Interactions between the *S*-Domain Receptor Kinases and AtPUB-ARM E3 Ubiquitin Ligases Suggest a Conserved Signaling Pathway in Arabidopsis[1][W][OA]

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The Arabidopsis (*Arabidopsis thaliana*) 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, is known to mediate the self-incompatibility (SI) response in *Brassica*. *S*-receptor kinase 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 SI 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 plant 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 *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 (*Nicotiana tabacum*) 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 redistribution to the plasma membrane of BY-2 cells when either treated with ABA or coexpressed 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 potential involvement of these proteins in ABA responses.

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, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin ligase. By far, the E3 ubiquitin ligase is the largest group of these enzymes that 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 41 E2 enzymes annotated in the Arabidopsis (*Arabidopsis thaliana*) 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 1,300 predicted E3 ligase genes in the Arabidopsis genome (Smalle and Vierstra, 2004). The larger known Arabidopsis families include the RING family with approximately 469 predicted proteins and the F-box family with approximately 700 predicted proteins (Gagne et al., 2004; Stone et al., 2005). 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 the 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 five 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 (*Oryza sativa*) genome (Mudgil et al., 2004; Samuel et al., 2006). 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

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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 the *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 (*Nicotiana tabacum*) ACRE276 protein appear to be positive regulators of cell death and defense responses because 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 hypersensitive response development and disease resistance (González-Lamothe et al., 2006). A role in plant hormone responses has also been reported with the potato (*Solanum tuberosum*) PHOR1 protein being 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 (SI) response where it functions downstream of the 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 *Arabidopsis lyrata* SRK genes (P. Liu et al., 2007). Last, 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 (Morris and Walker, 2003; Haffani et al., 2004). The *Brassica* SRK, which interacts with ARC1, is very closely related to the Arabidopsis *S*-domain-1 (SD1) 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 and Nasrallah, 1996), whereas 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 NtPUB4, is most closely related to members of the Arabidopsis SD1 receptor kinase subfamily (Kim et al., 2000). Cosuppression 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).

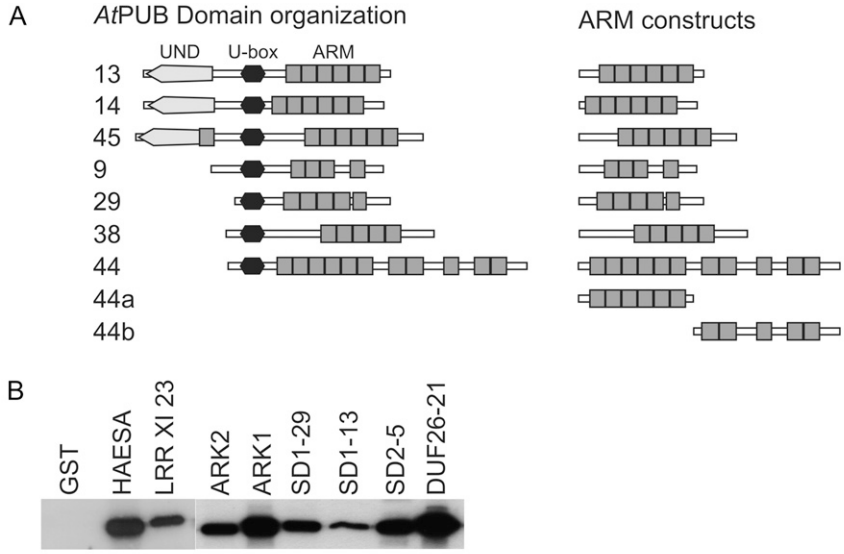
The observed interaction between *Brassica* SRK-ARC1 and tobacco CHRK1-NtPUB4 and the conservation of signaling components across *Brassica* and Arabidopsis suggested to us that the Arabidopsis *S*-domain receptor kinase family could potentially utilize the numerous AtPUB-ARM family members as their downstream signaling components. 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 (ABA) 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 that 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; J. Salt, M.A. Samuel, and D.R. Goring, unpublished data).

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 Leu-rich repeat (LRR) receptor kinases, LRR XI-16 (HAESA) and LRR XI-23. In addition, related *Brassica* SD1 receptor kinases, SFR1, SFR2, and SRK_{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 glutathione *S*-transferase (GST):kinase fusion proteins. The kinase domains were found to have strong autophosphorylation activity as shown in Figure. 1B or as previously shown (Mazzurco et al., 2001). Protein expression of all the constructs in



C		Receptor Kinases (Intracellular domains only)																
		Arabidopsis													*Brassica*			
		S-Domain									DUF26		LRR		S-Domain			C
		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)	DUF26-4 (RLK3)	LRR XI-16 (HAESA)	LRR XI-23	SFR1	SFR2	SRK910	Plamin
*At*PUB-ARMs (ARMs only)	13	+++	+++	++++	+++	+++	++	+++	-	-	+	-	-	-	++	++++	++++	-
	14	++	++	++++	+++	++	++	++	-	-	-	-	-	-	++	++++	++++	-
	45	-	-	-	++	-	-	-	-	nd	-	nd	-	-	+	++	+	-
	9	++	++	++	++	++	++	++	-	-	-	-	-	-	+	+	+	-
	29	-	-	-	+++	-	-	+	-	-	-	-	-	-	+	+	+	-
	38	++	++	++	+++	-	-	-	-	-	++	-	-	-	++	+++	+	-
	44	-	-	nd	+++	-	nd	-	-	nd	-	nd	-	-	-	-	-	-
	44a	-	-	nd	+++	-	nd	-	-	nd	-	nd	-	-	-	-	-	-
	44b	-	-	nd	+++	-	nd	-	-	nd	-	nd	-	-	-	-	-	-
C	VP16	-	-	nd	-	-	nd	-	-	nd	-	nd	-	-	-	-	-	-

