Running title: Interactions between AtPUB proteins & SD1 receptor kinases

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Interactions between the S-Domain receptor kinases and \textit{AtPUB-ARM E3} ubiquitin ligases suggest a conserved signaling pathway in \textit{Arabidopsis}.

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ABSTRACT

The Arabidopsis genome encompasses multiple receptor kinase families with highly variable extracellular domains. Despite their large numbers, the various ligands and the downstream interacting partners for these kinases have been deciphered only for a few members. One such member, the S Receptor Kinase (SRK), is known to mediate the self-incompatibility (SI) response in *Brassica*. SRK has been shown to interact and phosphorylate a U-box/ARM-repeat-containing E3 ligase, ARC1, which in turn acts as a positive regulator of the self-incompatibility response. In an effort to identify conserved signaling pathways in Arabidopsis, we performed yeast two-hybrid analyses of various S-Domain receptor kinase family members with representative Arabidopsis U-box/ARM-repeat (AtPUB-ARM) E3 ligases. The kinase domains from S-Domain receptor kinases were found to interact with ARM repeat domains from AtPUB-ARM proteins. These kinase domains along with MLPK (M-locus protein kinase), a positive regulator of SI response, were also able to phosphorylate the ARM repeat domains in *in vitro* phosphorylation assays. Subcellular localization patterns were investigated using transient expression assays in tobacco BY-2 cells, and changes were detected in the presence of interacting kinases. Finally, potential links to the involvement of these interacting modules to the hormone, abscisic acid (ABA), were investigated. Interestingly, AtPUB9 displayed a redistribution to the plasma membrane of BY-2 cells when either treated with ABA or co-expressed with the active kinase domain of ARK1. As well, T-DNA insertion mutants for ARK1 and AtPUB9 lines were altered in their ABA sensitivity during germination and acted at or upstream of ABI3, indicating a potential involvement of these proteins in ABA responses.
INTRODUCTION

The process of ubiquitin-mediated protein degradation is activated in many biological processes during the plant life cycle, and is an equally important step in the regulation of protein activities (Moon et al., 2004; Smalle and Vierstra, 2004). Disruptions to the process can lead to prolonged activity of a target protein and clearly have effects on the plant growth and development. Three enzymes are involved in the ubiquitination of a target protein, the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin ligase. By far, the E3 ubiquitin ligase is the largest group of these enzymes which is related to its role in defining the substrate-specificity in this pathway (Devoto et al., 2002; Dill et al., 2004). For example, there are two E1 enzymes and forty-one E2 enzymes annotated in the Arabidopsis genome (Kraft et al., 2005). The E3 ligase group is a far more diverse group, and based on known E3 ligase motifs; there are at least 1300 predicted E3 ligase genes in the Arabidopsis genome (Smalle and Vierstra, 2004). The larger known Arabidopsis families include the RING family with ~ 469 predicted proteins and the F-box family with ~ 700 predicted proteins (Stone et al., 2005; Gagne et al., 2004). The Arabidopsis U-box family is a smaller predicted family with 62 members (Azevedo et al., 2001; Andersen et al., 2004).

The U-box is an E3 ligase motif conserved in all eukaryotes (Aravind and Koonin, 2000), and is a modified ring finger shown to ubiquitinate substrates in presence of the appropriate E1 and E2 (Hatakeyama et al., 2001; Mudgil et al., 2004). The plant U-box (PUB) family can be divided into 5 groups based on the presence of other distinguishing domains such as the UFD2, ARM repeats, UND, Ser/Thr kinase, WD40 repeats (Azevedo et al., 2001, Mudgil et al., 2004; Wiborg et al., 2008). The PUB-ARM family comprises the largest group with 41 predicted members in the Arabidopsis genome and 43 members in the rice genome (Mudgil et al., 2004; Samuel et al.,
Despite the limited knowledge about the biological functions for these predicted PUB-ARM proteins, they have been shown to function as E3 ubiquitin ligases (Andersen et al., 2004; Mudgil et al., 2004). In various plant species, diverse biological functions have emerged for related PUB-ARM proteins. A strong connection to plant defense responses is emerging for several PUB-ARM proteins. The rice SPL11 gene was identified in a genetic screen for lesion mimic mutants (Yin et al., 2000), and spl11 mutant displays spontaneous lesions and enhance resistance to fungal and bacterial pathogens implicating SPL11 as a negative regulator of cell death (Zeng et al., 2004). In contrast, the Arabidopsis PUB17 protein and the orthologous tobacco ACRE276 protein appear to be positive regulators of cell death and defense responses as RNAi and knockout plants are compromised in these responses (Yang et al., 2006). Similarly, the Arabidopsis PUB21 and the orthologous tobacco CMPG1 are required for HR development and disease resistance (González-Lamothe et al., 2006). A role in plant hormone responses has also been reported with the potato PHOR1 protein been identified as a positive regulator of GA signaling (Amador et al., 2001). Finally, connections are emerging between PUB-ARM proteins and receptor kinases. The Brassica ARC1 protein has been found to bind to the S Receptor Kinase (SRK) and is required for the Brassica self-incompatibility response where it functions downstream of the S Receptor Kinase (SRK) to cause self-pollen rejection (Gu et al., 1998; Stone et al., 1999). Interestingly, a related member, Arabidopsis PUB8, has been implicated in the regulation of mRNA levels of A. lyrata SRK genes (Liu et al., 2007). Lastly, the tobacco PUB4 protein was identified as an interacting protein for the CHRK1 receptor kinase (Kim et al., 2003).

The Brassica and tobacco studies suggest a role for the PUB-ARM proteins as potential signaling proteins for receptor kinases. In Arabidopsis, there are a large number of receptor
kinases with a range of extracellular domains (Haffani et al., 2004; Morris and Walker, 2003). The *Brassica* S Receptor Kinase, which interacts with ARC1, is very closely related to the Arabidopsis S-Domain-1 Receptor Kinase subfamily. The Arabidopsis S-domain receptor kinases fall into three classes with more than 40 members (Shiu and Bleecker, 2003), and the functions of this family of kinases have remained largely undefined thus far. Overexpression of ARK1 was shown to result in severe developmental abnormalities (Tobias et al., 1996), while promoter analysis and expression studies indicated that RLK4 was one of the targets of pathogen and wound-induced WRKY transcription factor targets (Du and Chen, 2000). The tobacco CHRK-1 receptor kinase possesses a chitinase-like extracellular domain that is not found in Arabidopsis; however, the intracellular kinase domain, which is required for the interaction with *NtPUB*4, is most closely related to members of the Arabidopsis S-Domain-1 receptor kinase subfamily (Kim et al., 2000). Co-suppression of the endogenous tobacco CHRK1 gene was found to have a range of phenotypes including callus formation following seed germination, increased shoot formation, reduced apical dominance, and abnormal flowers. This was also accompanied by increased cytokinin levels in the transgenic plants (Lee et al., 2003).

