Functional test of *Brassica* self-incompatibility modifiers in *Arabidopsis thaliana*

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The self-incompatibility (SI) system of the Brassicaceae is based on allele-specific interactions among haplotypes of the *S* locus. In all tested self-incompatible Brassicaceae, the *S* haplotype encompasses two linked genes, one encoding the *S*-locus receptor kinase (SRK), a transmembrane kinase displayed at the surface of stigma epidermal cells, and the other encoding its ligand, the *S*-locus cysteine-rich (SCR) protein, which is localized in the pollen coat. Transfer of the two genes to self-fertile *Arabidopsis thaliana* allowed the establishment of robust SI in several accessions, indicating that the signaling cascade triggered by this receptor–ligand interaction and the resulting inhibition of "self" pollen by the stigma have been maintained in extant *A. thaliana*. Based on studies in *Brassica* species, the membrane-tethered kinase MLPK, the ARM repeat-containing U-box protein ARC1, and the exocyst subunit Exo70A1 have been proposed to function as components of an SI signaling cascade. Here we tested the role of these molecules in the SI response of *A. thaliana SRK-SCR* plants. We show that the *A. thaliana ARC1* ortholog is a highly decayed pseudogene. We also show that, unlike reports in *Brassica*, inactivation of the *MLPK* ortholog *AtAPK1b* and overexpression of *Exo70A1* neither abolish nor weaken SI in *A. thaliana SRK-SCR* plants. These results do not support a role for these molecules in the SI response of *A. thaliana*.

receptor signaling | *S* locus receptor kinase

In the self-incompatibility (SI) system of the Brassicaceae (crucifer) family, the ability of cells of the stigma epidermis to discriminate between "self" and "nonself" pollen grains and to inhibit "self" pollen is based on allele-specific interactions between two highly polymorphic proteins encoded by the *S*-locus haplotype: the *S*-locus receptor kinase (SRK), a single-pass transmembrane serine/threonine kinase displayed at the surface of stigma epidermal cells (1), and the *S*-locus cysteine-rich (SCR) protein, a small secreted protein located in the pollen coat (2, 3), which is the ligand for SRK (4, 5). The binding of SCR to the extracellular domain of its cognate SRK activates the receptor kinase (4–6) and is thought to trigger a response within the stigma epidermal cell that culminates in the inhibition of pollen tube development.

The molecular events precipitated by activation of SRK are currently poorly understood, and the immediate cause of self-pollen inhibition is not known. Nevertheless, a few candidate molecules have been proposed to function as downstream effectors in the SRK signaling pathway in *Brassica* species. The *M*-locus protein kinase (MLPK), identified by a map-based cloning approach (7), is thought to correspond to the gene disrupted by the *mod*, or *m*, mutation, which causes complete loss of SI in the stigmas of the *Brassica rapa* variety Yellow Sarson (8). Although a stable complementation experiment has not been reported, particle bombardment-mediated transient expression of *MLPK* in the stigma epidermal cells of *mod* plants was reported to complement the self-fertile phenotype of these cells (7). Because the two isoforms produced by the *MLPK* gene colocalize to the plasma membrane and interact with SRK, they are thought to function in SRK-mediated signaling (9).

Another candidate effector of SRK-mediated signaling is the ARM-repeat containing 1 (ARC1) protein, which was identified in a yeast two-hybrid screen as a protein that interacts with, and is phosphorylated by, the kinase domain of a *Brassica napus* SRK protein (10). Antisense down-regulation of *ARC1* transcripts in transgenic *B. napus* has been associated with partial breakdown of SI (11). Furthermore, ARC1, like other members of the plant U-box (PUB) proteins, is an E3 ubiquitin ligase that localizes to the proteasome and COP9 signalosome in an SRK-dependent manner (12). Recently, ARC1 was found to interact with Exo70A1 (13), a putative component of the exocyst complex, which functions in polarized secretion in yeast and animals (14, 15). Overexpression of Exo70A1 in stigma epidermal cells has been reported to cause partial breakdown of SI (13). Previous studies showed that Exo70A1 is required for compatible pollen-stigma interactions in *Arabidopsis thaliana* (16), suggesting that the exocyst is involved in the secretion of "compatibility factors" to the stigma surface (13). Together, these results suggest a model of SRK signaling in which the activated SRK, possibly together with MLPK, results in recruitment and phosphorylation of ARC1, which then targets Exo70A1 for ubiquitination and degradation, thereby precluding the secretion of compatibility factors and causing inhibition of pollen hydration, germination, and tube growth (13). Because a stigma epidermal cell can inhibit a self pollen grain while allowing the growth of a nonself pollen tube, this model requires that ARC1-mediated degradation of Exo70A1 and the resulting depletion of compatibility factors be confined to the region subtending the site of pollen–stigma contact, a process that has yet to be demonstrated.