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 *Escherichia coli* and subjected to an in vitro [γ^{32}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 and 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 β-galactosidase 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 β-galactosidase activity (blue color) was interpreted as no interaction and indicated as (−). C, Control; nd, not determined.

the transformed yeast (*Saccharomyces cerevisiae*) 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 AtPUB-ARM proteins while very low-level or no interactions were observed with non-SD1 receptor kinase family members (Fig. 1C). For example, AtPUB13, 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. AtPUB38 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 AtPUBs tested (Fig. 1C). Thus, AtPUB-ARM proteins tended to show interaction patterns that were largely confined to the SD1 subfamily of receptor kinases. However, within the SD1 receptor kinase subgroup, there appeared to be less specificity with several AtPUB-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 *M*-Locus Protein Kinase

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* SI, 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 SI response, although MLPK's cellular mechanism in this response remains unknown (Murase et al., 2004; Kakita et al., 2007a, 2007b). 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 and AtPUB9) 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-phospho-Thr 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* SRK_{910} 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 SRK_{910} (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, whereas 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 toward 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 toward AtPUB9 (Fig. 2C).

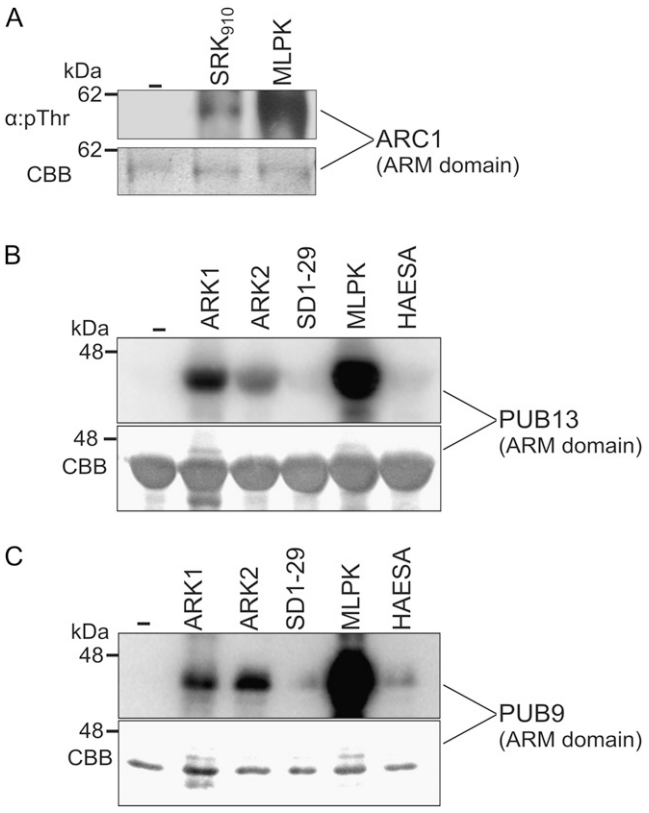


Figure 2. In vitro phosphorylation of ARM domains by SD1 receptor kinases and MLPK. A, Phosphorylation of the ARC1 ARM domain by SRK_{910} and MLPK. The top image shows the phosphorylation of the ARC1 ARM domain as detected by anti-phospho-Thr antibodies. Even loading of the ARC1 ARM domain is shown in the bottom image 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 AtPUB13 (B) and AtPUB9 (C) by active kinases. The top images show the autoradiogram of the [γ^{32}P]-labeled phosphorylation of AtPUB13 and AtPUB9 ARM domains. Even loading of the ARM domains is shown in the bottom image 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.

The Effect of Active Kinases on the Subcellular Localization of PUB-ARM Proteins

We have previously shown that transient coexpression of *Brassica* SRK_{910} with *Brassica* ARC1 in tobacco BY-2 cells resulted in relocalization of *Brassica* ARC1 from the cytosol/nucleus to endoplasmic reticulum (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, AtPUB9, and AtPUB13 in combination with SRK_{910}, ARK1, ARK2, or MLPK (Fig. 3). ARC1 and AtPUB13

