Review

Arabidopsis PPP family of serine/threonine protein phosphatases: many targets but few engines

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The major plant serine/threonine protein phosphatases belong to the phosphoprotein phosphatase (PPP) family. Over the past few years the complement of *Arabidopsis thaliana* PPP family of catalytic subunits has been cataloged and many regulatory subunits identified. Specific roles for PPPs have been characterized, including roles in auxin and brassinosteroid signaling, in phototropism, in regulating the target of rapamycin pathway, and in cell stress responses. In this review, we provide a framework for understanding the PPP family by exploring the fundamental role of the phosphatase regulatory subunits that drive catalytic engine specificity. Although there are fewer plant protein phosphatases compared with their protein kinase partners, their function is now recognized to be as dynamic and as regulated as that of protein kinases.

Phosphatases to rival kinases?

Protein phosphorylation is an ancient regulatory mechanism that over the course of evolution has become one of the dominant means of controlling protein function, regulating most biological processes, and operating across the domains of life [1–3]. Mass spectrometry studies have revealed that more than 70% of all human proteins are phosphorylated and that most have multiple phosphorylation sites [4]. Furthermore, the complement of protein kinases and phosphatases constitutes approximately 2–4% of the protein-encoding genes of most eukaryotes, including humans, yeast, and plants [1,5]. Given the large size of the protein kinase and phosphatase families across the eukaryotes, it is likely that phosphorylation is also as common in other eukaryotes, including plants. Eukaryotic phosphoproteins have been found in abundance in mitochondria [6], chloroplasts [7], nuclei [4], and the cytosol [4], and even extracellularly [8]. Phosphoproteomic analyses have revealed that even in the absence of classic tyrosine kinases, plant proteomes have a much higher abundance of phospho-tyrosine than originally thought, with similar proportions of phospho-serine, -threonine, and -tyrosine (~84–86%, 10–12%, and 2–4%, respectively) to those found in other higher eukaryotes [9–12].

For historical, and sometimes technical, reasons research studies have tended to focus more attention on investigating protein kinases than protein phosphatases [13]. *In vitro*, protein kinases show substrate specificity based on protein primary sequence, whereas protein phosphatase catalytic subunits are typically non-discriminate in the absence of additional protein binding partners [14], which led to the notion that protein phosphatases lack specific regulation and simply maintain a 'housekeeping' function. However, studies across a range of model eukaryotes have confirmed that the phosphatases are not passive players in the (de)phosphorylation balance and are as dynamic and highly regulated as their partner kinases. Biochemical and genetic studies have uncovered key roles for the plant PPP family in a wide range of biological contexts. In this review, we provide a framework for understanding this group of serine/threonine phosphatases in plants by exploring the fundamental role of the phosphatase regulatory subunits that drive catalytic engine specificity and discuss several recent advances.

Protein phosphatase families

Protein kinases, with only a few exceptions, group into one superfamily [15]. However, the protein phosphatases are divided into four groups based on primary sequence and catalytic mechanism [14,16–20] (Box 1): the PPP, the Mg^{2+}- or Mn^{2+}-dependent protein phosphatase (PPM)/protein phosphatase 2C (PP2C), the phosphotyrosine phosphatase (PTP), and the aspartate (Asp)-dependent enzyme families. It is thought that the PPPs catalyze over 90% of the protein dephosphorylation reactions in eukaryotic cells [14]. However, the proportion may be slightly lower in plants owing to the number of serine/threonine-specific PP2C enzymes [21].

Unlike the PPP and PPM families, the PTP and Asp-dependent enzymes are a mixture of phospho-tyrosine, -serine/-threonine, and dual-specificity phosphatases [22–24]. The dual-specificity phosphatases were named based upon the ability of the first mitogen-activated protein kinase (MAPK) phosphatase to be characterized to dephosphorylate both residues of the TxY motif in the MAPK activation loop [25]. Other 'dual-specificity' enzymes show a variety of phosphatase activities that are directed towards messenger RNA, phosphoinositides, complex carbohydrates, and other phosphorylated molecules [22,24,26,27]. Although several of the dual-specificity enzymes are not

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Box 1. Nomenclature, history, and phylogeny

The major serine/threonine protein phosphatases of eukaryotes were first detected and named based on the biochemical properties of mammalian enzymes [13,92]. They were designated type 1 (PP1) if the heat-stable proteins I1 and I2 inhibited their activity. If activity was not inhibited by I1 or I2, the protein phosphatase was designated type 2 and further divided into type 2A (PP2A) if there were no divalent cation requirements in the assay, type 2B (PP2B/PP3, which does not exist in plants) if the activity was stimulated by calcium, or type 2C (PP2C/PPM) if the activity was magnesium-dependent. Subsequent purification, cloning, sequence analysis, degenerate PCR to isolate related members, and finally genomics has identified highly conserved versions of PPP family members (now known to be composed of PP1, PP2 (PP2A), PP4, PP5, PP6, and PP7) in all eukaryotes [2]. Several novel (non-mammalian) PPP enzymes have recently been added to this list (see Figure 1 in main text). Phylogenetic analysis of these catalytic subunits (or catalytic engines) places the PP2C enzymes in their own family (now called PPM). In terms of sequence, the PPP family is unrelated to the PP2C enzymes and it is thought that each group (PPP and PPM) evolved independently to dephosphorylate phospho-serine and -threonine. The FCP/SCP [TFIIF (transcription initiation factor IIF)-associating component of CTD (C-terminal domain) phosphatase/small CTD phosphatase] enzymes from the Asp-dependent family were discovered later and have been found to control the serine/threonine phosphorylation status of RNA polymerase II [2,13,18].

protein phosphatases, they are grouped here based solely upon sequence identity.

