidopsis thaliana. *Proc. Natl. Acad. Sci. U. S. A.* 114, E4884–E4893.

94. Antoni, R, *et al.* (2013) PYRABACTIN RESISTANCE1-LIKE8 plays an important role for the regulation of abscisic acid signaling in root. *Plant Physiol.* 161, 931–941.

95. Hocq, L. *et al.* (2017) Connecting Homogalacturonan-Type Pectin Remodeling to Acid Growth. *Trends Plant Sci.* 22, 20-29.

96. Dietrich, D. *et al.* (2017) Root hydrotropism is controlled via a cortex-specific growth mechanism. *Nat. Plants* 3, 17057.

97. Boursiac, Y. *et al.* (2013) ABA transport and transporters. *Trends Plant Sci.* 18, 325–333.

98. Merilo, E. *et al.* (2015) The role of ABA recycling and transporter proteins in rapid stomatal responses to reduced air humidity, elevated CO2, and exogenous ABA. *Mol. Plant* 8, 657–659.

591 99. Ren, H. *et al.* (2018) A subset of plasma membrane-localized PP2C.D phosphatases
592 negatively regulate SAUR-mediated cell expansion in Arabidopsis. *PLoS Genet.* 14,
593 e1007455.
594 100. Spartz, A.K. *et al*. (2017) Constitutive Expression of Arabidopsis SMALL AUXIN UP
595 RNA19 (SAUR19) in Tomato Confers Auxin-Independent Hypocotyl Elongation. *Plant*
596 *Physiol.* 173, 1453–1462.
597 101. Fendrych, M. *et al*. (2016) TIR1/AFB-Aux/IAA auxin perception mediates rapid cell wall
598 acidification and growth of Arabidopsis hypocotyls. *Elife* 5, e19048.
599 102. Walcher, C.L. and Nemhauser, J.L. (2012) Bipartite promoter element required for auxin
600 response. *Plant Physiol.* 158,273-282.
601 103. Cosgrove, D.J. (2015) Plant cell wall extensibility: connecting plant cell growth with cell
602 wall structure, mechanics, and the action of wall-modifying enzymes. *J. Exp. Bot.* 67,
603 663–676.
604 104. Maris, A. *et al.* (2011) Differences in enzymic properties of five recombinant xyloglucan
605 endotransglucosylase/hydrolase (XTH) proteins of Arabidopsis thaliana. *J. Exp. Bot.* 62,
606 261–271.
607 105. Micheli, F. (2001) Pectin methylesterases: cell wall enzymes with important roles in plant
608 physiology. *Trends Plant Sci.* 6, 414–419.
609 106. Schweighofer, A. *et al.* (2004) Plant PP2C phosphatases: emerging functions in stress
610 signaling. Trends Plant Sci. 9, 236–243.
611 107. Soon, F.F. *et al.* (2012) Molecular mimicry regulates ABA signaling by SnRK2 kinases
612 and PP2C phosphatases. *Science* 335, 85–88.
613 108. Belin, C. *et al.* (2006) Identification of features regulating OST1 kinase activity and OST1
614 function in guard cells. *Plant Physiol.* 141, 1316–1427.
615 109. Ng, L.M. *et al.* (2011) Structural basis for basal activity and autoactivation of abscisic
616 acid (ABA) signaling SnRK2 kinases. *Proc. Natl. Acad. Sci. U. S. A.* 108, 21259–21264.
617 110. Yoshida, T. *et al.* (2010) AREB1, AREB2, and ABF3 are master transcription factors that
618 cooperatively regulate ABRE-dependent ABA signaling involved in drought stress
619 tolerance and require ABA for full activation. *Plant J.* 61, 672–685.

