***Arabidopsis* inositol polyphosphate kinases regulate COP9 signalosome deneddylase functions in phosphate-homeostasis**

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29 **Author Contributions**

30 Y.W. and S.B. conceived and designed the research. D.L. and G.S. generated transgenic line
 31 used here. Y.W. and S.B. performed most of the experiments. Y.W., M.K., K.D.I, S.P., and
 32 A.R. conducted and analyzed gene/protein expression changes. Y.W. and S.B. wrote the
 33 manuscript.

34 **Competing interest statement**

35 The authors declare no conflict of interest for this study.

36 **Keywords**

37 Inositol polyphosphates, COP9 signalosome, CSN5, IP5K, IP6K1, IPK1, ITPK1

Abstract

Targeted protein degradation is essential for physiological development and adaptation to stress. Mammalian INOSITOL PENTAKISPHOSPHATE 2-KINASE (IP5K) and INOSITOL HEXAKISPHOSPHATE KINASE 1 (IP6K1) pair generates inositol polyphosphates (InsPs) to modulate association/dissociation equilibrium of Cullin RING Ubiquitin E3 ligases (CRLs) on the COP9 signalosome (CSN) platform. Deneddylase activity of the CSN5 subunit protects cullins from self-ubiquitination ensuring their functional continuity. In plants, similar regulations by InsP-kinases are not known. Here, we show conserved interactions of *Arabidopsis thaliana* INOSITOL PENTAKISPHOSPHATE 2-KINASE 1 (IPK1) and INOSITOL 1,3,4-TRISPHOSPHATE 5/6-KINASE 1 (ITPK1), counterparts of the above mammalian InsP-kinase pair, with selective CSN subunits. In *ipk1* or *itpk1* mutants, deneddylation deficiencies not only cause increased neddylated Cullin1 (CUL1^{Nedd8}) pools more prone to degradation but also impair CSN5 entry/exit shuttles on the CSN holo-complex. Constitutive phosphate-starvation response (PSR), previously known for these mutants are suppressed by pharmacological inhibition of neddylation thus linking CSN-CRL functions to phosphate (Pi)-sensing. Similarly, in wild-type plants exposed to compounds that impair CSN5 deneddylase function affects its dynamics and mimic PSR properties of the mutants. We further show that under Pi-deprivation more CSN5 retentions on the CSN holo-complex and the resulting enhanced CUL1^{Nedd8} pools is essential for induction of downstream Pi-starvation inducible (PSI) genes. Overall, with our data we present InsP-kinase involvements in maintenance of Pi-homeostasis in plants via CRL-CSN(5) functional synergism.

Significance

Neddylation modifications on Culling-RING E3 ligases (CRLs) while essential for their role in proteostasis, also threaten their own stability. Selective inositol polyphosphates (InsPs) aid

62 the constitutive photomorphogenesis 9 signalosome (CSN) functions in protecting,
 63 deneddylating, and facilitating CRL recycling. Here we demonstrate that plant mutants
 64 deficient in these InsPs have disturbed CSN subunit partitioning, are deficient in deneddylase
 65 activity, and hyperaccumulate neddylated cullins that lead to constitutive phosphate-starvation
 66 response (PSR). Inhibition of CSN functions/consequences mirror the InsP mutant properties
 67 indicating pivotal involvement of CSN in phosphate homeostasis. These raises promising
 68 possibilities of targeted intervention on CSN functions for nutritional benefit of plants.

Introduction:

The versatile functions of inositol polyphosphates (InsPs) in cellular processes of all eukaryotes are owed to its propensity for differential degree of phosphorylation catalyzed by relatively conserved set of InsP-kinases (Stevenson-Paulik *et al.*, 2005; Monserrate and York, 2010; Shears and Wang, 2019). In plants, selective InsPs have been linked to phytohormone networks, inorganic phosphate (Pi)-homeostasis, mRNA export, (a)biotic stress responses, among others (Stevenson-Paulik *et al.*, 2005; Murphy *et al.*, 2008; Gillaspay, 2013; Lee *et al.*, 2015; Williams *et al.*, 2015; Jia *et al.*, 2019). With all six-hydroxyl group of the *myo*-inositol ring phosphorylated, InsP₆ (myo-inositol-1,2,3,4,5,6-hexakisphosphate or phytic acid), produced by INOSITOL PENTAKISPHOSPHATE 2-KINASE 1 (IPK1) is sequentially (pyro)-phosphorylated by INOSITOL 1,3,4-TRISPHOSPHATE 5/6-KINASES (ITPK1/2) and then by DIPHOSPHOINOSITOL PENTAKISPHOSPHATE KINASES (VIH1/2) to generate energy-rich phosphoanhydride bond-containing inositol pyrophosphates InsP₇ and InsP₈, respectively (Desai *et al.*, 2014; Laha *et al.*, 2015; Adepoju *et al.*, 2019; Laha *et al.*, 2019). Though *bona fide* receptors of plant InsPs remain unidentified, their co-factor functions are being increasingly elaborated (Shears *et al.*, 2012; Williams *et al.*, 2015). While InsP₆, or more recently speculated InsP₇, promotes auxin-dependent degradation of AUX/IAA repressors, InsP₈ targets JASMONATE ZIM-DOMAIN (JAZ) repressors in a jasmonic acid (JA)-dependent manner (Tan *et al.*, 2007; Sheard *et al.*, 2010; Laha *et al.*, 2015; Laha *et al.*, 2020). Interestingly, the cognate substrate receptors TRANSPORT INHIBITOR RESPONSE 1 (TIR1) or CORONATINE INSENSITIVE 1 (COI1) belong to F-box family proteins and are components of typical SCF (ASK1/SKP1-Cullin-F-Box; SCF^{TIR1} or SCF^{COI1})-type E3 ubiquitin ligases (Tan *et al.*, 2007; Sheard *et al.*, 2010). The *ipk1* or *itpk1* mutants with reduced InsP₇ or *vih2* plants with reduced InsP₈, are auxin- or JA-insensitive, respectively (Laha *et al.*, 2015; Laha *et al.*, 2020).

InsPs have also been recently implicated as sensor of phosphate (Pi)-status in plants (Kuo *et al.*, 2018; Dong *et al.*, 2019; Ried *et al.*, 2019; Zhu *et al.*, 2019). Necessity of phosphates in central dogma processes, protein modifications, and most importantly as indicators of adenylate currencies risk detrimental consequences when cellular Pi levels are low (Szigyarto *et al.*, 2011; Wundenberg and Mayr, 2012). Under a low Pi-availability from soil, a highly organized adaptive cascade of events collectively termed as PSR is activated and includes increased remobilization of Pi from root to shoot, harnessing of organellar Pi-storage reserves, accompanied by upregulated expression of PSI genes for Pi-transporters (*PHTs*), phosphatases (*PAPs*), and several micro- (*MIR399*) or non-coding RNAs (*IPS1*) (Puga *et al.*, 2014; Ham *et al.*, 2018). In Arabidopsis, PSR-associated transcriptional changes are majorly orchestrated by the MYB-transcription factors PHOSPHATE STARVATION RESPONSE 1 (PHR1) and its closest homolog PHR1-LIKE (PHL1) (Bustos *et al.*, 2010). These transcription factors bind to partially conserved sequences (P1BS elements) present on PSI gene promoters to activate their transcriptions (Rubio *et al.*, 2001). A *phr1 phl1* double mutant is deficient in PSI-gene expressions both basally as well as upon exposure to Pi-deplete conditions (Kuo *et al.*, 2014).