The PPP family comprises Ser/Thr protein phosphatase type one (PP1), PP2 (PP2A), PP3 (PP2B), PP4, PP5, PP6, and PP7, which are sequence and structurally related (Figure 1 and Box 1) and maintain an identical catalytic mechanism [14,18,20]. The biological relevance of the PPP family is underscored by the finding that PPPs are targets for oncogenic viruses and for potent inhibitory small molecules, such as microcystin and okadaic acid [2,28]. Uniquely, plants lack PP3 (or PP2B) enzymes and, similar to several other eukaryotes, have additional PPPs: protein phosphatases with Kelch-like repeat domains- (PPKLs) and *Shewanella*-like protein (SLP) phosphatases, which are absent in mammals [5,29–31]. The functions and evolutionary relationships of these unique PPPs are now being unraveled.

Protein partners bring specificity to the PPP engines

The total number of protein phosphatase catalytic subunits pales in comparison to the number of protein kinase subunits found in eukaryotes: for example, *Arabidopsis* (*Arabidopsis thaliana*) has ~150 protein phosphatases and ~1050 protein kinase catalytic subunits [5,32]. The PPP

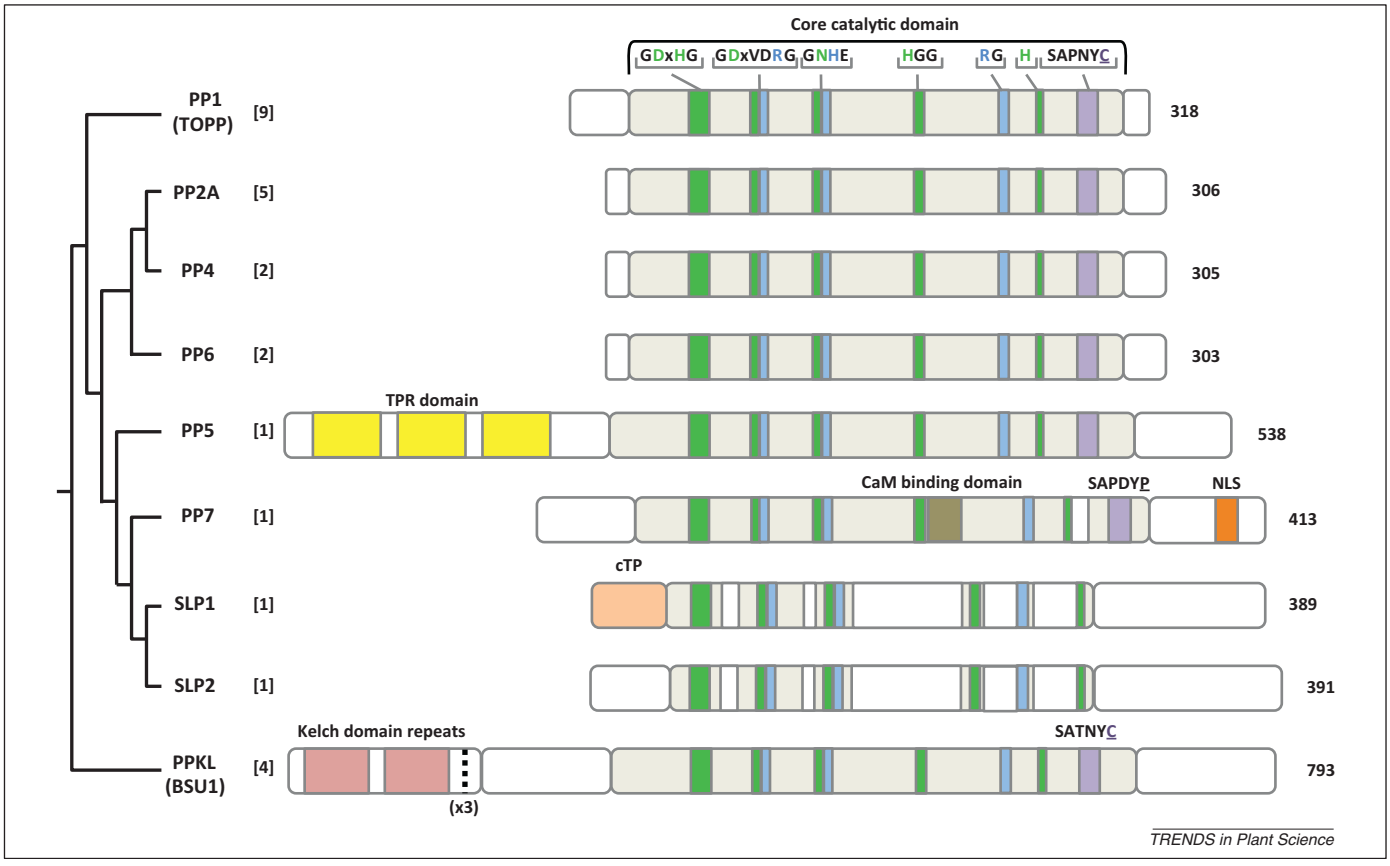

Figure 1. Phylogenetic and architectural depiction of the plant phosphoprotein phosphatase (PPP) family. The highly conserved core catalytic domain of each PPP subfamily is depicted in gray with signature aspects of each motif highlighted. Green and blue represent amino acids involved in metal ion coordination and phosphate binding, respectively. Also described is the microcystin inhibition docking motif SAPNYC (purple), highlighted by a reactive cysteine (C) to which microcystin covalently attaches. PP7 maintains this motif, but lacks the reactive C, whereas *Shewanella*-like protein (SLP) phosphatases completely lack this motif. Within these motifs 'x' represents any amino acid. Unique features of each subfamily are also depicted: TPR (tetratricopeptide repeat), NLS (nuclear localization signal), and cTP (chloroplast transit peptide). The *Arabidopsis* sequences used to compile the phylogenetic tree are: PP1 (TOPP1; At2g29400), PP2A-1 (At1g59830), PP4-1 (At4g26720), PP5 (At2g42810), PP6-1 (At1g50370), PP7 (At5g58500), SLP1 (At1g07010), SLP2 (At1g18480), and PPKL (protein phosphatase with Kelch-like repeat domain; BSU1; At1g03445). No canonical PP2B (calcineurin-A) has been found to be encoded by plants. Tree topology was obtained through alignment in ClustalX 2.0.12 and visualization in FigTree v1.3.1. The total number of amino acids for each enzyme is shown on the right and, for presentation purposes only, two (×3) of the six Kelch repeat motifs of the Kelch-like domain are shown for BSU1. The number of genes encoding each subfamily of the PPP family in *Arabidopsis* is shown in square brackets (e.g., [5]). Although the architecture of a specific gene product is depicted (i.e., TOPP1), each additional protein maintains the same motifs and domains.