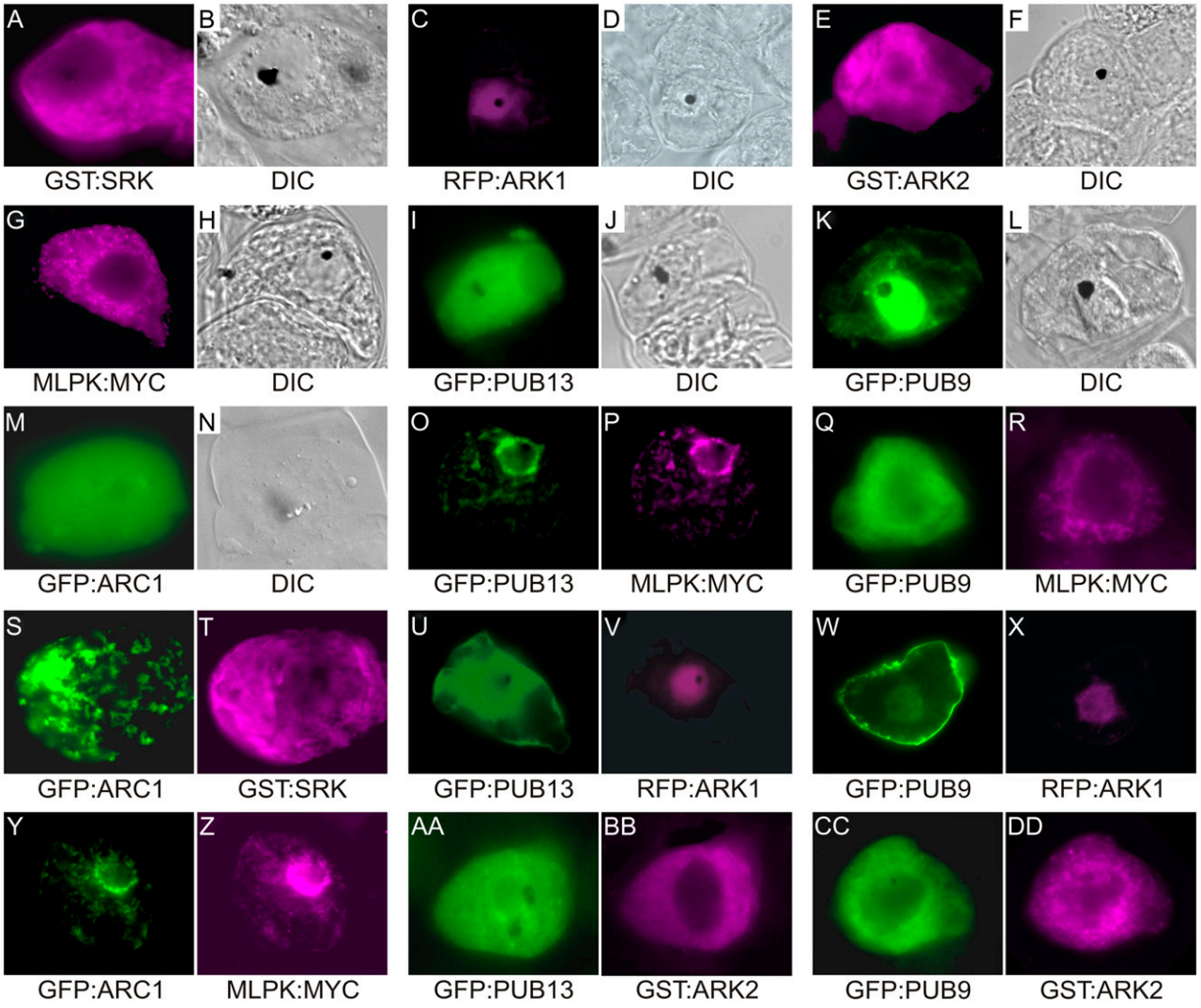


Figure 3. Subcellular localizations of the ARC1, AtPUB13, and AtPUB9 proteins in the presence or absence of SD1 receptor kinases and MLPK. A to N, BY-2 cells transiently expressing the following single constructs: (A) GST:SRK_{910}, (C) RFP:ARK1, (E) GST:ARK2, (G) MLPK:MYC, (I) GFP:PUB13, (K) GFP:PUB9, and (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, and N). O to DD, BY-2 cells cotransformed with two constructs and visualized for both corresponding proteins. O and P, GFP:PUB13 and MLPK:MYC. Q and R, GFP:PUB9 and MLPK:MYC. S and T, GFP:ARC1 and GST:SRK_{910}. U and V, GFP:PUB13 and RFP:ARK1. W and X, GFP:PUB9 and RFP:ARK1. Y and Z, GFP:ARC1 and MLPK:MYC. AA and BB, GFP:PUB13 and GST:ARK2. CC and DD, GFP:PUB9 and 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.

have a similar domain organization with the UND: U-box:ARM domains, whereas AtPUB9 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 red fluorescent protein (RFP):ARK1, GST:ARK2, and MLPK:MYC fusion proteins. As expected, the tagged cytosolic kinase domains were localized to the cytosol for SRK, ARK2, and MLPK (Fig. 3, A, 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, AtPUB13 and ARC1 displayed diffused expression throughout the cell and could be observed both in the cytosol and nucleus (Fig. 3, I and M), whereas AtPUB9 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).

Cotransformations 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 coexpressed, SRK_{910} remained cytosolic (Fig. 3T), whereas 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 coexpression of both MLPK and ARC1 resulted in both proteins targeted to the perinuclear region (Fig. 3, Y 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 coexpression of MLPK:MYC with AtPUB13 resulted in both proteins being targeted to the perinuclear region (Fig. 3, O and P). This suggests that MLPK has a similar effect on the two UND-containing proteins, ARC1 and AtPUB13. In contrast, the coexpression of either ARK1 or ARK2 with GFP:PUB13 resulted in no alterations to either AtPUB13 (Fig. 3, U and AA) or the kinases (Fig. 3, V 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. 3, Q and CC). In approximately 40% of these cells, AtPUB9 was also found in punctuate structures in the cytosol. Interestingly, when ARK1 was coexpressed 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 whether 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 knock out the respective activities. An AtPUB9 E3 ligase activity-deficient (ld) mutant was created by mutating a conserved Val previously shown to be required for E3 ligase activity (V91R; Zeng et al., 2004). When cotransformed 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 Lys-547 residue was changed to Ala (K547A) because this had been previously shown to abolish kinase activity of ARK1 (Tobias and Nasrallah, 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. 4, D and E). Therefore, the redistribution of AtPUB9 to the plasma membrane is a phosphorylation-dependent process. Interestingly, we have previously shown that SRK_{910} induces a phosphorylation-dependent relocalization 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 whether 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 1 h (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 h. ABA treatment resulted in complete loss of nuclear localization and the remobilization 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 1-aminocyclopropane-1-carboxylic acid (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 Act at or Upstream of ABI3

