Review

AGCVIII kinases: at the crossroads of cellular signaling

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AGCVIII kinases regulate diverse developmental and cellular processes in plants. As putative mediators of secondary messengers, AGCVIII kinases potentially integrate developmental and environmental cues into specific cellular responses through substrate phosphorylation. Here we discuss the functionality and regulation of AGCVIII kinases. Specifically, we question the view that activities of AGCVIII kinases, like their animal counterparts, are regulated by a common regulator, 3-phosphoinositide-dependent protein kinase-1 (PDK1). Instead, increasing evidence suggests that Ca^{2+} and phospholipids regulate AGCVIII kinases, by altering their activities or by affecting their subcellular localization. As AGCVIII kinases are at the crossroads of plant cellular signaling, they and the signaling networks in which they participate are keys to a better understanding of plant development and of interactions with their environment.

AGC kinases: mediators of cellular signaling

Phosphorylation is the most common and universal way of regulating protein function in eukaryotes. Reversible phosphorylation regulates protein activity, subcellular localization, stability and interaction with other proteins [1,2]. The AGC kinases belong to one of the six serine/threonine kinase superfamilies in plants [1]. AGC kinases are collectively named to include cAMP-dependent protein kinases (PKA), cGMP-dependent protein kinases (PKG), various types of protein kinase C (PKC), protein kinase B (PKB), 3-phosphoinositide-dependent protein kinase-1 (PDK1), and the ribosomal protein S6 kinases [3]. In animals and yeast, AGC kinases are critical mediators that perceive and interpret signaling initiated from secondary messengers such as cAMP, cGMP, Ca^{2+} and phospholipids through substrate phosphorylation [3]. Although most members of the plant AGC kinases have no assigned function, analysis of some AGC kinase mutants showed that they play essential roles in various cellular and developmental processes (Table 1), such as root hair and pollen tube growth [4–7], auxin transport [8–11], light sensing [12,13] and abiotic and biotic stresses [14–16]. That plant AGC kinases are involved in diverse cellular and developmental processes, together with analogy to the roles played by their animal and yeast counterparts, indicates that plant AGC kinases are at key positions to initiate cellular responses upon perception of developmental cues and extracellular stimuli. Placing AGC kinases in the context of signaling networks by identifying their regulatory mechanisms and downstream components will undoubtedly facilitate our general understanding of plant development and of interactions with their environment.

Plant AGC kinases were initially characterized into several subfamilies, including AGCVIII kinases, AGCVII kinases, AGCVI kinases, AGC other, and homologs of animal PDK1 [17]. The largest AGC kinase subfamily, AGCVIII kinases, was later sub-divided into four groups, named AGC1-AGC4 [18]. In this article, we focus on the AGCVIII subfamily because our understanding of other AGC kinases has not been significantly advanced since their initial characterization in 2003 [17]. We discuss the molecular mechanisms underlying the functionality of AGCVIII kinases in light of recent findings. Specifically,

Glossary

Ade motif: A highly conserved motif of 4 to 5 amino acids, [FD(X)$_{1-2}$Y/F], within the C-terminal tail of most animal AGC kinases, which is responsible for PDK1 binding. However, plant AGC kinases do not have this motif.
EF-hand motif: A helix-loop-helix domain engaged in Ca^{2+}-binding. An EF-hand motif is found in many Ca^{2+}-regulated proteins such as calmodulin, Ca^{2+}-dependent protein kinases and calcineurin B.
C2 domain: A Ca^{2+} and phospholipid-binding domain found in many types of proteins. Ca^{2+} and phospholipid binding to C2 domains regulate protein subcellular targeting and activity.
IP$_3$: Inositol 1,4,5-trisphosphate, a soluble phospholipid regulating the release of Ca^{2+} from internal stores such as the endoplasmic reticulum. It can be generated from another phospholipid PI(4,5)P$_2$ through the activity of phospholipase C, and can be dephosphorylated through the activity of inositol polyphosphate 5-phosphatases.
LOV domain: Light, oxygen, voltage domain. Two LOV domains are located upstream of the catalytic domain of phototropins. Blue light induces phosphorylation changes of LOV domains and of sequences between the two LOV domains, leading to autophosphorylation and activation of phototropins.
PA: Phosphatidic acid, a phospholipid generated either by phospholipase D or by diacylglycerol kinase.
PI3P: Phosphatidylinositol 3-phosphate, a membrane phospholipid enriched at endosomes. PI3P regulates protein sorting at endosomes and regulates vesicle fusions at target membrane compartments by recruiting protein complexes.
PIP$_3$: Phosphatidylinositol 3,4,5-trisphosphate, a membrane phospholipid produced upon extracellular stimuli by the activity of class I PI3P kinases. PIP$_3$ is an instructive signal for actin polymerization. Plants do not have class I PI3P kinase. PIP$_3$ has not been detected in plants.
PDK1: 3-phosphoinositide-dependent protein kinase-1. PDK1 is an evolutionarily conserved ser/thr kinase whose activity and subcellular localization can be regulated by specific phospholipids. Animal PDK1s bind to phosphatidylinositol 3,4,5-trisphosphate (PIP$_3$), which is absent in plants. Although Arabidopsis PDK1 showed binding to diverse phospholipids, only PA significantly increased PDK1 activity *in vitro* and *in vivo*.
PIF: PDK1-interacting fragment. The PIF motif is a highly conserved hydrophobic motif at the C-terminal tail of most AGC kinases, including plant AGC kinases.
TLI: T-loop insertion sequence, a 50 to 80 amino acid sequence present in the activation loop of all plant AGCVIII kinases but absent in animal AGC kinases. A domain-swapping experiment showed that the TLI was critical for subcellular localization of AGCVIII kinases, likely through protein-protein interactions.

