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Running head: ARK-PUB9 module regulates lateral root development

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Research Area: Signaling and Response

The *S*-domain Receptor Kinase *AtARK2* and the U-box/ARM-repeat-Containing E3 Ubiquitin Ligase *AtPUB9* Module Mediates Lateral Root Development Under Phosphate Starvation in Arabidopsis

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One sentence summary:

The ARK2-PUB9 module is required for lateral root development under phosphate starvation in *Arabidopsis thaliana*

ABSTRACT

When plants encounter nutrient limiting conditions in the soil, the root architecture is redesigned to generate numerous lateral roots that increase the surface area of roots, promoting efficient uptake of these deficient nutrients. Of the many essential nutrients, reduced availability of inorganic phosphate (Pi) has a major impact on plant growth due to the requirement of Pi for synthesis of organic molecules such as nucleic acids, ATP and phospholipids that function in various crucial metabolic activities. In our screens to identify a potential role for the S-domain receptor kinase 1-6 (SDK1-6 (ARK2)) and its interacting downstream signalling partner, the U-box/ARM repeat containing E3 ligase AtPUB9, we identified a role for this module in regulating lateral root (LR) development under phosphate starved conditions. Our results show that *Arabidopsis* double mutant plants lacking At*PUB*9 and At*ARK*2 (*ark2-1/pub9-1*) display severely reduced lateral roots when grown under phosphate-starved conditions. Under these starvation conditions, these plants accumulated very low to no auxin in their primary root and LR tips as observed through expression of the auxin reporter DR5::*uidA* transgene. Exogenous auxin was sufficient to rescue the LR developmental defects in the *ark2-1/pub9-1* lines indicating a requirement of auxin accumulation for this process. Our subcellular localization studies with tobacco suspension-cultured cells indicate that interaction between ARK2 and AtPUB9 results in accumulation of AtPUB9 in the autophagosomes. Inhibition of autophagy in wild-type plants resulted in reduction of LR development and auxin accumulation under phosphate starved conditions suggesting a role for autophagy in regulating LR development. Thus, our study has uncovered a previously unknown

signalling module (ARK2-PUB9) that is required for auxin-mediated lateral root development under phosphate-starved conditions.

INTRODUCTION

Roots are one of the most important adaptations of land plants as they provide anchorage, facilitate absorption of water and minerals and also aid in speciality functions such as storage of food and water, and vegetative reproduction in some plant species. Plants have the unique ability to alter their root morphology depending on resource availability in soil. Under nutrient starved conditions, this allows them to modify the roots to efficiently explore the heterogeneous soil environment for nutrients. Phosphorous in its inorganic form is often present at low concentrations and hence heavily supplemented through fertilizers in agriculture. Inorganic phosphate (Pi) is the main form of plant-assimilated phosphorous present in the soil. Pi starvation is an increasing global problem in agriculture and even in fertile soils Pi concentration rarely exceeds 10 µM (Bieleski 1973). To cope with these chronically low Pi levels, plants have developed highly specialized physiological and biochemical mechanisms to efficiently acquire and utilize Pi from the environment. This involves changes in the root architecture, increase in root-shoot ratio and root hair development (Williamson et al. 2001; López-Bucio et al. 2002; Al-Ghazi et al. 2003). When plants perceive Pi starvation, the most common response is the induction of numerous lateral roots, inhibition of primary roots and formation of denser root hairs, thus re-structuring the roots for exhaustive exploration of the top-soil for Pi (Raghothama, 1999; Lynch and Brown, 2001). During Pi deficiency, plant root hairs account for 90% of the total Pi uptake by the plants (Raghothama, 1999). Phosphate is acquired by high affinity Pi transporters and loaded into the xylem in roots. Pi moves freely through both xylem and phloem. When Pi is not limiting, Pi efflux by anion

channels ensures Pi homeostasis. The high and low affinity Pi transporters also mediate phosphate transport across the plasma membrane and tonoplast, which is powered by the membrane proton ATPase (Raghothama, 2000).

Multiple signals and molecular mechanisms are initiated when plants are exposed to Pi starvation. These include transcriptional regulation of gene expression, gene silencing by miRNAs and post-translational modification like sumoylation and ubiquitination (reviewed in Rojas-Triana et al. 2013). Although the complete signalling network through which plants cope with Pi limitation has not been deciphered, there is precedence for role of hormones in altering the root system architecture during Pi-starvation. Studies suggest both auxin-dependent and independent mechanisms during adaptive response to Pi limitation (Williamson et al. 2001; López-Bucio et al. 2002; Al-Ghazi et al. 2003; Nacry et al. 2005). However, cytokinins are known to negatively regulate this response (Martin et al. 2000; Wang et al. 2006). In addition to this, cross-talk between hormone signalling and sugar signalling pathways during response to Pi-starvation have also been reported. (Hammond and White 2011).

Several ubiquitin (Ub) conjugating and deconjugating enzymes are known to function in controlling adaptive response to Pi-starvation. FBX2 proteins which contain both WD40 and F-box motifs negatively regulates responses like root hair formation, expression of phosphoenolpyruvate carboxylase kinase (*PPCK*1 and *PPCK*2) and anthocyanin accumulation during Pi starvation (Chen et al. 2008). FBX2 also interacts with bHLH32 *in vitro*, another negative regulator of Pi-starvation responses (Chen et al. 2008). Recently, a rice U-box containing E3 Ub ligase, Os*UPS* that is induced by Pi-starvation has also been isolated (Hur et al. 2012).