The self-fertile model plant *A. thaliana* lacks functional *S* haplotypes (17–20); however, it can be made to express SI on transformation with any of several *SRK-SCR* gene pairs isolated from its close self-incompatible relatives *Arabidopsis lyrata* and *Capsella grandiflora* (21–23). *SRK-SCR* transformants of some *A. thaliana* accessions, such as Col-0, exhibit transient SI (i.e., their stigmas express a robust SI response during a narrow developmental window, but subsequently lose their ability to inhibit self pollen) (22, 23) due to the presence in these accessions of a hypomorphic allele of a modifier locus that causes reduced levels of SRK at late stages of stigma development (24). In contrast, *SRK-SCR* transformants of other accessions, such as C24 and Cvi-0, express developmentally stable SI (17, 23). Notably, the SI response of *A. thaliana SRK-SCR* transformants, whether stable or transient, is indistinguishable from the SI response of naturally self-incompatible *A. lyrata*, *C. grandiflora*, and *Brassica* species; an *A. thaliana* stigma that expresses adequate levels of SRK will inhibit the germination and tube growth of self pollen and typically will allow the growth of up to five self pollen

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tubes, while supporting abundant WT pollen tube growth. The fact that *A. thaliana* can express a robust SI response on transformation with just the *SRK* and *SCR* gene pairs demonstrates that all other factors required for SI, including components of the SI signaling pathway, have been maintained in the species. Thus, the *A. thaliana SRK-SCR* self-incompatible model is an excellent platform for analysis of the SRK-mediated signaling that underlies crucifer SI.

Highly polymorphic and physically linked *SRK* and *SCR* genes have been identified in all self-incompatible members of the Brassicaceae analyzed to date, including *A. lyrata*, *Arabidopsis halleri*, and *C. grandiflora* (21–23). It thus stands to reason that the SRK-mediated signaling pathway would be conserved across the Brassicaceae, and that orthologs of any downstream effectors of SRK signaling identified in *Brassica* species also would function in the SI response of other self-incompatible species of the Brassicaceae. Here we report on our use of *A. thaliana SRK-SCR* plants to test the aforementioned model of SI signaling, and in particular the involvement in SI of the *A. thaliana* orthologs of *Brassica MLPK*, *ARC1*, and *Exo70A1.* Our results do not support a role for these genes in *A. thaliana* SI. We discuss the implications of these results for our understanding of the SI signaling pathway.

Results and Discussion

Inactivation or Down-Regulation of *AtAPK1b* and *AtAPK1a* Does Not Disrupt the SI Response in *A. thaliana SRKb-SCRb* Plants. The *AtAPK1b* (At2g28930) gene is considered to be the ortholog of the *B. rapa MLPK* (*BrMLPK*) gene. Both genes belong to the RLCK VII subfamily of protein kinases, and of all *A. thaliana* members of this subfamily, *AtAPK1b* shares the greatest sequence identity with *BrMLPK* (*ca*. 76% at the amino acid level) (Table S1). Furthermore, the *A. thaliana* chromosomal region containing this gene shows high synteny with the *Brassica* chromosomal region that encompasses *BrMLPK* (Fig. 1*A*). Also like *BrMLPK* (9), *AtAPK1b* exhibits alternative use of the first exon and is predicted to produce transcripts encoding two distinct protein products (Fig. 1*B*; http://www.arabidopsis.org/servlets/TairObject?id=33491&type=locus).

To test the involvement of *AtAPK1b* in SI, we obtained a Col-0 strain carrying a T-DNA insertion in the gene. This strain (Salk_055314) produces no detectable *AtAPK1b* transcripts in either leaves or stigmas (9, 25). This strain was crossed with a Col-0 plant homozygous for the *A. lyrata*-derived *SRKb-SCRb* transgenes (23), which expresses an intense SI reaction in stigmas from stage-13 and early stage-14 floral buds (22). F_2 plants were generated, and the accumulation of *AtAPK1b* transcripts in stigmas was analyzed. As shown in Fig. 1*C* and Table S2, *AtAPK1b* transcripts were not detected in plants that inherited the *SRKb-SCRb* transgenes and were homozygous for the T-DNA insertion, based on segregation analysis in F_3 progenies (e.g., plants 8, 25, and 27). In contrast, *AtAPK1b* transcripts were readily detected in plants containing the *SRKb-SCRb* transgenes and lacking the *AtAPK1b* T-DNA insertion (e.g., plants 5, 18, and 20). Pollination assays showed that all of these plants expressed robust SI in stage-13 stigmas (the stage at which the SI response of Col-0[*SRK-SCR*] plants is most strongly expressed),