family has evolved to exploit the promiscuity of catalytic subunits (coupled with their serine/threonine specificity) through specific interactions with a multitude of regulatory subunits that confer substrate specificity and allow for enormous substrate diversity. Indeed, one of the hallmarks of the PPPs is additional subunits. For example, several hundred PP1 regulatory subunits have been identified in humans [14,17,18,33,34], and several have now been characterized in plants [35–38].

Protein phosphatase 1 – targeted at last

PP1 is remarkably conserved across eukaryotes with nine genes in *Arabidopsis* [5]. Given that PP1 substrate specificity is determined through the interaction with a variety of regulatory subunits, characterization of these subunits has been of paramount importance. Of the 200 known PP1 binding partners in humans, the vast majority dock PP1 through a conserved binding site defined as 'RVxF', which maintains the consensus (R/K)(R/K)(V/I)x(F/W) [14,18]. Numerous human PP1 interactors have orthologs in plants with conserved RVxF motifs and, thus, are likely to associate with PP1 [36,39]. The amino acids on PP1 coordinating the RVxF motif are also conserved across eukaryotes, supporting the view that this is an ancient protein docking site. Recent biochemical evidence has demonstrated that plant PP1 regulatory proteins interact through the RVxF motif as predicted [35–38]. Several secondary interaction motifs have also been identified on regulatory subunits, including the G/SILK and MyPhoNE motifs [14,40], which frequently reside N-terminal to the RVxF site. In a few instances, the G/SILK motif has been noted as the sole PP1 docking site [41]. These associated binding partners function to either abolish PP1 activity by blocking access to the active site (e.g., Inhibitor-2, known as I2) or by recruiting substrates and/or controlling active site access [42]. From a cellular localization perspective, the nine *Arabidopsis* PP1 proteins, similar to human PP1 isoforms, have each been shown to localize within the nucleus and cytosol [35,36], and in plants are excluded from the plastid [35,43].

Using a combination of microcystin– or PP1–Sepharose® affinity chromatography, several PP1 interactors have been uncovered in *Arabidopsis* (Figure 2), including nuclear inhibitor of protein phosphatase 1 (NIPP1), SUPPRESSOR OF DIS2 (SDS22), GL2 expression modulator (GEM), and inhibitor-2 (I2) [36]. Other studies, with