The ability of the active ARK1 kinase domain and ABA treatment to cause membrane localization of 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 was confirmed through reverse transcription (RT)-PCR (Fig. 5, A 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 1 μM ABA when compared to wild-type Columbia (Col-0) seeds (Fig. 5C). Similar results were also observed for *ark1.2*$-/-$ mutants. To test whether 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*$-/-$

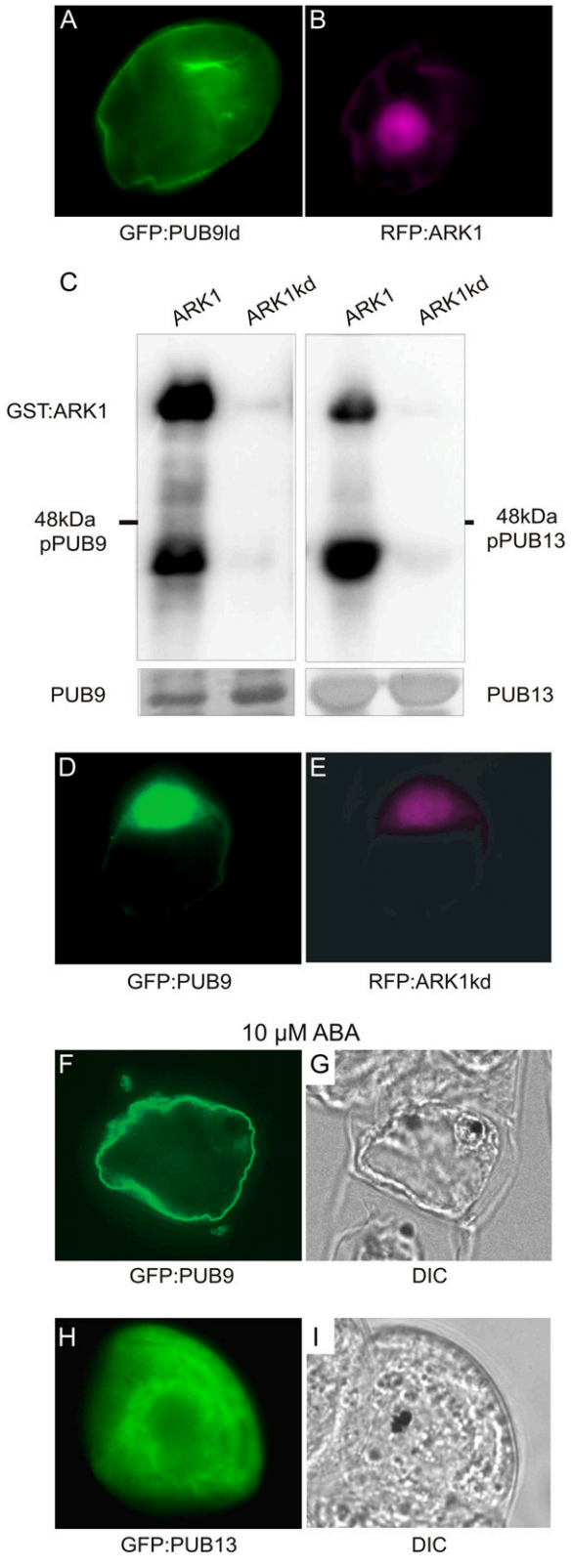


Figure 4. The ARK1-induced PUB9 relocalization to the plasma membrane requires an active ARK1 kinase and is mimicked by ABA treatment. A and B, BY-2 cells cotransformed with the ligase-deficient AtPUB9 (GFP:PUB9ld) and RFP:ARK1 constructs. C, In vitro phosphorylation of His-tagged ARM domains from AtPUB9 and AtPUB13 by the active ARK1 kinase domain and the ARK1 kinase-deficient (K547A) form. Top images show the autoradiogram of the [γ^{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. Bottom images show equal loading of CBB-stained His:PUB9 and His:PUB13 proteins. D and E, BY-2 cells cotransformed with the GFP:PUB9 and kinase-deficient ARK1 (RFP:ARK1kd) constructs. F to I, Subcellular localization patterns of GFP:PUB9 (F) and GFP:PUB13 (H), following treatment of BY-2 cells with 10 μM ABA for 2 h. 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.

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 *ark1−/−* and *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 (although to a lesser extent) cause plasma membrane relocalization of AtPUB9 in BY-2 cells. No phenotypic difference was observed between Col-0 and the *pub9−/−* and *ark1.1−/−* seedlings when grown on plates containing 30 μM ACC under dark or light conditions (Supplemental Fig. S2).

With both the *ark1−/−* and *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 *abi3-6* allele was chosen because it is in the same Col-0 background as *ark1* and *pub9* and displays a strong ABA-insensitivity phenotype. The *abi3-6* mutant is nondesiccating and can be readily identified through the selection of green (non-degreening) seeds (as well as PCR genotyping). Therefore, crosses between *abi3-6* and *ark1* or *pub9* were screened for green seeds in the T_2 generation. When the *abi3-6* and *ark1.1* mutants were crossed, *abi3-6−/−*, *ark1−/−* double-homozygous mutants could not be recovered. Upon closer examination, siliques from the *abi3-6+/−*, *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 *abi3-6* and *pub9* mutants did lead to the isolation of green seeds with the *abi3-6−/−*, *pub9−/−* double-homozygous genotype. When the *abi3-6−/−*, *pub9−/−* green seeds were tested for ABA responses during seed germination, they displayed an insensitive phenotype similar to *abi3-6* (Fig. 5C). This suggests that the ABA-insensitive phenotype of *abi3-6* is epistatic to *pub9* and indicates that PUB9 functions at or upstream of ABI3.