A class of stand-alone SPX (SYG1/Pho81/XPR1)-domain containing proteins SPX1/2 act as negative regulators of PHR1/PHL1 functions in maintaining phosphate balances in a cell. Following the first report that SPX domains can bind specific InsPs it was subsequently demonstrated that under Pi-replete conditions, SPX1 requires InsP₈ co-factor to bind PHR1, sequester it away from the nucleus, and suppress its activity (Wild *et al.*, 2016; Jung *et al.*, 2018; Dong *et al.*, 2019; Zhu *et al.*, 2019). When starved for Pi, intracellular InsP₈ levels reduce because of the phosphatase activity of VIH2 relieving SPX1-tethered inhibitions on PHR1, thus allowing successful transduction of PSR (Dong *et al.*, 2019). In InsP₈-deficient *ipk1-1*, *itpk1-2*, or *vih1 vih2* and in the *spx* mutants liberated PHR1 causes constitutive activation of PSI-gene expressions even under Pi-sufficient conditions accumulating high endogenous Pi

levels (Kuo *et al.*, 2014; Puga *et al.*, 2014; Dong *et al.*, 2019; Zhu *et al.*, 2019). Decreased InsP₈ levels that occur upon Pi-starvation also promotes ubiquitinylation of at least two SPX proteins (Lv *et al.*, 2014; Zhong *et al.*, 2018; Osorio *et al.*, 2019). Recent identification of E3 ubiquitin ligases that target rice SPX4 upon Pi-starvation (Ruan *et al.*, 2019) taken together with specific InsP-requirement in determination of fate of hormonal repressors, InsP₇/InsP₈ clearly are also biochemical regulators of stimulus-dependent substrate stabilities.

In a typical eukaryotic cell, ~20% of targeted protein degradations are mediated by Cullin RING ubiquitin ligases (CRLs), the largest member among the E3 ubiquitin ligase family (Soucy *et al.*, 2009; Yi Sun, 2020). A modular CRL contains a Cullin (CUL) scaffold (CUL1-4 in Arabidopsis), an E2-interacting RING-type Rbx/Roc protein, CUL member-specified substrate adaptor and a ubiquitination target-specified substrate receptor. CRLs require covalent modification of a Nedd8 moiety on CULs (a process termed as neddylation) for function (Yi Sun, 2020). Neddylation improves CRL efficiencies in complex formation with the ubiquitin E2 enzyme for subsequent degradation of targeted substrates. In absence of cognate substrates self-ubiquitinylation-vulnerable CRLs are protected on Constitutive photomorphogenesis 9 signalosome (CSN), an evolutionarily conserved eight-subunit (CSN1-8) macromolecular complex (Chamovitz and Segal, 2001; Dubiel *et al.*, 2015). The CSN5 subunit in this holo-complex contains an MPN+/JAMM metalloprotease motif that catalytically removes Nedd8 (deneddylation) from CULs (Cope *et al.*, 2002) causing CRL disassembly and promoting newer associations with substrate receptors and adaptors required for continuum of its functions. Interaction changes between CRL-CSN affect cellular ratio of neddylation: unneddylation (CUL^{Nedd8}:CUL) (Scherer *et al.*, 2016). Biochemical modes of a CSN operation including its dynamic rearrangements upon CRL binding have been gradually unraveled at structural and functional levels (Enchev *et al.*, 2012; Lingaraju *et al.*, 2014; Dubiel *et al.*, 2015). From these studies, it is evident that optimal CRL activity

necessitates regulated neddylation/deneydylaton cycles of CULs. Nedydylated CRLs are captured on the CSN holo-complex only in deneydylaton-deficient *csn* mutants inferring that deneydylaton-dependent disassembly is very rapid (Enchev *et al.*, 2012). The ‘CSN paradox’ (Dubiel, 2009), an apparent contradiction between genetic requirement of a CRL for CSN-protection at the cost of its deneydylaton-dependent inactivation is now better understood at a molecular level. Recent breakthroughs show that highly locale-specific coordinated activities of mammalian IP5K with IP6K1, functional homologs of Arabidopsis IPK1 and ITPK1, provide InsP₆ to strengthen and InsP₇ to loosen CRL-CSN associations (Rao *et al.*, 2014; Scherer *et al.*, 2016; Lin *et al.*, 2020). Through intricate orchestrations, a CRL tether-promoting InsP₆ is converted to the liberator InsP₇ by an inert IP6K1 that gets activated at CSN only upon a stimulus. This synchronization ensures functional demands of CRLs meet the response requirements proportionately without a compromise on its own stabilities. With reported interactions between Arabidopsis ITPK1 and CSN1 (Qin *et al.*, 2005), direct implication of (any) InsP-kinase on CSN-activities from a plant system has not been forthcoming.

Here we show that Arabidopsis IPK1-ITPK1 pair retains conserved CSN interactions and modulate CUL1 deneydylaton efficiencies impacting association/dissociation kinetics of CSN5 on the CSN holo-complex. Unlike noted in animal cells, CRL-CSN associations remain mostly unaffected in *ipk1* or *itpk1* mutants. Nevertheless, enhanced level of CUL^{Nedd8} result in constitutive PSR in these mutants. Further, we reveal that Pi-deprivation in wild-type plants is associated with CSN(5) perturbations and increased CUL1 neddylation that precedes PHR1/PHL1 role in inducing PSI gene expressions. Mutational or pharmacological inhibitions of CSN5 result in constitutive PSR even under sufficient Pi-availability. However, when actually Pi-starved, PSR responses in these plants or in the *ipk1* or *itpk1* mutants are suppressed indicating that equilibrium shifts of CSN5 deneydylase activity rather than its complete inhibition is essential to execute PSR. Our investigations overall reveal activities of plant IPK1-

ITPK1 pair in regulating CSN dynamics, its consequences on CRLs functions and requirement in maintaining Pi-homeostasis or responding to its deficiencies.

Results

Arabidopsis IPK1 and ITPK1 interact with CSN holo-complex subunits.