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Table 1. Overview of AGCVIII kinases with characterized functions

AGCVIII kinases	Expression specificity	Sub-cellular localization	Function
PINOID (PID)	Primordia of vegetative and floral organs [8,9] Embryo [9,28] Root hairs [11] Auxin-inducible [8]	Plasma membrane [11,28] Punctate vesicles, [11,27] Cell periphery [23]	Embryogenesis [9,10,28,61] Organogenesis and auxin efflux [8–10,28,61] Root hair growth [11]
PINOID2 (PID2)/AGC1-10/AGC3-4	Provascular tissue during embryogenesis [61]	ND	Embryogenesis [61]
WAG1/PK3At and WAG2/AGC1-11	Root tips and lateral root primordia at the seedling stage [6] Cotyledon primordia during embryogenesis [61]	Cytoplasm and nucleus (WAG1) [23]	Root development [6] Embryogenesis [61]
PHOTOTROPIN1(PHOT1) PHOTOTROPIN2 (PHOT2)	Cortical cells in the apical hook and in the root elongation zone in etiolated seedlings [30,33]	Plasma membrane, Punctate vesicles [30,33]	Phototropism, chloroplast relocation, stomata opening, rapid inhibition of growth, leaf expansion and solar tracking [62]
AGC1.5 AGC1.7	Pollen [7]	Cytoplasm (AGC1.7) [23] Cytoplasm (AGC1.5)[a] [7]	Polar and directional growth of pollen tubes [7]
AGC1.6	Root hairs [7,63]	ND	Root hair growth [63]
AGC2-1/OXIDATIVE SIGNAL-INDUCIBLE1 (OXI1)	Seedling and roots [16] Root hairs [4] H2O2, cellulase and wounding-inducible [16]	Developmentally- regulated, vesicles and cytoplasm [4]	Responses to oxidative stress [14,16] Root hair growth [4]
D6 protein kinases (D6PKs: AGC1.1, AGC1.2, AtPK5, AtPK7)	Roots [34]	Basal plasma membrane of root cells Cytoplasm upon BFA treatment [34]	Auxin-regulated lateral root initiation, gravitropism and axillary shoot differentiation [34]
BARREN INFLORESCENCE2 (BIF2)	Axillary meristem and lateral primordia [29]	Nucleus, cytoplasm and cell periphery [35]	Organogenesis [29,64]
AvrPto-dependent Pto-interacting protein 3 (Adi3)	ND	ND	Programmed cell death [15]

Abbreviations: ND, not determined.
[a]YFP-AGC1.5 fusion protein degradation was observed.

we address how their activities are regulated, whether AGCVIII kinases, like their animal counterparts, are signaling mediators of secondary messengers, and with what downstream components they interact. For readers interested in the involvement of AGC kinases in auxin-mediated plant development, two recent reviews are recommended [18,19].

Is PDK1 a master regulator of AGCVIII kinases?