One common response of plant cells to nutrient-starvation is the induction of autophagy or 'self-eating' in which autophagosomes are used for bulk degradation of cellular organelles in order to maintain the cell at a low metabolic state (Bassham 2007). Depending on the size of the cytoplasmic material engulfed for degradation, plant autophagy can be classified as either micro or macroautophagy (Bassham et al. 2006). At*ATG*18a (autophagy gene) in Arabidopsis is required for autophagic response during sucrose/nitrogen starvation and senescence (Xiong et al. 2005). RNAi lines with reduced expression of At*ATG*18a are hypersensitive to sucrose and nitrogen starvation (Xiong et al. 2005). Autophagy is also known to play a role in nutrient remobilization. Autophagy mutants of Arabidopsis are reduced in their nitrogen remobilization efficiency leading to lower biomass and yield (Guiboileau et al. 2012). Similarly, disruption of Arabidopsis *ATG*5 prevents formation of autophagosomes and causes sensitivity to nitrogen starvation (Thompson et al. 2005). Under Pi starvation, ubiquitin-like protein ATG8 has been shown to be up-regulated in root tips and disruption of *ATG*5 leads to early consumption of root meristem (Sakhonwasee and Abel 2009). Despite all these evidence for requirement of autophagy for maintaining root architecture, the mechanisms by which autophagy regulates this process during Pi starvation remain unclear.

AtPUB9 belongs to the armadillo (ARM)-repeat containing proteins with a U-box that is commonly present in many E3 ligases (Mudgil et al. 2004). While the biological functions of most of these proteins are still unknown, the roles of the other PUB family proteins range from self-incompatibility, hormone responses to defense and abiotic stress responses (reviewed in Yee and Goring 2009). *S*-domain receptor kinases (SDK) are known to function as upstream activators of PUBs (Samuel et al. 2008). Previously it has

been shown that one of the SDK1-6 (ARK2) could efficiently phosphorylate AtPUB9 *in vitro* (Samuel et al. 2008) and when co-expressed in tobacco suspension-cultured cells, this interaction led to localization of AtPUB9 in punctate structures (Samuel et al. 2008). In this report, we show that these punctate structures are lytic compartments or autophagosomes. Since autophagosomes are induced under nutrient starved conditions, we investigated *pub9-1* and *ark2-1* mutant phenotypes under nutrient limiting conditions. We found out that under Pi starvation, *ark2-1/pub9-1* double mutants were deficient in their ability to develop lateral roots. Our further investigation revealed that this defect is likely due to lack of auxin accumulation at the LR initiation sites. The LR defects in the double mutants could be rescued through by complementation with either *ARK*2 or *PUB*9, as well as by application of exogenous auxin.

RESULTS

PUB9 localizes to punctate structures and co-localizes with the autophagosomal marker ATG8 in the presence of ARK2

Plant U-box/ARM repeat proteins (AtPUBs) have been shown to interact with the Arabidopsis S-domain receptor kinases (Samuel et al. 2008) and likely function as downstream signaling molecules to these kinases. This interaction is known to both lead to phosphorylation of the AtPUBs and their alteration of cellular localization (Samuel et al. 2008). In tobacco BY2 cells, PUB9 localization could be influenced by its interaction with the cytosolic kinase domains of ARK1 and ARK2; while ARK1 re-distributes PUB9 from nucleus to the plasma membrane, ARK2 interaction leads to localization of PUB9 in punctate structures in 40% of cells (Samuel et al. 2008). To identify these sub-cellular

structures, we performed co-localization studies in the same system with fluorescent marker proteins or dyes that localize on intracellular punctate structures. When PUB9 and ARK2 were co-transformed with RFP-tagged oleosin (major structural protein associated with oil bodies), they largely localized independent of each other (Fig. 1A). When PUB9 and ARK2 were co-expressed with either lysotracker Red, an acidotropic fluorescent dye that labels acidic compartments like vacuoles or with RFP-SYP21, which is predominantly located on the membranes of the pre-vacuolar compartments, co-localization could be observed in the punctate structures (Fig. 1B, C). This suggested to us that these compartments could be lytic vacuoles that are destined to the central vacuole. When PUB9 and ARK2 were co-expressed with ATG8, an autophagosome marker, complete co-localization PUB9 and ATG8 could be observed on the punctate sub-cellular compartments (Fig. 1D).

Plants lacking both *AtPUB*9 and *ARK*2 are defective in LR formation under phosphate starvation

The localization pattern of AtPUB9 in the presence of ARK2 to autophagic compartments prompted us to investigate the *in vivo* role of these proteins under conditions that induced autophagosomes. For this, homozygous T-DNA insertional lines of *PUB9* (*pub*9-1) and *ARK2 (ark*2-1) and the homozygous double mutant, *ark2-1/pub9-1* carrying both the mutations were used (Fig. 2A, B). When root tissue was examined for expression of these genes, the double mutant line *ark2-1/pub9-1* lacked *PUB*9 or *ARK2* expression compared to wild-type (Col-0) as revealed by RT-PCR (Fig. 2C). When 3-day old seedlings of the single as well as the double mutants were transferred to nutrient rich

(+Pi/+Suc) medium and allowed to grow vertically for 7 days, there was no observable difference in lateral root density (number of lateral roots per cm of primary root length) (Fig. 2D). In contrast, when 3-day old seedlings were grown in medium lacking Pi and sucrose (–Pi/–Suc), *ark2-1/pub9-1* had severely reduced number of lateral roots compared to *pub*9-1, *ark*2-1 or Col-0. The primary root length was also inhibited in the double mutant relative to Col-0 and the single mutants (Fig. 2E). To verify that the observed lateral root developmental defect in the double mutant is due to lack of these respective genes, complementation of *ark2-1/pub9-1* with either *PUB9* or *ARK2* was performed. Overexpression (*35S:: PUB9*) of *PUB*9 in *ark2-1/pub9-1* led to the rescue of the LR defect phenotype observed under –Pi/–Suc conditions (Supplemental Fig. 1). However, the primary root length was reduced as a result of *PUB*9 overexpression in *ark2-1/pub9-1* independent of the nutrient status (Supplemental Fig. 1). Expression of *ARK*2 similarly rescued the LR defect observed in *ark2-1/pub9-1* under –Pi/–Suc condition. (Supplemental Fig. 1). These results indicate that both ARK2 and PUB9 are required for induction of LR under Pi starvation. These two diverse interacting partners likely play a redundant role in regulating LR development under phosphate- starved conditions.

