Guard cell sensory systems: recent insights on stomatal responses to light, abscisic acid, and CO₂

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Abstract

By controlling the opening and closure of the stomatal pores through which gas exchange occurs, guard cells regulate two of the most important plant physiological processes: photosynthesis and transpiration. Accordingly, guard cells have evolved exquisite sensory systems. Here we summarize recent literature on guard cell sensing of light and drought (via the phytohormone abscisic acid (ABA)). New advances in our understanding of how guard cells satisfy the energetic and osmotic requirements of stomatal opening and utilize phosphorylation to regulate the anion channels and aquaporins involved in ABA-stimulated stomatal closure are highlighted. Omics and modeling approaches are providing new information that will ultimately allow an integrated understanding of guard cell physiology.

Introduction

Guard cells define and regulate stomatal pores, the portals through which terrestrial plants exchange gases with the atmosphere. Guard cells therefore regulate photosynthetic CO₂ capture and plant water status and, accordingly, have evolved a multitude of mechanisms for sensing and responding to both physical and biotic stimuli. Here we briefly summarize some of the recent discoveries regarding guard cell responses to light, ABA and CO₂, and highlight one particular aspect of guard cell function: anion channel regulation by phosphorylation.

Stomatal opening: blue and red light

Red and blue light promote stomatal opening, thus ensuring CO₂ availability to mesophyll photosynthesis. The phototropins PHOT1 and PHOT2 are the major blue light-specific photoreceptors in guard cells (for details on other blue light photoreceptors, see [1]). Blue light perception initiates a signaling cascade that is not yet completely elucidated but includes phototropin-mediated phosphorylation of the protein kinase BLUE LIGHT SIGNALING 1 (BLUS1) [2,3], activation of protein phosphatase 1 (PP1), and recruitment of 14-3-3 proteins to AHA H⁺ ATPases [1,4]. Consequent H⁺ ATPase activation drives H⁺ extrusion that hyperpolarizes the guard cell membrane, which provides a driving force for K⁺ uptake through KAT1 K⁺ channels and increases their open probability. Accumulation of K⁺, the counterions Cl⁻ and malate, and sugars drives water uptake, guard cell inflation, and stomatal opening. Malate and sugars can be derived from starch hydrolysis and a recent study showed that phototropins control activation of amylases BAM1 and AMY3 in guard cells [5]. Stomatal opening is energetically demanding and recent measurements of lipid droplets and H⁺ extrusion in wild-type vs triacylglycerol metabolism mutants during light-stimulated stomatal opening indicate that, in addition to starch breakdown and glycolysis [6], triacylglycerol breakdown and subsequent beta oxidation of fatty acids is an important source of ATP [7].

Mesophyll photosynthesis reduces intercellular CO₂ concentrations and, as summarized in [8], it has been debated whether “red light-specific” stomatal opening is actually a CO₂ response rather than a light response. In recent work, it has been observed that a mutant in the protein kinase HIGH TEMPERATURE 1 (HT1) is impaired in both red light and low CO₂-induced stomatal opening [9]. However, double mutants of the β carbonic anhydrases (CA) CA1 and CA4 are CO₂-hyposensitive yet exhibit stomatal opening in response to red light [9], indicating that the red light response is not simply a CO₂ response. Red light signaling relies on guard cell chlorophyll [8,10,11], but few specific pathway components have been identified.

Stomatal closure: ABA

ABA inhibits H⁺ extrusion [12,13] and activates Ca²⁺-uptake channels [14-16] as well as both slow “S-type” (SLAC1) and rapid “R-type” (AtALUMINUM ACTIVATED MALATE TRANSPORTER 12/QUICK ANION CHANNEL 1 (AtALMT12/QUAC1)) anion efflux channels [17-21]. Consequent membrane depolarization activates a voltage-gated outward K⁺ channel, GORK, that mediates K⁺ efflux [22]. Combined anion and K⁺ efflux drives water efflux, guard cell deflation, and stomatal closure. ABA signal transduction has been intensively studied. ABA binding by PYR1/PYL/RCAR soluble ABA receptors [23-25] inhibits PP2C phosphatases such as ABI1, ABI2 and HAB1 [26-28], which otherwise negatively regulate the serine-threonine kinase OST1 [29,30]. OST1 activation stimulates NADPH oxidase (AtrbohD/F) –dependent ROS production, which triggers nitric oxide (NO) production, MAPK activation, GHR1-dependent opening of Ca²⁺ permeable channels, and cytosolic Ca²⁺ elevation [14,16,31-

34]. Between these early events and the endpoint of ion channel activation numerous intracellular signaling elements reside [35], too many to review here. Below we focus on some newly-identified components.