111. Peirats-Llobet, M. *et al.* (2016) A Direct Link between Abscisic Acid Sensing and the Chromatin-Remodeling ATPase BRAHMA via Core ABA Signaling Pathway Components. *Mol Plant.* 9, 136–147.
112. Osakabe, Y. *et al.* (2014) ABA control of plant macroelement membrane transport systems in response to water deficit and high salinity. *New Phytol.* 202, 35–49.
113. Cherel, I. *et al.* (2002) Physical and functional interaction of the Arabidopsis K(+) channel AKT2 and phosphatase AtPP2CA. *Plant Cell* 14, 1133–1146.
114. Lee, S.C. *et al.* (2009) A protein kinase-phosphatase pair interacts with an ion channel to regulate ABA signaling in plant guard cells. *Proc. Natl. Acad. Sci. U. S. A.* 106, 21419–21424.
115. Maierhofer, T. *et al.* (2014) Site- and kinase-specific phosphorylation-mediated activation of SLAC1, a guard cell anion channel stimulated by abscisic acid. *Sci. Signal.* 7, ra86.
116. Brandt, B. *et al.* (2015) Calcium specificity signaling mechanisms in abscisic acid signal transduction in Arabidopsis guard cells. *Elife* 4, e03599.

Glossary

ABI1, HAB1 and PP2CA/AHG3: ABA INSENSITIVE1, HYPERSENSITIVE TO ABA1 and PROTEIN PHOSPHATASE 2CA/ABA-HYPERSENSITIVE GERMINATION3 are clade A PP2Cs that function as negative regulators of ABA signalling. The ABI1 name originates from the phenotype of the *abi1-1D* allele.

BAK1/SERK3: BRASSINOSTEROID INSENSITIVE1 also known as SOMATIC EMBRYOGENESIS RECEPTOR KINASE3 is a leucine-rich repeat receptor kinase that has diverse functions in plant development and immunity, which are brought about through its binding to a large number of receptors including BRI1.

BIN2: BRASSINOSTEROID INSENSITIVE2 is a GSK3-like kinase that functions as a key negative regulator of BR signalling in Arabidopsis.

BRI1: BRASSINOSTEROID INSENSITIVE1 is a leucine-rich repeats receptor kinase, which is the major receptor of the plant BR hormones.

BSU1: BRI1 SUPPRESSOR1 is a member of the plant-specific family of protein phosphatases with Kelch-like domains. It is widely believed that BIN2 is inhibited through dephosphorylation by BSU1.

BZR1 and BES1/BZR2: BRASSINAZOLE RESISTANT1 and BRI1-EMS-SUPPRESSOR1/BZR2 are key BR transcription factors. Dephosphorylated BZR1 and BES1/BZR2 bind BRRE (BR RESPONSE ELEMENT)/E-box-containing promoters to regulate expression of thousands of BR-responsive genes important for plant growth and development.

CDG1: CONSTITUTIVE DIFFERENTIAL GROWTH1 is a member of the RLCK family that is involved in activation of BR signalling.

KIB1: KINK SUPPRESSED IN BZR1-1D is an F-box E3 ubiquitin ligase that promotes the degradation of BIN2 while blocking its substrate access.

ost2: *open stomata 2*, the *ost2-1D* and *ost2-2* alleles encode constitutively active versions of *AHA1*.

PP2A: PROTEIN PHOSPHATASE 2A is a type 2A serine/threonine protein phosphatase. PP2A activates BR-responsive gene expression and plant growth by dephosphorylating BZR1 and BES1/BZR2.

PP2C-A and PP2C-D: Clade A and D, respectively Protein Phosphatases Type 2C.

Qabi2-2: a *hab1-1abi1-2abi2-2pp2ca-1* loss-of-function mutant impaired in 4 PP2C-A.

PYR/PYL/RCARs: PYRABACTIN RESISTANCE1/PYR1-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS perceive ABA and negatively regulate PP2C-A.

RLCKs: RECEPTOR-LIKE CYTOPLASMIC KINASES lack extracellular ligand-binding domains and they have emerged as a major class of signalling proteins that regulate plant cellular activities in response to biotic/abiotic stresses and endogenous extracellular signalling molecules.

SAURs: auxin and BR-induced SMALL AUXIN UP-RNA proteins, a certain subset of SAURs interacts with and inhibits PP2C-D.