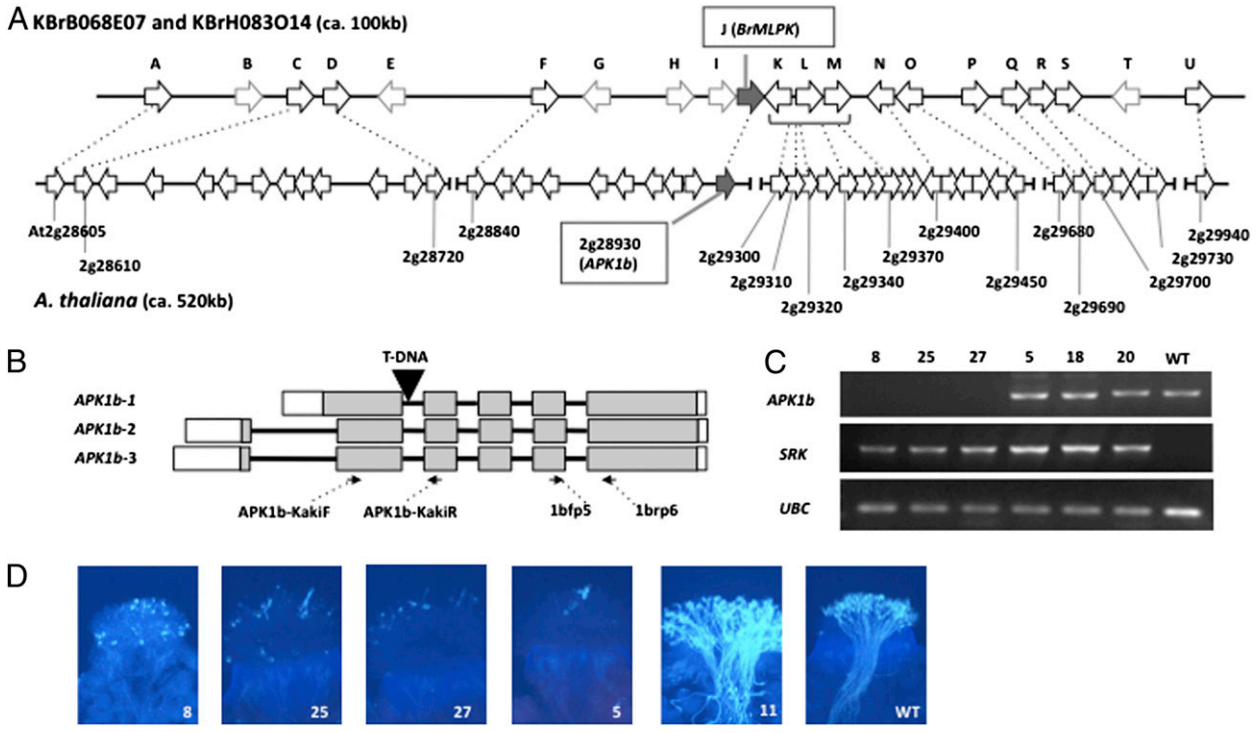

Fig. 1. Functional analysis of a T-DNA insertion allele of *AtAPK1b*. (*A*) Synteny of the *Brassica* and *A. thaliana* chromosomal regions containing *MLPK* and *APK1b*, respectively. E-values are listed in Table S5. (*B*) Structure of the *AtAPK1b* gene showing the two alternative transcript forms produced by the gene and the location of the T-DNA insertion in the SALK_055314 strain. The boxes indicate exons; shaded boxes represent protein-coding regions, and white boxes designate untranslated regions. The arrows below the diagram indicate the location of the primers (F and R) used for RT-PCR. (*C*) RT-PCR analysis of stigma RNA isolated from F_2 plants derived from a cross of the SALK_055314 T-DNA insertion strain and a Col-0[*SRKb-SCRb*] plant. Plants 8, 25, and 27 are homozygous for the T-DNA allele and do not express *AtAPK1b* transcripts, whereas plants 5, 18, and 20 lack the T-DNA insertion and express *AtAPK1b* transcripts at levels equivalent to those in WT Col-0. All of these F_2 plants express *SRKb* transcripts. *UBC* transcripts were used as a control. (*D*) SI phenotype of F_2 plants. Stigmas from stage 13 floral buds were pollinated with SCRb-expressing pollen. Plants 8, 25, and 27, which are homozygous for the T-DNA insertion in *AtAPK1b* and contain *SRKb-SCRb,* exhibit as robust an SI response as that observed in plant 5, which produces *AtAPK1b* transcripts and contains *SRKb-SCRb*. In contrast, plant 11, which is homozygous for the T-DNA allele but lacks *SRKb-SCRb,* did not exhibit SI, similar to WT Col-0.

and no attenuation of SI was observed in plants homozygous for the T-DNA insertion relative to sibs lacking the insertion allele (Fig. 1*D*). This result indicates that *AtAPK1b* is not required for the SI response of *A. thaliana.*