Mammalian IP5K co-precipitates with CSN2/5 whereas IP6K1 interacts with CSN1/2 (Rao *et al.*, 2014; Scherer *et al.*, 2016). Arabidopsis ITPK1 associates with CSN1 (Qin *et al.*, 2005). Interactions of Arabidopsis IPK1 with CSN subunits have not been investigated earlier. We immuno-enriched Myc-IPK1 or ITPK1-GFP from *ipk1-1:Myc-IPK1* and *itpk1-2:ITPK1-GFP* transgenic lines, respectively and probed for the presence of selective CSN subunits, CUL1 or the substrate adaptor ASK1. These transgenic lines restore high endogenous Pi and elevated PSI gene *SPX1* expressions to wild-type (Col-0) levels implicating the functionality of the corresponding fusion proteins (Laha *et al.*, 2020) (Supplemental Figure 1). In immunoprecipitation assays, CSN1/2/4/5, CUL1 and ASK1 co-purified with IPK1, whereas ITPK1 enrichments detected CSN1/2 and ASK1 (Figure 1A). Through Bimolecular Fluorescence Complementation (BiFC) assays, IPK1 or ITPK1 association with CSN1 and CSN2 are detected as nucleocytoplasmic complexes similar to localization of the individual proteins (Adepoju *et al.*, 2019) (Figure 1B). A negative control β -Glucuronidase (GUS) protein does not interact with IPK1, ITPK1 or CSN1/2 (Supplemental Figure 2). These data suggest that likewise to animals, CSN associations of IPK1 and ITPK1 are conserved in Arabidopsis. Although biochemically coupled by substrate-product relationship, interaction between IPK1 and ITPK1 has not been demonstrated *in planta*. To this end, BiFC detects IPK1-ITPK1 complexes at nucleocytoplasmic locales (Figure 1B). Further, we performed co-immunoprecipitation assays via transient co-expression of Myc-IPK1 and GFP-ITPK1 in

Nicotiana benthamiana leaves. Enrichment of GFP-ITPK1 with anti-GFP affinity beads co-elutes Myc-IPK1 indicating their associations *in vivo* (Figure 1C). Overall, our results recapitulate IPK1 and ITPK1 interactions with CSN and with each other.

To determine cellular proportions of IPK1 or ITPK1 that associate with the CSN holo-complex, we subjected Col-0, *ipk1-1:Myc-IPK1* and *itpk1-2:ITPK1-GFP* extracts to size-exclusion chromatography followed by immunoblots for representative CSN subunits, Myc-IPK1 or ITPK1-GFP. The elution patterns of molecular weight standards and selective CSN subunits with demarcation of CSN holo-complex fractions, as noted in earlier reports are shown (Gusmaroli *et al.*, 2004; Dohmann *et al.*, 2005) (Figure 2A). CSN1, exclusively associates with the CSN holo-complex and elutes accordingly in higher molecular weight pools (>600-400 kDa). CSN4 and CSN5 fractionates both as a part of CSN holo-complex as well as in lower molecular weight pools (monomers, 100-50 kDa). Partitioning profiles of CSN5 and CSN4 are comparable between Col-0, *ipk1-1:Myc-IPK1* and *itpk1-2:ITPK1-GFP* (Figure 2B; Supplemental Figure S3A). Remarkably, neither Myc-IPK1 nor ITPK1-GFP proteins are detected in fractions corresponding to the CSN holo-complex implying that their associations are either transient or represent only marginal proportions of these proteins (Figure 2A). Co-elution of Myc-IPK1 and ITPK1-GFP in overlapping fractions is likely indicative of their *in vivo* associations we noted earlier.

CSN5 deneddylase deficiencies cause enhanced CUL1^{Nedd8} pools in *ipk1* or *itpk1* plants.

Depletion of IP5K or IP6K1 reduces CRL-CSN associations in mammalian systems (Rao *et al.*, 2014; Lin *et al.*, 2020). To examine whether in *ipk1-1* or *itpk1-2* lower CUL1 pools co-fractionate with the CSN holo-complex, we immuno-enriched CUL1 from Col-0, *ipk1-1* or *itpk1-2* extracts and probed for the presence of CSN1/2. Although slightly lower CUL1 was immunoprecipitated from *ipk1-1* or *itpk1-2* than Col-0, nonetheless proportionately similar

amounts of CSN1/2 as in Col-0 co-eluted suggesting that CRL-CSN associations are likely not perturbed in these mutants (Figure 2C). Surprisingly, when we compared elution profiles, CSN5 but not CSN4 in *ipk1-1* or *itpk1-2* extracts showed increased presence in CSN holo-complex fractions with marked decrease in monomeric elutions, relative to Col-0 (Figure 2B). Total CSN5 levels however remained comparable between all plants.

Resident CSN5 performs the deneddylase role in a CSN holo-complex (Cope *et al.*, 2002). With changed partitioning of CSN5 noted in *ipk1-1* or *itpk1-2* we tested whether deneddylation efficiencies on CULs are impacted. In mammalian system depleting IP5K or IP6K1 causes strong increments in CUL^{Nedd8} levels (Rao *et al.*, 2014; Scherer *et al.*, 2016). Remarkably, in *ipk1-1* or *itpk1-2* lysates, enhanced CUL1^{Nedd8} levels relative to Col-0 are strikingly prominent (Figure 2D). These increases are specific to *ipk1-1* or *itpk1-2* and extracts from *ipk2β-1* with low InsP₆ levels as *ipk1-1* (Stevenson-Paulik *et al.*, 2005), *vih2-4* plants with elevated InsP₇ (Laha *et al.*, 2015), or *itpk4-1* with low InsP₆ and InsP₇ levels (Kuo *et al.*, 2018) do not show elevated CUL1^{Nedd8} pools (Supplemental Figure S3B). In the respective complemented lines of *ipk1-1* or *itpk1-2*, CUL^{Nedd8}:CUL ratios are restored comparable to Col-0 (Figure 2D).

Global ubiquitinylation is enhanced due to loss of mammalian IP6K1 (Rao *et al.*, 2014). As a possible consequence to increased CUL^{Nedd8} levels, we tested relative levels of ubiquitinylation in Col-0, *ipk1-1*, or *itpk1-2* extracts. Clear increase in global ubiquitin-conjugates in appearance of a smear when probed with anti-ubiquitin antibodies is detected in the mutant plants than Col-0 (Figure 2D). In complemented lines, wild-type ubiquitinylation levels are noted. Further, in-lysate deneddylation assay to determine deneddylase defects (Lin *et al.*, 2020) reveal decreased efficiencies of CUL1-deneddylation by endogenous CSN5 simultaneous with increased CUL1 turnover rates in *ipk1-1* or *itpk1-2* extracts (Figure 2E). Decreased CUL1 stability likely accounts for the reduced immuno-enrichments from the mutants observed earlier. Taken together, our results parallel animal studies demonstrating

impaired CSN5 deneddylase activity in *ipk1-1* or *itpk1-2* that leads to augmented CUL1^{Nedd8} levels prone to increased turnover due to self-ubiquitinylation.