Phosphate starvation leads to PUB9 localization on punctate sub-cellular structures *in planta*

To determine if PUB9 has a role in autophagy, the roots of *pub*9-1 constitutively expressing GFP-tagged *PUB*9 (*pub9-1/GFP-PUB9*) were analyzed by confocal microscopy. Under normal nutrient-rich conditions (+Pi/+Suc), GFP-PUB9 is distributed

throughout the cytosol in root epidermal cells (Fig. 3A). When GFP-PUB9 was observed under Pi/Suc-starvation (-Pi/-Suc), a few punctate structures could be observed on the edge of the large central vacuole (Fig. 3B). To observe the autophagic compartments, the transgenic seedlings were treated with concanamycin A (concA), a specific inhibitor of vacuolar ATPase which blocks vacuolar degradation and has been previously used to reveal autophagosomes (Thompson et al. 2005; Chung et al. 2010). Following ConcA treatment, PUB9 could be observed to localize on to punctate structures under nutrient-rich conditions (Fig. 3C). Interestingly, the number of these punctate structures dramatically increased in concA-treated and Pi/Suc-starved root cells (Fig. 3D, Supplemental Fig. 2). The punctate structures observed were 0.5-1.5 µm in diameter and appeared to be mobile and in the vacuolar lumen. The increase in the number of these structures following phosphate starvation likely indicates the occurrence of some degree of basal autophagy under normal conditions, which gets elevated upon starvation.

Inhibiting autophagy leads to defective LR formation under phosphate starvation in wild-type plants

To determine if autophagy has a role in regulating LR development under phosphate starvation, we transferred 3-day old seedlings of wild-type Col-0 to either a nutrient rich (+Pi/+Suc) or starved medium (–Pi/–Suc) supplemented with 1mM 3-methyladenine (3-MA), a known autophagy inhibitor and allowed to grow for 7 days. Interestingly only the seedlings grown on –Pi/-Suc medium with 3-MA showed severely reduced number of lateral roots phenocopying the *ark2-1/pub9-1* double mutants (Fig. 2E, 3E) The seedlings grown in nutrient rich media did not display any observable LR defects (Fig. 3E). These

results indicate that autophagy is required for LR formation under phosphate starvation and the LR defect in *ark2-1/pub9-1* double mutants could be a result of a defective autophagy process in this mutant.

ark2-1/pub9-1 plants lack DR5 promoter activity in root tips under starvation

Since auxin accumulation is tightly associated with root development, we aimed at investigating any possible role of auxin in ARK2-PUB9-mediated regulation of LR development. For this, *pub*9-1, *ark*2-1 and *ark2-1/pub9-1* lines expressing *GUS* under the control of the auxin-responsive DR5 promoter (DR5::*uidA*) were generated. When 10-day old seedlings grown in either rich medium or –Pi/–Suc medium were subjected to GUS assays, similar GUS staining could be observed in the primary root tip across all genotypes under nutrient-rich condition (Fig. 4A, upper panel). This indicated that auxin accumulation response was similar in all these lines under unchallenged conditions. Under Pi/Suc-starved condition, the *ark2-1/pub9-1* primary roots did not exhibit any GUS staining while Col-0 and other single mutants displayed GUS staining (Fig. 4A, bottom panel). This indicates severely reduced levels of auxin in *ark2-1/pub9-1* primary root tips under nutrient starved condition (Fig. 4A, bottom panel). A similar pattern was observed for LR tips with severely reduced GUS staining in *ark2-1/pub9-1* lines in the LR tips analyzed (Fig. 4B).

Autophagy is required for sustained auxin accumulation in root tips under starvation.

To investigate whether autophagy has a role in controlling auxin accumulation under phosphate starvation, we carried out GUS assays with 10-day old DR5::*uidA* expressing Col-0 seedlings grown in either rich medium (+Pi/+Suc) with 1mM 3-MA or –Pi/–Suc medium with 1 mM 3-MA. We observed that GUS staining was reduced in the primary and lateral root tips of plants grown in the presence of 3-MA under –Pi/-Suc starved condition (Supplemental Fig. 3) compared to plants grown in the presence of 3-MA under nutrient rich condition (Supplemental Fig. 3). These results indicate that auxin accumulation is controlled by autophagy under Pi starvation and reduced GUS staining observed in *ark2-1/pub9-1* lines could be a result of the defective autophagy.

Exogenous auxin rescues phosphate starvation induced LR defect in *ark2-1/pub9-1*

Based on the lack of auxin accumulation in root tips of *ark2-1/pub9-1*, following phosphate starvation, we hypothesized that the interaction between these two proteins could mediate auxin accumulation in root tips. If a defect in auxin accumulation is the sole reason for the observed LR developmental phenotypes in these lines, then supplementation of auxin should rescue this phenotype. To test this, 3-day old seedlings of Col-0 and *ark2-1/pub9-1* initially germinated on 0.5 X MS medium with 1% sucrose were either transferred to +Pi/+Suc or –Pi/–Suc medium with or without the addition of various concentrations of NAA (0.001 µM to 1 µM) (Fig. 5A, B). Addition of 0.001 µM NAA was sufficient to rescue the *ark2-1/pub9-1* LR defect in –Pi/–Suc medium (Fig. 5C). Both 0.001 and 0.01 µM NAA were the optimal concentrations for the rescue of LR phenotype in *ark2-1/pub9-1* lines without much effect on the primary root length in both NAA concentrations in Col-0 (Fig.5 D). Treatment with 0.1 and 1 µM NAA resulted in a

significant increase in the number of LR in both nutrient rich and nutrient starved conditions and both Col-0 and *ark2-1/pub9-1* produced similar number of LR under these conditions but had an inhibitory effect on the primary root elongation (Fig. 5C,D, Supplemental Fig. 4). The rescue of LR defect in *ark2-1/pub9-1* by auxin supplementation suggests that auxin is required for LR development under nutrient starved conditions and these two proteins control the accumulation of auxin under phosphate-starved conditions.