SERKs: SOMATIC EMBRYOGENESIS RECEPTOR KINASES are leucine-rich repeat receptor kinases involved in several, seemingly unrelated, plant-signalling pathways. In

678 *Arabidopsis thaliana*, the four SERK proteins have overlapping functions but each performs a
679 specific subset of signalling roles.
680 **Subfamily III SnRK2s**: ABA-ACTIVATED SNF1-RELATED PROTEIN KINASES, this
681 subfamily includes 3 kinases that play a key role for ABA signalling, i.e. SnRK2.2/SnRK2D,
682 SnRK2.3/ SnRK2I and SnRK2.6/ SnRK2E/Open Stomata 1 (OST1).
683 ***112458***: a *pyr1-1 pyl1 pyl2 pyl4 pyl5 pyl8* loss-of-function mutant that is blind to ABA
684 perception.
685
686 **BOX 1. Auxin, the acid growth theory, fluorescent pH indicators and hydrotropism.**
687 Auxin is fundamental to plant growth and development through regulation of cell expansion,
688 division and differentiation [17]. Particularly, cell expansion is limited by the cell wall, which
689 provides structural integrity to plant cells but also constrains them; therefore, cell-wall
690 loosening enzymes are required to enable cell expansion [103]. Cell wall loosening requires
691 apoplastic acidification, which is achieved by activation of PM H^+-ATPase. The
692 hyperpolarization of plasma membrane generated by the PM H^+-ATPase also enhances K^+
693 uptake, which facilitates water uptake and maintains turgor pressure for cell expansion (Figure
694 2). Cell wall extension requires PM H^+-ATPase activity, because low apoplastic pH triggers a
695 group of cell wall-related enzymes, such as expansins that disrupt hydrogen bonds between
696 polysaccharides [103], xyloglucan endotransglycosylase/hydrolases that cut and rejoin
697 xyloglucan chains [104], or pectin methylesterases that catalyse pectin demethylesterification
698 [105]. Moreover, cell expansion, in addition to proton-loosened and turgor-stretched cell wall,
699 requires exocytosis of certain proteins, enzymes and wall precursors. All these processes are
700 activated by auxin [77]. Thus, the acid growth theory provides a reasonable interpretation on
701 auxin-stimulated cell expansion in plant shoots but the model was heavily debated for roots,
702 mainly because of technical limitations in investigating root apoplastic pH at cellular resolution
703 [93]. Recently, the introduction of a suitable fluorescent pH indicator (HPTS, 8-hydroxypyrene-
704 1,3,6-trisulfonic acid) has enabled to confirm that cell wall acidification triggers cellular root
705 expansion through auxin signalling in root epidermal cells [93]. HPTS penetrates the root
706 apoplast without entering the root cells, which is crucial for specific assessment of pH in the

cell wall. HPTS has protonated and deprotonated forms, which are visualized by excitation wavelengths of 405 and 458 nm, respectively [93]. In acidic medium, there are more protonated than deprotonated molecules of HPTS and therefore, the lower 458/405 value represents lower pH and more H^+ efflux [93]. By using HPTS in a root hydrotropism assay, asymmetric H^+ extrusion was observed because the fluorescence of HPTS in the convex (dry) side showed lower 458/405 value than that of the concave (moist) side of the bending root, indicating a lower apoplastic pH in the dry side [90].