Sequence features of AGCVIII kinases indicate that they are closely related to PKAs [17,20]. PKAs, as well as other animal AGC kinases, are regulated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) through phosphorylation of their activation loop (T-loop). PDK1 is a serine/threonine (S/T) kinase that is highly conserved among eukaryotes. In animals, the production of phosphatidylinositol 3,4,5-trisphosphate (PIP_3), upon perception of an extracellular stimulus, recruits PDK1 through a pleckstrin homology (PH) domain to the plasma membrane, where PDK1 phosphorylates several AGC kinases to initiate signaling cascades [21]. The interaction between PDK1 and animal AGC kinases involves a C-terminal hydrophobic motif named PIF (PDK1-interacting fragment) in AGC kinases [21]. The C-terminal PIF motifs can be either a hexapeptide FXXFS/TY/F, in which the S/T residues are subjected to phosphorylation, or a tetrapeptide FXXF. Docking of PDK1 at the PIF motifs of AGC kinases results in phosphorylation of AGC kinases at a conserved threonine in the T-loop, and their subsequent activation [21]. Recently, a highly conserved motif (Ade motif, see Glossary) within the C-terminal tail [20] of AGC kinases was identified as a second site for PDK1 interaction [22]. According to structural modeling, the ATP-binding pocket and the C-terminal tail of PDK1 interact with the Ade motif and with the PIF motif of its substrate AGC kinase respectively [22], making multiple layers of regulation possible.

The Ade motif [FD$(X)_{1\text{-}2}$Y/F], although highly conserved among animal AGC kinases [20,22], is not present in AGCVIII kinases (our unpublished observations). However, most AGCVIII kinases contain a C-terminal motif similar to the PIF motif of PKAs [17]. Studies of several AGCVIII kinases indicated that plant PDK1s dock on AGCVIII kinases through the C-terminal PIF. One study using an *in vitro* pull-down assay showed that all 15 *Arabidopsis* (*Arabidopsis thaliana*) AGCVIII kinases [17], also designated AGC1/3 [18], interacted with PDK1 [23]. Yeast two hybrid screening also showed an interaction between PDK1 and an *Arabidopsis* AGCVIII kinase, AGC2.1/OXI1 (Table 1) [4]. The interaction between AGCVIII kinases and PDK1 most likely relied on the presence of the PIF motif because PDK1 docking on AGCVIII kinases was abolished by mutating the PIF motif FXXF to AXXA [4], or by replacing the PIF motif with an unrelated short peptide [4,15], or by deleting the PIF motif [14]. Some recent data indicated that the PIF motif might not be critical for interaction with PDK1 [23,24], although alternative interpretations suggest that the question is still open. For example, it was shown that replacing FXXF of the PIF motif in PID (Table 1) with VXXV did not abolish the interaction between PID and PDK1 [24]. However, an F to V mutation would not abolish the hydrophobicity of the PIF that is critical for PDK1 docking [21], as would an F to

A mutation [5]. In the other study, several AGCVIII kinases that do not contain a C-terminal PIF still interacted with PDK1 *in vitro* [23], but, as pointed out [23], the authors did not exclude the possibility that there was an internal PIF motif that accounted for this interaction; this possibility needs to be tested.

Correlated with their promiscuous interaction with AGCVIII kinases, plant PDK1s were able to significantly increase both autophosphorylation and trans-phosphorylation of many AGCVIII kinases *in vitro* [14,15,23,24]. A Thr residue within the T-loop of animal AGC kinases is a phosphorylation site for animal PDK1s [25]. In AGCVIII kinases an invariant Ser at the same position, together with a second Ser two amino acids upstream, are phosphorylation sites for plant PDK1s [4,15,24], suggesting an evolutionarily conserved regulatory mechanism of AGCVIII kinases by PDK1.

However, we think it is still unresolved as to whether PDK1 is the master regulator for AGCVIII kinases for the following three reasons [17]. (i) To regulate AGCVIII kinases, PDK1 expression should overlap with expression of AGCVIII kinases, which are expressed in different tissues and at diverse developmental stages (Table 1). Although microarray data suggest a constitutive expression pattern for PDK1 [26], detailed analysis of its expression by promoter–reporter constructs is missing. To safely conclude biological relevance, *in vitro* physical interaction between PDK1 and AGCVIII kinases [4,15,23,24] will need the support of overlapping expression patterns. (ii) The specificity of the signaling output initiated by a given kinase is also defined by spatial information within a cell. Multiple AGCVIII kinases can be present in the same cell, for example in root hairs [4,7,11,27,28], but in different subcellular compartments. To date, AGCVIII kinases were detected in the cytoplasm [7,23,29,30], at the plasma membrane [11,18,28,30–34], in the nucleus [35], and at punctate vesicles (possibly endosomes or trans-Golgi network (TGN)) [4,27,33,36]. Although it is possible that the spatiotemporal localization of PDK1 in a given cell is dynamically regulated by different extracellular stimuli, co-localization at the same subcellular compartment would have to be demonstrated to establish PDK1 as a bona fide regulator of a particular AGCVIII kinase with which it showed *in vitro* interaction and phosphorylation. (iii) Although PDK1 increased the autophosphorylation and trans-phosphorylation activity of many AGCVIII kinases *in vitro* [14,15,23,24], so far PDK1 activity has only been shown to be required in two AGCVIII kinase-regulated cellular processes *in vivo*, that is, biotic and abiotic stresses, as demonstrated by PDK1 down-regulation by RNAi [14,15]. AGC1.5 and AGC1.7, two AGCVIII kinases involved in pollen tube directional growth, were activated by co-incubation with PDK1 *in vitro* [23]. PDK1 was expressed in pollen tubes and it interacted with AGC1.5 in a yeast two-hybrid (Y2H) assay (our unpublished data). Over-expression of a AGC1.5 mutant, in which the second conserved Ser within the T-loop was mutated to Asp, caused twisted tube morphology, which was different from the phenotype of the AGC1.5 loss-of-function mutant, suggesting that the S to D mutant had altered kinase activity (Figure 1). However, over-expression of PDK1 in pollen tubes did not interfere with tube morphology (Figure 1). Although there might be alternative explanations for this result, we use this example to emphasis the necessity of *in vivo* analysis before establishing PDK1 as the upstream regulator of AGCVIII kinases. Based on these thoughts, we speculate that PDK1 is not the master regulator for all AGCVIII kinases, but instead acts as an upstream regulator for a subset of AGCVIII kinases *in vivo*.