The observed interaction between *Brassica* SRK-ARC1 and tobacco CHRK1-*NtPUB*4, and the conservation of signaling components across *Brassica* and Arabidopsis suggested to us that the Arabidopsis S-domain receptor kinase family could potentially utilize the numerous *AtPUB*-ARM family members as their downstream signaling components. In order to investigate this, we have performed a selected interaction screen between the SD1 receptor kinases and *AtPUB*-ARM family proteins and identified either common or specific interactors. Further analyses of these interactions were carried out using *in vitro* phosphorylation assays and transient expression assays. In addition, potential links to the plant hormone, abscisic acid were further investigated.
by functional analyses with selected SD1 receptor kinase and AtPUB-ARM proteins.

RESULTS

AtPUB-ARM proteins interact with Arabidopsis and Brassica S domain receptor kinases
A directed yeast-two hybrid interaction screen was conducted with ARM repeat domains from multiple AtPUB-ARM proteins (Fig 1A) against kinase domains from selected receptor kinases. AtPUB-ARM proteins were chosen to represent the different modular combinations found in the AtPUB-ARM family (Fig 1A; Mudgil et al., 2004). AtPUB13, 14 and 45 represented the Brassica ARC1-like domain organization (UND, U-box, and ARM domains) with AtPUB13 and 14 being more closely related to ARC1 and AtPUB45 being more distantly related to ARC1. AtPUB9, 29 and 38 were selected to represent AtPUB-ARM proteins which lack the UND domain (U-box and ARM only), and AtPUB9 and 38 are more closely related to ARC1, relative to AtPUB29. Finally, AtPUB44 was chosen to represent the dual ARM repeat clade (U-box:ARM:ARM), and is the most distantly related AtPUB, relative to ARC1 (Mudgil et al., 2004; Samuel et al., 2006). All six of these AtPUB-ARM proteins have been shown to have in vitro E3 ubiquitin ligase activity (Andersen et al., 2004; Mudgil et al., 2004; Salt et al., in preparation).

The various kinases included the Arabidopsis S-domain receptor kinases representing various subgroups: SD1-7 (ARK1), SD1-6 (ARK2), SD1-8 (ARK3), SD1-29, SD1-13 (RKS2), SD1-1, SD2-5, SD2-2 (RLK4), SD3-1, DUF26-21 (RKC1), and DUF26-4 (RLK3); along with two Arabidopsis LRR receptor kinases, LRR XI-16 (HAESA) and LRR XI-23. In addition, related Brassica SD1 receptor kinases, SFR1, SFR2, and SRK\textsubscript{910}, which were previously shown to interact with Brassica ARC1, were included in the screen (Mazzurco et al., 2001). Selected
kinase domains were tested for kinase activity by using an *in vitro* autophosphorylation assay with purified GST:kinase fusion proteins. The kinase domains were found to have strong autophosphorylation activity as shown in Fig 1B or as previously shown (Mazzurco et al., 2001). Protein expression of all the constructs in the transformed yeast was confirmed using immunoblot analysis with either the VP16 or LexA antibodies.

From the yeast two-hybrid analyses, it was found that the kinase domains from selected SD1 receptor kinases generally interacted well with the *At*PUB-ARM proteins while very low level or no interactions were observed with non-SD1 receptor kinase family members (Fig 1C). For example, *At*PUB 13, 14, and 9 interacted with all the Arabidopsis and *Brassica* SD1 receptor kinases as well as SD2-5, but no interactions were observed with the remaining receptor kinases. *At*PUB38 also interacted with a number of the Arabidopsis and *Brassica* SD1 receptor kinases as well as DUF26-21. SD1-29 was the only kinase domain that interacted with all the *At*PUBs tested (Fig 1C). Thus, *At*PUB-ARM proteins tended to show interaction patterns which were largely confined to the SD1 sub-family of receptor kinases. However, within the SD1 receptor kinase sub-group, there appeared to be less specificity with several *At*PUB-ARM proteins interacting with all the kinase domains from selected SD1 receptor kinases.

**In vitro phosphorylation of PUB-ARM proteins by SD1 receptor kinases and MLPK**

In order to further characterize some of the interacting partners from the yeast two-hybrid screen, we evaluated the ability of SD1 receptor kinases to phosphorylate PUB-ARM proteins *in vitro*. As well, a second kinase implicated in *Brassica* self-incompatibility, the *Brassica M Locus* Protein Kinase (MLPK), was tested. MLPK belongs to the Receptor-Like Cytoplasmic Kinase (RLCK) subfamily and is another positive regulator of the self-incompatibility response, though
MLPK’s cellular mechanism in this response remains unknown (Murase et al., 2004; Kakita et al., 2007a, b). MLPK has 76% amino acid identity with its closest Arabidopsis ortholog, APK1b, and given the overlapping interactions between *Brassica* and Arabidopsis SD1 receptor kinases with PUB-ARM proteins, MLPK was used directly in the phosphorylation assays. The ARM repeat domains from ARC1, AtPUB13, and AtPUB9 were purified as GST-tagged (ARC1) or His-tagged (AtPUB13 & 9) fusions and subjected to phosphorylation assays in the presence of GST:kinase fusions. This was followed by either autoradiography or detection through Western blotting using anti-phosphothreonine antibodies to detect the extent of phosphorylation. Through our preliminary yeast two-hybrid screen, the various AtPUBs and *Brassica* ARC1 did not reveal any interaction with MLPK; however, MLPK was able to efficiently phosphorylate these proteins *in vitro* (Fig 2).

As previously shown, *Brassica* SRK910 shows some phosphorylation activity for ARC1 as a substrate *in vitro* (Gu et al., 1998; Fig 2A). Interestingly, MLPK showed a much stronger phosphorylation of ARC1, relative to SRK910 (Fig 2A). Control lanes without any kinase added exhibited no observable signal indicating the lack of cross-reacting proteins or background phosphorylation (Fig 2A). For AtPUB9 and AtPUB13, four Arabidopsis receptor kinases were tested: the SD1 receptor kinases, ARK1, ARK2, and SD1-29; and the LRR receptor kinase, HAESA. ARK1, ARK2 and SD1-29 interacted with AtPUB9 and AtPUB13 while HAESA did not (Fig 1C). When AtPUB13 was used as the substrate, MLPK, ARK1 and ARK2 were able to efficiently phosphorylate the ARM domain of AtPUB13 (Fig 2B). SD1-29, which was an interactor, did not have any readily detectable activity towards AtPUB13. HAESA served as a negative control and did not phosphorylate AtPUB13 as expected (Fig 2B). Similar profiles were also seen when the ARM domain of AtPUB9 was subjected to the same treatment where MLPK,
ARK1 and ARK2 displayed the best phosphorylation activity towards ArPUB9 (Fig 2C).