Pi-starvation alters dynamics of CSN5 and its deneddylase efficiencies.

Known consequences in *ipk1-1* and *itpk1-2* are auxin-insensitivity and constitutive PSR (Kuo *et al.*, 2014; Kuo *et al.*, 2018; Laha *et al.*, 2020; Riemer *et al.*, 2020). Reduced InsP₈ in these mutants likely disrupt SPX1-PHR1 interactions that lead to activation of PSI-genes (Ried *et al.*, 2019). MLN4924, a small molecule pharmacological inhibitor of CUL neddylation (Soucy *et al.*, 2009) is functional in Arabidopsis, inhibits degradation of CRL substrates and confers auxin insensitivity (Hakenjos *et al.*, 2011). To test whether increased PSI-gene expressions in *ipk1-1* or *itpk1-2* are consequences of enhanced CUL1^{Nedd8} levels via augmenting CRL functions, we treated these mutants with MLN4924. Remarkably, upregulated expression of PSI-genes such as *IPSI*, *SPX1*, or *PHT1;3* as well as high endogenous Pi levels in *ipk1-1* or *itpk1-2* were suppressed by MLN4924-treatments (Figure 3A-B; Supplemental Figure 4A). On Col-0 grown under Pi-sufficient conditions, MLN4924 treatments did not cause detectable change in endogenous Pi-levels or *IPSI* expression (Figure 3A-B). Most surprisingly, MLN4924 treatments on Col-0 suppressed the induction of *PHT1;3* or *IPSI* when subjected to Pi-starvation implying that neddylation processes drive PSI-gene expressions (Figure 3C).

To test whether neddylation efficiencies of cullins are affected upon Pi-deprivation, extracts from Col-0 grown under Pi-replete or Pi-deplete conditions were immunoblotted with anti-CUL1 antibodies. Prominent increase in CUL1^{Nedd8} levels, sensitive to MLN4924 inhibition, was noted under Pi-starvation (Figure 3D). Interestingly, these enhancements were induced even in the *phr1phl1* plants when subjected to Pi-deplete conditions implying that neddylation readjustments are upstream of PHR1/PHL1 role in inducing PSI-gene expressions. Already enhanced CUL1^{Nedd8} levels in *ipk1-1* or *itpk1-2* remained unaffected under Pi-deplete

exposures (Supplemental Figure 4B). Taken together, our results identify CUL1 hyperneddylation as an early response to Pi-starvation essential for transducing downstream signaling.

As tested earlier for *ipk1-1* or *itpk1-2*, to correlate increased CUL1^{Nedd8} levels to decreased CSN5 deneddylase activity, we performed in-lysate deneddylation assay on Pi-replete or starved Col-0. Diminished CSN5 deneddylase activity was clearly apparent in Pi-deprived Col-0 extracts (Figure 4A). In the same extracts CUL1 instabilities were not evident perhaps reflecting tight regulations between its increased utilization and turnover rates. When profiled, CSN5 elution patterns under Pi-deplete conditions were peculiarly similar to *ipk1-1* or *itpk1-2* with more engagements on the CSN holo-complex and lower monomeric pools (Figure 4C). Total CSN5 protein remained comparable between Pi-replete and Pi-deplete extracts. Profiles of CSN1 remained unaltered by the Pi-changes (Supplemental Figure 4C). Resupplied phosphate restored both CSN5 partitioning changes and increased CUL1^{Nedd8} amounts to Pi-replete pattern suggesting that observed changes were due to Pi-starvation (Figure 4B, C). Curiously, unlike *ipk1-1* or *itpk1-2*, global ubiquitinylation was not enhanced upon Pi-starvation although resupplying phosphate did cause a modest increase (Figure 4D). Together, these results suggest intricate coordination between CSN-CRL activities to maintain specificity in responding to reduced Pi-availability. Also, our data indicates that plants modulate CSN5 deneddylase activity and affect its dynamics during Pi-deprivation to induce PSI-gene expressions, a phenomenon that is constitutive in *ipk1-1* or *itpk1-2*. In Pi-starved *ipk1-1:Myc-IPK1* or *itpk1-2:ITPK1-GFP* plants, marked increase in ITPK1-GFP, but not very prominent for Myc-IPK1 protein, was detected in comparison to non-starved extracts (Supplemental Figure 4D). These at least are not due to increased transcriptions as reported earlier (Riemer et al., 2020) and may instead be in accordance with increased stability the mammalian IP6K1 acquires when associated with an active CRLs (Rao et al., 2014).

Functional inhibition of CSN5 phenocopy *ipk1* or *itpk1* responses.

To determine whether PSI-gene expressions are globally affected by CSN functions, we mined the available microarray data of differentially expressed transcripts in *csn* null mutants (*csn3*, *csn4* and *csn5ab*) (Dohmann *et al.*, 2008). Strong upregulation in expressions compared to wild-type plants are especially noted in genes responsible for Pi-remobilization (*PAP1*, *PAP10*, *PAP23*, *DFR*, *F3H*) and several Pi-transporters (*PHT3;2*, *PHT3;3*, *OCT1*) (Supplemental Figure 5). Expressions of *PHR1* or *PHL1* remain unaffected in these mutants similar to *ipk1-1* or *itpk1-2* (Kuo *et al.*, 2014; Kuo *et al.*, 2018). Also, neither *IPK1* nor *ITPK1* transcriptions are changed in the *csn* mutants. Thus, CSN holo-complex dysfunctions affect several PSI-gene expressions likely because of its deneddylation deficiencies. To explore CSN5 roles further, we utilized the *csn5a-2* plants harboring a T-DNA insertion in the last exon of the *CSN5A* isoform (*At1lg22920*). Similar to *ipk1-1* or *itpk1-2*, *csn5a-2* extracts accumulate higher CUL1^{Nedd8} levels and display auxin-insensitivity (Gusmaroli *et al.*, 2004; Dohmann *et al.*, 2005; Dohmann *et al.*, 2008; Laha *et al.*, 2020). Under Pi-replete growth, increased basal Pi-levels accompanied by mild up-regulated expressions of *PHT1;3* or *SPX1* is detected in the *csn5a-2* plants (Figure 5A-B). Also, primary root lengths of *csn5a-2* plants are slightly, but not statistically shorter than Col-0 in this growth media (Supplemental Figure 6). These features are very similar to *ipk1-1* or *itpk1-2* (Riemer *et al.*, 2020). Growth under Pi-starvation led to strong inhibition of primary root length in Col-0, while in *csn5a-2* (and in *ipk1-1* or *itpk1-2*) plants these were prominently less. With exposure to Pi-deplete conditions, *csn5a-2* plants are completely impaired in inducing *PHT1;3* or *SPX1* (Figure 5B). These results resemble PSI gene induction deficiencies in *itpk1-2* when subjected to Pi-starvation (Riemer *et al.*, 2020) suggesting that CSN5 functions are necessary in maintenance of Pi-homeostasis and successful transduction of PSR.

