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The *ARC1* E3 Ligase Gene Is Frequently Deleted in Self-Compatible Brassicaceae Species and Has a Conserved Role in *Arabidopsis lyrata* Self-Pollen Rejection[W]

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Self-pollen rejection is an important reproductive regulator in flowering plants, and several different intercellular signaling systems have evolved to elicit this response. In the Brassicaceae, the self-incompatibility system is mediated by the pollen S-locus Cys-Rich/S-locus Protein11 (SCR/SP11) ligand and the pistil *S* Receptor Kinase (SRK). While the SCR/SP11-SRK recognition system has been identified in several species across the Brassicaceae, less is known about the conservation of the SRK-activated cellular responses in the stigma, following self-pollen contact. The ARM Repeat Containing1 (ARC1) E3 ubiquitin ligase functions downstream of SRK for the self-incompatibility response in *Brassica*, but it has been suggested that ARC1 is not required in *Arabidopsis* species. Here, we surveyed the presence of *ARC1* orthologs in several recently sequenced genomes from Brassicaceae species that had diversified ~20 to 40 million years ago. Surprisingly, the *ARC1* gene was deleted in several species that had lost the self-incompatibility trait, suggesting that *ARC1* may lose functionality in the transition to self-mating. To test the requirement of *ARC1* in a self-incompatible *Arabidopsis* species, transgenic *ARC1* RNA interference *Arabidopsis lyrata* plants were generated, and they exhibited reduced self-incompatibility responses resulting in successful fertilization. Thus, this study demonstrates a conserved role for *ARC1* in the self-pollen rejection response within the Brassicaceae.

INTRODUCTION

Many flowering plants have reproductive controls to avoid inbreeding when self-related male pollen appears on the female pistil. Self-incompatibility is one such system as it causes self-pollen rejection, and in so doing, allows pollination and fertilization by other genetically distinct plants. In the Brassicaceae (mustard family), the self-incompatibility response is regulated by the pistil-specific *S* Receptor Kinase (SRK) and the pollen S-locus Cys-Rich/S-locus Protein11 (SCR/SP11) ligand (Schopfer et al., 1999; Takasaki et al., 2000; Takayama et al., 2000; Silva et al., 2001). While polymorphic SRK and SCR/SP11 genes have been identified in several Brassicaceae species, including *Arabidopsis lyrata* (Kusaba et al., 2001; Schierup et al., 2001) and *Capsella grandiflora* (Paetsch et al., 2006; Boggs et al., 2009; Guo et al., 2009), their functions have been best defined in *Brassica* crop species (reviewed in Ivanov et al., 2010; Tantikanjana et al., 2010; Iwano and Takayama, 2012). Following self-pollination, the pollen grain contacts a stigmatic papilla at the top of the pistil, and the SCR/SP11 ligand on the pollen binds to the haplotype-specific SRK present in the papilla (Kachroo et al., 2001; Takayama et al., 2001; Shimosato et al., 2007). SRK then activates a pollen rejection pathway to rapidly block pollen hydration or pollen tube penetration into the stigmatic surface (reviewed in Chapman and Goring, 2010; Ivanov et al., 2010).

Downstream components required for self-pollen rejection in the SRK signaling pathway have also been identified in *Brassica* species, and two positive regulators that interact with SRK are the *M*-locus Protein Kinase (MLPK) and the ARM-Repeat Containing1 (ARC1) E3 ubiquitin ligase. *Brassica rapa* MLPK is required for the self-incompatibility response, and *mlpk* mutants are unable to reject self-pollen (Murase et al., 2004). MLPK belongs to the receptor-like cytoplasmic kinase subfamily and is located in the plasma membrane with SRK (Murase et al., 2004). MLPK has been shown to interact transiently with SRK at the plasma membrane, potentially with the inactive SRK complex before activation via SCR/SP11 (Kakita et al., 2007a, 2007b). *B. napus* ARC1 was originally identified through a yeast two-hybrid screen and binds to the phosphorylated SRK kinase through its C-terminal Armadillo (ARM) repeats (Gu et al., 1998). ARC1 is able to be phosphorylated in vitro by both SRK and MLPK, suggesting that perhaps ARC1 may be recruited to a SRK-MLPK complex at the plasma membrane (Samuel et al., 2008). ARC1 is a member of the Plant U-box (PUB) family of E3 ubiquitin ligases and is required for the self-incompatibility response (Stone et al., 1999, 2003; Azevedo et al., 2001). The U-box domain in ARC1 classifies it as an E3 ligase and has been shown to bind the E2 conjugating enzyme in the closely related