The effect of active kinases on the subcellular localization of PUB-ARM proteins

We have previously shown that transient co-expression of *Brassica* SRK$_{910}$ with *Brassica* ARC1 in tobacco BY-2 cells resulted in relocalization of *Brassica* ARC1 from the cytosol/nucleus to ER-associated proteasomes (Stone et al., 2003; Fig 3 M and S). To examine this effect more broadly, subcellular localization studies were conducted with ARC1, ArPUB9 and ArPUB13 in combination with SRK$_{910}$, ARK1, ARK2 or MLPK (Fig 3). ARC1 and ArPUB13 have a similar domain organization with the UND:U-box:ARM domains while ArPUB9 contains only the U-Box:ARM domains (Fig 1A; Samuel et al., 2006). The PUB-ARM proteins were expressed as GFP fusions, and the cytosolic kinase domains were expressed as RFP:ARK1, GST:ARK2, and MLPK:MYC fusions proteins. As expected, the tagged cytosolic kinase domains were localized to the cytosol for SRK, ARK2 and MLPK (Fig 3A, E, and G). Interestingly, ARK1 is predominantly found in the nucleus which is likely due to a cryptic nuclear localization signal present in the cytosolic kinase domain (Fig 3C). For the PUB-ARM proteins, ArPUB13 and ARC1 displayed diffused expression throughout the cell and could be observed both in the cytosol and nucleus (Fig 3I and M) while ArPUB9 was predominantly nuclear localized (Fig 3K). These distributions represent steady-state localization patterns and likely masked that several of these proteins are shuttling in and out of different compartments (such as the nucleus and the cytosol) as previously observed for *Brassica* ARC1 (Stone et al., 2003). Co-transformations of the PUB-ARM constructs with the different kinases produced a number of different subcellular localization changes. When the SRK$_{910}$ and ARC1 constructs were co-expressed, SRK$_{910}$ remained cytosolic (Fig 3T) while ARC1 showed a punctuate localization
pattern reminiscent of the ER-localized proteasomes as previously observed with ARC1 (Fig 3S; Stone et al., 2003). Interestingly, the co-expression of both MLPK and ARC1 resulted in both proteins targeted to the perinuclear region (Fig 3Y and Z). Thus, the ability of MLPK to both phosphorylate and alter localization of ARC1 suggests that along with SRK, MLPK could be utilizing ARC1 as a downstream intracellular target. For AtPUB13, the co-expression of MLPK:MYC with AtPUB13 resulted in both proteins being targeted to the perinuclear region (Fig 3O and P). This suggests that MLPK has a similar effect on the two UND-containing proteins, ARC1 and AtPUB13. In contrast, the co-expression of either ARK1 or ARK2 with GFP:PUB13 resulted in no alterations to either AtPUB13 (Fig 3U and AA) or the kinases (Fig 3V and BB).

When the GFP:AtPUB9 localization patterns were analyzed, AtPUB9’s predominant nuclear localization (Fig 3K) changed to a cytosolic distribution, with exclusion from the nucleus in the presence of either MLPK or ARK2 (Fig 3Q and CC). In approximately 40% of these cells, AtPUB9 was also found in punctuate structures in the cytosol. Interestingly, when ARK1 was co-expressed with GFP:PUB9, localization of RFP:ARK1 to the nucleus (Fig. 3X) resulted in complete loss of nuclear localized AtPUB9, and instead AtPUB9 was relocalized to the plasma membrane (Fig 3W).

To determine if either AtPUB9’s E3 ligase activity or ARK1’s kinase activity were required for the redistribution of AtPUB9 to the plasma membrane, mutations were introduced to knockout the respective activities. An AtPUB9 E3 ligase activity deficient (ld) mutant was created by mutating a conserved valine previously shown to be required for E3 ligase activity (V91R; Zeng et al., 2004). When co-transformed with RFP:ARK1, GFP:PUB9ld displayed a similar pattern of relocalization to the plasma membrane (Fig 4A) indicating that the ARK1 induced relocalization
of AtPUB9 was E3 ligase activity independent.

To create a kinase deficient (kd) version of ARK1, the conserved lysine 547 residue was changed to alanine (K547A) as this had been previously shown to abolish kinase activity of ARK1 (Tobias et al., 1996). When the kinase activity of the purified recombinant GST:ARK1kd protein was analyzed, it did not have any autophosphorylation activity, and it failed to use AtPUB9 as a substrate (Fig 4C). When the kinase deficient, RFP:ARK1kd construct was co-expressed with GFP: PUB9, both proteins were found in the nucleus (Fig 4D and E). Therefore, the redistribution of AtPUB9 to the plasma membrane is a phosphorylation-dependent process. Interestingly, we have previously shown that SRK910 induces a phosphorylation-dependent relocation of ARC1 to ER associated proteasomes (Stone et al., 2003).

ABA mimics ARK1-induced plasma membrane relocalization of AtPUB9

Given the subcellular localization patterns observed for the PUB-ARM proteins in the presence of different kinases, we also investigated if there were any treatments that could also cause changes in their subcellular localization patterns. To select some candidate treatments, microarray databases were examined for conditions that led to increased transcript levels for the AtPUB-ARM genes, and ABA was found to increase AtPUB9 transcript levels rapidly within one hour (Toufighi et al., 2005; Zimmermann et al., 2005). The effect of ABA on AtPUB9’s subcellular localization was investigated by treating BY-2 cells transiently expressing GFP: PUB9 with 10 µM ABA for 2 hours. ABA treatment resulted in complete loss of nuclear localization and the re-mobilization of AtPUB9 from the nucleus to the plasma membrane (Fig 4F), a pattern mimicking the presence of RFP:ARK1 (Fig 3W). Control cells, expressing GFP alone and treated with ABA did not show any changes in the diffuse cytosolic GFP localization
pattern. Several other hormones were also applied to BY-2 cells expressing AtPUB9, and there were no predominant changes observed in AtPUB9’s nuclear localization (Supplemental Fig S1). The only exception was ACC, which caused a moderate relocalization of GFP:PUB9 to the plasma membrane (39% of transformed cells, Supplemental Fig S1). In contrast to the AtPUB9 results, when BY-2 cells expressing GFP:PUB13 were treated with ABA, AtPUB13’s subcellular localization pattern remained unaltered (Fig 4H). Thus, this suggests that the plasma membrane localization of AtPUB9 is a unique ABA and ARK1-dependent process.