New elements in ABA signaling

PIP2;1 was recently identified as a key aquaporin mediating water efflux during ABA-induced stomatal closure [36]. In oocytes, water transport by PIP2;1 is increased by co-expression with OST1. *in vitro*, OST1 phosphorylates PLASMA MEMBRANE INTRINSIC PROTEIN 2;1 (PIP2;1) at S121, and complementation of *pip2;1* mutants with PIP2;1 S121A results in loss of ABA-stimulated water permeability and in ABA hyposensitivity of stomatal closure. However, complementation with the phosphomimetic S121D PIP2;1 does not constitutively close stomata even though elevated basal water permeability is observed. Water permeability is not further increased by ABA yet, surprisingly, these lines still exhibit restoration of ABA-induced stomatal closure, which speculatively may arise from PIP2;1-mediated H₂O₂ uptake [36].

Previous research demonstrated that ABA stimulates sphingosine-1-phosphate (S1P) production [37], and that S1P stimulates stomatal closure and activation of slow anion channels and inhibits stomatal opening and KAT1 K⁺ uptake channels [37-40]. These responses are lost in null mutants of the sole canonical G protein α subunit, GPA1, demonstrating that S1P signaling is G-protein dependent [37]. S1P can be synthesized by alkaline ceramidases, and new research shows that mutants of *ARABIDOPSIS TURGOR REGULATION DEFECT 1 (TOD1)*, a Golgi-localized alkaline ceramidase display hyposensitivity of ABA-induced stomatal closure and restoration of closure by exogenous S1P [41].

Several other newly identified ABA signaling elements have yet to be fully positioned within the signaling network. Recent genetic and electrophysiological analyses showed that inhibition of the calcium-dependent protein kinase, CPK33, by THI1, a thiamine thiazole synthase (an essential enzyme in vitamin B1 synthesis) is required for full ABA responsiveness [42]. Another CPK, CPK8, activates the catalase CAT3 by phosphorylation at S261, which presumably provides a brake on ABA-induced ROS elevation [43]. *cpk8* and *cat3* mutants show elevated H₂O₂ levels which might be expected to promote stomatal closure (given that ABA elevates ROS in guard cells), yet these mutants exhibit reduced ABA and Ca²⁺ inhibition of inward K⁺ channels, impaired ABA, H₂O₂, and Ca²⁺ induced stomatal closure, and enhanced drought sensitivity [43]. Apparently, it is not simply absolute ROS levels but their dynamic regulation that is essential for faithful transduction of the ABA signal.

Kinase regulation of anion channels

Numerous roles of kinases and phosphatases as *intermediaries* in guard cell ABA signaling are known, as reviewed elsewhere [32,44,45]. Below we briefly discuss *direct* phospho-regulation of anion channels (Table 1), especially as revealed from experiments in which the relevant channel and candidate regulatory protein(s) are expressed in *Xenopus* oocytes for electrophysiological analysis. In brief, the SLAC1 anion channel is activated by direct phosphorylation via two calcium-independent kinases: the above-mentioned OST1, which is a SnRK2 type kinase (sucrose nonfermenting-1 (Snf1)-related protein kinase 2.6), and the receptor-like kinase GHR1 [31]. SLAC1 channels are also activated by CPKs, and by CIPKs, which are protein kinases activated upon Ca²⁺ binding by interacting calcineurin B-like (CBL) protein partners [44]. As reviewed in [46], PP2C-type phosphatases are implicated in the converse regulatory event, SLAC1

dephosphorylation. In addition to SLAC1, guard cells also express another kinase-activated S-type anion channel, SLAH3 [47,48], although mutant phenotypes of *slah3* knockout plants have yet to be observed [47].

Challenges to understanding ion channel phosphoregulation

One universal challenge in decoding kinase regulation is the issue of target site specificity. For example, phosphoproteomic analysis of *in vitro*-phosphorylated SLAC1 peptides indicates that OST1 can also phosphorylate CPK's preferred site, S59, [49] and that CPK6 can also phosphorylate OST1's preferred site, S120, [50] suggesting lack of absolute specificity. Indeed, one recent study showed that mutation of S59 suffices to reduce SLAC1 activation in oocytes by any of the three kinases OST1, CPK6 or CIPK23/CBL1 [48] although another group reported full activation of S59A SLAC1 by OST1 in oocytes [50].