The *A. thaliana* genome contains a gene, *AtAPK1a* (At1g07570), which exhibits 82.3% amino acid sequence identity to *AtAPK1b*. *AtAPK1a* is unlinked to *AtAPK1b* and is located on chromosome 1 (Fig. 2*A*), and it produces three transcript forms predicted to encode distinct protein products (Fig. 2*B*; www.arabidopsis.org/servlets/TairObject?id=29045&type=locus). However, unlike *BrMLPK* and *AtAPK1b, AtAPK1a* is not preferentially expressed in stigmas (9), and it is poorly expressed in these structures; *AtAPK1a (APK1a-1*, *-2*, and *-3*) transcripts levels are 12.3-fold lower than *AtAPK1b (APK1b-1*, *-2*, and *-3*) transcripts (Fig. 2*C*). Furthermore, *B. rapa* contains a transcribed gene that exhibits 86.1% amino acid sequence identity to *AtAPK1a.* This gene is located on BAC AC172873 (KBrH010M08), which is derived from a chromosomal region showing extensive synteny with the *AtAPK1a*-spanning region of the *A. thaliana* genome (Fig. 2*A*), and it clearly does not function redundantly with *BrMLPK*, because it cannot overcome the self-fertility phenotype effected by homozygosity for the *mod* mutation. Together, these features indicate that *AtAPK1a* is not the ortholog of *BrMLPK.*

Nevertheless, we explored the possibility that *AtAPK1a* might have assumed the function of *AtAPK1b* in *A. thaliana* SI. Several T-DNA strains annotated as containing insertions in *AtAPK1a* (Salk_56259, Salk_96327, and Salk_22547) were obtained, but all of these strains still expressed *AtAPK1a* transcripts at appreciable levels. Therefore, we attempted to suppress expression of *AtAPK1a* in stigmas of the C24 accession using artificial miRNA (26) driven by the promoter of the *AtS1* gene, which is active specifically in stigma epidermal cells (27). Of several transformants analyzed, a transgenic plant (T45) showed significant reduction in the already-low levels of stigma *AtAPK1a* transcripts (Fig. 2*D*). This plant was crossed to a C24 plant homozygous for the *SRKb-SCRb* transgenes, which exhibits strong and developmentally stable SI in stigmas starting from stage-13 floral buds and into late-stage flowers (23). Among F_2 progenies derived from this cross, several plants that inherited the *SRKb-SCRb* transgenes showed reduced *AtAPK1a* transcript levels relative to WT, with reduction levels ranging from 60% to 40% (Fig. 2*D*). However, all of these *AtAPK1a*-suppressed plants retained the strong and developmentally stable SI phenotype characteristic of C24[*SRKb-SCRb*] plants (Table S2).

***A. thaliana* Ortholog of *Brassica ARC1* Is a Fragmented Pseudogene Lacking Most of the Coding Region.** We identified the *A. thaliana* ortholog of *Brassica ARC1* by comparative analysis of *A. thaliana*, *A. lyrata*, and *B. rapa* genomic sequences. The region of *A. thaliana* chromosome 2 between At2g25910 and At2g35510 was found to share extensive synteny with the *ARC1*-containing region of the *B. rapa* A04 linkage group (28, 29) (Fig. 3*A*). A BLAST search of this region with the *B. napus BnARC1* (AF024625.1) did not retrieve any hits; however, a BLAST search with the *AlARC1* cDNA (XM_002879443.1, which is 73% identical to *BnARC1*), resulted in a hit in the At2g34250-At2g34290 region of the *A. thaliana* Col-0 genome (Fig. 3*B*). Alignment of the Col-0 *ARC1*-related sequences with the *AlARC1* cDNA sequence identified only a highly degraded *ARC1* sequence lacking the initiating methionine codon (Fig. 3*C*) and consisting of three small fragments interspersed with other genes (Fig. 3*B*). These fragments exhibited 90%–100% nucleotide sequence identity with regions spanning nucleotides 4–169, 309–334, and 2101–2152 in *AlARC1* cDNA.

A BLAST search of the C24 genome sequence, which covers 96% of the Col-0 reference genome (ref. 30; available at www.1001genomes.org) also identified a fragmented *ARC1* sequence identical to the Col-0 *ARC1* sequence within Scaffold-1680 (Fig. 3 *B* and *C*). Thus, the *ARC1* gene of *A. thaliana* is a highly