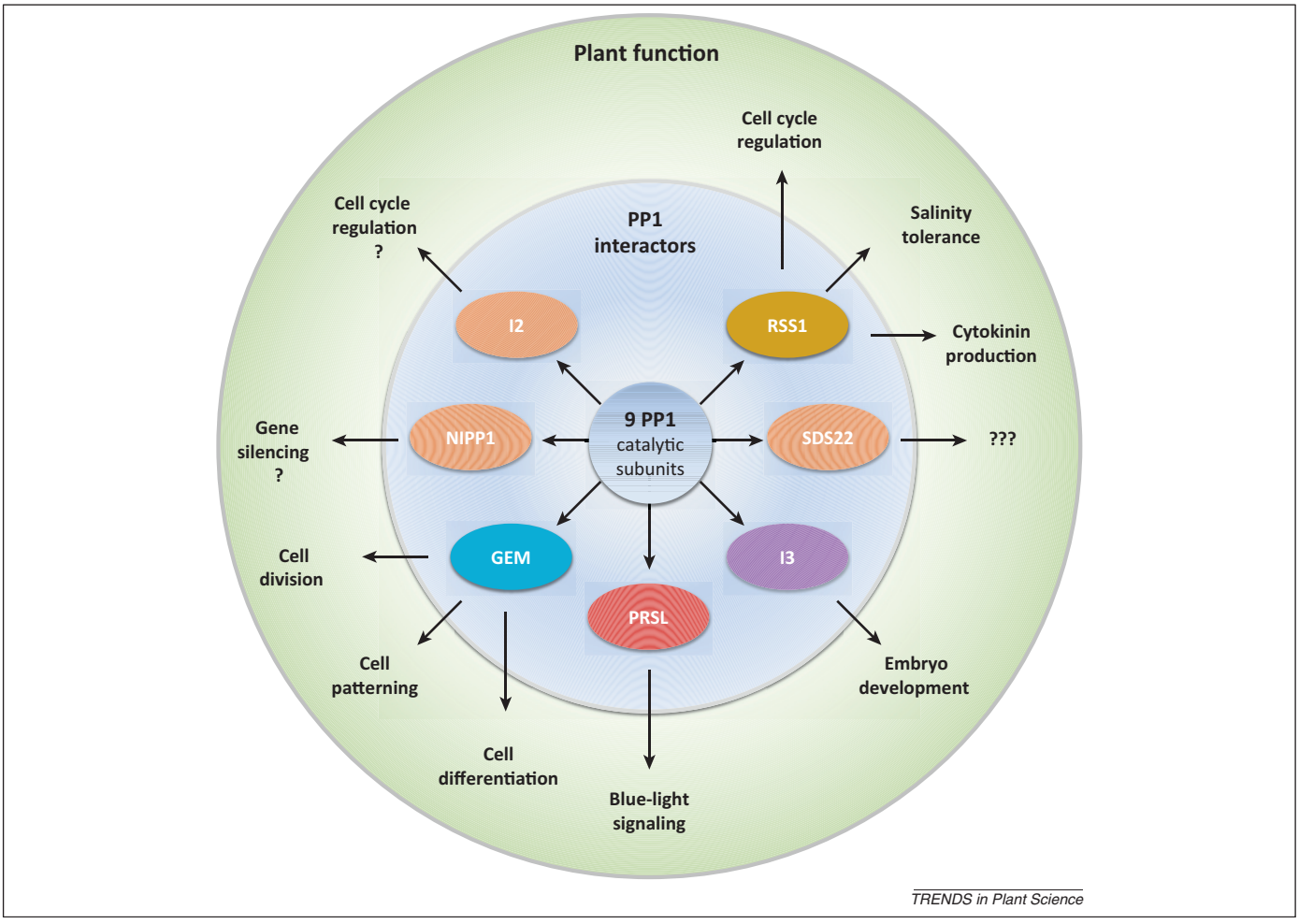

Figure 2. Plant PP1 (protein phosphatase type one): from nonspecific catalytic subunits to specialized cellular functions. PP1 is encoded in the genomes of all eukaryotes as a bare catalytic subunit that requires the interaction of regulatory proteins to drive its specificity within the cell. Interaction between PP1 catalytic subunits and regulatory proteins is most often facilitated by a regulatory protein binding motif called the RVxF motif. Depicted are the documented plant PP1 protein interactors that, with the exception of SUPPRESSOR OF DIS2 (SDS22), all possess an RVxF motif. With human PP1 maintaining several hundred regulatory proteins, this list of documented plant PP1 regulatory proteins will probably expand significantly. Plant functions with question marks (?) are defined roles for these PP1 complexes in humans and are yet to be confirmed in plants.

the aid of genetic approaches, have also identified RVxF-containing plant PP1 partners inhibitor-3 (I3) [35] and the rice (*Oryza sativa*) protein RICE SALT SENSITIVE 1 (RSS1) [38]. Of the PP1 protein regulators identified to date, I2 is thought to be one of the most ancient [44–46]. AtI2 was found to contain a conserved RVxF motif but lacks the human I2 specific G/SILK motif, and showed an ability to inhibit all nine *Arabidopsis* PP1 isoforms. Given the remarkable conservation of key regions, AtI2 is likely to play an important role in the plant cell cycle as in other eukaryotes [45]. Using the inhibitory properties of I2, it has been shown that PP1 regulates stomatal opening downstream of the blue-light sensing kinase phototropin but acts upstream of the H^+-ATPase [47]. Yeast two-hybrid screening for PP1 interactors has identified several RVxF-containing proteins (Figure 2), including PRSL1 (PP1 regulatory subunit 2-like protein 1), which, similar to GEM, binds PP1 in an RVxF-dependent manner [37]. Although yet to be confirmed, these results suggest that PRSL1 targets PP1 to regulate blue-light sensing. The RVxF-containing PP1 regulatory protein RSS1 was also identified recently through a combined approach of genetic screening for salt tolerance in rice and yeast two-hybrid screening; the loss of RSS1 results in short root and dwarf phenotypes under high salt [38]. RSS1 accumulates as cells progress through the S-phase of the cell cycle where it is required for the maintenance of proliferative cells in meristematic tissues. With a handful of plant PP1 binding partners now uncovered, the question still remains: is PP1 the major serine/threonine phosphatase in plants controlling hundreds of processes as in mammals [14].

PP2A, PP4, and PP6 – from brassinosteroid signaling to metabolism

When phylogenetically compared with the other members of the PPP family, PP2A, PP4, and PP6 form a distinct cluster suggestive of a common ancestor [2] (Figure 1). In addition, all eukaryotic catalytic subunits of PP2A, PP4, and PP6 contain a C-terminal YFL motif that allows for potential regulatory C-terminal leucine methylation [48]. Consistent with these findings was the discovery that *Arabidopsis* PP2A, PP4, and PP6 can each bind TAP46 (α4 in humans and TAP42 in yeast) with varying affinities independently of their other regulatory subunits [49]. Similar to α4 and TAP42, TAP46 was found to be a substrate of the target of rapamycin (TOR) protein kinase and through RNA-induced gene silencing, TAP46 was shown to be crucial for cell growth and survival, autophagy, and protein synthesis [49] (Figure 3A).