A second challenge is that while oocytes assays are invaluable for mechanistic studies, they obviously cannot replicate the complexity of the guard cell itself. For example, maximal SLAC1 currents were observed in oocytes upon co-expression of both OST1 and CPK6 [50], suggesting that loss of just one of these kinases' key target sites *in planta* would impair ABA activation of the channels. However, contrary to this expectation, complementation of *slac1* Arabidopsis knockouts with either S120A or S59A SLAC1 mutants restores full anion channel activation and stomatal closure by ABA [50]. Double S59/S120 mutation does engender ABA insensitivity of both guard cell SLAC1 currents and stomatal closure [50].

A similar dichotomy between oocyte and guard cell electrophysiology is seen upon genetic manipulation of the kinases themselves. For example, in guard cells, *ost1* knockout suffices to block ABA activation of slow anion currents [51], even though the GHR1, CPK, and CIPK/CBL signaling systems are still present. One possibility is that in guard cells OST1 is required to activate the other kinases, either directly or indirectly via OST1 regulation of cytosolic Ca^{2+} [52]. Some data point toward the latter mechanism, as ABA activation of Ca^{2+} permeable channels is also impaired in *ost1* [51]. On the other hand, GHR1 is a Ca^{2+} -independent kinase and some CPKs (e.g. CPK6 and CPK23) [52,53] are active at resting cellular Ca^{2+} levels, which would perhaps suggest a contribution by the former mechanism.

Similar to the loss of ABA activation of S-type currents upon sole knockout of OST1 in Arabidopsis [51], double knockout of CPKs 3 and 6 [54] or quadruple knockout of CPKs 5, 6, 11, and 23 [50] suffices to abrogate ABA-activation of S-type currents in guard cells, despite the presence of GHR1, OST1 and CIPK/CBL kinase systems. Moreover, despite loss of S-type current activation, in the quadruple *cpk* mutant, ABA-induced stomatal closure is only partially impaired [50], perhaps because ABA activation of the R-type anion channel QUAC1/AtALMT12 [19] still occurs. AtALMT12 is directly activated by OST1 in oocytes [55] although a phosphosite(s) has yet to be identified, and knockout of *OST1* results in reduced activation of R-type anion currents in guard cells [55].

In another surprising twist, Arabidopsis loss of function mutants of CIPK23 exhibit ABA hypersensitivity of stomatal closure, as opposed to the anticipated ABA hyposensitivity given CIPK23 activation of SLAC1 (Table 1), perhaps because *cipk23* mutants show elevated transcript levels of other key ABA signaling elements [48].

A third challenge is posed by the complexity of the kinase families themselves, exemplified by the 34-member CPK family [56]. The ABA-hypersensitivity of *cpk33* insertional mutants [42] discussed earlier illustrates a negative role of a *cpk* in ABA signaling in addition to positive roles (cf. Table 1). Moreover, not all CPKs are involved in ABA signal transduction: *cpk10*, *cpk4cpk11* show wild-type ABA-induced stomatal closure, as do *cpk7 cpk8 cpk32* triple mutants [57]. The latter result is unexpected given the ABA hyposensitivity of the *cpk8* single mutant [43], possibly suggesting opposing roles of CPK8 and CPK7/32. In short, much work remains to dissect guard cell phosphoregulation. Quantitative phosphoproteomic studies, *in vivo* kinase assays, and parallel measurements of stomatal response in null, kinase-dead, and kinase phosphomimetic lines may provide paths forward.