315 To directly relate CSN5 deneddylase role in Pi-homeostasis, we tested the effects of recently
 316 reported CSN5 inhibitors Azaindole#6 and CSN5i-3 on Arabidopsis (Schlierf *et al.*, 2016;
 317 Altmann *et al.*, 2017). In animal cell lines, these inhibitors interfere with the CSN5 deneddylase
 318 activity, and trap CRLs in their neddylated states. Because they remain untested on any plant
 319 systems, we first determined their efficacy on Col-0 seedlings. Parallel MLN4924 treatments
 320 were used as neddylation-inhibition controls. Dose-dependent increments in CUL1^{Nedd8} levels
 321 with collateral decrease in unneddylated CUL1 was detected at 24hr post-treatment with
 322 CSN5i-3 (Figure 5C). At 48-hrs post-treatment, efficiency of CSN5i-3 inhibitions intensified
 323 (Supplemental Figure 7A). At all doses tested, Azaindole#6 remained ineffective in changing
 324 CUL1^{Nedd8}:CUL1 ratios. As expected, MLN4924 treatments in a dose-dependent manner
 325 reduced CUL1^{Nedd8} levels. For subsequent experiments, we continued with 10μM dose of
 326 CSN5i-3 and the same for MLN4924, as used in our earlier assays. On Pi-replete Col-0, CSN5i-
 327 3 caused a modest increase in basal Pi levels and upregulation in expression of *PHT1;3*, but
 328 not significantly for *SPX1* or *IPS1* (Figure 5D-E; Supplemental Figure 7B-C). Under Pi-deplete
 329 exposure, CSN5i-3 suppressed the induction of *PHT1;3*, *SPX1* or *IPS1* in Col-0 and mirrored
 330 MLN4924 effects (Figure 5D-E; Supplemental Figure 7B-C). CSN5i-3-treated Col-0 extracts
 331 when fractionated, CSN5 was markedly enriched in the CSN holo-complex pools with
 332 negligible detection in monomeric elutions, a pattern resembling *ipk1-1*, *itpk1-2*, or Pi-starved
 333 profiles (Figure 6A). With these results, we reason strong correlation between CSN5
 334 deneddylation deficiencies to its enhanced retention on the CSN holo-complex, and trigger of
 335 PSR as observed in *ipk1-1* or *itpk1-2* plants. Most interestingly, *ipk1-1* or *itpk1-2* seedlings
 336 grown under sufficient Pi when treated with CSN5i-3 had reduced *IPS1* expressions than
 337 untreated plants indicating that skewed kinetics of CSN5 activity rather than its complete
 338 inhibition were attributed to these mutants (Supplemental Figure 7D). MLN4924-treatments
 339 on *ipk1-1* or *itpk1-2* in Pi-deplete medium offered relief by elevating *IPS1* expressions further

supporting our hypothesis that inadequacies in these mutants to support PSR is related to dynamics of CSN5-regulated neddylation/deneylation cycles of CRLs (Supplemental Figure 7E). From these data, it is suggestive that IPK1/ITPK1 functions influence deneylase efficiencies and shuttling modes of CSN5 thereby affecting CRL requirements in executing PSR.

IP6K1-like activity, but not InsP₆ regulates CSN5 deneylase functions of CSN.

InsP₆ is implicated as the physiological glue in potentiating CSN2-CRL associations (Scherer *et al.*, 2016). That a kinase-dead IP5K interacts weakly with CSN2/5 further indicates that InsP₆ augmenting these interactions is produced locally. Supplementing InsP₆ to IP5K-depleted cell lysates strengthens CRL-CSN2 associations, and increases deneylation activities of recombinant CSN on purified neddylation cullins (Scherer *et al.*, 2016). Apparently, the externally added metabolite gets access its designated binding pocket at the CRL-CSN2 interface. Even though CRL-CSN associations are not affected in *ipk1-1* or *itpk1-2*, we investigated whether adding InsP₆ to lysates recover deneylation deficiencies in these mutants, or augments it in Col-0. In lysate deneylation assays showed that supplemented InsP₆ did not alter deneylation deficiencies or CUL1 instability in *ipk1-1* or *itpk1-2* (Supplemental Figure 8). In similar assays on Col-0 extracts, InsP₆ addition did not change deneylation or CUL1 turnover rates indicating that unlike mammalian systems, CSN functions in plants are not influenced by InsP₆.

As a CRL-CSN release factor, InsP₇ when supplemented to mammalian cell extracts does not cause complex disassembly meaning that IP6K1 activity is very tightly regulated spatiotemporally (Rao *et al.*, 2014). With observed ITPK1-CSN2 interactions, this instigated us to investigate for a similar mechanism in plants. The cell-permeable compound N2-(m-(trifluoromethyl)benzyl) N6-(p-nitrobenzyl) purine (TNP) inhibits animal IP6K1 functions and

reduce InsP₇ levels (Padmanabhan *et al.*, 2009). TNP effects so far have remained untested on Arabidopsis InsPs. In extracts of 10μM TNP-treated Col-0, distinct increase in CUL1^{NEDD8} levels were observed although less potent than a similar dose of CSN5i-3 (Figure 6B). To improve TNP efficacies, we used 25μM doses for subsequent assays. In Col-0 grown in Pi-sufficient media, TNP presence replicated CSN5i-3 results in augmenting basal Pi levels, upregulation of *IPS1* or *PHT1;3* expressions, and more retention of CSN5 in the CSN holo-complex pools (Figure 6A-C-D). TNP also suppressed endogenous deneddylation rates with increased CUL1 instability (Figure 6E). And likewise, to CSN5i-3, TNP also suppressed PSI-gene inductions in Col-0 under Pi-deplete circumstances (Figure 6D). Curiously, CSN5i-3, TNP or MLN4924 treatments on *itpk1-2::ITPK1-GFP* resulted in increased ITPK1-GFP levels, similar to observed in Pi-deplete media (Supplemental Figure 9). Although direct inhibitions of TNP on Arabidopsis ITPK1 or other InsP₍₆₎-kinases still remains to be tested, nevertheless our results imply that similar to mammals an IP6K1-like activity affects CSN5 performance on regulating CUL1 deneddylation rates and its consequences on PSR. Overall, with our data we present localized coordinated involvement of InsP-kinase pair in modulating CSN dynamics, defining molecular concept of the CSN paradox, and underlining CSN role in phosphate stress responses of plants.