Gene expression analysis in LRs suggests an auxin-dependent mechanism

To further confirm our hypothesis that ARK2-PUB9 module regulates auxin accumulation, quantitative RT-PCR was performed using key genes involved in auxin signaling and transport. For this, 3-day old seedlings were transferred to either +Pi/+Suc or –Pi/–Suc condition and were allowed to grow vertically for 4 days. The LR initiation zone, 2-2.5 cm away from the primary root region was used as the tissue for expression analysis. The expression of *IAA*28, an AUX/IAA repressor of auxin-responsive genes was found to be upregulated in Pi/Suc-starved *ark2-1/pub9-1* compared to Col-0 (Fig. 6). However, the auxin transport genes *PIN*1, *PIN*3, *PIN*5, *PIN*6 and *PIN*7 were significantly down regulated several folds in Pi/Suc-starved *ark2-1/pub9-1* compared to Col-0 (Fig. 6), with *PIN*5 showing the lowest level of expression, being reduced by 3.03-folds and *PIN*2 displaying a moderately enhanced expression (Fig. 6). Under nutrient-rich conditions, the expression of these genes in *ark2-1/pub9-1* was similar to that of Col-0 (Supplemental Fig. 5). These observations are indicative of the requirement of ARK2-PUB9 module in

maintaining the expression of the *PIN* transport proteins and the IAA28 repressor under nutrient starved conditions.

DISCUSSION

Despite identification of 417 receptor kinases with an extracellular domain in Arabidopsis, the functions of most of these kinases and their downstream interacting partners remain unknown (Shiu and Bleecker 2001a, 2001b). Previously it was shown that the Arabidopsis *S*-domain-1 (SD1) receptor kinase subfamily could potentiate signaling through the U-box/ARM repeat proteins (PUBs) (Samuel et al. 2008). In particular, PUB9 is a short E3 Ub ligase containing a U-box domain and ARM repeats and is expressed in nearly all tissue types except leaves (Mudgil et al. 2004). ARK1, ARK2 and MLPK are capable of phosphorylating PUB9 based on *in vitro* phosphorylation assays (Samuel et al. 2008). Phosphorylation of AtPUB9 by ARK2 led to its re-localization from the nucleus to the cytosol and 40% of the cells formed punctate structures (Samuel et al. 2008). In the present study, we have identified these compartments as autophagic bodies based on its co-localization with markers for lytic compartments and autophagosome marker ATG8 (Fig. 1). At*PUB*9 in root epidermal cells of transgenic *pub9-1*::GFP-*PUB*9 plants localized to punctate structures in the vacuole under starvation and upon concA treatment (Fig. 3D). This suggests that PUB9 localizes to autophagic bodies following phosphate starvation. Previous studies have shown that ATG8 localizes to similar compartments upon concA treatment (Thompson et al. 2005; Chung et al. 2010). ConcA is a specific inhibitor of vacuolar type H+ATPase (Dröse et al. 2001) that increases the pH in the vacuoles preventing the activity of

vacuolar proteases, thus enabling the visualization of GFP in the vacuole (Tamura et al. 2003).

When examined for a role for the ARK2-PUB9 module in mediating stress responses under nutrient starved conditions, we identified that the LR development phenotype manifested only during phosphate-starved conditions in the *ark2-1/pub9-1* double mutants (Fig 2). Our results with 3-MA using Col-0 seedlings also confirmed the need for autophagy to mediate LR development under phosphate-starved conditions (Fig. 3E). The developmental plasticity of the root system allows it to respond and adapt to external environment by modifying itself through a cascade of hormone-mediated events. We observed that the lack of LR formation is due to the inability of *ark2-1/pub9-1* plants to accumulate auxin in the root tips under phosphate starvation (Fig 4). The phytohormone auxin and the associated polar auxin transport are the principal stimulators of LR initiation, primordium development and emergence (for review refer to Lavenus et al. 2013). Previous studies have shown that exogenous application of auxin promotes the production of numerous LRs (Himanen et al. 2002; Malamy and Ryan 2001). Likewise, mutation or overexpression of genes involved in auxin biosynthesis, metabolism, transport and signalling led to an altered number of LRs (Hobbie and Estelle 1995; Celenza et al. 1995; Rogg et al. 2001; Fukaki et al. 2002; Swarup et al. 2008; Mashiguchi et al. 2011). Interestingly, *IAA*28, a repressor of auxin responsive genes was upregulated in *ark2-1/pub9-1* compared to wild-type under Pi-starvation. *IAA*28 functions as a repressor of LR development since gain-of-function mutant *iaa28-1* is defective in LR development and *GATA23* expression can rescue this phenotype (Rogg et al. 2001; De Rybel et al. 2010). While the repressor IAA28 can regulate auxin signalling, auxin

gradients facilitated by PIN efflux carrier proteins using both root and shoot-derived auxin is crucial for LR development (Bénkova et al. 2003). During Pi starvation, expression levels of *PIN*1, *PIN*3, *PIN*5, *PIN*6 and *PIN*7 were found to be downregulated in *ark2-1/pub9-1* plants, providing further evidence that both auxin efflux carriers and the signalling proteins are affected, which could lead to lack of auxin accumulation in the root tips of *ark2-1/pub9-1* mutants. Recent studies have also unravelled a link between E3 ligases and the Ub proteasome system during LR development and are known to function through modulating auxin accumulation and signalling under limited phosphate availability. Pi-starvation increases the expression of the auxin receptor TRANSPORT INHIBITOR RESPONSE1 (TIR1) which triggers ubiquitination and subsequent degradation of AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) repressors thus relieving the AUXIN RESPONSE FACTORs (ARFs) and regulating the expression of genes involved in LR formation (Pérez-Torres et al. 2008).