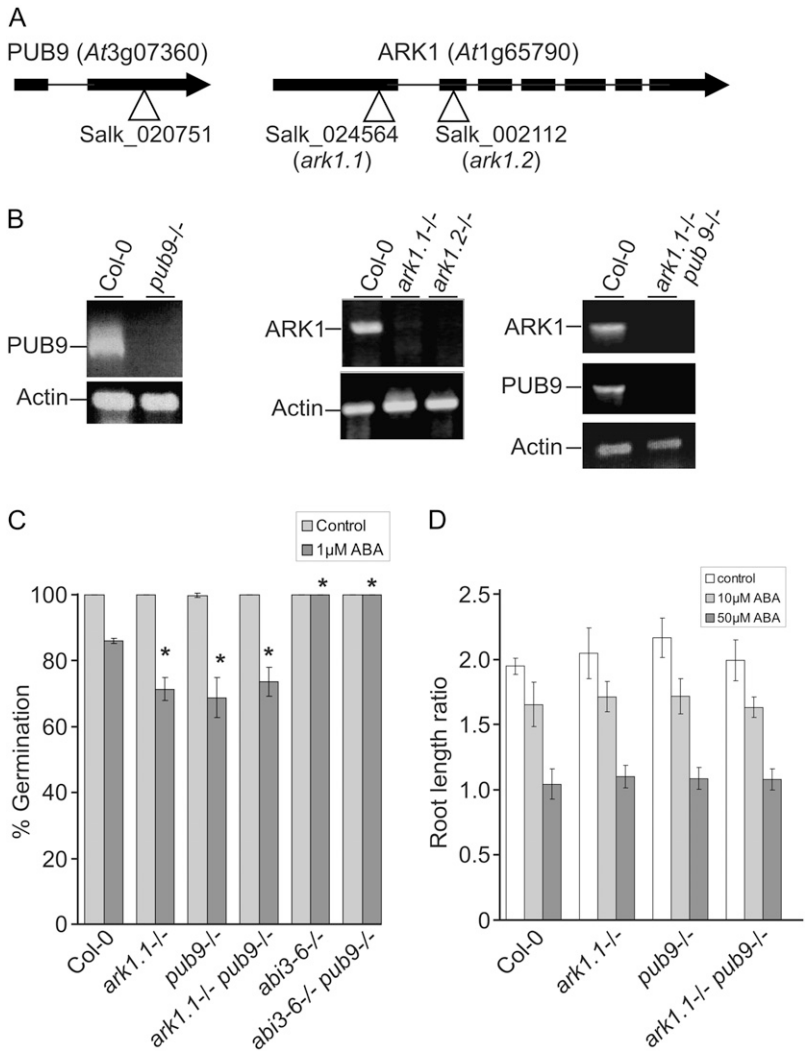


Figure 5. Loss of PUB9 and ARK1 results in altered sensitivity to ABA during seed germination. A, Schematic of the Arabidopsis PUB9 and ARK1 genes displaying the T-DNA insertion points for the various Salk lines. B, RT-PCR analysis using gene-specific primers to show the lack of PUB9 and ARK1 mRNA expression in the T-DNA insertion lines (top). Actin primers were used as a positive control (bottom). 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 d followed by germination under light. Seed germination rates were measured on day 5, poststratification. The values are represented as the percentage of germination. The values represent the mean ± SE (n = 4). D, Root growth assays for Col-0; the single mutants, *pub9−/−* and *ark1.1−/−*; and the double mutant, *ark1.1−/− pub9−/−*, in the presence of exogenous ABA. Seedlings were grown vertically on 0.5× MS plates for 5 d, followed by transfer to 0.5× MS control plates or plates with 10 or 50 μM ABA, and grown vertically for an additional 5 d. The inhibitory effect of ABA on root lengths were expressed as a ratio of millimolar root growth on ABA over millimolar root growth on 0.5× MS plates prior to transfer to ABA plates. The values represent the mean ± SE ($n > 10$).

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; Gomez-Gomez and Boller, 2000; Clouse, 2002; 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 that 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 SI response (Stone et al., 1999, 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 article, 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. Whereas 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 that 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. Because 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 interaction 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 NtPUB4 protein was identified as an interacting partner for CHRK1 (Kim et al., 2003).

In this article, we also investigated potential connections with the MLPK, which had previously been identified in the *Brassica* SI response (Murase et al., 2004). MLPK is a positive regulator of *Brassica* SI and has been proposed to act along with SRK in mediating ARC1 activation or localization (Goring and Walker, 2004). MLPK belongs to the 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 AtPUB9 and AtPUB13 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 AtPUB 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. (2007b), 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., 2007a).