Discussion

In animal systems, CRL-CSN tether is augmented by InsP₆ and released via stimulus-driven InsP₇ synthesis. Here, we show that although Arabidopsis IPK1 and ITPK1 retain conserved CSN interactions, InsP₆/InsP₇ changes in the corresponding mutants do not overall affect CRL1-CSN associations. Thus, either the residual levels of these InsPs suffice at the CRL-CSN junctures or the association/dissociation cycles involve an altogether different InsP(s) not

locally altered in the above mutants. Unlike mammalian results, that added InsP₆ does not rescue *ipk1-1* or even alter CUL1 deneddylation rates/stability in Col-0 supports our speculation. Further, an *ITPK4* mutant (*itpk4-1*) with reduced InsP₆/InsP₇ as in *ipk1-1* or *itpk1-2* has normal PSR (Kuo *et al.*, 2018) and matches Col-0 ratio of CUL1^{Nedd8}:CUL1 also lends credence to our hypothesis. One striking distinction in *ipk1-1* or *itpk1-2* is that these mutants accumulate increased levels of Ins(3,4,5,6)P₄ which neither the *itpk4-1* nor Col-0 subjected to PSR does (Kuo *et al.*, 2018). Whether this increased abundance of Ins(3,4,5,6)P₄ or another unidentified InsP altered in *ipk1-1* or *itpk1-2* satisfy CRL-CSN interaction requirements remains a possibility to explore further. Nevertheless, analogous to mammals our observations overall parallel that decreased CSN5 deneddylase performance with lower CUL1 deneddylation rates associate with the loss of IPK1 or ITPK1 and implicates their distinct roles on CSN functions.

Mammalian ITPK1 over-expression partitions more monomeric CSN5 free from the CSN holo-complex (Sun *et al.*, 2002). We demonstrate here that absence of Arabidopsis ITPK1 or TNP inhibitions antagonize CSN5 release from the CSN holo-complex. Even with yet to be tested TNP effects on ITPK1 (or another InsP-kinase) functions in plants, nonetheless a mammalian IP6K1-like activity in choreographing CSN5 entry-exit shuttles seems to be conserved in Arabidopsis. Overall structural integrity of an inert CSN holo-complex is remarkably maintained without CSN5, albeit for deneddylation CSN5 incorporation is mandatory (Lingaraju *et al.*, 2014). When CSN5 is integrated into the holo-complex, its interfaces with CSN4-CSN6 inhibit deneddylase activation in the absence of a bound CRL (Enchev *et al.*, 2012; Lingaraju *et al.*, 2014). Upon binding a neddylated CRL, a series of conformational change prompted by CSN4 orients CSN5 active site towards the neddylated cullins. Closely proximal to this active site, conserved lysines in CSN2 alpha helices coordinate bound InsP₆ to bridge for more stable associations with CRL. The bound InsP₆ in this

electropositive environment is uniquely oriented with its 5'-phosphate exposed, amenable for subsequent pyrophosphorylation. Contrastingly, when an InsP₇ is modeled into the same active site strong steric clashes especially at CRL-CSN junctures are noted (Lin *et al.*, 2020). These steric intolerances may underlie InsP₇-triggered CSN5 deneddylase activation, followed by CRL and perhaps CSN5 release from the CSN holo-complex. When added to cell lysates, since free InsP₇ fails to enhance cullin neddylation or disrupt CRL-CSN associations (Rao *et al.*, 2014), its localized synthesis achieved through site-specific availability of InsP₆, and its accessibility to CSN5 active site is hence crucial for its proposed role. Thus, similar to the mammalian IP6K1 a plant ITPK1 activity (via InsP₇ or an unknown InsP) may possess propensity to reconfigure CSN, initiating deneddylation coupled to CRLs and CSN5 disassembly from the holo-complex. These roles may be supportive of the recent implication that a plant ITPK1 has evolutionarily integrated functional signatures of both mammalian ITPK1 and IP6K1 (Whitfield *et al.*, 2020).

With obvious InsP₈ reductions in *ipk1-1*, *itpk1-2* or upon VIH2 phosphatase activity during Pi-starvation (Dong *et al.*, 2019; Zhu *et al.*, 2019), PHR1 liberated from SPX1 causes activation of PSI-gene expressions. Here we demonstrate that a mode of perception of Pi-deprivation in addition to the above also occurs at CSN, involving changes in associated InsP-kinase activities, and is responded by decreased deneddylation rates with pronounced CSN5 retentions on the CSN holo-complex. Since the resulting increase in CUL1^{Nedd8} levels remain independent of PHR1/PHL1 presence, these observations likely indicate parallel responsive routes of a Pi-starved plant to facilitate more available pools of neddylated CRLs to ubiquitinylate substrates including the recently identified SPX4, released from PHR1-hold (Osorio *et al.*, 2019). These features we show are constitutive in *ipk1-1*, *itpk1-2*, and mimicked in wild-type plants with CSN5i-3 or TNP treatment, and suppressed by the neddylation inhibitor MLN4924. Our observation that phosphate re-supplementation post-starvation restores CSN5 partitioning and

CUL^{Nedd8}:CUL1 ratios to Pi-replete patterns in wild-type plants implies that the noted shifts are only transitory adaptations. That, unlike in *ipk1-1* or *itpk1-2*, global polyubiquitin-conjugates remains unchanged in Col-0 under low-Pi availability additionally emphasize on the strictly regulatory PSR mechanisms to controls ubiquitinylation-specificities on intended targets. Not the least, observed failure in *itpk1-2* (Riemer *et al.*, 2020) or *csn5a-2* to respond to low-Pi by anticipated augmentation of already upregulated PSI-gene expressions, or in CSN5i-3 or TNP treated Col-0 indicates that CSN5 partitioning re-adjustments are only shifts in neddylation/deneydylolation equilibrium and not a complete inhibition of the CSN5 deneydylolase. Also, that MLN4924 presence causes sub-optimal induction of PSI-gene expressions in Pi-depleted Col-0, suggests continued requirements of CRL neddylation/deneydylolation cycles to successfully transduce PSR. Thus, sustained and successful response to Pi-starvation is likely achieved by a shift in equilibrium of CSN5-mediated deneydylolation dynamics on CUL1 and not complete inhibition of its activities as described in our model (Figure 7). Similar phenomenon is mirrored by the shuttling requirements of CSN5 in cell proliferation wherein its perturbations cause complete arrest (Tomoda *et al.*, 2002; Yoshida *et al.*, 2010).