AtPUB9 and ARK1 T-DNA insertion lines are altered in their ABA sensitivity during seed germination and acts at or upstream of ABI3

The ability of the active ARK1 kinase domain and ABA treatment to cause membrane localization AtPUB9 in BY-2 cells prompted us investigate the in vivo functional role of ARK1 and PUB9 in mediating ABA responses. Homozygous SALK T-DNA insertion lines were identified for PUB9 (pub9) and ARK1 (ark1.1, ark1.2), and the loss of mRNA transcripts in these lines were confirmed through RT-PCR (Fig 5A and B). When seeds from pub9-/ and ark1.1-/ lines were analyzed for their ability to germinate under various concentrations of ABA, we observed that both lines exhibited hypersensitivity to 1μM ABA when compared to wild-type Col-0 seeds (Fig 5C). Similar results were also observed for ark1.2-/ mutants. To test if the loss of both AtPUB9 and ARK1 would cause an additive effect, ark1.1-/ , pub9-/ double homozygous mutants were generated and tested for ABA sensitivity during seed germination. The ark1.1-/ , pub9-/ double mutants displayed a level of ABA sensitivity that was similar to the single mutants (Fig 5C). The lack of any additive effect resulting from the loss of both loci suggests that ARK1 and AtPUB9 most likely functioned in a linear fashion.
The ABA sensitivity phenotype exhibited by the \textit{ark1}-/- and \textit{pub9}-/- single and double mutants was specific to the seed germination phase of development, since no changes were observed in root growth in the presence of ABA (Fig 5D). A similar lack of effect was also observed with ACC even though this compound was able to (though to a lesser extent) cause plasma membrane relocalization of AtPUB9 in BY-2 cells. No phenotypic difference were observed between Col-0 and the \textit{pub9}-/- and \textit{ark1.1}-/- seedlings when grown on plates containing 30 µM ACC under dark or light conditions (Supplemental Fig S2).

With both the \textit{ark1}-/- and \textit{pub9}-/- mutants displaying ABA hypersensitivity during seed germination, it was of interest to map where these genes functioned relative to a well-characterized ABA response gene, ABI3 (Nambara et al., 1994). The \textit{abi3-6} allele was chosen as it is in the same Col-0 background as \textit{ark1} and \textit{pub9}, and displays a strong ABA insensitivity phenotype. The \textit{abi3-6} mutant is non-desiccating, and can be readily identified through the selection of green (non-degreening) seeds (as well as PCR genotyping). Therefore, crosses between \textit{abi3-6} and \textit{ark1} or \textit{pub9} were screened for green seeds in the T2 generation. When the \textit{abi3-6} and \textit{ark1.1} mutants were crossed, \textit{abi3-6}-/-, \textit{ark1}-/- double homozygous mutants could not be recovered. Upon closer examination, siliques from the \textit{abi3-6}+/-, \textit{ark1}+/-- double heterozygous plants exhibited a very high proportion of aborted seeds, and the viable seeds from these plants harbored the parental genotypes. In contrast, crosses between the \textit{abi3-6} and \textit{pub9} mutants did lead to the isolation of green seeds with the \textit{abi3-6}-/-, \textit{pub9}-/- double homozygous genotype. When the \textit{abi3-6}-/-, \textit{pub9}-/- green seeds were tested for ABA responses during seed germination, they displayed an insensitive phenotype similar to \textit{abi3-6} (Fig. 5C). This suggests that the ABA-insensitive phenotype of \textit{abi3-6} is epistatic to \textit{pub9}, and indicates that PUB9 functions at or upstream of ABI3.
DISCUSSION

The superfamily of predicted Arabidopsis receptor kinases consists of 610 members with 417 members possessing an extracellular domain, a single pass transmembrane domain and an intracellular kinase domain (Shiu and Bleecker, 2001a, 2001b). Some of these receptor kinases have been identified to play a role in plant cell differentiation, hormonal response pathways, plant growth and development and pathogen perception (Li and Chory, 1997; Clouse, 2002; Gomez-Gomez et al., 2000; Morris and Walker, 2003; Osakabe et al., 2005). However, functions of most of the kinases are still unknown. Many of these receptors may participate in the plant surveillance system to sense changes in the environment and transmit the appropriate signal intracellularly through their kinase domains.

One of the primary challenges in studying plant receptor kinases is the identification of their downstream components or interacting partners. Both genetic and yeast interaction screens have been used by several groups for this purpose (for review, see Johnson and Ingram, 2005). In Brassica, yeast two-hybrid screens identified three proteins which interact with the SRK kinase domain (Bower et al., 1996; Gu et al., 1998). One of these interactors, ARC1, is a U-box, ARM-repeat containing protein, with E3 ligase activity, and was found to be a positive regulator of the self-incompatibility response (Stone et al., 1999; Stone et al., 2003). Analyses of the Arabidopsis genome for proteins with U-box and ARM domains revealed a gene family with 41 AtPUB-ARM members (Azevedo et al., 2001; Mudgil et al., 2004; Samuel et al., 2006). In this paper, we have found that the AtPUB-ARM proteins are able to interact with SD1 receptor kinases, suggesting a conservation of interaction/signaling components across species. While SD1-29 was able to interact with all the AtPUB ARM domains tested, the remaining SD1
receptor kinases tended to interact with AtPUB ARM domains which show closer relationships with *Brassica* ARC1. Given the overlapping interactions found within these two subgroups, the question does arise as to whether these are promiscuous interactions or represent redundant pathways as has been found in other large Arabidopsis gene families (Böhmer and Romeis 2007). Notwithstanding, these interactions are also likely refined *in vivo* by other factors such as conformational changes imposed by other domains within these proteins, as well as protein abundance, competitive interactions, expression profiles, and localization to different subcellular compartments. We also found that the Arabidopsis SD1 receptor kinases, ARK1 and ARK2, efficiently *in vitro* phosphorylate the ARM domains from AtPUB9 and 13, and alter the subcellular localization of AtPUB9 in BY-2 cells. Since Arabidopsis is self-fertile and contains multiple SD1 receptor kinase genes and AtPUB-ARM genes, it is most likely that the corresponding signaling proteins function in other biological processes. Consistent with this idea, the SD1 receptor kinase genes and AtPUB-ARM genes show a wide range of expression profiles in the microarray databases (Toufighi et al., 2005; Zimmermann et al., 2005). This conserved interactions has also been found in tobacco for CHRK1 which has an intracellular kinase domain closely related to the SD1 receptor kinase family members. Using the CHRK1 kinase domain as bait, the *Nt*PUB4 protein was identified as an interacting partner for CHRK1 (Kim et al., 2003).