BOX 2. PP2C-A and ABA signalling in plasma membrane

PP2C-A (clade A protein phosphatases type 2C) consist of 9 members out of 76 Arabidopsis PP2Cs, which are classified in 7 major subgroups (A to G) and other leftover PP2Cs [105]. PP2C-A can regulate the activity of subclass III SnRK2s by physically blocking the kinase active site and dephosphorylating the conserved Ser residue (Ser^{175} for SnRK2.6) in the activation loop of the kinase [107-109]. Structural comparison of receptor-phosphatase and substrate (SnRK2)-phosphatase complexes has revealed a molecular mimicry mechanism whereby the hormone receptor and the kinase alternate the binding to the PP2C-A [107]. Upon increase of endogenous ABA levels by abiotic stresses, PYR/PYL/RCARs inhibit competitively the PP2C-A and release subclass III SnRK2s that act as positive regulators in ABA signalling [64, 65, 80]. Subclass III SnRK2s phosphorylate numerous targets, including ABFs/AREBs transcription factors and the chromatin-remodeler ATPase BRAHMA, for activation of ABA transcriptional response [110, 111]. However, ABA signalling also plays a fundamental role in the plasma membrane (PM) for regulation of ion and water transport [112]. These PP2C-A- and SnRK2-dependent changes in PM transport are not restricted to guard cells only, for example, regulation of K^+ transport, anion efflux and activity of PM H^+-ATPase also occur in Arabidopsis roots, although their connection with plant physiology has been less studied [85, 90]. Although frequently overlooked, PP2C-A also have important targets in the PM, such as the S-type anion channel SLAC1, K^+ transporters and PM H^+-ATPase [90, 113-116]. PP2C-A rapidly dephosphorylate SLAC1, which together with down regulation of SnRK2s prevents unspecific

735 Ca^{2+} signalling in PM in the absence of ABA [116]. The recent role of ABI1 in regulation of
736 PM H^+-ATPase activity, further extends the role of PP2C-A in PM.
737
738 **Figure Legends** (250 words per legend)
739
740 **Figure 1. Cartoon representation of BRI1, AHA2, ABI1 and TMK1 based on reported**
741 **crystal structures.** Structures of the leucine-rich repeat (LRR) domain of the BR receptor BRI1
742 (PDB code 3RGX) and BRI1 kinase domain (PDB code 5LPZ), AHA2 (PDB code 5KSD) and
743 C-ter of AHA2 was created by the program MODELLER version 10.1
744 (http://salilab.org/modeller/) using 2O98 as a template, LRR domain of TMK1 (PDB code
745 4HQ1) and TMK1 kinase domain created by MODELLER version 10.1 using 5LPZ as a
746 template. The cytosolic ABI1 (PDB code 3JRQ) interacts with the R domain of AHA2. TM,
747 transmembrane; PM, plasma membrane; BRI1, BRASSINOSTEROID INSENSITIVE1; ABI1,
748 ABA INSENSITIVE1; AHA2, Arabidopsis PM Proton Pump H^+-ATPase2; TMK1,
749 TRANSMEMBRANE KINASE1; A domain, Actuator domain acts as an intrinsic phosphatase,
750 which dephosphorylates the P (phosphorylation) domain during each catalytic cycle of P-type
751 ATPases; N domain, Nucleotide-binding domain binds ATP and phosphorylates the P domain;
752 R domain, C-terminal regulatory domain, consisting of approximately 100 amino acid residues;
753 N-ter, N- terminus; C-ter, C-terminus; AMPPNP or AMPPCP, Non-hydrolysable analogues of
754 ATP**.**
755
756 **Figure 2. Working model of AHA2-mediated proton (H^+) extrusion regulated by**
757 **brassinosteroids and auxin.** In the absence of brassinosteroids (BRs) and auxin (left), BRI1 is
758 inactive. Hence, the constitutively active BIN2 kinase phosphorylates the BZR family of
759 transcription factors and negatively regulates their activity through multiple mechanisms [29].
760 Aux/IAA proteins bind to ARFs and inhibit their transcriptional activity as well. Then, The
761 SAURs are not expressed and PP2C-D interacts and dephosphorylates the C-terminus of AHA2
762 to keep its basal activity and to limit cell expansion by suppressing H^+ extrusions. In the
763 presence of BRs, BRI1 is activated resulting in induction of the SAURs proteins through