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 SRK_{910} causes ARC1 to relocalize to ER-associated proteasomes (Stone et al., 2003). This sorting was dependent on the ability of SRK to phosphorylate ARC1 because a truncated version of SRK did not lead to the proteasomal distribution of ARC1 (Stone et al., 2003). Similar studies with CHRK1 and NtPUB4 also resulted in the relocalization of NtPUB4 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 coexpression 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 toward 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 10 μM 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 because 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 al., 2002; Roelfsema et al., 2004). A plasma membrane-localized, putative G protein-coupled receptor has also been proposed to be an ABA receptor (X. Liu et al., 2007a, 2007b), 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, which encode protein phosphatase 2C, have been proposed to be negative regulators of ABA signaling (Leung et al., 1994, 1997; Meyer et al., 1994; 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. AIP2 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 because *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, whereas 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, whereas the ABI1 and ABI2 protein phosphatases act at or upstream of ERA1 (Brady et al., 2003). 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 (*At*3g46510), AtPUB14 (At3g54850), AtPUB45 (At1g27910), AtPUB9 (At3g07360), AtPUB29 (At3g18710), AtPUB38 (At5g65200), and AtPUB44 (At1g20780), were cloned in the pVP16 vector. For the Arabidopsis (*Arabidopsis thaliana*) receptor kinases from various subfamilies, 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 SRK_{910} 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 (*Saccharomyces cerevisiae*) strain using the Gietz and Woods (2002) protocol. 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 to 200 mL of 2× YT containing 100 μg mL^{-1} ampicillin was inoculated with 1/100th volume of an overnight culture and grown to an OD_{600} of 0.8 at 37°C. Isopropyl β-D-thiogalactoside was added to a final concentration of 0.5 mM induced at 37°C for 5 to 6 h, pelleted, resuspended in 10 mL of binding buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 5 mM imidazole, 5% glycerol, 500 μM phenylmethylsulfonyl fluoride [PMSF], and 1 mM benzanidine), and followed with sonication. Triton X-100 was added to a final concentration of 0.01%, and the samples were spun at 17,000 rpm for 10 min at 4°C. To the supernatant, 1 mL of 50% (v/v) nickel nitrilotriacetic acid 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 PMSF, and 1 mM 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 PMSF, and 1 mM 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 to 20 mL of G-lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol, 200 μM 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% (v/v) 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, was subcloned 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 into 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 p-Lys strain of *Escherichia 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 AtPUB9 and 13 ARM domains were constructed in PET15b, whereas 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 0.1 μg of the active GST:kinase fusion proteins were mixed with 0.5 μg of the ARM domain fusion proteins in a 20-μL reaction with 20 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μg/mL aprotinin, and either 100 μM ATP or 5 μCi of [γ^{32}P]ATP, and incubated for 60 min at 30°C. The proteins were separated on a 12% SDS-PAGE gel. Unlabeled protein gels were transferred and immunoblotted with an anti-phospho-Thr antibody (New England Biolabs), whereas [γ^{32}P]-labeled protein gels were subjected to autoradiography. Coomassie Brilliant 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 (*Nicotiana tabacum*) 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 cauliflower mosaic virus 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, whereas 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 20 h 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 2 h, fixed, and visualized as described above. Control cells were treated either with water or 0.0001 N 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, was used to cross both *ark1.1* and *pub9* plants. Both desiccated seeds and seeds with green embryos (a nondesiccating 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 (approximately 75–100) 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 0.5× Murashige and Skoog (MS) plates containing varying concentrations of ABA, stratified in the dark for 3 d, followed by germination under light. Germination was measured on days 4 and 5, poststratification, and plotted as the germination ratio relative to wild-type Col-0. The values represent the mean ± SE ($n = 4$). 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 0.5× MS plates for 5 d, followed by transfer to ABA plates and grown for 5 d. The root lengths were measured 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 0.5× MS plates prior to transfer to ABA plates. The values represent the mean ± SE ($n > 10$). For ethylene treatments, seeds from the various lines were plated on 0.5× MS plates with 30 μM ACC, stratified for 4 d, and left at 22°C either in the dark or under light for 5 d before observation for ethylene responses.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Hormonal regulation of AtPUB9 localization.

Supplemental Figure S2. Loss of ARK1 and PUB9 does not alter ethylene responses.

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LITERATURE CITED

Amador V, Monte E, Garcia-Martinez JL, Prat S (2001) Gibberellins signal nuclear import of PHOR1, a photoperiod-responsive protein with homology to Drosophila armadillo. Cell **106:** 343–354

Andersen P, Kragelund BB, Olsen AN, Larsen FH, Chua NH, Poulsen FM, Skriver K (2004) Structure and biochemical function of a prototypical Arabidopsis U-box domain. J Biol Chem **279:** 40053–40061

Aravind L, Koonin EV (2000) The U box is a modified RING finger—a common domain in ubiquitination. Curr Biol **10:** 132–134

Azevedo C, Santos-Rosa MJ, Shirasu K (2001) The U-box protein family in plants. Trends Plant Sci **6:** 354–358

Böhmer M, Romeis T (2007) A chemical-genetic approach to elucidate protein kinase function *in planta.* Plant Mol Biol **65:** 817–827

Bower MS, Matias DD, Fernandes-Carvalho E, Mazzurco M, Gu T, Rothstein S, Goring DR (1996) Two members of the thioredoxin-h family interact with the kinase domain of a *Brassica* S-locus receptor kinase. Plant Cell **8:** 1641–1650

Brady SM, Sarkar SF, Bonetta D, McCourt P (2003) The ABSCISIC ACID INSENSITIVE 3 (ABI3) gene is modulated by farnesylation and is involved in auxin signaling and lateral root development in Arabidopsis. Plant J **34:** 67–75

Clouse SD (2002) Brassinosteroids. Plant counterparts to animal steroid hormones? Vitam Horm **65:** 195–223

Devoto A, Nieto-Rostro M, Xie D, Ellis C, Harmston R, Patrick E, Davis J, Sherratt L, Coleman M, Turner JG (2002) COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in Arabidopsis. Plant J **32:** 457–466