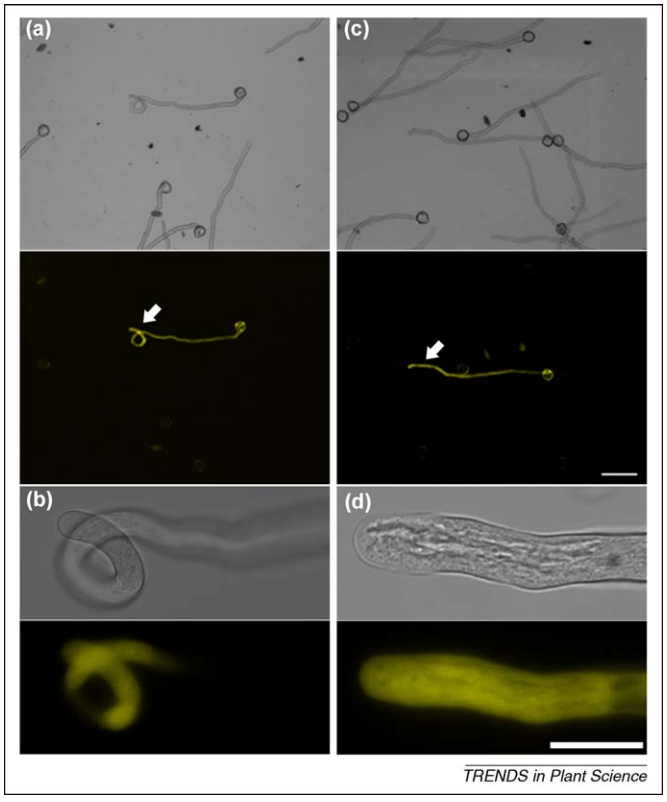

Figure 1. Over-expression of *Arabidopsis* PDK1 in tobacco (*Nicotiana tabacum*) pollen tubes did not phenocopy over-expression of AGC1.5-CA. Related bright field and fluorescent images are shown vertically. **(a-b)** Over-expression of a yellow-fluorescent protein-fused AGC1.5 mutant, YFP-AGC1.5-S408D, in which the second Ser within the T-loop was mutated to Asp. **(c-d)** Over-expression of YFP-PDK1. The same result was obtained when YFP was fused to the C-terminal end of PDK1 (data not shown). Arrows indicate the apical tip of transformed pollen tubes. Non-transformed pollen tubes in **(a)** and **(c)** are not seen in fluorescent images. Scale bar for **(a)** and **(c)** = 100 μm. Scale bar for **(b)** and **(d)** = 20 μm.

Secondary messengers: who is talking and how?

If PDK1 is not an upstream regulator of some AGCVIII kinases, what are these upstream regulators? The most obvious answer would be secondary messengers. Secondary messengers, including cAMP, cGMP, Ca^{2+} and phospholipids, regulate kinase activity, subcellular localization and substrate specificities of animal AGC kinases, mostly through specific sensor domains within AGC kinases, such as the phospholipid-binding PH domain in PKB and the Ca^{2+}/lipid-binding C2 domain in PKCs. Vast amounts of data suggest that secondary messengers are equally important for plant cell signaling (Box 1), but whether AGCVIII kinases function downstream of secondary messengers was unclear.

Now there is some evidence that AGCVIII kinases mediate signaling initiated from secondary messengers, specifically, Ca^{2+} and phospholipids. AGCVIII kinases do

Box 1. Secondary messengers in plants

Secondary messengers, including cAMP, cGMP, Ca^{2+} and phospholipids, are important for cell growth and survival. They regulate the activities, turnover, and subcellular localization of proteins that contain their respective sensor domains. Although most secondary messengers are present in both animals and plants, an understanding of the signaling cascades they initiate in plants is far behind what is known in animals.