Evolutionary conservation of anion channel phosphoregulation

A new line of research that could yield additional insights into kinase-dependent regulation of SLAC/SLAH channels is evolutionary analysis. Most core elements of the ABA pathway, including SLAC-type channels, OST1-like SnRK2s and ABI1 phosphatases, predate the emergence of mosses [58]. SLAC-type channels have been found in all plant genomes assessed to date [20] and the family appears to have originated in prokaryotes [59]. However, N-terminal sites implicated in kinase regulation of SLAC1 first appear in moss SLAC/SLAH orthologs. Indeed, SLAC/SLAH channels of more evolutionary ancient taxa (liverwort and Streptophyte alga) are not activated by OST kinases [60] while a moss (*Physcomitrella patens*) SLAC ortholog PpSLAC1 is activated by Arabidopsis OST1 or by a *Physcomitrella* OST ortholog [60]. Activation can be blocked by co-expression with ABI1 phosphatases from either species [60]. These findings suggest the evolution of ABA-dependent regulation of SLAC channels might coincide with the appearance of ABA-dependent regulation of stomatal closure in the mosses [61-63]. Activation of moss SLACs by CPKs or CIPKs has not yet been examined, but both types of kinases and the CBLs required for CIPK function are also found in *Physcomitrella* [64,65].

The S59 CPK/CIPK phosphorylation site lies in a region of low conservation between evolutionarily-distant SLAC/SLAH channels, making it difficult to judge conservation of this phosphosite across lycophytes, mosses, and angiosperms. Interestingly, the most conserved N-terminal motif present in Arabidopsis kinase-activated SLAC/SLAH channels (SLAC1, SLAH2 and SLAH3; see Table 1 and [66]) includes the S120 OST1 phosphosite of SLAC1 and the putative CPK21 T187 phosphosite of SLAH3. An alignment of this motif (Figure 2) shows that the T187 site is highly conserved, suggesting that phosphorylation here might generally activate SLAC family channels, and it would be intriguing to examine this possibility further. There are several additional broadly-conserved Ser and Thr residues in the region between the SLAH3 T187 and SLAC1 S120 sites that could also potentially serve as phosphorylation sites. Some of these are more conserved than the S120 site itself, which interestingly is a Glu in PpSLAC1, the channel that is readily activated by OST kinases [60]. Thus OST-dependent activation must involve an alternate mechanism in PpSLAC1, suggesting that our knowledge of physiologically-relevant OST1 phosphosites in SLAC1 is incomplete. Consistent with this idea, a phosphomimetic substitution at S120 is not sufficient to activate Arabidopsis SLAC1 [67]. Detailed examination of phosphorylation-dependent activation of evolutionarily diverse SLAC/SLAH channels could lead to new mechanistic insights into kinase regulation of S-type anion currents.

Stomatal closure: elevated CO₂ concentrations

ABA and high CO₂ target similar *ion transport* mechanisms [68-72]; however, the extent to which ABA and high CO₂ *signaling* pathways overlap is a current point of debate, starting with the essentiality of ABA itself. One recent report suggests essentiality, showing CO₂ insensitivity in ABA quadruple receptor mutants and ABA synthesis mutants [73] while another report shows that the same quadruple receptor mutant has slowed but functional CO₂ responses [71].

New research suggests that ABA-stimulated water efflux and CO₂/HCO₃⁻ entry both utilize PIP2;1: expression of this aquaporin in oocytes along with CA4, SLAC1 and SLAC1-phosphorylating kinases OST1 or CPK6, allows SLAC1 activation by extracellular CO₂/HCO₃⁻ [74]. However, PIP2;1 is unlikely to be the only route for CO₂/HCO₃⁻ entry into guard cells given that Arabidopsis *pip2;1* mutants show wild-type stomatal closure kinetics in response to elevated CO₂ [36].

The full complement of guard cell CO₂/HCO₃⁻ sensors remains unknown. Stomatal CO₂ hyposensitivity of *calca4* double mutants implicates carbonic anhydrases CA1 and CA4 as CO₂/HCO₃⁻ sensors or transducers [75], and a MATE-type transporter is also implicated [76]. CA4 localizes at the plasma membrane and CA1 in chloroplasts [77]. However, cytosolic mammalian CAII suffices to restore stomatal CO₂ sensitivity to *calca4* mutants [77], which might suggest that the role of CAs is provision of HCO₃⁻ which is then sensed by a different receptor. Elevated intracellular CO₂/HCO₃⁻ can activate SLAC1 in oocytes, if OST1, CPK6, or CPK23 is co-expressed [74], implying CO₂-sensing by either the channel or kinase. Complementation of *slac1* knockout Arabidopsis plants with truncated SLAC1 proteins that lack the N and C termini (which harbor OST1 and CPK phosphorylation sites [78]) restored CO₂ responsiveness but not ABA responsiveness of SLAC1 activation and stomatal closure [79]. This dichotomy suggests either an ABA-independent component of the CO₂ response, or an ABA-responsive sub-network upstream of SLAC1 that is sufficient to restore the CO₂ response but insufficient to restore the ABA response. Conversely, when Arabidopsis *slac1* mutants were complemented with a construct expressing SLAC1 mutated at one of two tyrosine residues, Y243 or Y462, ABA-induced stomatal closure was restored but CO₂-induced stomatal closure was reduced as compared to complementation with wild-type SLAC1. To date, no evidence for phosphorylation of either residue has been obtained [79], so how these sites regulate CO₂ responsiveness is an intriguing question.