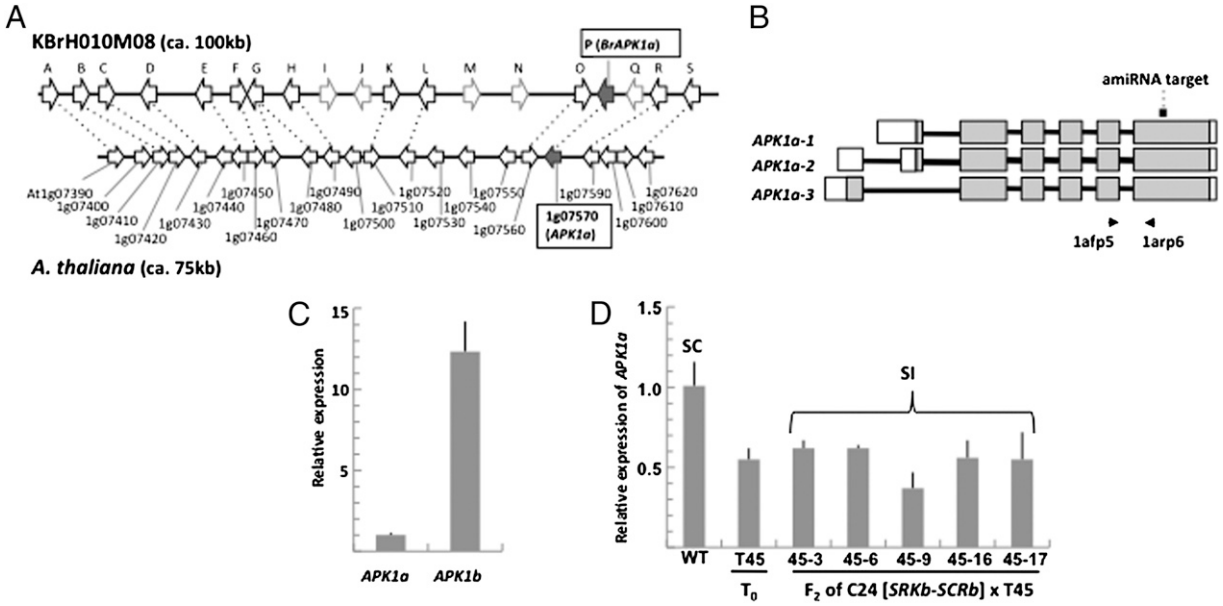

Fig. 2. Expression analysis of *AtAPK1a*-suppressed plants. (*A*) Synteny of *APK1a*-containing chromosomal regions in *Brassica* and *A. thaliana*. E-values are listed in Table S5. (*B*) Structure of the *AtAPK1a* gene showing its three alternative transcript forms and the location of the sequence targeted by the artificial microRNA (amiRNA target) designed to down-regulate the gene. The arrows below the diagram indicate the location of the primer pairs used for transcript quantitation. (*C*) Quantitative real-time PCR analysis of *AtAPK1b* and *AtAPK1a* transcripts in the stigmas of WT C24 stigmas. (*D*) Quantitative real-time PCR analysis of *AtAPK1a* transcripts in the stigmas of WT C24, the T45 *AtAPK1a*-suppressed primary transformant, and F_2 plants derived from crossing T45 with C24[*SRKb-SCRb*]. All of the F_2 plants analyzed contain the *SRKb-SCRb* transgenes but express *AtAPK1a* transcripts at lower levels than WT. For all real-time PCR experiments, relative gene expression levels were generated by normalizing the signal using the ubiquitin-conjugating (*UBC*) gene (At5g25760). SDs from triplicate experiments are indicated by error bars.