Based on structural simulations [57] and *in vitro* biochemical assays [4], AGCVIII kinases resemble animal PKA. However, plant genomes do not encode several components required for cAMP signaling. Although *in vitro* chemical treatment indicated that cAMP regulates pollen tube growth [58], this should be supported by other evidence to substantiate its *in vivo* function.

In animals, cGMP functions downstream of nitric oxide (NO) through binding to PKG, cyclic nucleotide-gated channels (CNGCs), and phosphodiesterases (PDEs) [59]. Both CNGCs and PDEs are encoded in plant genomes and their members have been functionally analyzed [59], suggesting an analogous signaling pathway. Because NO regulates diverse plant developmental and cellular processes, including responses to biotic and abiotic stresses, to hormonal modulation, or during polar cell growth [59], it is plausible that some AGCVIII kinases may function as animal PKG equivalents, mediating NO-initiated cGMP signaling.

In contrast to cAMP and cGMP, Ca^{2+}-mediated signaling in plants has been extensively studied. Ca^{2+} regulates various processes including development and cell growth, biotic and abiotic stresses, hormone responses, and environmental sensing [60]. The perception of Ca^{2+} is achieved through sensor domains such as EF hands, calmodulin, and C2 domains. Cytosolic Ca^{2+} levels can be elevated by most physiological stimuli [60], most of which were also shown to regulate AGCVIII kinases. Whether Ca^{2+} regulation of AGCVIII kinases is a general feature requires more study.

The functional significance of phospholipids, most importantly phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), phosphatidylinositol 3-phosphate (PI3P) and phosphatidic acid (PA), is starting to be appreciated in plants [41]. Phospholipids can be rapidly interconverted by kinases, phosphatases, and phospholipases [41] and therefore are ideal temporal signals. Phospholipids also provide spatial information to proteins with phospholipid-binding domains, such as pleckstrin homology (PH) domains, phox homology (PX) domains, and FYVE domains [39]. Although AGCVIII kinases do not contain recognizable phospholipid-binding domains, unconventional sequences may direct their phospholipid binding, as was shown for PID [23].

not contain canonical Ca^{2+}-sensing motifs. However, Ca^{2+} sensors were found in interacting proteins of several AGCVIII kinases. For example, two PID interactors, TOUCH 3 and PID BINDING PROTEIN 1 (PBP1) both contain EF-hand motifs and showed Ca^{2+}-dependent interactions with PID [8]. The N-terminal sequence of a kinesin-like calmodulin-binding protein (KCBP) interacted with the AGC kinase KIPK, although it is not clear whether this interaction was Ca^{2+}-dependent [37]. However, these proteins that interacted with AGCVIII kinases were not phosphorylation substrates for their interacting AGCVIII kinases, suggesting they might act upstream of AGCVIII kinases upon Ca^{2+} sensing. Indeed, PID activity was inhibited by Ca^{2+} *in vitro* [24] and by Ca^{2+} or by an inhibitor of calmodulin *in vivo* [38]. In addition to this direct evidence that Ca^{2+} regulates the activities and interacting profiles of AGCVIII kinases, Ca^{2+} might also affect the activity of AGCVIII kinases through cross-talk with other signaling pathways that directly regulate AGCVIII kinases, such as auxin [4,9,10,28], biotic stresses and abiotic stresses (Table 1) [14–16].

Despite the lack of discernible sensor domains [39] for phospholipids, at least one AGCVIII kinase, PID, was able to bind to various phospholipids *in vitro* [23], suggesting that AGCVIII kinases might use unconventional sequences for phospholipid binding. Phospholipid binding might affect the activity of AGCVIII kinases through activation of PDK1 or through cross-talk with other signaling pathways. Plant PDK1 s have amino acid changes in the PH domain that should abolish binding to PIP_3 [40]. Indeed, *Arabidopsis* PDK1 showed promiscuous binding to diverse phospholipids [40], among which phosphatidic acid (PA) (see Box 1) significantly increased its activity [4,14]. At least one AGCVIII kinase, AGC2.1/OXI1, showed increased activity upon PA treatment, which was PDK1-dependent [14]. Additionally, phospholipids are rapidly produced upon diverse environmental stimuli, such as osmotic stress, drought, wounding, pathogen invasion, and oxidative stress [41]. Among these phospholipid-promoting stimuli, pathogen invasion and oxidative stress were shown to regulate the activities of several AGCVIII kinases [4,14,15], suggesting a potential link between phospholipids and AGCVIII kinase activity.