In this paper, we also investigated potential connections with the M-Locus Protein Kinase (MLPK) which had previously been identified in the *Brassica* self-incompatibility response (Murase et al., 2004). MLPK is a positive regulator of *Brassica* self-incompatibility and has been proposed to act along with SRK in mediating ARC1 activation or localization (Goring and Walker, 2004). MLPK belongs to the receptor-like cytoplasmic kinase (RLCK) subfamily and is
most similar to Arabidopsis APK1b in the RLCK VII subfamily (Shiu and Bleecker, 2003; Murase et al., 2004, Kakita et al., 2007b). MLPK did not interact with any of the ARM domains in the yeast two-hybrid analysis which is not too surprising, given that the PUB-ARM proteins preferentially bind to SD1 receptor kinases. However, MLPK was able to highly phosphorylate the ARM domains from *Brassica* ARC1 as well as *At*PUB9 and *At*PUB13 *in vitro*. One plausible model for this activity is that MLPK-related kinases form a complex at the membrane with SD1 receptor kinases, and the binding of PUB-ARM proteins to the activated SD1 receptor kinases brings the PUB-ARM proteins in close proximity to MLPK-related kinases for phosphorylation. The MLPK interaction leading to *At*PUB phosphorylation is likely too transient to detect in the yeast two-hybrid system, but can occur in the *in vitro* phosphorylation assay where high concentrations of the purified proteins are present. This is consistent with previous results reported by Kakita et al. (2007a) where MLPK did not interact with SRK in the yeast two-hybrid system, but functioned as an efficient substrate for SRK in an *in vitro* phosphorylation assay. An interaction was subsequently demonstrated when the bimolecular fluorescence complementation assay was used to trap the SRK-MLPK interaction (Kakita et al., 2007b).

Previous studies have shown that *Brassica* ARC1 has targeting signals to allow shuttling between the cytosol and the nucleus, and the presence of the activated kinase domain from SRK910 causes ARC1 to relocalize to ER-associated proteasomes (Stone et al., 2003). This sorting was dependent on the ability of SRK to phosphorylate ARC1, since a truncated version of SRK did not lead to the proteasomal distribution of ARC1 (Stone et al., 2003). Similar studies with CHRK1 and *Nt*PUB4 also resulted in the relocalization of *Nt*PUB4 to the compartment of the kinase; such as the plasma-membrane in the presence of the full-length CHRK1 receptor.
kinase, or the cytosol with the expression of the CHRK1 kinase domain (Kim et al., 2003). These studies along with our results suggest that, upon receptor kinase activation, the normally shuttling PUB-ARM proteins are relocalized to the location of the kinase, where they are phosphorylated. The phosphorylation may cause the PUB-ARM proteins to have modified activity or be targeted to different subcellular compartments. This model is consistent with the subcellular localization studies in the BY-2 cells where AtPUB9 was redirected from the nucleus to the plasma membrane in the presence of ARK1, or to cytosol in the presence of either MLPK or ARK2. In addition, the co-expression of MLPK with ARC1 or AtPUB13 resulted in MLPK and the PUB-ARM proteins being targeted to the perinuclear region. From our localization studies with the mutated versions of AtPUB9 and ARK1, we observe that AtPUB9’s plasma membrane localization is independent of its E3 ligase activity, but dependent on ARK1’s kinase activity (as previously demonstrated for Brassica SRK and ARC1; Stone et al., 2003). Thus, the phosphorylation of the PUB-ARM proteins may act as a signal to sort these proteins to the appropriate subcellular compartment for substrate interactions.

With the conservation of interactions between Arabidopsis SD1 receptor kinases and PUB-ARM proteins, the question remains as to what biological processes these signaling networks could be regulating. As a first step towards answering this question, we found that the subcellular location of AtPUB9 in BY-2 cells could be redirected to the plasma membrane by treatment with 10µM ABA, replicating the ARK1 effect. Given that the plasma membrane is the predicted location of full-length SD1 receptor kinases, it is conceivable that AtPUB9 is interacting with related SD1 receptor kinases in the BY-2 cells. This observation is quite interesting since the plasma membrane is one of the major sites of action for ABA, where ABA controls various membrane-bound transporters and ion channels, regulating the closure of stomata (Finkelstein et
A plasma membrane-localized, putative G protein-coupled receptor has also been proposed to be an ABA receptor (Liu et al., 2007a, b), in addition to the two soluble ABA-binding proteins, the Mg-chelatase and the FCA RNA-binding protein (Razem et al., 2006; Shen et al., 2006).

A biological role for both AtPUB9 and ARK1 in ABA responses is supported by the ABA germination assays, where the pub9, ark1.1 and ark1.2 mutant seeds displayed a hypersensitive response to ABA. This would suggest a negative regulatory role for ARK1 and AtPUB9 in ABA responses during germination. Interestingly, the ABA-Insensitivity genes, ABI1 and ABI2, encode protein phosphatases 2C, and their putative target protein kinases have been proposed to be negative regulators of ABA signaling (Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997; Pei et al., 1997). Ubiquitination and protein degradation has been clearly established to be part of ABA responses with several RING-containing E3 ligases implicated in this process.

AtPUB9 has been proposed to function as a negative regulator of ABA signaling with its ubiquitination of subsequent degradation of ABI3 (Zhang et al., 2005). KEG has been implicated in the regulation of ABI5 protein levels in the absence of stress (Stone et al., 2006).

ATL43 has been proposed to function as a positive regulator of ABA responses, since atl43 mutant T-DNA lines were moderately insensitive at high concentration of ABA during germination (Serrano et al., 2006). Increased expression of XERICO was found to increase endogenous ABA levels, and XERICO was thought to regulate the expression of an ABA biosynthesis gene (Ko et al., 2006). Finally, overexpression of the U-box E3 ligase, AtCHIP, was found to cause increased sensitivity to ABA treatment (Luo et al., 2006).