Perspectives

This brief review has touched on only a subset of known guard cell signaling cascades. For example, guard cell responses to pathogens are not covered [80], and we have summarized signaling downstream of the PYR/PYL/RCAR ABA receptors, but other ABA binding proteins also exist [81-84]. Besides protein phosphorylation, nitrosylation and other redox modifications are emerging as important post-translational regulator elements [45,85,86]. We also have not discussed signal integration. Both continuous and discrete modeling approaches [4,35,87] are providing novel insights into inter-connected guard cell signaling networks and their impacts on ion transport. For example, new predictions of ABA-red light antagonism arose from a multi-level discrete model that integrated red light, blue light, CO₂, and ABA regulatory networks [4], while a kinetic model of solute fluxes and cytosolic buffering successfully predicted indirect effects of *SLAC1* knockout on K⁺ currents of guard cells [88]. Modelling approaches will become increasingly essential as knowledge regarding this vital cell type continues to proliferate.

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Figure Legends

Figure 1. Kinase dependent regulation of SLAC/SLAH anion channels. (A) Schematic diagram of an individual SLAC/SLAH subunit based on structural analysis [59]. Five transmembrane domains (purple cylinders) define a central pore and are surrounded by an outer shell of 5 additional transmembrane domains (blue cylinders). N- and C- termini are cytoplasmic and may lie in close proximity because they emerge from adjacent transmembrane domains. Important identified sites for kinase-dependent regulation of plant SLAC1/SLAH3 channels are labeled in pink: 1,2) the SLAC1 S59 and SLAH3 T187 phosphosites for CPKs, 3) the SLAC1 S120 phosphosite for OST1, and 4) a C-terminal SLAC1 motif involved in OST1-dependent regulation of *Physcomitrella* SLAC channels [60]. (B) Summary of CPK, CIPK/CBL and OST1-dependent activation of Arabidopsis SLAC/SLAH channels observed in *Xenopus* oocytes. Checks indicate activation has been observed, an X indicates activation does not occur, and n.d. indicates not done.

Figure 2. Evolutionary conservation of an N-terminal motif implicated in kinase-dependent activation of SLAC/SLAH channels. Identical or conservatively substituted residues present in at least 5 sequences are shaded. Green indicates conserved serine and threonine residues and blue indicates conserved basic residues. The putative CPK phosphosite T187 of SLAH3 and the OST1 phosphosite S120 of SLAC1 are boxed in red. Channels are divided into SLAC1 and SLAH2/3 groups observed in phylogenies [20]; the placement of Ppat_Pp3C9_17220 and Ppat_Pp3C14_15640 relative to these groups is uncertain. Gene names are given at the left (Atha, *Arabidopsis thaliana*; Smel, *Sellaginella mellindorfi*; Ppat, *Physcomitrella patens*) and amino acid positions are given at the right. Phytozome [89] sequence identifiers are given in the *Sellaginella* and *Physcomitrella* channel names. PpSLAC1 and PpSLAC2 are named as in [60]; Phytozome identifiers are Pp3c1_33890 and Pp3c2_26550, respectively. For Smel_31887 and Smel 122390, the motif shown was not present in current gene predictions but could arise given assumed intron splicing.

Table 1. Diverse kinases activate S-type (SLAC1, SLAH3) and R-type (QUAC1/AtALMT12) anion channels of Arabidopsis guard cells.