Phospholipids have restricted membrane distributions in a given cell [42]. Therefore, AGCVIII kinases might be targeted to particular membrane compartments through binding to a specific phospholipid. For example, several AGCVIII kinases, including PHOT1 [36], PHOT2 [33,43] and PID [27], showed stimuli-induced changes of subcellular localization from the plasma membrane to punctate vesicles that resembled endosomes. Endosomes are phosphatidylinositol 3-phosphate (PI3P)-enriched (Box 1), highly dynamic membrane systems, which orchestrate trafficking of both proteins and lipids among diverse subcellular compartments [44]. Endosomes are not only compartments used to attenuate receptor-initiated signals but can also serve as platforms for signal amplification and specification [45]. It is interesting that PHOT1 interacted with two ADP-ribosylation factors (ARFs), which are small GTPases involved in membrane trafficking [46]. Further studies using co-localization with fluorescent markers and application of chemicals that interfere with endosome activities are needed to verify whether these AGCVIII kinases are translocated to endosomes upon extracellular stimuli.

Phospholipid-binding might also regulate the proteins with which AGCVIII kinases interact. Specific phospholipid-binding can stabilize protein–protein interactions, thereby adding another layer of regulation for signaling specificities [42]. A well-studied example is the interaction between animal PDK1 and PKB. Both were recruited to the plasma membrane upon PIP_3 production, resulting in spatially restricted activation of PKB by PDK1 [21]. A similar mechanism has not been shown for AGCVIII kinases, but one study hinted at such a possibility. The basal membrane localization of the AGCVIII kinase D6PK in *Arabidopsis* root cells was abolished when the cells were treated with a fungal toxin brefeldin A (BFA) [34]. BFA inhibits exocytosis and interferes with protein/lipid composition at the plasma membrane [47], suggesting that D6PK localization relies on certain proteins/lipids at the plasma membrane.

Phototropins are distinct among AGCVIII kinases because they seem to regulate Ca^{2+} and phospholipid signaling but not *vice versa*. PHOT1 mediates blue light-induced Ca^{2+} influx through plasma membrane Ca^{2+} channels [31,48,49]; PHOT2 might regulate Ca^{2+} release from internal stores, since the *phot2* mutant was less sensitive to the inhibition of inositol 1,4,5-trisphosphate (IP_3) [31]. PHOT1 might also regulate IP_3–mediated Ca^{2+} release, because increased IP_3 levels (by suppressing an inositol polyphosphate 5-phosphatase) rescued some phototropic defects of the *phot1* and *phot1 phot2* mutants [50].

Substrates of AGC kinases: tip of an iceberg

Signaling cascades initiated by kinases are reflected by phosphorylation of their downstream substrates. Only a few plant AGCVIII kinases have known substrates. PTI1-2, a S/T kinase, was identified as an interacting partner of AGC2.1/OXI1 using a yeast two hybrid (Y2H) approach [14]. PTI1-2 was phosphorylated by AGC2.1/OXI1 *in vitro* and showed a phosphorylation response similar to that of AGC2.1/OXI1 upon extracellular stimuli [14]. This result suggested an interesting scenario where AGC2.1/OXI1 regulates an extracellular stimuli-induced phosphorylation cascade, through which different signal inputs can be integrated to ensure specificities of the signaling outputs.

Substrates of several AGCVIII kinases were recently identified based on the rationale that protein kinases and their substrates function in the same pathway, and therefore should show similar mutant phenotypes. Mutations in PID as well as in several D6PKs resulted in growth defects in organogenesis similar to those seen with the auxin efflux carrier PIN-FORMED (PIN) mutants [8–10,34]. These phenotypic resemblances prompted studies on the genetic and physical interactions between PIN1 and these AGCVIII kinases. Indeed, PIN1 was phosphorylated by PID [28] and D6PK [34] both *in vitro* and *in vivo*. The phosphorylation of PIN1 affected its dynamic subcellular localization at different plasma membrane domains and at endosomes [28]. This regulatory mechanism is apparently evolutionarily conserved, as the maize ortholog of PID, BARREN INFLORESCENCE2 (BIF2), phosphorylated ZmPIN1a *in vitro* and regulated its subcellular localization *in vivo* [51]. Interestingly, BIF2 also phosphorylated a basic helix-loop-helix transcription factor, BARREN STALK1 (BA1), *in vitro* [35]. The possibility that activation of BIF2 results in transcriptional changes through the phosphorylation of BA1 is certainly worthy of future investigation.