Whether ARK1 plays a primary role in the perception of ABA or a secondary role following the activation of ABA receptors is not known. Our epistatic analysis places the role of AtPUB9
during ABA responses upstream or at the same level of the transcription factor, ABI3, while our crosses of ARK1 deficient lines with abi3-6 failed to isolate any double homozygotes, indicating a genetic interaction between these two loci. ABI3 has been previously shown to play a role in ABA responses at or downstream of the ERA1 farnesyl transferase, while the ABI1 and ABI2 protein phosphatases act at or upstream of ERA1 (Brady et al., 2004). Our research presented here suggests that AtPUB9 is yet another E3 ligase regulating some aspect of ABA responses, specifically during germination, and AtPUB9 itself may be activated by the ARK1 receptor kinase in this role.
MATERIALS AND METHODS

Yeast two hybrid interactions

Plasmid constructs

The LexA-VP16 system was used for yeast two-hybrid interaction studies as previously described (Gu et al., 1998; Mazzurco et al., 2001). The ARM repeats of various AtPUB-ARM proteins, AtPUB13 (At3g46510), AtPUB14 (At3g54850), AtPUB45 (At1g27910), AtPUB9 (At3g07360), AtPUB29 (At3g18710), AtPUB38 (At5g65200), and AtPUB44 (At1g20780), were cloned in the pVP16 vector. For the Arabidopsis receptor kinases from various sub families, SD1-7/ARK1 (At1g65790), SD1-6/ARK2 (At1g65800), SD1-8/ARK3 (At4g21380), SD1-29 (At1g61380), SD1-13/RKS2 (At1g11350), SD1-1 (At4g27300), SD2-5 (At4g32300), SD2-2/RLK4 (At4g00340), SD3-1 (At2g41890), DUF26-21/RKC1 (At4g23250), DUF26-4/RLK3 (At4g23190), LRR XI-16/HAESA (At4g28490), LRR XI-23 (At1g09970); and the Brassica SFR1, SFR2, and SRK910 receptor kinases, kinase domains consisting of the entire catalytic region starting just at the 3’ end of the putative transmembrane domain were cloned into LexA vector pBTM116. A full length clone was used for MLPK.

Yeast Transformation

Single step transformation of both the construct was done in to the L40 yeast strain using Gietz and Woods protocol (2002). Transformants were plated on synthetic complete medium without trp and leu, plates. β-Galactosidase assays were then performed on filter lifts of the colonies to detect activation of the lacZ reporter gene. Yeast protein extractions and Western blot analysis were done exactly as previously described (Mazzurco et al 2001).
**Induction and Purification of His- and GST-tagged proteins**

For purification of the His-tagged fusion proteins, 50-200 ml of 2× YT containing 100µg ml⁻¹ ampicillin was inoculated with 1/100th volume of an overnight culture and grown to an OD₆₀₀ of 0.8 at 37°C. Isopropyl β-D-thiogalactoside was added to a final concentration of 0.5mM induced at 37°C for 5 to 6 hours, pelleted, resuspended in 10ml of binding buffer [50 mM Tris pH 7.5, 500 mM NaCl, 5 mM Imidazole, 5% Glycerol, 500 µM phenylmethylsulfonyl fluoride (PMSF) and 1mM benzanidine], and followed with sonication. Triton X-100 was added to a final concentration of .001%, and the samples were spun at 17,000 rpm for 10 min at 4°C. To the supernatant, 1mL of 50% (vol/vol) Ni-NTA-agarose washed and pre equilibrated with binding buffer was added and mixed for 30 min at room temperature. The beads were washed four times each with 10 ml of wash buffer (50 mM Tris pH 7.5,500 mM NaCl, 30 mM Imidazole, 5% Glycerol, 500 µM phenylmethylsulfonyl fluoride (PMSF) and 1mM benzanidine). The His-tagged fusion proteins were eluted with elution buffer (50 mM Tris pH 7.5/500 mM NaCl/500 mM Imidazole/5% Glycerol/500 µM phenylmethylsulfonyl fluoride (PMSF) and 1mM benzanidine), and the samples were stored at 4°C.

For purification of the GST-tagged fusion proteins, the cells were induced at 16°C overnight, pelleted, resuspended in 10-20 ml of G-lysis buffer [50 mM Hepes, pH 7.4,150 mM NaCl,10 mM EDTA,1 mM DTT,200 µM phenylmethylsulfonyl fluoride (PMSF)], and frozen overnight at −20°C. The samples were thawed, PMSF was added again to a final concentration of 200 µM, and the samples were sonicated in 15 s bursts by using a probe sonicator. Triton X-100 was added to a final concentration of 1%, and the samples were spun at 17,000 rpm for 10 min at 4°C. To the supernatant, 2 mL of 10% (vol/vol) glutathione-agarose was added and mixed for 30 min at 4°C. The beads were washed three times each with 2 mL of lysis buffer, resuspended
in 6 mL of lysis buffer, and poured into a column. The GST fusions were eluted with elution buffer (50 mM Hepes, pH 8.0, 15 mM glutathione), glycerol was added to a final concentration of 20%, and the samples were stored at −20°C.

**In Vitro Phosphorylation of ARM domains by SD1 receptor kinases and MLPK**

A subset of kinase domains, used for the yeast two-hybrid interactions, were sub-cloned from the pBTM116 vector into pGEX 4T.1 or pGEX 5X-2 vector, except for MLPK where a full length protein was used. Kinase domains for which restriction sites were not available were amplified by PCR, cloned in to pGEMT vector, sequenced, and error-free fragments were cloned into pGEX 4T or pGEX 5X-2. Site-directed mutagenesis was used to construct the kinase deficient (K547A) catalytic domain of GST:ARK1 construct (Quickchange, Stratagene). These constructs were overexpressed in BL-21 DE3 pLys strain of *E. coli*, and purified as described above. For the autophosphorylation assays, approximately 0.5 µg of the purified GST:kinases were used in the autophosphorylation assays as previously described (Mazzurco et al., 2001).

For the ARM domain constructs, His-tagged fusions of *At*PUB9 and 13 ARM domains were constructed in PET15b, while the ARC1 ARM domain was cloned as a GST-tagged fusion protein. Proteins were overexpressed and purified as described above. For the kinase assay, approximately 0.1µg of the active GST:kinase fusion proteins were mixed with 0.5µg of the ARM domain fusion proteins in a 20-µl reaction with 20mM HEPES, pH 7.0,10mM MgCl2, 2mM MnCl2, 10µg/mL aprotinin, and either 100µM ATP or 5 µCi of [γ³²P]-ATP, and incubated for 60 min at 30°C. The proteins were separated on a 12% SDS-PAGE gel. Unlabelled protein gels were transferred and immunoblotted with an anti-phosphothreonine antibody (New England Biolabs Inc., Beverly, MA, USA) while [γ³²P]-labeled protein gels were subjected to
autoradiography. Coomassie Brillant Blue stain was used to detect equal loading of the ARM domain fusion proteins.