The lack of auxin accumulation in the root tips of *ark2-1/pub9-1* seedlings under phosphate starvation and the rescue of the LR phenotype by exogenous auxin (Fig 5), are indicative of ARK2-PUB9 module regulating auxin accumulation in the root tips during phosphate starvation. In accordance with this, 3-MA treatment of Col-0 plants showed a reduction in auxin accumulation in LRs following phosphate starvation (Supplemental Fig. 3), suggesting a role for autophagy in controlling auxin accumulation. Our results suggest that lack of these two proteins could block autophagy under phosphate-starved conditions and possible degradation of repressors of auxin accumulation. Alternatively, activation of PUB9 by ARK2 could lead to ubiquitination of repressors of auxin accumulation, which are subsequently targeted to the autophagosomes through a selective

autophagy process. In this scenario, *ark2-1/pub9-1* would have normal formation of autophagosomes except selective targeting of proteins through the ARK2-PUB9 module would be absent. Identifying potential interactors of PUB9 would provide clues to possible downstream targets of this module that could negatively regulate auxin accumulation.

Whether these two proteins play a redundant role in parallel pathways or whether they form one of the many SD1 kinase-PUB modules that are necessary for this process is not known. At the kinase level, ARK2 has the ability to interact with and phosphorylate multiple PUBs (Samuel et al. 2008). Kinase activity of ARK1 has been shown to be essential for ARK1 mediated re-localization of PUB9 from the nucleus to the plasma membrane, while in our study ARK2 interaction with PUB9 results in localization of PUB9 to autophagosomes (Fig 1). ARK1 and PUB9 function in a linear pathway, negatively regulating ABA responses since both the single and double *ark1/pub9-1* mutants exhibited similar hypersensitivity to ABA during seed germination (Samuel et al. 2008). In this study, only lack of both ARK2 and PUB9 resulted in the LR phenotypes suggestive of genetic interaction between these two protein products playing a functionally redundant role during phosphate starvation. Thus, it is likely that PUB9 can be utilized by multiple upstream kinases to mediate unique tissue specific responses. Nevertheless, our study has uncovered a previously unknown, redundant role for these highly diverse interacting partners and provides further evidence for plant U-box/ARM proteins functioning as potential downstream signalling targets for *S*-domain receptor kinases.

MATERIALS AND METHODS

Plant Materials and Genetic Analysis

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 was used as the wild-type for all experiments. T-DNA insertion lines for At*PUB*9 (SALK_020751) and At*ARK*2 (SAIL_594_A06) were obtained from Arabidopsis Biological Resource Center. Homozygous lines were generated by PCR genotyping using primers listed in Supplemental Table 1. Double homozygotes *(ark2-1/pub9-1)* were created crossing these single mutant lines.

Transient Expression using BY2 Cells

The full-length At*PUB*9 (At3g07360) and the kinase domain of At*ARK*2 (At1g65800) were cloned into pRTL2 as GFP-tagged and GST-tagged constructs under the control of cauliflower mosaic virus 35S promoter (CaMV35S), respectively as described in Samuel et al. 2008. RFP-Olesoin and SYP21 (At5g16830) were kindly provided by Dr. Robert Mullen (University of Guelph, Canada). Lysotracker Red (Invitrogen, USA) staining was performed as per manufacturer's instructions. Cultured tobacco (*Nicotiana tabacum*) Bright Yellow-2 (BY2) cells were used to perform biolistic bombardment as described previously (Stone et al. 2003). Cells were fixed in 4% paraformaldehyde and visualized directly through Leica DMR epifluorescence microscope for detecting GFP and RFP (Stone et al. 2003).

Cloning in Binary Vectors and Plant Transformation

*Arabidopsis thaliana PUB*9 with GFP fused to its N-terminus and *ARK*2 with YFP fused to its C-terminus were cloned into pCAMBIA binary vectors pCAM-ter and pCAMBIA1301 respectively. The absence of single nucleotide in *ARK*2 (RAFL09-14-P09, RIKEN) was corrected by site-directed mutagenesis using primers listed in Supplemental Table 1. These constructs were mobilized into *Agrobacterium tumefaciens* strain GV3101 separately and used for transforming *ark2-1/pub9-1* plants by floral dip method (Clough and Bent 1998). The transgenic plants were obtained by selecting the harvested seeds on MS medium (Murashige and Skoog 1962) with kanamycin for *PUB*9 and hygromycin for *ARK*2.

Phosphate Starvation and Root Growth Assay

Vapour-phase sterilization of seeds was carried out by adding 3 ml concentrated HCl to 75 ml commercial bleach (Clorox) and allowed to sit for 4 h in a desiccator jar. Seeds were then plated on 0.5 X Murashige and Skoog (MS) medium supplemented with 1% (w/v) sucrose and 0.75% (w/v) agar. After stratifying for 3 d in dark, the plates were oriented vertically and allowed to germinate under light. After 3 d, the seedlings were transferred either to nutrient-rich (+Pi/+Suc) or to phosphate starvation (–Pi/–Suc) medium.