764 downstream BZR-dependent signalling [52]. It remains to be determined if BRI1 directly
765 activates AHA2 via phosphorylation (dashed line). In the presence of auxin, TMK1 binds the
766 PM H^+-ATPase and phosphorylates the penultimate Thr residue in the C-terminus within
767 seconds [15, 16]. SAURs are also induced by auxin through a $SCF^{TIR1/AFB}$-mediated signalling
768 pathway. SAURs bind directly to the PM-localized PP2C-D2/PP2C-D5/PP2C-D6 to repress
769 their phosphatase activities, thus preventing Thr^{947} dephosphorylation and keeping the PM H^+-
770 ATPases in an active state [53]. Ultimately, the increased proton pump activity acidifies the
771 extracellular space, activating cell wall-related enzymes to loosen the cell wall. PM, plasma
772 membrane; BRI1, BRASSINOSTEROID INSENSITIVE1; BIN2, BR INSENSITIVE2; BZR,
773 BRASSINAZOLE-RESISTANT; SAURs, AUXIN-INDUCED SMALL AUXIN UP-RNAs;
774 PP2C-D, Clade D PP2Cs; AHA, Arabidopsis PM H^+-ATPase; SCF, Skp1/Cullin1/F-box
775 PROTEIN UBIQUITIN LIGASE; TIR1/AFB, TRANSPORT INHIBITOR RESPONSE1/
776 AUXIN SIGNALING F-BOX PROTEIN.
777
778 **Figure 3. Working model of AHA2-mediated proton (H^+) extrusion regulated by ABA.** (A)
779 When ABA signalling is turned-off, PP2C-A have different targets, e.g. subfamily III SnRK2s
780 and PM targets such as PM H^+-ATPase AHA2. For example, the phosphatase ABI1 interacts
781 with and dephosphorylates Thr^{947} at the C-terminus of AHA2 to decrease its activity [90]. (B)
782 When ABA levels rise, nM increases can be perceived by PYL/PYL/RCAR ABA receptors,
783 which form a ternary complex (receptor-ABA-phosphatase) with PP2C-A. Hence, PP2C-A is
784 inhibited and becomes unable to bind and dephosphorylate AHA2, which maintains Thr^{947}
785 phosphorylation. The dashed line indicates a possible phosphorylation of the C-terminus of
786 AHA2 by SnRK2.2, which has not been demonstrated yet in vivo [85]. Activation of AHA2
787 leads to apoplastic acidification, and the subsequent PM hyperpolarization drives PM transport
788 processes. For example, K^+ uptake by K^+ channels and anion symporters. The influx of solutes
789 maintains the water flux into the cell, which maintains turgor pressure. Therefore, activation of
790 AHA2, in addition to acidification of the apoplast to favour cell wall extensibility (loosening is
791 facilitated by acid-activated apoplastic enzymes), also leads to influx of water, promoting cell
792 expansion. PM, plasma membrane; PYR, PYRABACTIN RESISTANCE; SnRK2s, ABA-
793 ACTIVATED SNF1-RELATED PROTEIN KINASES2; PP2C-A, Clade A PP2Cs.