Kinase	Phosphosite	<i>In vitro</i> phosphorylation: Recombinant proteins/peptides	Oocyte electrophysiology assay	Arabidopsis mutant: guard cell currents	Arabidopsis mutant: stomatal phenotype	Reference
SLAC1 anion channel						
GHR1	unknown	Gel autoradiography on SLAC1 N- and C- termini showed phosphorylation of N-terminus (a.a. 1-186) but not C- terminus (a.a. 501-556)	SLAC1 activated by GHR1 coexpression, not by kinase-dead K798E GHR1 coexpression	Reduced ABA/H ₂ O ₂ activation of S-type currents in <i>ghr1</i> guard cells	Reduced ABA/H ₂ O ₂ activation of stomatal closure/inhibition of stomatal opening in <i>ghr1</i> guard cells	[31]
OST1	unknown	Gel autoradiography on SLAC1 N- and C- termini showed phosphorylation of both N- and C- termini	SLAC1 activated by OST1 coexpression, not by kinase-dead K50N OST1 coexpression			[78]
OST1	S120	Gel autoradiography, MS/MS on SLAC1 ¹⁻¹⁸⁶ N terminus			<i>S120F slac1</i> tilling mutant deficient in ozone-induced stomatal conductance decreases	[49]
OST1	S120	Gel autoradiography on SLAC1 N- and C- termini; Peptide arrays of mutant N- and C- termini identified S120 candidate phosphosite	S120A SLAC1 not activated by OST1 co-expression, but S120D SLAC1 (phosphomimetic) does not suffice to activate SLAC1 without OST1 coexpression; SLAC1 weakly activated by OST1 homologs SnRK2.2 2.3, 2.8	Reduced ABA activation of SLAC1 currents in <i>ost1</i> guard cells		[67]
OST1	S120		S120A SLAC1 not activated by OST1 co-expression	Complementation of <i>slac1</i> mutant with <i>S120A SLAC1</i> fully restored ABA	Complementation of <i>slac1</i> mutant with <i>S120A SLAC1</i> fully restored ABA	[50]

				activation of S-type currents	activation of stomatal closure	
OST1	S59	Gel autoradiography; MS/MS on SLAC1 ¹⁻¹⁸⁶ N terminus				[49]
OST1	S59		S59A SLAC1 activated to the same level as wild-type by OST1 co-expression	Complementation of <i>slac1</i> mutant with S59A SLAC1 partially restored ABA activation of S-type currents	Complementation of <i>slac1</i> mutant with S59A SLAC1 fully restored ABA activation of stomatal closure	[50]
OST1	S59		S59A SLAC1 activated at a reduced level by OST1 coexpression			[48]
CPK3 and CPK6	unknown			No ABA activation of SLAC1 currents in <i>cpk3 cpk6</i> guard cells	Partial activation of stomatal closure by ABA in <i>cpk3 cpk6</i> guard cells	[54]
CPK3, CPK6	unknown	Gel autoradiography on SLAC1 N-terminus showed phosphorylation by CPK3, CPK6, CPK21, or CPK23	SLAC1 activated by CPK6 and by Ca ²⁺ -independent CPK3deLEF coexpression			[52]
CPK6	S120	<i>In vitro</i> phosphorylation followed by MS/MS	S120A SLAC1 activated to the same level as wild-type by CPK6 coexpression			[50]
CPK6	S59	In gel autoradiography shows <i>in vitro</i> phosphorylation of SLAC1 N-terminus but not C-terminus. MS/MS identified S59 as a phosphorylation site.	SLAC1 activated by coexpression of CPK6 or CPK23 but not CPK3 or CPK12 or CPK31; S59A SLAC1 not activated by CPK6 coexpression; Phosphomimetic S59D SLAC1 did not show constitutive activation			[90]
CPK6	S59	Gel autoradiography on SLAC1 N-terminus (also with CPK23)	SLAC1 activated by CPK6 or CPK5 coexpression; S59A SLAC1 not activated by CPK6 coexpression (nor by CPK5, CPK23 coexpression)	<i>cpk5/6/11/23</i> quadruple mutant lacked ABA activation of S-type currents; complementation	<i>cpk5/6/11/23</i> quadruple mutant was hyposensitive to ABA-induced stomatal closure; complementation of	[50]