The +Pi/+Suc was composed of 0.5 X MS medium supplemented with 1.5% (w/v) sucrose and 0.75% (w/v) agar, whereas, the –Pi/–Suc medium contained 3 µM $CaCl_2$, 3 µM $MgSO_4.7H_2O$, 5 µM KCl, 2 µM MES hydrate, 3 µM KNO_3 1X micronutrient salt (Sigma, USA) and 1X vitamins (Sigma, USA) supplemented with 0.75% (w/v) agar. The plates were placed vertically to allow root growth on the agar

surface at 22°C in a plant growth chamber with a photoperiod of 16 h of light and 8 h of dark. The seedlings were photographed after 7 d; the number of lateral roots was counted and the primary root lengths were measured using ImageJ software (Abramoff et al. 2004). For root growth assay with autophagy inhibitor 3-Methyladenine, seedlings were grown in similar media composition as described above (+Pi/+Suc and –Pi/-Suc) with the addition of 1 mM 3-Methyladenine (Sigma, USA) in the respective media.

ConcA Treatment of *Arabidopsis* seedlings and Confocal Microscopy

Two different lines of *Arabidopsis pub9-1* seedlings constitutively expressing GFP-tagged *PUB*9 under the control of CaMV35S promoter were grown vertically for 3 d in 0.5 X MS medium with 1% sucrose and 0.75% (w/v) agar. The seedlings were then transferred either to +Pi/+Suc or –Pi/-Suc liquid media and incubated for 24 h at 22°C in light. Ten to twelve seedlings were then transferred to 24-well ELISA plates with +Pi/+Suc or –Pi/-Suc liquid media either with or without 2 µM concA (Santa Cruz Biotechnology, USA; 1 mM stock in DMSO). In treatments without concA, same volume of DMSO was added to the liquid media. The seedlings were then incubated for 12-16 h in dark at room temperature, mounted in an aqueous environment and visualized using a confocal microscopy. A Leica TCS SP5 confocal microscope system was used in this study. Quantification was carried out by counting the number of punctate structures (0.5-1.5 µm) in three different 100 μm^2 areas of at least two different root epidermal cells for each treatment.

Histochemical GUS Assay

The mutant lines *pub9-1*, *ark2-1* and *ark2-1/pub9-1* containing the transgene DR5::*uidA* and the wild-type Col-0 expressing DR5::*uidA* were grown under +Pi/+Suc or –Pi/–Suc as described above, were incubated overnight in GUS reaction buffer (0.5 mg/ml 5-bromo-4-chloro-3-indolyl-β -D-glucuronide, 0.1 mM $K_4[Fe(CN)_6]$, 0.1 mM $K_3[Fe(CN)_6]$, Triton X-100 in 100 mM sodium phosphate buffer, pH-7.2). The seedlings were then cleared in 70% ethanol, mounted on 50% glycerol and visualized under Leica DMR epifluorescence microscope bright-field optics. For GUS assay in the presence of autophagy inhibitor 3-MA, similar protocol was followed using wild-type plants (Col-0) expressing the transgene DR5::*uidA* and grown under +Pi/+Suc or –Pi/–Suc with 1mM 3-Methyladenine in the media.

Auxin Treatment of *Arabidopsis* seedlings

For hormonal complementation of *ark2-1/pub9-1* lateral root defect, 3 d old seedlings were transferred to +Pi/+Suc or –Pi/–Suc medium supplemented with 0.001 to 1 µM concentrations of NAA. After 7 days, the seedlings were photographed and analyzed by ImageJ software.

Quantitative Reverse Transcription PCR

RNA was isolated from the lateral root forming zone (LRZ) of Col-0 and *ark2-1/pub9-1* seedlings either grown under +Pi/+Suc or –Pi/–Suc condition for 4 d using TRIzol (Invitrogen, USA). First-strand cDNA was synthesized from 800 ng of DNase-treated total RNA using oligo $(dT)_{12-18}$ primer and SuperScript II Reverse Transcriptase (Invitrogen, USA) following manufacturer's instructions. The qPCR was performed

using StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Primer pairs are listed in Table 1. Each PCR reaction contained 1X Fast SYBR Green Master Mix (Applied Biosystems, USA), 200 nM of each primer, and 0.5 µl cDNA in a final volume of 20 µl. PCR amplification was performed for 40 cycles at 95ºC, 3 s and 60ºC, 30 s with a preceding initial enzyme activation of 20 s at 95ºC. Relative expression levels were calculated by Δ-ΔCt method, and all quantifications were normalized using *Ubq*10 mRNA as an internal control. For each target gene, the reactions were carried out in duplicate and for two biological replicates.

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LITERATURE CITED

Abramoff MD, Magalhaes PJ, Ram SJ (2004) Image processing with ImageJ. Biophot Int **11**: 36-42

Al-Ghazi Y, Muller B, Pinloche S, Tranbarger TJ, Nacry P, Rossignol M, Tardieu F, Doumas P (2003), Temporal responses of *Arabidopsis* root architecture to phosphate starvation: evidence for the involvement of auxin signalling. Plant Cell Environ **26**: 1053–1066

Bassham DC, Laporte M, Marty F, Moriyasu Y, Ohsumi Y, Olsen LJ, Yoshimoto K (2006) Autophagy in development and stress responses of plants. Autophagy **2**: 2-11

Bassham DC (2007) Plant autophagy—more than a starvation response. Curr Opin Plant Biol **10**: 587-593

Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertová D, Jürgens G, Friml J (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell **115**: 591-602

Bieleski RL (1973) Phosphate pools, phosphate transport, and phosphate availability. Annu Rev Plant Physiol **24**: 225-252

Celenza JL Jr., Grisafi PL, Fink GR (1995) A pathway for lateral root formation in *Arabidopsis thaliana*. Genes Dev 9: 2131–2142