Distinct increase in ITPK1 protein but not gene expression under Pi-starvation (Riemer *et al.*, 2020) and as shown here with CSN5i-3, TNP or MLN4924 treatments is conceivably similar to the gain of stability the mammalian IP6K1 acquires upon CRL binding (Rao *et al.*, 2014). Thus, similar to CUL1 vulnerability for self-ubiquitinylation, ITPK1 fates may also be regulated in the absence of CRL substrates. Increased stabilities hence may therefore reflect ITPK1's priority to accommodate stimulus-appropriate co-factor requirements in downstream responses not limited only for modulating CRL functions. The mammalian phosphate exporter Xenotropic and polytropic retrovirus receptor 1 (XPR1) relies on InsP₇ synthesized by IP6K1/2 for function (Wilson *et al.*, 2019). Arabidopsis XPR1 ortholog PHO1 does not complement the

pho1 mutant if mutations are introduced in its InsP₇-binding pocket (Wild *et al.*, 2016). And as is known during PSR, augmented auxin responses involving SCF^{TIR1} also require InsP₇ (Laha *et al.*, 2020). Whether these new engagements of ITPK1 are adaptation strategies to transiently deprive CSN (and CSN5 release from the holo-complex) and shift balances towards more neddylation CRL availability and/or for providing InsP₇ for other cellular processes are encouraging possibility to explore. In conclusion, with our data we highlight remarkable conservation, albeit with slight distinction, in plant InsP-kinase roles in regulating CRL-CSN functions and its implications in responding to Pi-imbalances.

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Figure legends

Fig. 1. IPK1 and ITPK1 interact with each other and with selective CSN or CRLs

components *in planta*. (A) Immuno-enrichments of IPK1 (*top panel*) or ITPK1 (*bottom panel*) from corresponding complemented lines (*ipk1-1:Myc-IPK1* or *itpk1-2:ITPK1-GFP*, respectively) was probed with anti-CSN1/2/4/5, anti-CUL1, or anti-ASK1 antibodies. IgG-agarose enrichments were used as negative controls. (B) Bimolecular Fluorescence Complementation (BiFC) assays showing *in planta* IPK1 or ITPK1 interaction with CSN1/2 and with each other. Indicated binary vectors combinations were co-expressed via *Agrobacterium* in *Nicotiana benthamiana* leaves and fluorescence observed under a confocal microscope with YFP filter. Scale bar=50 μ m. (C) Co-immunoprecipitation of transiently expressed GFP-ITPK1 with Myc-IPK1 from *N. benthamiana* leaves. Anti-GFP pulldown was probed with anti-GFP or anti-Myc antibodies.

Fig. 2. CSN5 deneddylase deficiencies with increased holo-complex retention in *ipk1-1* or

***itpk1-2* cause enhanced CUL1^{Nedd8} levels.** (A) IPK1 or ITPK1 does not co-elute with the CSN holo-complex. (B) Enhanced CSN5 engagements on the CSN holo-complex in *ipk1-1* and *itpk1-2*. Extracts from marked plants fractionated by size-exclusion chromatography were immuno-blotted with indicated antibodies. Fraction numbers and elution positions of molecular weight standards (in kDa) are shown. Fractions corresponding to CSN holo-complex and CSN subunit monomeric elutions are marked. (C) Immuno-enrichment of CUL1 detects stoichiometrically similar amounts of CSN1 and CSN2 in *ipk1-1* or *itpk1-2* to Col-0. Pulldowns with either IgG or anti-CUL1 antibodies were probed with anti-CUL1, anti-CSN1 or anti-CSN2 antibodies. (D) Increased CUL1^{Nedd8} levels (*top panel*) and enhanced global polyubiquitin-conjugates (*bottom panel*) in *ipk1-1* or *itpk1-2*. Total protein extracts were probed with anti-CUL1, anti-actin, or anti-Ubiquitin antibodies as indicated. (E) CSN5-deneddylation deficiencies and lower CUL1 half-life in *ipk1-1* or *itpk1-2*. In-lysate CUL1

deneddylation assay (Lin *et al.*, 2020) at the indicated time points (minutes post-incubation; mpi) probed with anti-CUL1 antibodies. Migration position of molecular weight standards (in kDa) and neddylated (CUL1^{Nedd8}) or unneddylated (CUL1) Cullin1 are shown. Anti-actin or Ponceau S staining demonstrates loadings controls.

Fig. 3. Enhanced CUL1^{Nedd8} levels associated with PSR is essential for PSI-gene inductions. MLN4924 suppresses (A) elevated PSI-gene expressions, and (B) higher endogenous Pi-levels in *ipk1-1* or *itpk1-2*. (C) Induction of *PHT1;3* or *IPSI* expressions in Col-0 upon Pi-starvation is inhibited by MLN4924. (D) Increments in CUL1^{Nedd8} levels during Pi-deprivation is PHR1/PHL1-independent. Plants grown in Pi-replete medium or transferred to Pi-deplete medium were treated with or without MLN4924. Gene expression changes were relative to *MON1* transcripts and reported as fold-change to Col-0 levels. Data is mean $\pm$ SD (n=3 for qPCRs; n=6-7 for Pi-estimations). Statistical significance indicated with different alphabets is according to post-hoc Tukey's test (p<0.05). For *PHT1;3* or *SPX1* expression data, statistical comparisons were to their respective DMSO-treated Col-0. For immunoblots, anti-CUL1 antibodies were used. Ponceau S staining shows loadings controls.

Fig. 4. Pi-starvation reduces endogenous CSN5 deneddylase activity and is restored upon phosphate resupply. (A) Extracts at indicated time points (minutes post-incubation; mpi) from in-lysate CUL1 deneddylation assay on Col-0 from Pi-replete or Pi-deplete media were probed with anti-CUL1 antibodies. (B) Restoration of enhanced CUL1^{Nedd8} levels occur upon Pi-resupply post-starvation. (C) Increased retention of CSN5 on the CSN holo-complex under Pi-deplete exposure is reversed with Pi-resupply. (D) Global levels of polyubiquitin-conjugates remain unchanged during PSR. For these assays, plants were subjected to Pi-starvation for 3 days and phosphate re-supplementation was done for 12hrs. Comparable loadings are shown by Ponceau S staining. Elution positions of molecular weight standards (in kDa) are indicated.

Fig. 5. Inhibition of CSN5 functions activates PSR under Pi-replete but confers non-responsiveness under Pi-deplete growth. *csn5a-2* plants have (A) elevated endogenous Pi levels, (B) increased *PHT1;3* or *SPX1* transcript levels under Pi-replete growth but suppressed expression upon Pi-starvation. (C) CSN5i-3 inhibits Arabidopsis CUL1 deneddylation, (D) elevates endogenous Pi-levels, and (E) suppresses *PHT1;3* induction during phosphate-deprivation. Immunoblots were probed with anti-CUL1 antibodies. Ponceau S staining shows loading controls. Inhibitor treatments were done for 24 hrs and doses used are indicated. Expression levels of *MON1* transcripts was used as an internal reference for qPCRs and reported as fold-change relative to DMSO-treated Col-0 under Pi-replete growth. Error bars are mean $\pm$ SD (n=3 for qPCRs; n=6-7 for Pi-estimations). Different alphabets denote statistical significance determined by post-hoc Tukey's test (p<0.05). *PHT1;3* and *SPX1* data are compared to respective Pi-replete Col-0.