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Arabidopsis thaliana PUB14 protein (Stone et al., 2003; Andersen et al., 2004). In addition to the ARM repeat domain and the U-box domain, ARC1 contains a U-box N-terminal domain (UND) domain (Stone et al., 2003; Mudgil et al., 2004; Samuel et al., 2006). The UND domain is found in a subset of PUB proteins and may participate in protein–protein interactions (Mudgil et al., 2004; Samuel et al., 2006). More recently, the ARC1 UND domain was used in a yeast two-hybrid screen resulting in the identification of Exo70A1 as a target for ARC1 (Samuel et al., 2009). Exo70A1 is required in the stigma to accept compatible pollen and is proposed to function as part of the exocyst complex to deliver secretory vesicles to the stigmatic papillar plasma membrane under the pollen contact site, thereby providing resources to the compatible pollen for hydration and pollen tube penetration into the stigmatic surface. ARC1 is predicted to promote self-pollen rejection in the self-incompatibility response by negatively regulating Exo70A1 and blocking the delivery of secretory vesicles to the pollen contact site (Samuel et al., 2009).

Outside of the *Brassica* spp in the Brassicaceae, several population and functional studies have been performed using *A. lyrata* and *A. thaliana*. Surveys on self-incompatible *A. lyrata* populations across North America and Europe have shown a similar diversity in the polymorphic *SCR*/*SP11* and *SRK* genes when compared with *Brassica* spp (Schierup et al., 2001, 2006; Mable et al., 2005; Prigoda et al., 2005; Mable and Adam, 2007). *A. thaliana* is a fully self-compatible species, and the loss of *A. thaliana SCR*/*SP11* and *SRK* genes (and self-incompatibility) has been studied in various ecotypes through analyses on the genomic region encompassing these genes (Kusaba et al., 2001; Bechsgaard et al., 2006; Tang et al., 2007; Shimizu et al., 2008; Tsuchimatsu et al., 2010; Guo et al., 2011). Transgenic studies with *SCR*/*SP11* and *SRK* genes have also been conducted to restore self-incompatibility in *A. thaliana*. The transformation of *A. lyrata SRK* and *SCR*/*SP11* genes has been reported to confer self-incompatibility in some *A. thaliana* ecotypes, but not all ecotypes tested (Nasrallah et al., 2004; Boggs et al., 2009). Additionally, the transformation of a functional $SCR/SP11_a$ allele into the *A. thaliana* Wei-1 ecotype, which has an intact endogenous SRK_a allele, resulted in a self-incompatibility phenotype (Tsuchimatsu et al., 2010). In these *A. thaliana* transgenic experiments, the signaling pathway activated by SRK is unknown as MLPK and ARC1 do not appear to function in the reconstituted self-incompatibility response in transgenic *A. thaliana* (Rea et al., 2010; Kitashiba et al., 2011). For example, while an ARC1 ortholog has been identified in the *A. lyrata* genome, ARC1 exists as a pseudogene in *A. thaliana* (Kitashiba et al., 2011). Thus, Kitashiba et al. (2011) proposed that an alternate SRK signaling pathway functions in *Arabidopsis* species, perhaps related to the fact that *Brassica* and *Arabidopsis* belong to different lineages that have been estimated to have diversified ~20 to 40 million years ago (Franzke et al., 2011). However, it is difficult to know if the SRK signaling pathway(s) has become altered in *A. thaliana* with the transition to self-compatibility. In this study, we set out to investigate the presence of *ARC1* orthologs in other members of the Brassicaceae and use RNA interference (RNAi) suppression to examine the role of *ARC1* in the naturally occurring self-incompatible *A. lyrata*. The growing availability of sequenced genomes for members of the Brassicaceae allowed us to take an evolutionary genetics approach to conduct a more widespread examination of the *ARC1* gene in regards to self-incompatibility. Our results indicate that *ARC1* is frequently deleted in self-compatible Brassicaceae species and that *ARC1* is required for the self-incompatibility response in *A. lyrata*.