The PP4 and PP6 catalytic subunits are conserved across eukaryotes, including plants [39,50,51], with several regulatory subunits now identified [39,52,53]. Although no clear roles for plant PP4 have been defined, PP6 appears to play a role in the phosphorylation of PIN-FORMED (PIN) proteins and auxin efflux [51], as well as abscisic acid (ABA) signaling in *Arabidopsis* [50]. Loss-of-function mutants, overexpression lines, and interaction studies suggest that when ABA levels drop, PP6 dephosphorylates the transcription factor Abscisic Acid Insensitive 5 (ABI5) leading to its degradation. This allows initiation of seed germination and post-germination growth [50].

The PP2A holoenzyme is a trimer, consisting of a catalytic (C), scaffolding (A), and regulatory (B) subunit [54]. *Arabidopsis* encodes five catalytic, three scaffolding, and 17 regulatory B subunits, which can interact in a variety of combinations to exert different regulatory outcomes [55,56]. The 17 B subunits group into B, B′, and B″ subunit families, and recent structural analysis confirm biochemical data that B subunits control substrate access to the catalytic subunit active site [20]. Several studies have identified the *Arabidopsis* PP2A–A1 subunit [ROOTS CURL IN NAPHTHYLPHTHALAMIC ACID 1 (RCN1)] and several B subunits as direct players in stress signaling (B′γ) [55] and root growth (B″α) [57]. Metabolic links have also been established through B″ (α and β) as negative regulators of 3-hydroxy-3-methylglutaryl Coenzyme A reductase (HMGR), a key enzyme that regulates the isoprenoid biosynthesis pathway [58] and B55 (α and β) through nitrate reductase activation [59].

Aspects of PP2A function also indicate a regulatory link between different post-translational modifications, such as phosphorylation–acetylation [39] and phosphorylation–methylation [60]. Recently, PP2A was found to co-purify with the histone deacetylase HDA14 and the histone acetyltransferase ELP3 [39]. HDA14 was shown to deacetylate α-tubulin and is the ortholog of human histone deacetylase 6 (HDAC6), which also targets α-tubulin [39]. Although the specific function of this interaction remains unknown, PP2A has been implicated in the control of microtubule function [61] and may regulate the binding and trafficking of kinesins [39]. There is evidence to suggest that HDA14 may be a regulatory 'B' subunit owing to its direct interaction with scaffolding A subunits of PP2A; however, further experimentation is required to confirm this hypothesis (Figure 3B).

Similar to PP1, PP2A has recently been implicated in regulating aspects of light signaling. In particular, yeast two-hybrid and *in vitro* pull-down assays uncovered a direct interaction between RCN1 (PP2A-A1) and phototropin 2 (PHOT2) [62]. This work, and that by other research groups [39,63,64], have highlighted that the scaffolding A subunits do not just bind the C and B subunits of PP2A but are also likely to bind other protein partners. The association of RCN1 (PP2A-A1) and PHOT2 was found to downregulate phototropism and stomatal opening through the dephosphorylation of PHOT2 under blue-light conditions (Figure 3C) [62].

PP2A is also involved in regulating aspects of plant hormone signaling, with recent evidence demonstrating the influence of PP2A over the auxin transport system and cell polarity [65,66]. PP2A–C, more specifically subfamily 2, and an opposing protein kinase (PINOID) were found to be responsible for regulating the phosphorylation state of the auxin efflux PIN proteins in roots (Figure 3D) [67]. It is hypothesized that within plant vasculature, PP2A–C (isoform 4) is coexpressed with and dephosphorylates PIN1, to fine-tune normal auxin transport to the root tip [66]. Interestingly, this regulatory mechanism also seems to be conserved in the leaf epidermis, controlling the patterning of pavement cells [65].

Perhaps the most significant finding in the past few years with regard to the PPP family was the discovery that