Chen ZH, Jenkins GI, Nimmo HG (2008) Identification of an F-Box protein that negatively regulates Pi starvation responses. Plant Cell Physiol **49**:1902-1906

Chung T, Phillips AR, Vierstra RD (2010) ATG8 lipidation and ATG8-mediated autophagy in Arabidopsis require ATG12 expressed from the differentially controlled *ATG12A* AND *ATG12B* loci. Plant J **62**: 483–493

Clough SJ, Bent AF (1998) Floral dip: a simplified method for*Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J **16**:735–743

De Rybel B, Vassileva V, Parizot B, Demeulenaere M, Grunewald W, Audenaert D, Van Campenhout J, Overvoorde P, Jansen L, Vanneste S et al. (2010) A novel Aux/IAA28 signaling cascade activates GATA23- dependent specification of lateral root founder cell identity. Curr Biol **20**: 1697-1706

Dröse S, Boddien C, Gassel M, Ingenhorst G, Zeeck A, Altendorf K (2001) Semisynthetic derivatives of concanamycin A and C, as inhibitors of V- and P-type ATPases: Structure-activity investigations and developments of photoaffinity probes. Biochemistry **40**: 2816–2825

Fukaki H, Tameda S, Masuda H, Tasaka M (2002) Lateral root formation is blocked by a gain-of-function mutation in the *SOLITARY-ROOT/IAA14* gene of*Arabidopsis*. Plant J **29**: 153–168

Guiboileau A, Yoshimoto K, Soulay F, Bataillé MP, Avice JC, Masclaux-Daubresse C (2012) Autophagy machinery controls nitrogen remobilization at the whole-plant level under both limiting and ample nitrate conditions in Arabidopsis. New Phytol **194**: 732-740

Hammond JP, White PJ (2011) Sugar signaling in root responses to low phosphorus availability. Plant Physiol **156**: 1033–1040

Himanen K, Boucheron E, Vanneste S, de Almeida Engler J, Inzé D, Beeckman T (2002) Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell **14:** 2339–2351

Hobbie L, Estelle M (1995) The axr4 auxin-resistant mutants of Arabidopsis thaliana define a gene important for root gravitropism and lateral root initiation. Plant J **7**: 211-220

Hur YJ, Yi YB, Lee JH, Chung YS, Jung HW, Yun DJ, Kim KM, Park DS, Kim DH (2012) Molecular cloning and characterization of OsUPS, a U-box containing E3 ligase gene that respond to phosphate starvation in rice (Oryza sativa). Mol Biol Rep **39**: 5883-5888

Lavenus J, Goh T, Roberts I, Guyomarc'h S, Lucas M, De Smet I, Fukaki H, Beeckman T, Bennett M, Laplaze L (2013) Lateral root development in Arabidopsis: fifty shades of auxin. Trends Plant Sci **18**: 450-458

López-Bucio J, Hernández-Abreu E, Sánchez-Calderón L, Nieto-Jacobo MF, Simpson J, Herrera-Estrella L (2002) Phosphate availability alters architecture and causes changes in hormone sensitivity in the Arabidopsis root system. Plant Physiol **129**: 244–256

Lynch JP, Brown KM (2001) Topsoil foraging: an architectural adaptation of plants to low phosphorus availability. Plant Soil **237**: 225–237

Malamy JE, Ryan KS (2001) Environmental regulation of lateral root initiation in Arabidopsis**.** Plant Physiol **127**: 899-909

Martín AC, Del Pozo JC, Iglesias J, Rubio V, Solano R, De La Peña A, Leyva A, Paz-Ares J (2000) Influence of cytokinins on the expression of phosphate starvation responsive genes in Arabidopsis. Plant J **24**: 559-567

Mashiguchi K, Tanaka K, Sakai T, Sugawara S, Kawaide H, Natsume M,Hanada A, Yae no T, Shirasu K, Yao H, et al. (2011) The main auxin biosynthesis pathway in *Arabidopsis.* Proc Natl Acad Sci USA **108**: 18512-18517

Mudgil Y, Shiu S-H, Stone SL, Salt JN, Goring DR (2004) A large complement of the predicted *Arabidopsis* ARM repeat proteins are members of the U-box E3 ubiquitin ligase family. Plant Physiol. **134**: 59-66

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant **15**: 473-497

Nacry P, Canivenc G, Muller B, Azmi A, Van Onckelen H, Rossignol M, Doumas P (2005) A role for auxin redistribution in the responses of the root system architecture to phosphate starvation in Arabidopsis. Plant Physiol **138**:2061–2074

Pérez-Torres C-A, López-Bucio J, Cruz-Ramírez A, Ibarra-Laclette E, Dharmasiri S, Estelle M, Herrera-Estrella L (2008) Phosphate availability alters lateral root development in *Arabidopsis* by modulating auxin sensitivity via a mechanism involving the TIR1 auxin receptor. Plant Cell **20**: 3258–3272

Raghothama KG (1999) Phosphate acquisition. Ann Rev Plant Physiol Plant Mol Biol **50**: 665-693

Raghothama KG (2000) Phosphate transport and signaling. Curr Opin Plant Biol **3**: 182-187

Rogg LE, Lasswell J, Bartel B (2001) A gain-of-function mutation in *IAA28* suppresses lateral root development. Plant Cell **13**: 465-480

Rojas-Triana M, Bustos R, Espinosa-Ruiz A, Prat S, Paz-Ares J, Rubio V (2013) Roles of ubiquitination in the control of phosphate starvation responses in plants. J Int Plant Biol 55: 40-53

Sakhonwasee S, Abel S (2009) Autophagy sustains the Arabidopsis root meristem during phosphate starvation. UC Davis: Department of Plant Sciences, UC Davis. Retrieved from: http://www.escholarship.org/uc/item/50t6w37f