RESULTS

Synteny of the *ARC1* and *PUB17* Genomic Regions in the Brassicaceae

Brassica ARC1 is a member of PUB family of E3 ubiquitin ligases and was previously found to be most closely related to *A. thaliana* PUB17 (At1g29340), a protein involved in plant defense responses (Azevedo et al., 2001; Yang et al., 2006). *Brassica ARC1* was initially thought to be an ortholog of *A. thaliana PUB17*, but more recently, a potential *Brassica ARC1* ortholog was identified in the *A. lyrata* genome, a self-incompatible species, while a corresponding pseudogene was found in the self-compatible *A. thaliana* Columbia-0 (Col-0) and C24 ecotypes (Rea et al., 2010; Kitashiba et al., 2011). *A. lyrata* is closely related to *A. thaliana* with speciation occurring ~5 to 10 million years ago (Koch et al., 2001; Charlesworth and Vekemans, 2005; Bailey et al., 2006; Wang et al., 2011), so this raised the question of whether *ARC1* orthologs have been lost in other Brassicaceae species and whether this loss was correlated with self-compatibility. We conducted a broader survey of potential *ARC1* orthologs using genome sequences now available for several other Brassicaceae species: *B. rapa* (Wang et al., 2011); *Thellungiella parvula* (Dassanayake et al., 2011); *Capsella rubella* (*Capsella rubella* Genome Project 2011; www.phytozome.net/capsella); *Thellungiella halophila* (*Thellungiella halophila* Genome Project 2011; www.phytozome.net/thellungiella); and *Sisymbrium irio*, *Leavenworthia alabamica* race a4, and *Aethionema arabicum* (Value-Directed Evolutionary Genomics [VEGI] consortium; biology.mcgill.ca/vegi). Of the aforementioned species, *B. rapa* is another self-incompatible species, while the remaining sequenced genomes were from self-compatible plants. With *PUB17* being the most closely related *ARC1* paralog, its presence or absence was also surveyed.

Predicted *ARC1* and *PUB17* genomic regions were first examined by synteny between *A. lyrata*, *T. halophila*, *A. thaliana*, *S. irio*, *L. alabamica*, *T. parvula*, *B. rapa*, and *A. arabicum* using the whole-genome orthologous alignment conducted by the VEGI consortium (http://grandiflora.eeb.utoronto.ca:8086) (Figure 1A). The most striking observation is that the predicted *ARC1* gene is not found in five of the genomes (*T. halophila*, *A. thaliana*, *L. alabamica*, *T. parvula*, and *A. arabicum*), while the *PUB17* gene is highly conserved in all eight genomes. This is unlikely to be an artifact of incomplete genome assemblies, as the flanking genes were identified in a syntenic block in all cases. Furthermore, all of the species that appear to be missing a predicted *ARC1* gene are self-compatible, and *S. irio* is the only self-compatible species in this set that shows conservation to *A. lyrata ARC1*. Interestingly, a gene flanking *ARC1*, the predicted *DUF1296-related* gene, is also absent in four of the genomes. Figure 1B illustrates the predicted genes present in the *ARC1* and *PUB17* genomic regions assembled from annotated

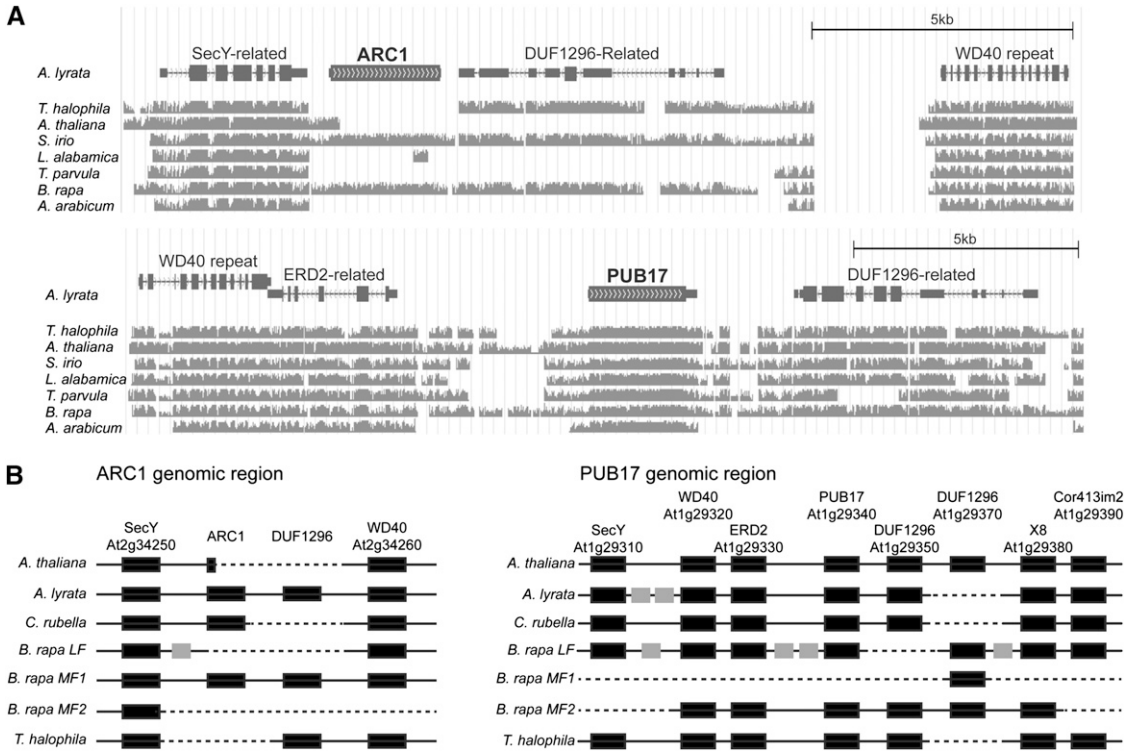

Figure 1. Synteny of the *ARC1* and *PUB17* Genomic Regions in the Brassicaceae.