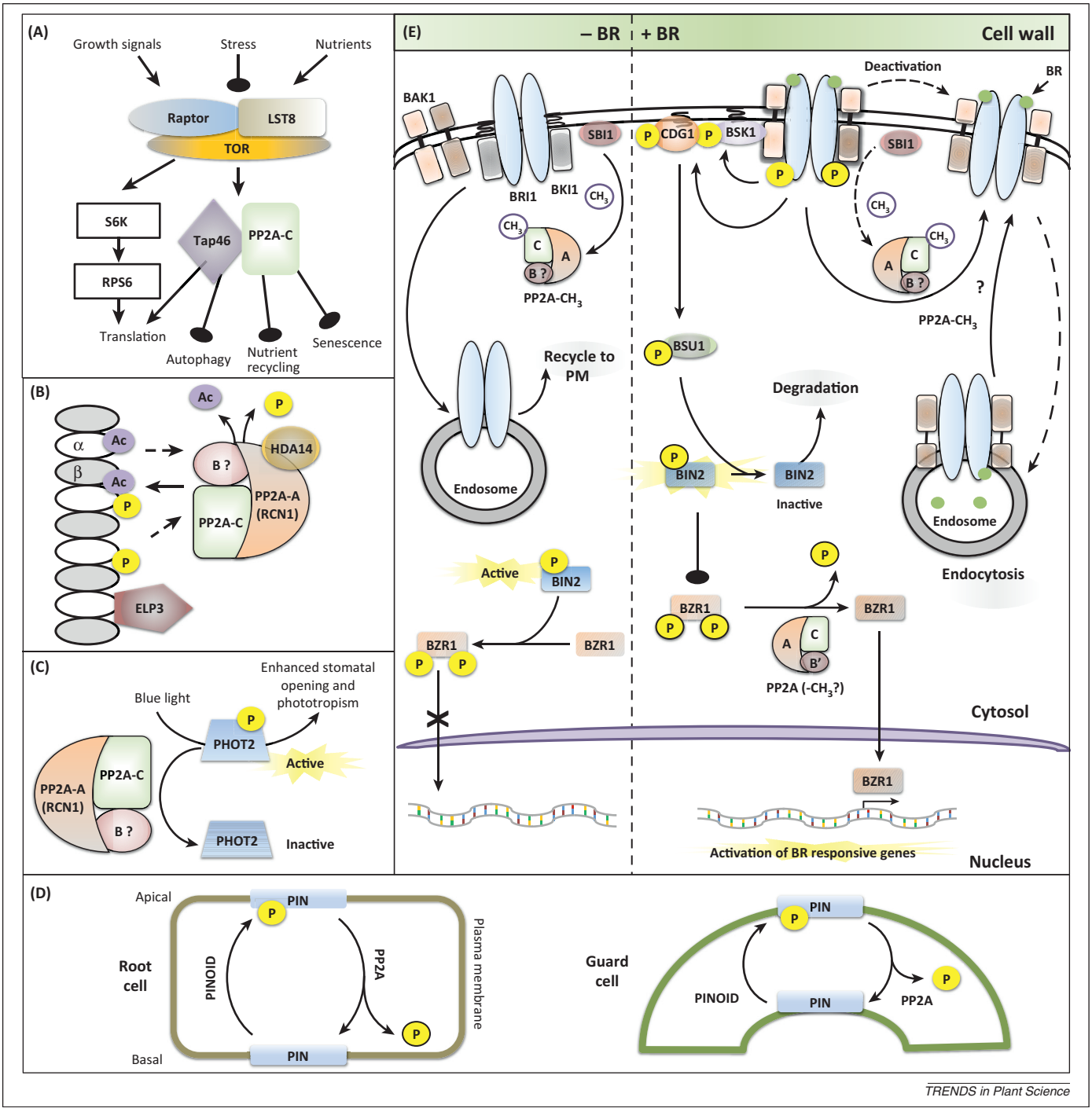

Figure 3. Cellular events regulated by PP2A (protein phosphatase 2A) in plants. **(A)** Many key cellular signals converge on target of rapamycin (TOR) in plants as in humans and yeast. PP2A–C interacts with regulatory subunit Tap46 (α4/Tap42), a target of TOR, to modulate downstream cellular events: protein translation, autophagy, nutrient cycling, and senescence in plants. Pointed and blunt arrowheads denote activation and inhibition of enzyme activity or cellular processes, respectively. **(B)** The plant PP2A-A1 [ROOTS CURL IN NAPHTHYLPHTHALAMIC ACID 1 (RCN1)] scaffolding subunit forms a specific complex with histone deacetylase 14 (HDA14) to deacetylate α-tubulin. Whether HDA14 represents a novel B targeting subunit or simply a protein interactor has yet to be determined. The role of histone acetyltransferase ELP3 (elongator complex protein 3) in conjunction with PP2A in modulating α-tubulin acetylation is also unknown. The identification of an HDA14–PP2A complex represents an interesting point of crosstalk between protein phosphorylation and acetylation. Broken arrows denote the possible influence of nearby post-translational modifications on the ability of a PP2A–HDA14 complex to bind tubulin. **(C)** PP2A specifically dephosphorylates light sensor phototropin 2 (PHOT2) to regulate phototropism and stomatal opening. The regulatory B subunit directing the specificity of these functions remains unknown. **(D)** PINOID kinase and PP2A regulate polar auxin transport through auxin efflux carrier PIN via phosphorylation and dephosphorylation, respectively. **(E)** Central to brassinosteroid (BR) signaling are PP2A-mediated events that control brassinosteroid receptor BRI1 turnover and transcription factor movement to the nucleus. For BRI1 turnover it is unclear if PP2A operates at the level of the plasma membrane or during the recycling of receptors from the endomembrane system. Kelch-like phosphatase BSU1 is also involved in the BR signaling pathway by facilitating nuclear enrichment of BZR transcription factors via BIN2 kinase dephosphorylation. Solid and broken arrows represent the activation and subsequent inactivation of BR signaling, respectively. Question marks (?) beside B subunits indicate that specific B subunits have yet to be identified. Abbreviations: Ac, acetylation; BAK1, BRI1-associated kinase 1; BKI1, BRI1 kinase inhibitor 1; BSK1, BR signaling kinase; LST8, Lethal with Sec Thirteen 8; P, phosphorylation; RPS6, ribosomal protein S6; S6K, S6 protein kinase. Double black line represents the plasma membrane. Parts (A), (D), and (E) were adapted from [49,67,93], respectively.

PP2A is intimately involved in regulating intracellular responses to brassinosteroids (BRs). Several studies have revealed the action of PP2A at two key points of this pathway: (i) during the dephosphorylation and turnover of the BR receptor BRI1 (BR insensitive 1) [60] (Figure 3E) and (ii) dephosphorylation of the transcription factors BZR1 and BES1 (BZR2) ([68] and Figure 3E). In the former process (i), a genetic screen for suppressors of bri1-5 identified a suppressor (sbi1) that accumulated BRI1 protein in the mutant plant. SBI1, a leucine carboxylmethyltransferase, specifically methylates the PP2A catalytic subunit C-terminal YFL motif leucine. It is not clear if PP2A (and SBI1) act at the plasma membrane or during receptor recycling through the endomembrane system. This marks BRI1 for degradation and thus switches off the BR signaling pathway [60]. Although PP2A and SBI1 operate at this level in the pathway, it has yet to be established if BRI1 is a direct substrate of PP2A. Interestingly, this is also another point of crosstalk between diverse post-translational modifications. In the later event (ii), after initiation of the intracellular BR signal cascade, PP2A activates BR-responsive gene transcription through the dephosphorylation and subsequent nuclear accumulation of the transcription factors BZR1 and BES1 (BZR2) ([68] and Figure 3E). The dephosphorylation of BZR1 and BES1 specifically employs a PP2A–B′ complex and is critical for the BR signaling cascade.