Samuel MA, Mudgil Y, Salt JN, Delmas F, Ramachandran S, Chilelli A, Goring DR (2008) Interactions between the S-domain receptor kinases and AtPUB-ARM E3 ubiquitin ligases suggest a conserved signaling pathway in *Arabidopsis*. Plant Physiol **147**: 2084–2095

Shiu SH, Bleecker AB (2001a) Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci USA **98**: 10763-10768

Shiu SH, Bleecker AB (2001b) Plant receptor-like kinase gene family: diversity, function, and signaling. Sci STKE **113**: re22

Stone SL, Anderson EM, Mullen RT, Goring DR (2003) ARC1 is an E3 ubiquitin ligase and promotes the ubiquitination of proteins during the rejection of self-incompatible *Brassica* pollen. Plant Cell **15**: 885–898

Swarup K, Benkova E, Swarup R, Casimiro I, Peret B, Yang Y, Parry G, Nielsen E, De Smet I, Vanneste S, et al. (2008) The auxin influx carrier LAX3 promotes lateral root emergence. Nat Cell Biol **10**:946–954

Tamura K, Shimada T, Ono E, Tanaka Y, Nagatani A, Higashi S, Watanabe M, Nishimura M, Hara-Nishimura I (2003) Why green fluorescent fusion proteins have not been observed in the vacuoles of higher plants. Plant J **35**: 545–555

Thompson AR, Doelling JH, Suttangkakul A, Vierstra RD (2005) Autophagic nutrient recycling in Arabidopsis directed by the ATG8 and ATG12 conjugation pathways. Plant Physiol **138**: 2097-2110

Wang X, Yi K, Tao Y, Wang F, Wu Z, Jiang D, Chen X, Zhu L, Wu P (2006) Cytokinin represses phosphate-starvation response through increasing of intracellular phosphate level. Plant Cell Environ **29**: 1924-1935

Williamson LC, Ribrioux SPCP, Fitter AH, Leyser HMO (2001) Phosphate availability regulates root system architecture in Arabidopsis. Plant Physiol **126**: 875–882

Xiong Y, Contento AL, Bassham DC (2005) AtATG18a is required for the formation of autophagosomes during nutrient stress and senescence in Arabidopsis thaliana. Plant J **42**: 535-546

Yee D, Goring DR (2009) The diversity of plant U-box E3 ubiquitin ligases: from upstream activators to downstream target substrates. J Exp Bot **60**: 1109-1121

FIGURE LEGENDS

Figure 1. Subcellular localization of transiently co-expressed GST::ARK2 (kinase domain) and GFP::PUB9 with (A) oil body marker RFP::Oleosin, (B) vacuolar marker Lysotracker Red, (C) prevacuolar marker RFP::SYP21 and (D) autophagy marker RFP-ATG8. 5-10 µg plasmids were biolistically transformed into tobacco BY2 cells and images were captured through epifluorescence microscopy. To determine the co-localization pattern the green and red channels were merged.

Figure 2. Absence of ARK2-PUB9 module leads to lateral root defect under Pi/Suc-deficient condition. (A) Schematic representation of the location of T-DNA inserts in *pub9-1* and *ark2-1* mutant lines. (B) Homozygous double mutant *ark2-1/pub9-1* line obtained by crossing *pub9-1* and *ark2-1* was confirmed by PCR genotyping. (C) RT-PCR analysis of At*PUB*9 *and* At*ARK*2 expression in *ark2-1/pub9-1* roots relative to Col-0. The lateral root density (calculated as the number of lateral roots per cm length of primary root) of *Arabidopsis* seedlings under (D) nutrient-rich (+Pi/+Suc) and (E) Pi/Suc-starved (-Pi/-Suc) condition of the various genotypes. 3-d-old seedlings were allowed to grow vertically in either +Pi/+Suc or -Pi/-Suc media for 7 d before being photographed. The values represent mean ± SE (n = 15). Scale= 1cm

Figure 3. Subcellular localization of GFP::PUB9 in stably transformed *Arabidopsis pub9-1* root epidermal cells grown under different nutrient conditions. Localization of GFP::PUB9 (A) under nutrient-rich condition without concA (B) under Pi/Suc-deficient condition without concA (C) under 2 μM concA-supplemented nutrient-rich condition and (D) under 2 μM concA-supplemented Pi/Suc-deficient condition (E) Effect of 3-MA on the lateral root formation of wild-type Col-0 under nutrient rich (+Pi/+ Suc) condition and under nutrient deficient (-Pi/-Suc) condition.

Figure 4. DR5::*uidA* expression in (A) primary root and (B) lateral root tips. GUS staining was performed to detect level of auxin accumulation in the PR and LR tips of seedlings of the various genotypes grown on medium with or without phosphate and sucrose. Scale= 16 μm.

Figure 5. Rescue of the LR defect phenotype by exogenous auxin supplementation. 3-d-old (A) Col-0 and (B) *ark2-1/pub9-1* seedlings were allowed to grow vertically for 7 d in either +Pi/+Suc or -Pi/-Suc media with or without 0.1 μM NAA. Graphical representation of the LR density (C) and PR length (D) for Col-0 and *ark2-1/pub9-1* grown on -Pi/-Suc media in the presence of various concentrations of NAA. The values represent mean ± SE (n = 6).

Figure 6. Altered expression pattern of auxin signaling and transport genes under Pi/Suc-deficient condition. Quantitative RT-PCR analysis was performed with RNA isolated from 7-d-old *Arabidopsis* seedlings that were subjected to Pi and Suc starvation for 4

days. Relative expression levels were calculated by Δ-ΔCt method, with Pi/Suc-starved Col-0 as the calibrator and all quantifications were normalized using *Ubq*10 mRNA as an internal control. Data represent mean ± SE (n = 4).