Dill A, Thomas SG, Hu J, Steber CM, Sun TP (2004) The *Arabidopsis* F-box protein SLEEPY1 targets gibberellin signaling repressors for gibberellin-induced degradation. Plant Cell **16:** 1392–1405

Du L, Chen Z (2000) Identification of genes encoding receptor-like protein kinases as possible targets of pathogen- and salicylic acid-induced WRKY DNA-binding proteins in Arabidopsis. Plant J **24:** 837–847

Finkelstein RR, Gampala SS, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell **14 (Suppl):** S15–S45

Gagne JM, Smalle J, Gingerich DJ, Walker JM, Yoo SD, Yanagisawa S, Vierstra RD (2004) Arabidopsis EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. Proc Natl Acad Sci USA **101:** 6803–6808

Gietz RD, Woods RA (2002) Transformation of yeast by the LiAc/SS Carrier DNA/PEG Method. Methods Enzymol **350:** 87–96

Gomez-Gomez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Mol Cell **5:** 1003–1011

González-Lamothe R, Tsitsigiannis DI, Ludwig AA, Panicot M, Shirasu K, Jones JD (2006) The U-box protein CMPG1 is required for efficient activation of defense mechanisms triggered by multiple resistance genes in tobacco and tomato. Plant Cell **18:** 1067–1083

Goring DR, Walker JC (2004) Self-rejection, a new kinase connection. Science **303:** 1474–1475

Gu T, Mazzurco M, Sulaman W, Matias DD, Goring DR (1998) Binding of an arm repeat protein to the kinase domain of the S-locus receptor kinase. Proc Natl Acad Sci USA **95:** 382–387

Haffani YZ, Silva NF, Goring DR (2004) Receptor kinase signalling in plants. Can J Bot **82:** 1–15

Hatakeyama S, Yada M, Matsumoto M, Ishida N, Nakayama KI (2001) U box proteins as a new family of ubiquitin-protein ligases. J Biol Chem **276:** 33111–33120

Johnson KL, Ingram GC (2005) Sending the right signal: regulating receptor kinase activity. Curr Opin Plant Biol **8:** 648–656

Kakita M, Murase K, Iwano M, Matsumoto T, Watanabe M, Shiba H, Isogai A, Takayama S (2007a) Two distinct forms of M locus protein kinase localize to the plasma membrane and interact directly with S locus receptor kinase to transduce self-incompatibility signaling in *Brassica rapa.* Plant Cell **19:** 3961–3973

Kakita M, Shimosato H, Murase K, Isogai A, Takayama S (2007b) Direct interaction between S-locus receptor kinase and M-locus protein kinase involved in Brassica self-incompatibility signaling. Plant Biotechnol **24:** 185–190

Kim M, Cho HS, Kim DM, Lee JH, Pai HS (2003) CHRK1, a chitinase-related receptor-like kinase, interacts with NtPUB4, an armadillo repeat protein, in tobacco. Biochim Biophys Acta **1651:** 50–59

Kim YS, Lee JH, Yoon GM, Cho HS, Park SW, Suh MC, Choi D, Ha HJ, Liu JR, Pai HS (2000) CHRK1, a chitinase-related receptor-like kinase in tobacco. Plant Physiol **123:** 905–915

Ko JH, Yang SH, Han KH (2006) Upregulation of an Arabidopsis RING-H2 gene, XERICO, confers drought tolerance through increased abscisic acid biosynthesis. Plant J **47:** 343–355

Kraft E, Stone SL, Ma L, Su N, Gao Y, Lau OS, Deng XW, Callis J (2005) Genome analysis and functional characterization of the E2 and RING-type E3 ligase ubiquitination enzymes of Arabidopsis. Plant Physiol **139:** 1597–1611

Lee JH, Takei K, Sakakibara H, Sun Cho H, Kim DM, Kim YS, Min SR, Kim WT, Sohn DY, Lim YP, et al (2003) CHRK1, a chitinase-related receptor-like kinase, plays a role in plant development and cytokinin homeostasis in tobacco. Plant Mol Biol **53:** 877–890

Leung J, Bouvier-Durand M, Morris PC, Guerrier D, Chefdor F, Giraudat J (1994) Arabidopsis ABA response gene ABI1: features of a calcium-modulated protein phosphatase. Science **264:** 1448–1452

Leung J, Merlot S, Giraudat J (1997) The *Arabidopsis* ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell **9:** 759–771

Li J, Chory J (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell **90:** 929–938

Liu P, Sherman-Broyles S, Nasrallah ME, Nasrallah JB (2007) A cryptic modifier causing transient self-incompatibility in Arabidopsis thaliana. Curr Biol **17:** 734–740

Liu X, Yue Y, Li B, Nie Y, Li W, Wu WH, Ma L (2007a) A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. Science **315:** 1712–1716

Liu X, Yue Y, Li W, Ma L (2007b) Response to Comment on ''A G protein–coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid''. Science **318:** 914d

Luo J, Shen G, Yan J, He C, Zhang H (2006) AtCHIP functions as an E3 ubiquitin ligase of protein phosphatase 2A subunits and alters plant response to abscisic acid treatment. Plant J **46:** 649–657

Mazzurco M, Sulaman W, Elina H, Cock JM, Goring DR (2001) Further analysis of the interactions between the Brassica S receptor kinase and three interacting proteins (ARC1, THL1 and THL2) in the yeast two-hybrid system. Plant Mol Biol **45:** 365–376