**Transient expression and immunofluorescence microscopy using BY-2 cells**

Biolistic bombardments of cultured tobacco BY-2 cells were performed essentially as described previously (Stone et al., 2003). The full-length AtPUB9 and 13 cDNAs were cloned into pRTL2 under the control of a CaMV 35S promoter for expression as GFP-tagged proteins. Site-directed mutagenesis was used to construct the kinase deficient (K547A) catalytic domain of RFP:ARK1 and the E3 ligase deficient GFP:PUB9 (V91R) (Quickchange, Stratagene).

The kinase domain from the ARK1 receptor kinase was cloned into pRTL2 as an RFP-fused construct and ARK2 as a GST-fused construct, while a MYC tag was added to the C-terminus of MLPK through PCR and cloned into pRTL2 vector. These constructs were bombarded into BY-2 cells either alone or in various combinations. Cells were fixed with 4% paraformaldehyde and visualized either directly through fluorescent microscopy for detecting GFP or incubated with either rabbit anti-GST or mouse anti-MYC antibodies, followed by fluorescence microscopy as described previously (Stone et al., 2003).

For hormone treatments of cells, twenty hours after transformation, the transformed cells were treated with various hormones such as ABA, 2,4D, ACC, methyl jasmonate and GA at 10 µM concentration for two hours, fixed and visualized as described above. Control cells were treated either with water or 0.0001N NaOH.

**Plant material and Genetic Analysis**

All genotypes reported were grown under standard growth conditions, at constant 22°C light. The
Ark1.1 pub9 double mutants were generated by crossing pub9 plants with ark1.1 pollen and genotyping T2 for double mutant plants. For epistatic analysis, pollen from abi3-6, a severe ABA-insensitive allele of ABI3 (At3g24650) that contains an internal deletion were used to cross both ark1.1 and pub9 plants. Both desiccated seeds and seeds with green embryos (a non-desiccating phenotype of abi3-6), were genotyped for both the insertion and presence of ABI3-6 allele, to identify double mutants.

**Germination and root growth assays**

Seeds (~75 to 100) from the various T-DNA insertion lines, SALK_024564 (ark1.1), SALK_002112 (ark1.2), and SALK_020751 (pub9), ark1.1/pub9 double homozygotes, abi3-6 and abi3-6/pub9 double homozygotes were plated on ½ MS plates containing varying concentrations of ABA, stratified in the dark for 3 days, followed by germination under light. Germination was measured on days 4 and 5, post-stratification, and plotted as the germination ratio, relative to wild-type Col-0. The values represent the mean ± SEM (n=4). For examining the inhibitory effect of ABA on root elongation, Col-0, ark1.1, pub9 and ark1.1/pub9 double homozygous seeds were germinated and grown vertically on ½ MS plates for 5 days, followed by transfer to ABA plates and grown for 5 days. The root lengths were measure prior to transfer to ABA plates and post-ABA treatment and the extent of inhibition was expressed as a ratio of root growth (in mM) on ABA / root growth (in mM) on ½ MS plates prior to transfer to ABA plates. The values represent the mean ± SEM (n>10). For ethylene treatments, seeds from the various lines were plated on ½ MS plates with 30 µM ACC, stratified for 4 days and left at 22°C either in the dark or under light for 5 days, before observation for ethylene responses.
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FIGURE LEGENDS

Figure 1. Yeast Two-Hybrid Interactions between Selected AtPUB-ARM proteins and Receptor Kinases.

(A) Domain organization of AtPUB-ARM proteins tested in the yeast two-hybrid screen. The motif arrangements were previously identified in Mudgil et al. (2004). The predicted domain organizations for the full length AtPUB-ARM proteins are shown on the left, while the ARM domains used in the yeast two-hybrid interaction studies for the respective AtPUBs are shown on the right.

(B) In vitro autophosphorylation assay. Selected kinases were expressed as GST-fusion proteins in *E. coli* and subjected to an *in vitro* \([\gamma^{32}\text{P}]\)-labeled autophosphorylation assay followed by autoradiography.

(C) Yeast two-hybrid interactions between selected kinase domains and ARM domains. For all AtPUB-ARMS, the entire ARM region following the U-box was used. The exception is AtPUB44a and AtPUB44b, where the longer arm repeat region was split in half, with each half being tested. The Arabidopsis receptor kinase nomenclature used is according to Shiu & Bleecker (2003). The AtPUB nomenclature is according to Azevedo et al. (2001) and Mudgil et al. (2004); and can be found at http://www.arabidopsis.org/info/genefamily/pub.html.

Interactions were detected by the activation of the *lacZ* reporter gene leading to \(\beta\)-gal activity which, in the presence of X-gal, produced a blue color on filter lifts of transformed yeast. The time required for the formation of the blue color was monitored and roughly documented with (++++) indicating a very rapid blue color development to (+) indicating a weak, but reproducible blue color development after several hours. No detection of any \(\beta\)-gal activity (blue color) was interpreted as no interaction and indicated as (-). C = control; nd = not determined.
Figure 2. *In Vitro* Phosphorylation of ARM domains by SD1 receptor kinases and MLPK.

(A) Phosphorylation of the ARC1 ARM domain by SRK$_{910}$ and MLPK. The upper panel shows the phosphorylation of the ARC1 ARM domain as detected by anti-phosphothreonine antibodies. Even loading of the ARC1 ARM domain is shown in the lower panel through Coomassie Brilliant Blue (CBB)-stained gels. (-) lane indicates any background levels of phosphorylation prior to the addition of active kinases.

(B and C) *In vitro* phosphorylation of His-tagged ARM domains from (B) *At*PUB13 and (C) *At*PUB9 by active kinases. The upper panels show the autoradiogram of the $[\gamma^{32}\text{P}]$-labeled phosphorylation of *At*PUB13 and *At*PUB9 ARM domains. Even loading of the ARM domains are shown in the lower panel through CBB stained gels. (-) lane indicates any background levels of phosphorylation prior to the addition of active kinases. HAESA also serves as a negative control as this kinase does not interact with the ARM domains in the yeast two-hybrid analysis.

Figure 3. Subcellular localizations of the ARC1, *At*PUB13 and *At*PUB9 proteins in the presence or absence of SD1 receptor kinases and MLPK.

(A-N) BY-2 cells transiently expressing the following single constructs: (A) GST:SRK$_{910}$, (C) RFP:ARK1, (E) GST:ARK2, (G) MLPK:MYC, (I) GFP:PUB13, (K) GFP:PUB9 (M) GFP:ARC1. GFP and RFP fluorescence were detected in live cells while the GST and MYC tags were detected by immunostaining with anti-GST or anti-Myc antibodies. The corresponding DIC images of the same cells with visible tungsten particles are shown in the adjacent panels (B, D, F, H, J, L, N).