Protein phosphatase 5 – a tetratricopeptide repeat containing phosphatase

The protein architecture of the PP5 subfamily involves a conserved N-terminal tetratricopeptide (TPR) domain and a C-terminal phosphatase catalytic domain (Figure 1). Subcellular localization analysis has revealed that the PP5 subfamily maintains a dual cytosolic–nuclear location similar to other members of the PPP family [69]. Of the two structural components that make up PP5, the TPR domain is implicated in facilitating protein–protein interactions as well as functioning as an autoinhibitory domain (Figure 1) [70,71]. Remarkably, the crystal structure of human PP5 shows that the carboxylate group of Glu76 of one TPR domain engages the active site in a manner remarkably similar to microcystin and okadaic acid binding to PP1 [72]. In light of this, the PP5 subfamily is characterized by low basal levels of phosphatase activity that increase in the presence of arachidonic acid or *in vitro* proteolytic truncation of the TPR domain [73]. In *Arabidopsis* and tomato (*Lycopersicon esculentum*), PP5 is encoded by a single gene that produces two alternatively spliced transcripts [69]: the larger isoform (62 kDa) maintains the additional exon and an endoplasmic reticulum localization, whereas the smaller isoform (55 kDa) exhibits the well-conserved dual cytosolic–nuclear subcellular localization [69].

In plants, documented functions of PP5 include roles in disease resistance [70,71], thermotolerance [74], and light detection [75]. Studies focused on disease resistance have revealed protein interactions among PP5, heat shock protein 90 (Hsp90), and ARGONAUTE1 (AGO1) RNA-induced silencing complexes [70], whereas enhanced thermotolerance has been shown to be related to PP5 chaperone activity stemming from the TPR domain [74]. Most recently, PP5 has been shown to function as a component of the tetrapyrrole-mediated plastid signaling pathway [76].

Protein phosphatase 7 – a role for calcium/calmodulin?

The PP7 subfamily is unique to plants and the domain architecture differs from that of other members of the PPP family. Unlike the comparable class of mammalian protein phosphatases with EF-hand domains (PPEFs), the phosphatases in the PP7 subfamily do not maintain any N- or C-terminal extensions [77]. Instead, they possess a charged insertional region of variable length within the catalytic domain in addition to a non-canonical C-terminal nuclear localization signal, which is required for constitutive nuclear localization (Figure 1) [19,78]. Despite the lack of EF-hand domains, it is likely that Ca^{2+} still influences PP7 *in vivo* because the catalytic insert region has been shown to bind calmodulin *in vitro* in a Ca^{2+}-dependent manner [79].

Functionally, the PP7 subfamily has been implicated in several sensory functions, in particular, light sensing through the regulation of cryptochrome and phytochrome [19,80,81]. Recently, light sensing has been directly tied to PP7 through stomatal aperture control via cryptochrome blue-light signaling and the dephosphorylation of a nuclear, ZZ-type zinc finger-containing protein called HYPERSENSITIVE TO RED AND BLUE 1 (HRB1) [81].

Novel protein phosphatases in the plant PPP family

Novel members of the PPP family include the PPKL and SLP phosphatases [5,30] (Figure 1). PPKLs are named after their tandem N-terminal Kelch repeats, which are likely to facilitate protein–protein interactions [31,82]. In *Arabidopsis* there are four PPKLs: BSU1, BSL1, BSL2, and BSL3 [2,61].

BSU1 was the first to be studied and is the only well-characterized PPKL phosphatase [82–84]. BSU1 is okadaic acid-sensitive and I2-insensitive [82]. Investigation of the other *Arabidopsis* Kelch repeat phosphatases, BSL1–BSL3, by using RNA interference-mediated suppression revealed that their functions largely overlap [82].

BSU1 was identified as a suppressor of the BR receptor BRI1, and it was suggested that BSU1 directly dephosphorylated the downstream BR-induced transcription factors BZR1 and BES1 (BZR2), resulting in their nuclear accumulation and activation of BR-induced gene transcription [82]. However, it was subsequently found that BSU1 indirectly fine-tunes the phosphorylation status of BZR1 and BZR2 through the dephosphorylation and inactivation of glycogen synthase kinase-3-like kinase BIN2 [83], and that the BZR1 and BZR2 dephosphorylation was catalyzed by PP2A [68]. It has recently been revealed that the conveyance of BRI1 plasma membrane-mediated BR signaling to BSU1 is facilitated by a cytosolic receptor-like kinase, CONSTITUTIVE DIFFERENTIAL GROWTH 1 (CDG1) [84]. Transgenic plant experiments have revealed that CDG1 positively regulates BR signaling, whereby BRI1 phosphorylates CDG1 on S234, which then in turn phosphorylates BSU1 on S764, resulting in BIN2 inactivation via its dephosphorylation (Figure 3E) [84]. Specific disruption of these phosphorylation sites through targeted site-directed mutagenesis impairs BR signaling [84].