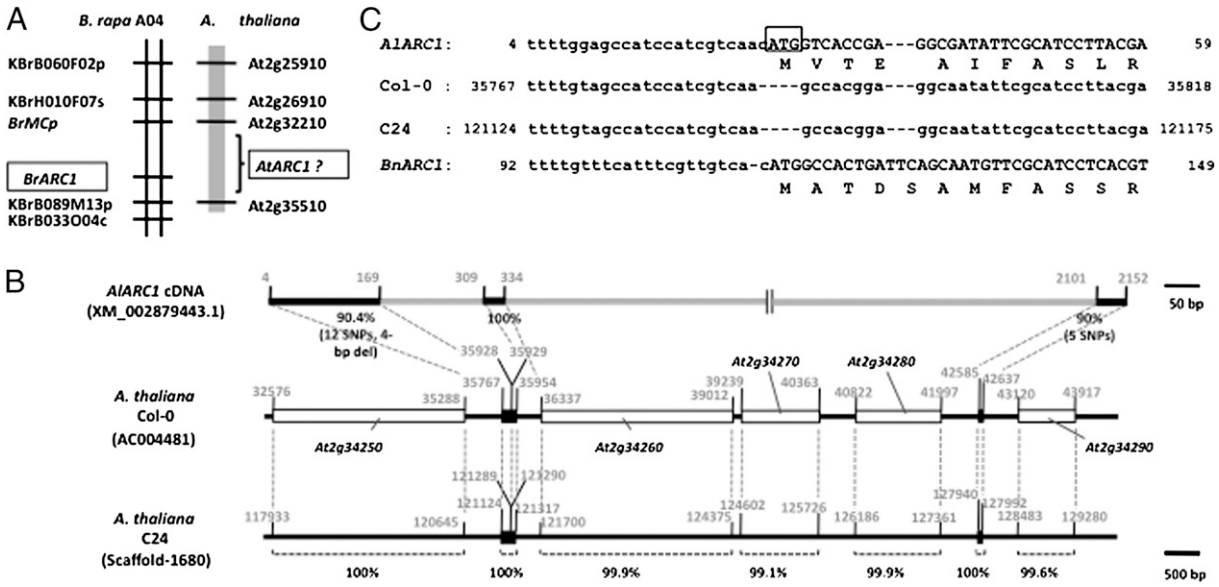

Fig. 3. The *A. thaliana* ortholog of *Brassica ARC1*. (*A*) Synteny of the *BrARC1*-containing region of *B. rapa* A04 linkage group with the region of *A. thaliana* chromosome 2 flanked by At2g25910 and At2g35510. (*B*) Structure of *A. thaliana ARC1* sequences in the Col-0 and C24 accessions compared with *A. lyrata ARC1* cDNA. Note the highly degraded structure of *AtARC1* in both Col-0 and C24. (*C*) Alignment of the 5′ end of the *AlARC1* cDNA sequence with the corresponding region of *AtARC1* from Col-0 and C24, and of *BnARC1*. Note the absence of the initiating methionine codon in the *AtARC1* sequences.

degraded pseudogene even in C24, an accession in which *SRK-SCR* transformants express a robust and developmentally stable SI. It is also unlikely that the *A. thaliana* genome contains another *ARC1*-like gene that functions in SI. Among the apparently intact *PUB* genes in *A. thaliana*, *AtPUB17* (At1g29340) is the most similar to *BnARC1* (58% amino acid sequence identity; Table S1). However, this gene shares 96.6% and 85.9% amino acid sequence identity, respectively, with sequences that are the presumed orthologs of *AtPUB17*: an *A. lyrata* sequence (XM_002890762.1) and a *B. rapa* sequence within BAC AC189240 (KBrB016E20), both of which are located in chromosomal regions syntenous with each other and with the *AtPUB17* region of *A. thaliana,* but not with the *ARC1* regions of *A. thaliana*, *A. lyrata*, and *B. rapa* (Fig. S1). Furthermore, we had previously assayed a T-DNA knockout allele of *AtPUB17* and found that this gene has no role in SI (25). Thus, we found no evidence for the involvement of an *ARC1*-like gene in the *A. thaliana* SI response.

Overexpression of AtExo70A1 Does Not Weaken SI in *SRKb-SCRb* Plants. Despite the lack of a functional *ARC1* gene in *A. thaliana,* we tested the potential involvement of *AtExo70A1* in the SI response of C24 plants expressing *SRKb* and *SCRb* transgenes. We transformed C24 plants with a construct, designated *AtS1pr::AtExo70A1*, consisting of the *AtS1* promoter fused to the transcriptional unit of *AtExo70A1,* which is the likely *A. thaliana* ortholog of *Brassica Exo70A1* based on synteny and sequence identity (Fig. S2 and Table S1). Several transformants were generated and crossed to C24 plants carrying *SRKb* and *SCRb* transgenes. Because *Exo70A1*, as a component of the exocyst, functions in secretion, we thought that modifying its expression in stigmas might possibly exert its reported weakening of SI by affecting SRK protein accumulation or targeting either directly or indirectly. Therefore, to allow detection of SRKb protein, we used an *SRK-SCR* construct designed to express an SRKb molecule containing an N-terminal YFP tag from the *AtS1* promoter. The YFP:SRKb fusion produced by the *AtS1pr::YFP:SRKb/SCRb* transgene is functional, as demonstrated by the SI response observed in pollination assays (Table S3). To test the involvement of *AtExo70A1* in SI, we focused on an *AtS1pr::AtExo70A1* transformant that carried a single integration of the *AtS1pr::AtExo70A1* transgene and exhibited an ∼15-fold increase in stigma *AtExo70A1* transcripts relative to WT. This strain was crossed with a plant homozygous for *AtS1pr:: YFP:SRKb/SCRb* transgenes and to a plant homozygous for the *SRKb-SCRb* transgenes, which consist of native promoters driving intact coding regions. Although the levels of *BnExo70A1* transcripts reported to cause weakening of SI in *B. napus* have not been given (13), we hypothesized that a more than 10-fold increase in *AtExo70A1* transcript levels should provide an adequate test of the role of *AtExo70A1* in the SI response of *A. thaliana* SRK-expressing plants.

We analyzed 64 F_2 plants derived from the *AtS1pr::YFP:SRKb/SCRb* × *AtS1pr::AtExo70A1* cross and 56 F_2 plants derived from the *SRKb-SCRb* × *AtS1pr::AtExo70A1* cross. Among hygromycin-resistant F_2 progenies, the numbers of plants containing the *SRKb/SCRb* and *AtS1pr::AtExo70A1* transgenes, *SRKb/SCRb* alone, and *AtS1pr::AtExo70A1* alone were 27:12:25 in the former cross and 27:10:19 in the latter cross. In both crosses, F_1 plants and all F_2 plants that inherited the *SRKb* transgenes, regardless of whether or not they contained the *AtS1pr::AtExo70A1* transgene, exhibited an intense SI response on pollination assays (Table S3), and we did not observe any promotion of pollen hydration and pollen tube penetration into the stigma epidermal cell wall in these assays.