Conclusions and perspectives

Genetic, biochemical and cellular approaches have shown that AGCVIII kinases are at the crossroads of plant cellular signaling, playing critical roles in integrating developmental and environmental cues into specific cellular responses through substrate phosphorylation. Substantial progress has been made in understanding their function, regulatory mechanisms and substrate specificity. Nonetheless, most AGCVIII kinases have yet to be assigned a function. Here, we highlight future research by which AGCVIII kinases and the signaling networks they regulate should be elucidated.

Spatiotemporally-specific expression is a prerequisite for functional specificity (Table 1). Genome-wide microarray and MPSS data are providing increasingly high-resolution expression data, which could be used to guide functional studies of AGCVIII kinases. Because plant genomes contain large numbers of AGCVIII kinases, some of which are highly similar [18], hierarchical mutants will need to be generated to overcome functional redundancy when expression profiles overlap, as was the case with AGC1.5/AGC1.7 during pollen tube growth [7], with WAG1/WAG2 during root development [6], and with D6PKs in auxin transport [34].

As an alternative, application of pharmacological inhibitors should improve our understanding about how AGCVIII kinases are regulated. Inhibitors that perturb Ca^{2+} fluxes or inter-conversion of phospholipids can be applied to mutants and wild type to identify potential response differences. For example, the inhibition of blue light (BL)-induced Ca^{2+} efflux from internal stores by inhibitors of phospholipase C was abolished in the *phot2* mutant, suggesting that PHOT2 is critical for the BL-induced increase of cytosolic Ca^{2+} [31]. It will also be revealing to measure the effect of inhibitors on kinase activities. The effectiveness of this strategy was nicely demonstrated in a study in which decreasing the production of PA, by treating tissues with inhibitors of phospholipase D, negatively affected the activities of PDK1 and AGC2.1/OXI1 [14], suggesting that PA activates PDK1 and AGC2.1/OXI1.

Additionally, expression-based strategies can be used as a complement to mutant studies to reveal regulatory mechanisms of AGCVIII kinases. Expression of engineered kinases, with truncations/deletions in certain motifs/domains, ideally under their native promoters and in corresponding mutant backgrounds, will be useful to understand their *in vivo* regulation. Such a strategy was elegantly employed to identify intramolecular inhibition of PHOT1/PHOT2 kinase activity by their N-terminal LOV2 domains [33,52]. In another case, by swapping the plant-specific T-loop insertion sequences (TLI) [17] between PID and AGC1.7, the TLI was demonstrated to be essential for their subcellular targeting [23]. Although mutations of one or both Ser residues within the T-loop to a non-phosphorylatable Ala residue showed different effects on the activity of AGCVIII kinases [4,15,24,53], phosphor-mimicking mutations (S to D or E) retained and mostly increased kinase activity [4,15,24,53], suggesting that it is feasible to generate more active versions of kinases. Expression of such mutant AGCVIII kinases should be informative regarding their *in vivo* function, especially for those without a discernable mutant phenotype due to functional redundancy.

Another little-explored aspect that requires future attention is the significance of multiple phosphorylation sites in AGCVIII kinases [15,24,54]. AGCVIII kinases contain two closely spaced Ser residues within the T-loop. A single Ser mutation to Ala abolished autophosphorylation of AGC2.1/OXI1 [4] but did not affect that of Adi3 [15], suggesting that phosphorylation of different AGCVIII kinases might be regulated differently. The two conserved Ser residues within the T-loop of PHOT1 also showed different effects on PHOT1 activity [53]. In addition,