Other unique plant protein phosphatases in the PPP family include the SLP phosphatases [2,5,29,30,85,86]. This PPP subfamily was first described in a bioinformatic survey of bacterial-like phosphatases encoded by eukaryotes and were named after their relatedness to phosphatases from *Shewanella* bacteria [30]. Unique to the PPP family, the SLP phosphatases of *Arabidopsis* (AtSLP1 and AtSLP2) were found to be completely insensitive to classic small molecule inhibitors okadaic acid and microcystin in addition to AtI2 [29,87]. Many differences have been uncovered between AtSLP1 and AtSLP2. Western blotting of *Arabidopsis* plant tissues revealed distinct green (rosettes) and non-green (roots) tissue-specific protein expression patterns for AtSLP1 and AtSLP2, respectively, which was largely mirrored transcriptionally [29]. Phylogenetic analysis has established that SLP phosphatases form two distinct groups (Group 1 – SLP1 and Group 2 – SLP2) based on the proportion of amino acid identity. These groups directly correlate with their respective chloroplast (SLP1) and cytosolic (SLP2) subcellular localization, based on a combination of consensus bioinformatic predictions and transient expression of fluorescently tagged protein constructs [29].

The future of protein phosphatase research

Initial studies of protein phosphatases were complicated by their broad substrate specificities and how surprisingly few catalytic subunits there are relative to protein kinases. These two findings contributed to the belief that protein phosphatases possessed only a simple housekeeping function and that protein kinases represented the main regulatory factor behind reversible protein phosphorylation. It is now understood that a given phosphatase catalytic subunit may have many regulatory subunits or protein partners that dictate function, substrate specificity, and allow recruitment to specific cellular complexes. As described here, one major and continuing advancement in the field has been assigning regulatory subunits to phosphatase catalytic engines, and with PP1 it is expected that hundreds of binding partners still await discovery (Box 2). It is also emerging that protein phosphatases are themselves regulated by covalent modification, including phosphorylation [17,40,60]. Although yet to be characterized in plants, several yeast and human PP1 binding partners have been found to reversibly recruit PP1 with phosphorylation near or within the RVS/TF docking motif (RVxF) [17,40] preventing phosphatase association in response to a signal. A quick survey of RVS/TF binding sites in phosphoproteomic datasets indicates that this is probably a more common means of controlling PP1 dis/association than is currently known.

The question most often asked about a particular protein phosphatase is: 'what is its substrate?' Given the broad *in vitro* specificity of many protein phosphatases, this has been a challenging question to tackle using biochemical approaches. Genetics, however, has played an instrumental role in uncovering specific protein phosphatases that function in regulating a particular cellular or biological event, but the technology to address 'what is the substrate' of a specific phosphatase complex has only emerged in the age of quantitative proteomics.

Box 2. Outstanding questions

- What role do metabolites play in defining protein phosphatase function and signaling events in general?
- How are dephosphorylation events integrated with other covalent modifications?
- What is the ancient origin of the PPP enzymes?
- Is the reversible association of PP1 with regulatory subunits a mechanism to control PP1 function in plants?
- Which plant enzymes are responsible for dephosphorylation of the abundant phosphotyrosine modifications in the absence of classic tyrosine phosphatases?

Mass spectrometry as the new driving force

Mass spectrometry coupled with TAP-tagged phosphatase catalytic subunits, immunoprecipitations, and now a method denoted 'fragmentome analysis' [88] will be the approaches to define the bulk of the remaining regulatory subunits of the PPP enzymes. This will also tie specific phosphatases to known or new cellular complexes and link phosphatase function to them. These approaches have been instrumental in animal and yeast systems providing many of the plant protein phosphatase regulatory subunit orthologs we know today.

Although we must be cautious when knocking out a phosphatase catalytic subunit that probably plays a role in many different processes, when coupled to quantitative mass spectrometry it is possible to identify substrates using quantitative phosphoproteomics. A recent study used this approach to search for protein phosphatase substrates and this has proven successful [89]. In this case, a specific PPP-phosphatase (PP4) was knocked down in human cells in culture and a global change in phosphorylation of individual peptides was monitored to reveal phosphatase substrates under a physiological condition (in this case DNA damage). This approach will be even more powerful when targeted knockdown of specific regulatory subunits is undertaken, emphasizing the need to first identify and catalog protein phosphatase regulatory subunits.

Interfacing multiple covalent modifications and metabolites in signal transduction

The next age or phase of signal transduction research will probably incorporate how such a vast array of possible covalent modifications on a single protein (phosphorylation, acetylation, methylation, etc.) interface with each other and control signaling output (Box 2). This has been explored with histone tails where phosphorylation, acetylation, and methylation events on nearby amino acids control recruitment of covalent modification-dependent binding partners (e.g., 14-3-3s) [90]. Protein recruitment via one modification is thought to control access to other modification sites or docking sites due to spatial limitations (e.g., phosphatase access to phospho-residues or bromodomain binding to acetyl-lysine). There is also documentation that specific metabolite levels allosterically control the conformation of target proteins and allow access to phosphorylation sites. This is best exemplified by the ability of the protein phosphatase PP2C to dephosphorylate the AMP-activated protein kinase (AMPK)

T-loop [91]. During cellular energy stress when ADP and AMP levels increase, binding of these metabolites to allosteric sites on AMPK abolishes access to the T-loop phosphorylation site by the protein phosphatase. This effect correspondingly reverses as ADP and AMP levels drop and ATP levels increase. It is likely that this represents the tip of the iceberg in terms of metabolite control over phosphatase substrate access (and other signal transduction proteins) and will form the foundation of much signaling research over the next decade, including protein phosphatases.

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