As expected based on the intense SI response observed in these plants, *AtExo70A1* overexpression had no significant effect on SRKb protein levels (Fig. 4*A*). However, the stigmas of these plants exhibited significantly increased *AtExo70A1* transcript levels (Fig. 4 *B* and *C*). In stigmas of F_1 plants derived from both crosses, *AtExo70A1* transcript levels were more than fivefold higher than in the stigmas of WT untransformed control plants. Among F_2 plants, the stigmas of plants that inherited only the *SRKb* transgene and lacked *AtS1pr::AtExo70A1* (e.g., plant 9 in Fig. 4*B* and plant 6 in Fig. 4*C*) accumulated *Exo70A1* transcripts at levels equivalent to the low levels observed in untransformed WT control plants, as expected. On the other hand, plants that inherited the *AtS1pr::AtExo70A1* transgene alone (e.g., plant 5 in Fig. 4*B* and plant 8 in Fig. 4*C*) or both the *SRKb* transgene and *AtS1pr::AtExo70A1* (e.g., plants 4, 6, 8, 13, and 14 in Fig. 4*B* and plant 7 in Fig. 4*C*) exhibited increased *AtExo70A1* transcript

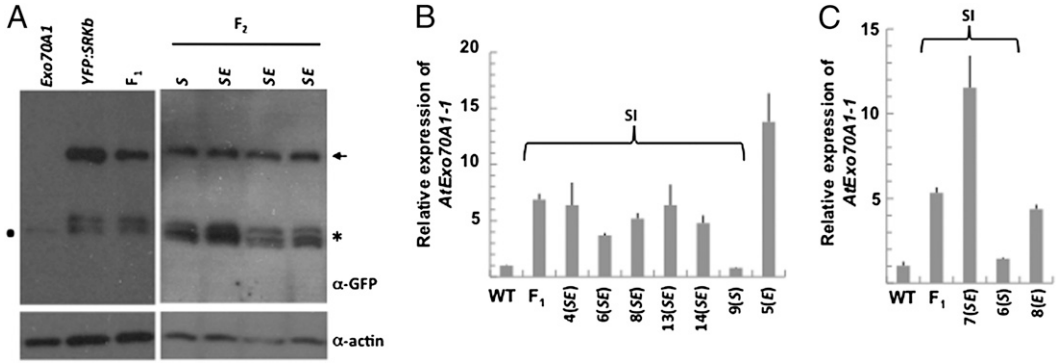

Fig. 4. Expression analysis of stigmas from progenies of the *AtS1pr::YFP:SRKb/SCRb* × *AtS1pr::AtExo70A1* and *SRKb-SCRb* × *AtS1pr::AtExo70A1* crosses. (*A* and *B*) Analysis of the *AtS1pr::YFP:SRKb/SCRb* × *AtS1pr::AtExo70A1* cross. (*A*) Stigma protein gel blot analysis of YFP:SRKb using anti-GFP monoclonal antibodies and anti-actin antibodies to detect actin as a loading control. The arrow indicates the full-length YFP:SRKb protein, and the asterisk indicates the smaller protein species that correspond to the extracellular domain of SRKb and are typically produced by *SRK* genes. The dot indicates a background protein detected in *A. thaliana* stigmas by the anti-GFP monoclonal antibody. (*B*) Quantitative real-time PCR analysis of *AtExo70A1* transcripts in stigmas from WT C24, an F_1 plant, and representative F_2 plants. For each F_2 plant, the presence or absence of each transgene is indicated: SE, plants containing both *AtS1pr::YFP:SRKb/SCRb* and *AtS1pr::AtExo70A*; S, plants containing only *AtS1pr::YFP:SRKb/SCRb*; E, plants containing only *AtS1pr::AtExo70A*. The variation in the relative levels of *AtExo70A1* transcripts among F_2 plants is due primarily to differences in gene dosage. Of all F_2 plants shown, only plant 5 was homozygous for the *AtS1pr::AtExo70A* transgene. Other sources of variability are stochastic variation in transgene expression between plants and differences in the developmental age of stigmas included in each sample. (*C*) Quantitative real-time PCR of *AtExo70A* in progenies of the *SRKb-SCRb* × *AtS1pr::AtExo70A1* cross. Relative expression levels in stigmas from WT C24, an F_1 plant, and representative F_2 plants are shown. The presence or absence of the transgenes in each F_2 plant are as shown in *B*. Normalization of signals and SDs are as shown in Fig. 2.

levels relative to plants lacking this transgene, ranging from ~5-fold to >10-fold. This variability in transcript levels reflects the dosage of the *AtS1pr::AtExo70A1* transgene and possibly differences in the developmental age of stigmas used for transcript quantitation. The *AtS1* promoter is developmentally regulated during stigma development, and stigmas at somewhat less advanced stages of maturity might have been included in some of the 25 stigma samples used in our analysis, which might have caused us to underestimate *AtExo70A1* transcript levels in some plants. In any case, our results indicate that overexpression of *AtExo70A1* transcripts in SRKb-expressing stigmas, even at >10-fold higher levels than those effected by the endogenous *AtExo70A1* transgene, has no significant effect on the SI response.