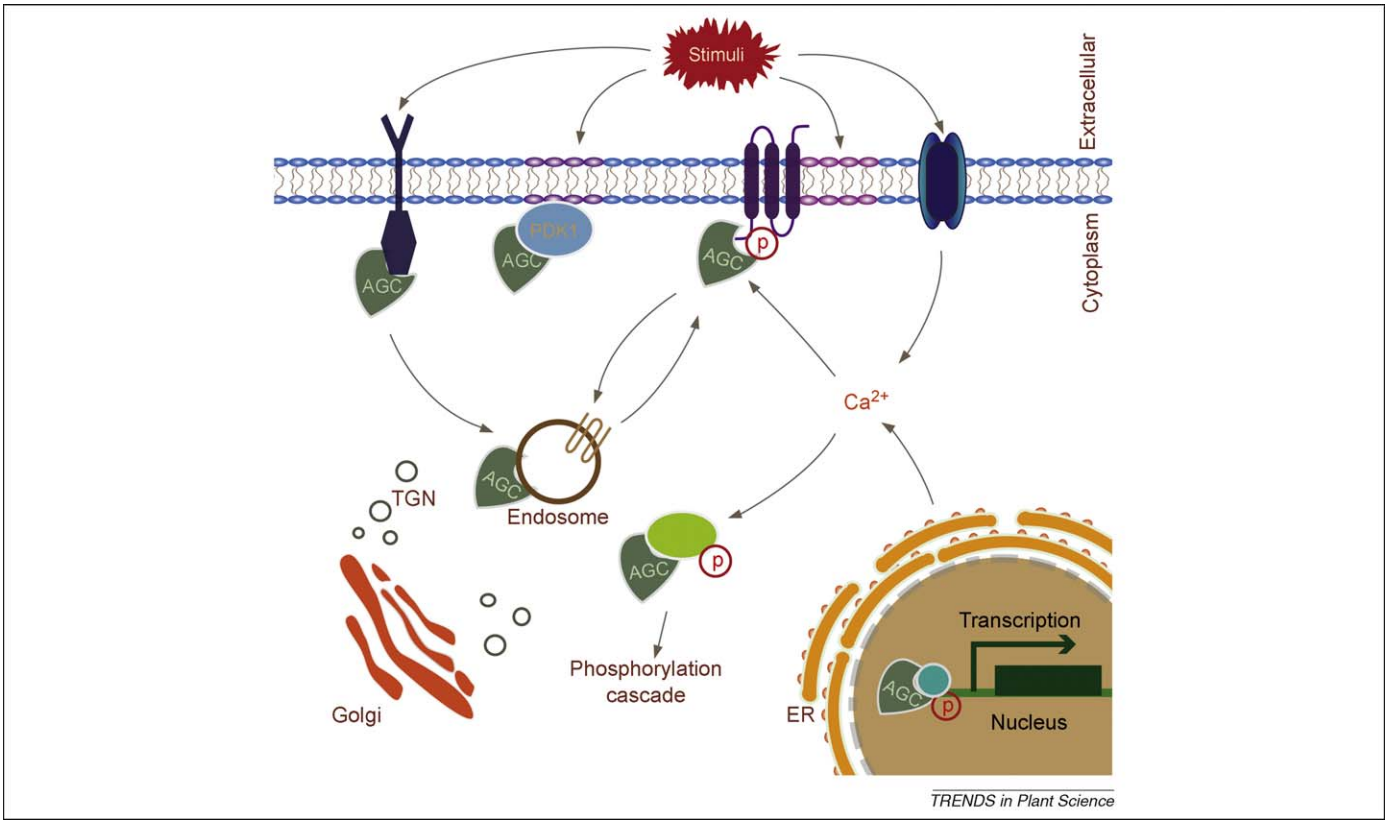

Figure 2. Cartoon illustrating putative signaling pathways mediated by AGCVIII kinases (AGC). Extracellular stimuli are perceived at the plasma membrane, likely by transmembrane proteins. As a response, cytosolic Ca^{2+} concentration and membrane lipid compositions change to affect the activity, subcellular localization and substrate accessibility of AGCVIII kinases. Such spatiotemporal regulation is amplified through phosphorylation (P) of AGCVIII kinase substrates, leading to specific signal outputs, such as phosphorylation cascades or initiation of transcription. ER, endoplasmic reticulum; TGN, trans-Golgi network.

PHOT1 contains multiple phosphorylation sites outside its catalytic domain and phosphorylations at these sites are regulated differently [46,53,55]. Distinct phosphorylation profiles of PHOT1 were reported to regulate different phototropic responses [53]. It is therefore a likely scenario that multiple phosphorylation sites within AGCVIII kinases serve to distinguish different signal inputs so that specificity of signaling outputs can be achieved. Mutagenesis of individual phosphorylation residues combined with available mutants, as shown in the case of PHOT1 [53], will be useful in solving this puzzle.

Last but not least, identification of substrates will be necessary to finally place AGCVIII kinases in a larger context in plant signaling networks. Proteins identified from genome-wide screening using the yeast two hybrid system yielded more upstream regulators of AGCVIII kinases [15,37,38] than substrates [14]. Instead, kinase-substrate pairs were identified based on similar mutant phenotypes, as in the case of the kinase-substrate pairs PID-PIN [10,28], D6PK-PIN [34], BIF2-ZmPIN1a [51], and BIF2-BA1 [35]. An alternative strategy using affinity tagging allowed genome-wide identification of kinase substrates. This method uses a bio-orthogonal ATPγS analog in phosphorylation reactions with an engineered, analog-sensitive kinase [56]. Phosphorylation substrates were then affinity-purified from cell extracts and analyzed by tandem mass spectrometry [56]. Once identified, AGCVIII kinase-substrate interactions ideally should be tested in planta using fluorescent imaging technologies such as Bimolecular Fluorescent Complementation, especially when dynamic spatial information is critical for understanding the consequences of substrate phosphorylation, as exemplified by the PID-PIN interaction [28].

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