Fig. 6. CSN5i-3 or TNP treatments on Col-0 mirrors *ipk1-1* or *itpk1-2* properties. (A) CSN5 partition shifts to CSN holo-complex pools upon CSN5i-3 or TNP treatment. TNP (B) enhances pools of CUL1^{Nedd8}, (C) increases endogenous Pi-content, (D) upregulates *IPSI* or *PHT1;3* expressions under Pi-replete, but suppresses them under Pi-deplete growth, and (E) decreases endogenous deneddylation rates with increased CUL1 instability. Immunoblots were probed with anti-CUL1 or anti-CSN5 antibodies as indicated. Ponceau S staining shows loading controls. Expression levels of *MON1* transcripts was used as internal reference for qPCRs. Error bars are mean $\pm$ SD (n=3 for qPCRs; n=6-7 for Pi-estimations). Statistical significance according post-hoc Tukey's test (p<0.05) is shown with different alphabets.

Fig. 7. Schematic representation of CRL-CSN dynamics in plants. CSN holo-complex platform protects CRLs in the absence of bound ubiquitinylation substrate (steric hindrance prevents E2 enzyme binding (Enchev *et al.*, 2012; Mosadeghi *et al.*, 2016) utilizing coordinated activities (bi-directional white arrows) of the IPK1-ITPK1 pair. Equilibrium is maintained

between CSN-mediated deneddylation cycles and CUL1^{Nedd8} requirements in steady-state CRL functions. Balanced assembly/disassembly cycles of CSN5 on the CSN holo-complex are also linked to these regulations. Perception (via unknown mode; question mark) of a stimulus, such as Pi-starvation, reduces CSN5 deneddylase functions, likely via more facilitation of the InsP-kinase functions to CRL-SCF complex roles in targeted ubiquitinylation (Ubⁿ) and degradation of specific substrates (such as SPX4 or Aux/IAAs). IPK1-ITPK1 pair provides necessary InsP co-factors to these activities. Inhibition modes of TNP, CSN5i-3 and MLN4924 are indicated at the respective processes. CSN subunits are presented as numbers; CUL1, Cullin1; RBX, RING H2 protein1; E2, Ubiquitin conjugating enzyme E2; SA, substrate adapter; SR, substrate receptor; N8, Nedd8; IPK1, Inositol pentakisphosphate 2-kinase1; ITPK1, Inositol 1,3,4-trisphosphate 5/6-kinase 1.

Materials and Methods

Plant material and growth conditions. Details of *Arabidopsis thaliana* (ecotype Col-0) T-DNA insertion lines *ipk1-1*, *itpk1-2*, *ipk2β-1*, *itpk4-1*, *csn5a-2*, *vih2-4*, *itpk1-2:ITPK1-GFP*, and *phr1 phl1* plants have been described earlier (Kuo *et al.*, 2014; Jia *et al.*, 2015; Kuo *et al.*, 2018; Laha *et al.*, 2020). T-DNA and respective gene-specific primers are listed in Supplementary Table 1. Seeds were stratified for 2-days at 4°C in dark and then sterilized using 30% bleach solution. They were then germinated either directly on soil or on ½ strength Murashige and Skoog (MS) Pi-replete (625 μM KH₂PO₄) agar plates in growth chambers maintained at 22°C with 70% Relative humidity (RH) and 16hrs: 8hrs; light: dark having light intensity 100 μmol μm⁻²s⁻¹. For fractionation, Pi-starvation assays, and inhibitor treatments, plants grown on media plates as above were transferred 7-days post-germination to liquid ½ MS medium in 12-well sterile culture plates are proceeded accordingly as indicated in

respective experiments. For immuno-precipitation assays 4-week-old soil-grown plants were used.

Size-exclusion chromatography of plant extracts. Gel-filtration protocol was according to Huang *et al* (2013). Briefly, 14-day-old plants ground to a fine powder with liquid nitrogen were homogenized in Gel filtration buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 10% Glycerol, 10mM MgCl₂, 1mM DTT, 1mM PMSF and 1X Protease inhibitor cocktail). Homogenates were centrifuged for 10 mins at 4°C and supernatant passed through 0.2 µm syringe filters. Total protein extracts were fractionated through Superdex 10/300 GL Column (GE- Healthcare, USA) and eluted in the fractionation buffer at 0.2mL/min flow rate. The eluants were TCA (Trichloroacetic acid) precipitated, washed with acetone and resuspended in 1X Laemmli loading dye and used for indicated immunoblots.

In-lysate deneddylation assay. In-lysate deneddylation assay was according to Lin *et al* (2020). Briefly, 14-day-old seedlings were quickly homogenized in deneddylation buffer (10mM Tris-HCl pH7.5, 150mM NaCl and 0.5% NP-40) in absence of protease inhibitors. The extracts were incubated at 37°C for indicated times, reaction stopped by adding Laemmli loading dye to 1X, and then used for immunoblots.

Phosphate-starvation/re-supplementation assays. After germination of seeds as described earlier, seedlings were transferred to liquid ½ MS medium in 12-well sterile culture plates with Pi-replete conditions (625 µM KH₂PO₄) and placed in the growth chamber. After 4-days, the plants were either maintained with Pi-replete or transferred to Pi-deplete (10 µM KH₂PO₄) liquid media and continued for another 3 days (total 14-days post-germination) before being used for Pi-estimation, qRT-PCR, total protein extraction, or fractionation assays. For Pi-resupply, plants in Pi-deplete medium were transferred to Pi-replete medium for 12 hrs before analysis.

Inhibitor treatments. Stock solutions of MLN4924 (Cayman Chemicals, USA), CSN5i-3 (Novartis Inc, Switzerland), or TNP (Sigma-Aldrich, USA) was prepared in DMSO. For treatments, 10 μ M final concentration of MLN4924 or CSN5i-3 were used. TNP was used at 25 μ M final unless otherwise indicated. For assays on Col-0, *ipk1-1*, or *itpk1-2* or for fractionations, inhibitors were treated for 24hrs. For effects on PSR, inhibitors were added during the shift to Pi-deplete medium.

Ubiquitination assays. For determining global ubiquitination levels, extracts from indicated plants prepared in Gel filtration buffer in the presence of 10 μ M proteasome inhibitor MG132 were incubated on ice for 2hrs. Immunoblotting was then performed with anti-ubiquitin antibodies (P4D1; SantaCruz Biotechnology, USA).

Statistical analysis. All Data presented here are representative of at least three biological and technical replicates for each. Post-hoc Tukey's test ($p < 0.05$) were applied for statistical analysis.

Additional details of cloning methodologies for generation of constructs, and transgenic lines, BiFC assays, immuno-enrichments, immunoblotting, Pi-estimations, and qRT-PCRs are described in *Supplementary Materials and Methods*.

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