Conclusions

SRK-SCR transformants of *A. thaliana* express an SI response that is as strong as that observed in naturally self-incompatible *A. lyrata* or *Brassica* species, either throughout stigma development, as in the C24 accession, or during a narrow window of stigma development, as in the Col-0 accession. This fact implies that the SI signaling pathway triggered by the SRK–SCR interaction, which culminates in the inhibition of self pollen, has been maintained in this species since the switch to self-fertility occurred. Thus, transgenic *A. thaliana SRK-SCR* plants are a suitable platform for assaying the involvement of specific molecules in the SI response. This study demonstrates that none of the *A. thaliana* orthologs (or closest paralogs) of the genes that have been implicated in the SI response of *Brassica* species function in the SI response of *A. thaliana SRK-SCR* plants. First, a null allele of *AtAPK1b* does not cause loss or weakening of SI in *SRKb*-expressing stigmas, unlike the reported loss of SI resulting from a mutation in the *B. rapa MLPK* gene. Second, the *A. thaliana* ortholog of the *B. napus ARC1* gene is clearly a pseudogene, and a null allele of its closest paralog, *PUB17,* does not disrupt SI in *SRK*-expressing stigmas (25). Finally, the observation that overexpression of *AtExo70A1* in SRK-expressing stigmas did not produce the weakening of SI reported on overexpression of *BnExo70A1* in *B. napus* indicates that the ARC1/Exo70A1-based model of SI signaling proposed for *Brassica* does not apply to *A. thaliana SRK-SCR* plants.

The discrepancies observed between *Brassica* and *A. thaliana* might suggest that the SRK–SCR interaction triggers different signaling cascades in the two taxa. Alternatively, the inhibition of self pollen in SI might be the cumulative outcome of multiple SRK-mediated signaling pathways (31) that are differentially utilized in different species of the Brassicaceae. Neither of these scenarios seems probable, however. Signaling pathways that underlie the same process are typically highly conserved over long evolutionary time spans (>100 million years), often across kingdoms (32–34). Indeed, it is precisely this conservation that justifies the use of model systems, such as *A. thaliana,* to understand biological processes in less tractable nonmodel organisms. It therefore seems unlikely that SRK-mediated signaling was drastically modified in the ~14–20 million years that separate *Brassica* and *Arabidopsis* (35, 36).

A possible explanation for the observed discrepancies is that other genes in the 46-member *RLCK VII* family and 42-member *PUB* family of *A. thaliana* (Table S1) might have assumed the role proposed for *MLPK* and *ARC1* (although the putative ARC1-like protein would have to act on a target other than Exo70A1). Additional data are needed to confirm the roles of *ARC1* and *MLPK* in *Brassica* SI. At present, the possibility exists that the weakening of SI observed in *B. napus* plants transformed with the antisense *ARC1* transgene (11) might have been caused by down-regulation not of *BnARC1* but of a related gene. Supporting this possibility, the stigmas of a *B. rapa* strain homozygous for a hypomorphic *ARC1* allele exhibit no weakening of SI despite expressing 10-fold less *ARC1* transcripts than other self-incompatible *B. rapa* strains (25). In the case of *MLPK*, it is important to prove that this gene does indeed function in *Brassica* SI through stable complementation of the *mod* mutation, rather than through the less robust method of transient expression via particle bombardment of stigmas (7). Resolution of the discrepancies revealed by the results reported here clearly requires an evaluation of the roles in SI of additional *A. thaliana MLPK*- and *ARC1*-like genes and reexamination of the roles of *MLPK*, *ARC1*, and *Exo70A1* in *Brassica* species.

Materials and Methods

The experiments described herein used *A. thaliana* Col-0 or C24 plants transformed with the *SRKb-SCRb* genes isolated from the *A. lyrata Sb* hap-

lotype (18). Because T-DNA insertional mutants are typically available in the Col-0 accession, we used Col-0[*SRKb-SCRb*] transformants to assess the effect of T-DNA insertional mutations on SI. However, for experiments involving transgenic down-regulation or overexpression of candidate genes, we used *SRKb-SCRb* transformants of the C24 accession, which allowed us to monitor the strength of SI by both microscopic examination of pollen tube growth and the amount of seed set.

The *SRK-SCR* transgenes and T-DNA mutant strains used in this study, along with the constructs designed to manipulate candidate gene expression, are described in *SI Materials and Methods*. The methods used for plant transformation, pollination assays, DNA gel blot analysis, immunoblot analysis, and expression analysis are also discussed in *SI Materials and Methods*. The primers used for genotyping by PCR and for expression analysis are listed in Table S4.

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