N-ter

LRR domain

N-ter

LRR domain

Extracellular Space

TM domain

TM domain

TM domain

Plasma Membrane

Cytoplasm

AMPPNP

C-ter

Kinase domain

N-ter

K^+

AMPPCP

Mg^{2+}

A domain

N domain

R domain

C-ter

P domain

C-ter

N-ter

C-ter

C-ter

Kinase domain

Extracellular Space

PM

Cytoplasm

Nucleus

BRI1

TMK1

BAK1

TM

PP2C-D

P

N

A

C-ter

LRR

Kinase

BSKs/ CDG1

BSU1

BIN2

bikinin

KIB1

Ub

E3

BZRs

14-3-3

PP2A

IAA

AUX/IAA

SCFs$^{TIR/AFB}$

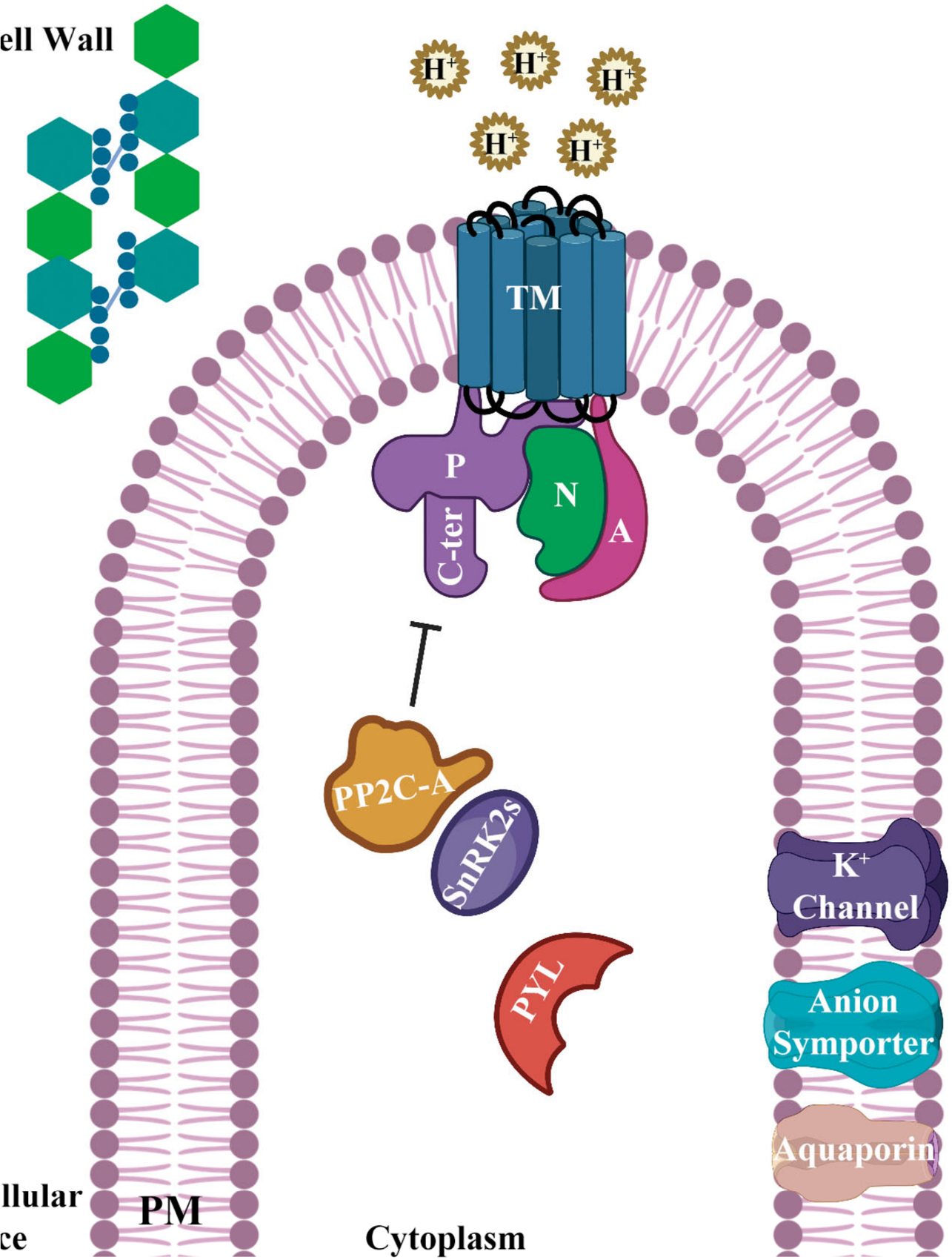
Auxin

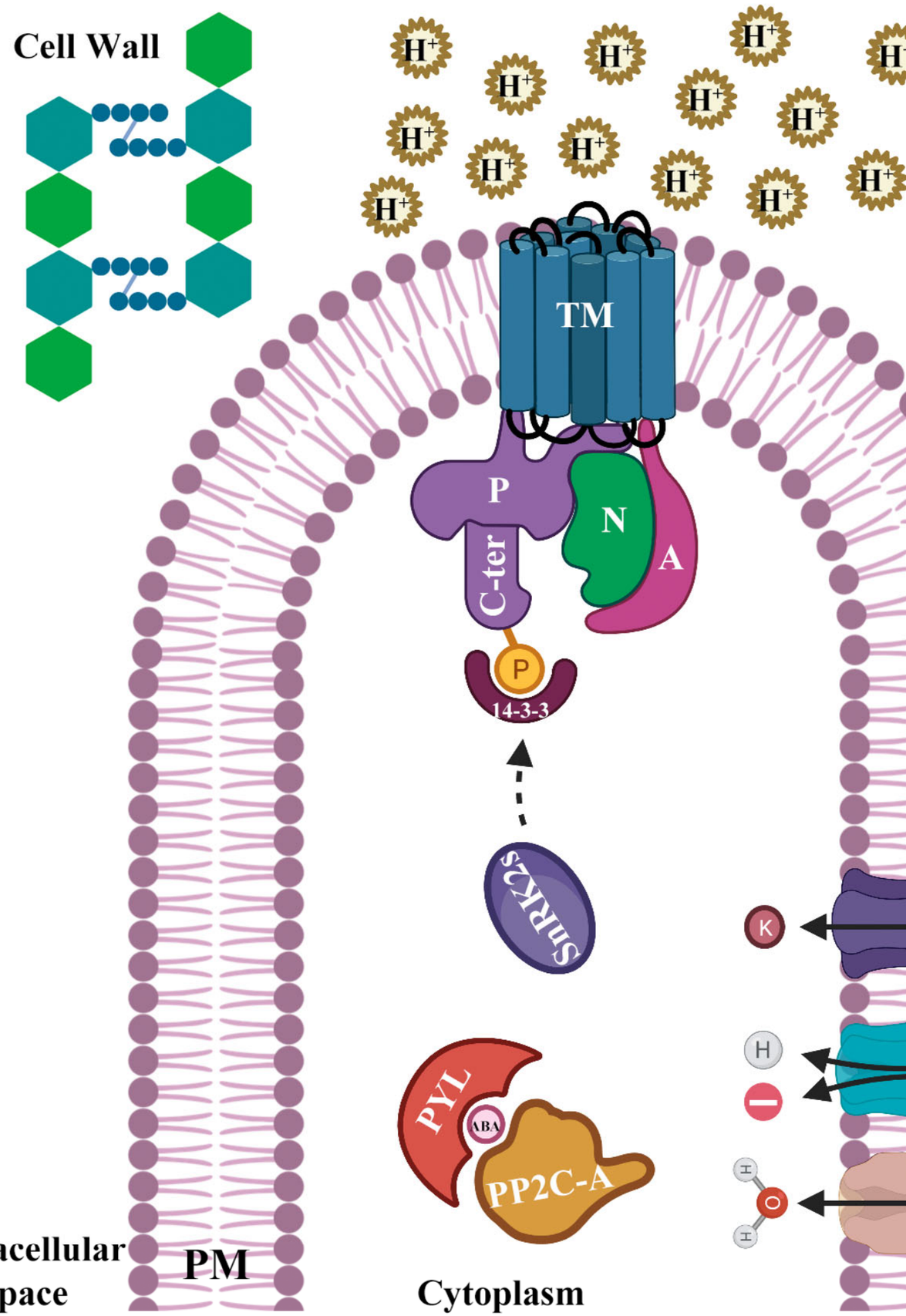
SAUR

H

Br

(B)


ell Wall
H+
TM
P
N
A
C-ter
PP2C-A
SnRK2s
PYL
K+ Channel
Anion Symporter
Aquaporin
llular ce
PM
Cytoplasm


Cell Wall
H+
TM
P
N
A
C-ter
P
14-3-3
SnRK2s
PYL
ABA
PP2C-A
K
H
Extracellular Space
PM
Cytoplasm