				of <i>slac1</i> mutant with S59A <i>SLAC1</i> partially restored ABA activation of S-type currents	<i>slac1</i> mutant with S59A <i>SLAC1</i> fully restored ABA activation of stomatal closure	
CPK23	unknown	Gel autoradiography on <i>SLAC1</i> N- and C-termini showed phosphorylation of the N-terminus only; CPK21 also phosphorylated the N-terminus (C-terminus not tested)	<i>SLAC1</i> activated upon CPK23 co-expression, not by kinase-dead D193A CPK23 coexpression; <i>SLAC1</i> activated at a reduced level by CPK21 and CPK31 does not activate			[53]
CPK23	S59		S120A <i>SLAC1</i> mutant activated by CPK23 coexpression; S59A <i>SLAC1</i> activated at a reduced level by CPK23			[48]
CIPK23 plus CBL1	S59		<i>SLAC1</i> activated by CIPK23 plus CBL1 or CBL9 coexpression but not by CIPK7 or CIPK15 coexpression (plus all 10 known CBLs); <i>SLAC1</i> not activated by kinase dead K60N CIPK23 plus CBL1; S120A <i>SLAC1</i> activated by CIPK23 plus CBL1 S59A <i>SLAC1</i> activated at a reduced level by CIPK23/CBL1			[48]
CPK6, CPK23, OST1, CIPK23 plus CBL1	T513 Candidate phosphosite identified from peptide arrays of [67]		Phosphomimetic T513D <i>SLAC1</i> showed low-level kinase-independent activity; phosphomimetic T513D <i>SLAC1</i> activated by coexpression of all of the listed kinases			[48]
SLAH3 anion channel						
CPK21, CPK23	unknown	Gel autoradiography on N- or C-terminal domains of <i>SLAH3</i> (amino acids 1 to 257 and 563 to 635) showed only N-	<i>SLAH3</i> not activated by OST1 coexpression; <i>SLAH3</i> activated by coexpression with Ca^{2+} -			[47]

		terminal phosphorylation. Peptide arrays identified Ser184, Thr187, Ser189, and Thr197 as candidate phosphosites.	independent CPK21DEF or CPK23DEF; Kinase-dead CPK21DEFD204A did not activate; Phosphomimetic T187D SLAH3 was constitutively active but S184D, S289D, T197D SLAH3 were not			
CIPK23 plus CBL1	unknown	Gel autoradiography on SLAC1 N-terminus shows phosphorylation by CIPK23 without a CBL.	SLAC1 activated by CIPK23 plus CBL1; kinase-dead K60N CIPK23 plus CBL1 did not activate			[48]
QUAC1/AtALMT12 anion channel						
OST1	unknown		QUAC1/AtAlmt12 activated by OST1 coexpression			[55]

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Figure 1

B

Kinase-dependent activation
in *Xenopus* oocytes

	<u>CPK</u>	<u>CIPK/CBL</u>	<u>OST1</u>
Atha_ SLAC1	✓	✓	✓
Atha_ SLAH2	✓	✓	n.d.
Atha_ SLAH3	✓	✓	✗

Figure 2

		CPK?															OST1														
SLAC1 Clade	Atha_SLAC1	T	D	F	S	M	F	R	T	K	S	T	-	-	L	S	K	Q	K	S	L	L	P	S	124						
	Smel_53336	T	S	Y	N	L	F	K	T	R	S	S	K	S	L	E	K	Q	I	P	W	L	S	K	66						
	Smel_53394	E	K	Y	V	L	F	R	T	R	S	S	K	-	I	E	K	Q	L	S	R	L	R	E	132						
	Smel_122390	N	G	Y	A	A	F	S	T	C	A	R	K	-	L	T	R	D	S	K	D	P	N	L	57						
	Smel_31887	D	T	Y	T	M	F	Q	T	R	S	P	-	-	M	S	R	L	S	F	N	S	R	N	108						
	PpSLAC1	G	T	Y	D	L	F	K	T	R	S	R	K	-	F	S	G	P	L	E	R	I	A	T	152						
	PpSLAC2	A	P	Y	D	K	F	R	T	R	S	T	R	-	Y	D	R	Q	G	S	S	A	R	V	155						
	Ppat_Pp3c9_17220	G	N	Y	G	L	F	R	T	R	S	T	S	-	G	S	R	G	N	S	L	T	N	V	299						
	Ppat_Pp3c14_15640	Q	D	H	P	P	H	K	H	R	H	S	-	-	L	D	K	S	S	L	D	A	H	Q	81						
SLAH3 Clade	Atha_SLAH2	K	R	Y	D	L	F	R	T	M	S	G	K	-	L	E	R	Q	I	S	N	L	R	G	89						
	Atha_SLAH3	N	R	Y	N	S	F	K	T	W	S	G	K	-	L	E	R	Q	F	T	R	K	P	A	201						