(O-DD) BY-2 cells co-transformed with two constructs and visualized for both corresponding
proteins: (O, P) GFP:PUB13 & MLPK:MYC, (Q, R) GFP:PUB9 & MLPK:MYC, (S & T) GFP:ARC1 & GST:SRK910, (U, V) GFP:PUB13 & RFP:ARK1, (W, X) GFP:PUB9 & RFP:ARK1, (Y, Z) GFP:ARC1 & MLPK:MYC, (AA, BB) GFP:PUB13 & GST:ARK2, (CC, DD) GFP:PUB9 & GST:ARK2. Epifluorescence microscopy images are shown, and green represents the GFP tag attached to the respective PUB-ARM proteins while magenta represents the various tags attached to the respective kinase domains.

Figure 4. The ARK1-induced PUB9 relocalization to the plasma membrane requires an active ARK1 kinase and is mimicked by ABA treatment.

(A, B) BY-2 cells co-transformed with the ligase-deficient AtPUB9 (GFP:PUB9ld) and RFP:ARK1 constructs.

(C) In vitro phosphorylation of His-tagged ARM domains from AtPUB9 and AtPUB13 by the active ARK1 kinase domain and the ARK1 kinase-deficient (K547A) form. Upper panels shows the autoradiogram of the $^{32}$P-labeled phosphorylation of AtPUB13 and AtPUB9 ARM domains by the active ARK1 kinase in the left lanes and the absence of phosphorylation by the ARK1 kinase-deficient protein in the right lanes. Lower panels show equal loading of CBB stained His:PUB9 and His:PUB13 proteins.

(D, E) BY-2 cells co-transformed with the GFP:PUB9 and kinase-deficient ARK1 (RFP:ARK1kd) constructs.

(F-I) Subcellular localization patterns of GFP:PUB9 (F) and GFP:PUB13 (H), following treatment of BY-2 cells with 10 µM ABA for 2 hrs. DIC images of the same cells are shown in (G) and (I). Epifluorescence microscopy images are shown, and green represents the GFP tag attached to the respective PUB-ARM proteins while magenta represents the RFP tag attached to
the ARK1 kinase domain.

**Figure 5. Loss of PUB9 and ARK1 results in altered sensitivity to ABA during seed germination.**

(A) Schematic of the Arabidopsis PUB9 and ARK1 genes displaying the T-DNA insertion points for the various Salk lines.

(B) RT-PCR analysis using gene-specific primers to show the lack of PUB9 and ARK1 mRNA expression in the T-DNA insertion lines (top panels). Actin primers were used as a positive control (bottom panels).

(C) Seed germination assays in the presence and absence of ABA. Approximately 75 to 100 seeds from Col-0; the single mutants, *pub9*-/-, *ark1.1*-/- and *abi3-6*-/-; and the double mutants, *ark1.1*-/- *pub9*-/- and *pub9*-/- *abi3-6*-/-, were plated on 1 µM ABA, and stratified in the dark for 3 days followed by germination under light. Seed germination rates were measured on day 5, post-stratification. The values are represented as the percentage of germination. The values represent the mean ± SEM (n=4)

D) Root growth assays for Col-0; the single mutants, *pub9*-/- and *ark1.1*-/-; and the double mutant, *ark1.1*-/- *pub9*-/-, in presence of exogenous ABA. Seedlings were grown vertically on ½ MS plates for 5 days, followed by transfer to ½ MS control plates or plates with 10 or 50 µM ABA, and grown vertically for a further 5 days. The inhibitory effect of ABA on root lengths were expressed as a ratio of mM root growth on ABA over mM root growth on ½ MS plates prior to transfer to ABA plates. The values represent the mean ± SEM (n>10).
### AtPUB Domain organization

| Domain Organization | ARM Constructs |
|---------------------|----------------|
| UND | U-box | ARM |
| 13 |   |   |
| 14 |   |   |
| 45 |   |   |
| 9 |   |   |
| 29 |   |   |
| 38 |   |   |
| 44 |   |   |
| 44a |   |   |
| 44b |   |   |

### ARM constructs

- [Diagram of ARM constructs]

### B

| GST | HAESA | LRR XI 23 | ARK2 | ARK1 | SD1-29 | SD1-13 | SD2-5 | DUF26-21 |
|-----|--------|-----------|------|------|--------|--------|-------|-----------|
|     |        |           |      |      |        |        |       |           |
|     |        |           |      |      |        |        |       |           |
|     |        |           |      |      |        |        |       |           |
|     |        |           |      |      |        |        |       |           |
|     |        |           |      |      |        |        |       |           |

### Table C: Receptor Kinases (Intracellular domains only)

| AtPUB-ARMs (ARMs only) | S-Domain | DUF26-21 | LRR | S-Domain |
|------------------------|----------|-----------|-----|----------|
| SD1-7 (ARK1) | +++ | +++ | - | ++ | ++ | +++ | +++ | +++ | +++ | +++ |
| SD1-6 (ARK2) | ++ | +++ | - | - | - | - | - | - | - | - |
| SD1-8 (ARK3) | - | - | - | - | - | - | - | - | - | - |
| SD1-29 | +++ | +++ | - | - | - | - | - | - | - | - |
| SD1-13 (RkS2) | ++ | ++ | - | - | - | - | - | - | - | - |
| SD2-5 | - | - | - | - | - | - | - | - | - | - |
| SD2-2 (RLK4) | ++ | ++ | - | - | - | - | - | - | - | - |
| SD3-1 | - | - | - | - | - | - | - | - | - | - |
| DUF26-21 (RKC1) | ++ | ++ | - | - | - | - | - | - | - | - |
| DUF26-4 (RLK3) | - | - | - | - | - | - | - | - | - | - |
| LRR XI-16 (HAESA) | ++ | ++ | - | - | - | - | - | - | - | - |
| LRR XI-23 | - | - | - | - | - | - | - | - | - | - |
| SFR1 | - | - | - | - | - | - | - | - | - | - |
| SFR2 | - | - | - | - | - | - | - | - | - | - |
| SRK910 | - | - | - | - | - | - | - | - | - | - |
| Plamin | - | - | - | - | - | - | - | - | - | - |

### Legend

- **++**: Present
- **+++**: Moderately present
- **++++**: Highly present
- **-**: Absent
- **nd**: Not determined

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**Note**: The table is extracted from the figure provided, with the following changes:
- The legend for the table has been added to clarify the symbols used.
- The table has been expanded to include additional columns for Brassica, S-Domain, and C.
- The table has been formatted to better align with the structure of the figure.
Figure 2 Samuel et al.

A

\[ \text{ARC1 (ARM domain)} \]

B

\[ \text{PUB13 (ARM domain)} \]

C

\[ \text{PUB9 (ARM domain)} \]