Meyer K, Leube MP, Grill E (1994) A protein phosphatase 2C involved in ABA signal transduction in Arabidopsis thaliana. Science **264:** 1452–1455

Moon J, Parry G, Estelle M (2004) The ubiquitin-proteasome pathway and plant development. Plant Cell **16:** 3181–3195

Morris ER, Walker JC (2003) Receptor-like protein kinases: the keys to response. Curr Opin Plant Biol **6:** 339–342

Mudgil Y, Shiu SH, 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

Murase K, Shiba H, Iwano M, Che FS, Watanabe M, Isogai A, Takayama S (2004) A membrane-anchored protein kinase involved in Brassica self-incompatibility signaling. Science **303:** 1516–1519

Nambara E, Keith K, McCourt P, Naito S (1994) Isolation of an internal deletion mutant of the Arabidopsis thaliana ABI3 gene. Plant Cell Physiol **35:** 509–513

Osakabe Y, Maruyama K, Seki M, Satou M, Shinozaki K, Yamaguchi-Shinozaki K (2005) Leucine-rich repeat receptor-like kinase1 is a key membrane-bound regulator of abscisic acid early signaling in *Arabidopsis*. Plant Cell **17:** 1105–1119

Pei ZM, Kuchitsu K, Ward JM, Schwarz M, Schroeder JI (1997) Differential abscisic acid regulation of guard cell slow anion channels in *Arabidopsis* wild-type and abi1 and abi2 mutants. Plant Cell **9:** 409–423

Razem FA, El-Kereamy A, Abrams SR, Hill RD (2006) The RNA-binding protein FCA is an abscisic acid receptor. Nature **439:** 290–294

Roelfsema MR, Levchenko V, Hedrich R (2004) ABA depolarizes guard cells in intact plants, through a transient activation of R- and S-type anion channels. Plant J **37:** 578–588

Samuel MA, Salt JN, Shiu SH, Goring DR (2006) Multifunctional arm repeat domains in plants. Int Rev Cytol **253:** 1–26

Serrano M, Parra S, Alcaraz LD, Guzman P (2006) The ATL gene family from Arabidopsis thaliana and Oryza sativa comprises a large number of putative ubiquitin ligases of the RING-H2 type. J Mol Evol **62:** 434–445

Shen YY, Wang XF, Wu FQ, Du SY, Cao Z, Shang Y, Wang XL, Peng CC, Yu XC, Zhu SY, et al (2006) The Mg-chelatase H subunit is an abscisic acid receptor. Nature **443:** 823–826

Shiu SH, Bleecker AB (2003) Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in Arabidopsis. Plant Physiol **132:** 530–543

Shiu SH, Bleecker AB (2001a) Plant receptor-like kinase gene family: diversity, function, and signaling. Sci STKE **113:** RE22

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

Smalle J, Vierstra RD (2004) The ubiquitin 26S proteasome proteolytic pathway. Annu Rev Plant Biol **55:** 555–590

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

Stone SL, Arnoldo M, Goring DR (1999) A breakdown of Brassica self-incompatibility in ARC1 antisense transgenic plants. Science **286:** 1729–1731

Stone SL, Hauksdottir H, Troy A, Herschleb J, Kraft E, Callis J (2005) Functional analysis of the RING-type ubiquitin ligase family of Arabidopsis. Plant Physiol **137:** 13–30

Stone SL, Williams LA, Farmer LM, Vierstra RD, Callis J (2006) KEEP ON GOING, a RING E3 ligase essential for *Arabidopsis* growth and development, is involved in abscisic acid signaling. Plant Cell **18:** 3415–3428

Tobias CM, Nasrallah JB (1996) An S-locus-related gene in Arabidopsis encodes a functional kinase and produces two classes of transcripts. Plant J **10:** 523–531

Toufighi K, Brady SM, Austin R, Ly E, Provart NJ (2005) The Botany Array Resource: e-northerns, expression angling, and promoter analyses. Plant J **43:** 153–163

Wiborg J, O'Shea C, Skriver K (2008) Biochemical function of typical and variant Arabidopsis thaliana U-box E3 ubiquitin-protein ligases. Biochem J (in press)

Yang CW, Gonzalez-Lamothe R, Ewan RA, Rowland O, Yoshioka H, Shenton M, Ye H, O'Donnell E, Jones JD, Sadanandom A (2006) The E3 ubiquitin ligase activity of *Arabidopsis* PLANT U-BOX17 and its functional tobacco homolog ACRE276 are required for cell death and defense. Plant Cell **18:** 1084–1098

Yin Z, Chen J, Zeng L, Goh M, Leung H, Khush GS, Wang GL (2000) Characterizing rice lesion mimic mutants and identifying a mutant with broad-spectrum resistance to rice blast and bacterial blight. Mol Plant Microbe Interact **13:** 869–876

Zeng LR, Qu S, Bordeos A, Yang C, Baraoidan M, Yan H, Xie Q, Nahm BH, Leung H, Wang GL (2004) Spotted leaf11, a negative regulator of plant cell death and defense, encodes a U-box/armadillo repeat protein endowed with E3 ubiquitin ligase activity. Plant Cell **16:** 2795–2808

Zhang X, Garreton V, Chua NH (2005) The AIP2 E3 ligase acts as a novel negative regulator of ABA signaling by promoting ABI3 degradation. Genes Dev **19:** 1532–1543

Zimmermann P, Hennig L, Gruissem W (2005) Gene-expression analysis and network discovery using Genevestigator. Trends Plant Sci **10:** 407–409