(A) Synteny in the predicted *A. lyrata ARC1* and *PUB17* genomic regions to *T. halophila*, *A. thaliana*, *S. irio*, *L. alabamica*, *T. parvula*, *B. rapa*, and *A. arabicum*. This image was generated in the University of California, Santa Cruz genome browser (Fujita et al., 2011) using a nine-species whole-genome orthologous alignment assembled by the VEGI consortium (http://grandiflora.eeb.utoronto.ca:8086). The *A. lyrata* annotated genes are shown at the top, and the degrees of similarity to the *A. lyrata ARC1* or *PUB17* genomic regions are shown in gray for each genome. Missing regions relative to the *A. lyrata ARC1* or *PUB17* genomic regions are shown as gaps.

(B) Schematic illustrating the gene conservation in the *ARC1* and *PUB17* genomic regions for *A. thaliana*, *A. lyrata*, *C. rubella*, *B. rapa* (LF, MF1, and MF2 subgenomes), and *T. halophila*. Annotated Brassicaceae genomes in the public genome databases were used to identify predicted genes present in the ARC1 and PUB17 genomic regions. The *A. thaliana* annotated genes are shown at the top, and the black boxes represent conserved orthologs annotated in the respective genomes. Gray boxes represent unconserved genes, and dashed lines represent deletions in the scaffolds.

genomes in the public genome browsers (Tables 1 and 2). The *ARC1* genomic regions can be identified by the flanking *SecY-related* (*A. thaliana* At2g34250) and *WD40-repeat* (*A. thaliana* At2g34260) genes, and these genes were found in a syntenic gene order with *ARC1* missing. This analysis also includes another self-compatible species, *C. rubella*, and three regions from the triplicated *B. rapa* genome. In *A. thaliana*, the first 143 bp of the *ARC1* coding region (with a missing start sequence) are present, but the rest of *ARC1* and the *DUF1296-related* gene are both deleted. Interestingly, the *C. rubella* genome does contain a predicted *ARC1* gene, despite being self-compatible, but the flanking *DUF1296-related* gene is deleted. In *B. rapa*, the *MF1* subgenome has all four genes present, while the *LF* and MF2 subgenomes only have the flanking *SecY-related/WD40-repeat* genes and the *SecY-related* gene, respectively. Finally, as indicated in Figure 1A, the self-compatible *T. halophila* genome is missing *ARC1* in this syntenic region, but still has the other three predicted genes. Thus, from these analyses, five of the seven self-compatible species appear to have lost the *ARC1* ortholog, and *S. irio* is the only self-compatible species that does not appear to have undergone any large deletions in this region. These results seem to suggest a link between the loss of *ARC1* and self-compatibility in these Brassicaceae species.

A similar analysis for the *PUB17* region shows that an ortholog can be readily identified in all the genomes (Figure 1B, Table 2), including two copies in *B. rapa* (*LF* and *MF1* subgenomes). *PUB17* (At1g29340) is flanked on the right by a *DUF1296-related* gene (*A. thaliana* At1g20350), and in both *A. thaliana* (At1g29370) and the *B. rapa LF* subgenome, a second copy of a *DUF1296-related* gene is also present. Further to the right, two more conserved genes (*A. thaliana* At1g29380 and At1g29390) are present across all genomes surveyed. On the left side of *PUB17*, there are three conserved genes (*A. thaliana* At1g29310, At1g29320, and At1g29330) present across all genomes with the exception of the *SecY-related* gene absent from the *B. rapa MF2* subgenome (and the MF1 subgenome that only contains a *DUF1296-related* gene). Thus, in contrast with the *ARC1* genomic region,

Table 1. Gene Identifiers and Accession Numbers for the ARC1 and PUB17 Genomic Regions

Species	Gene Identifiers and Accession Numbers			
A. thaliana	At2g34250; NP_180972	ARC1	DUF1296-related	At2g34260; NP_565782
A. lyrata	482367; XP_002879488	934022; XP_002879489	321144; XP_002881327	934024; XP_002879490
C. rubella	10023123	10025155	–	10023519
B. rapa LF	Bra005441	–	–	Bra005439
B. rapa MF1	Bra021898	Bra021899	Bra021900	Bra021901
T. halophila	10016599	–	10016218	10016919

Dashes represent the absence of a corresponding orthologue in the ARC1 genomic region.

the *PUB17* genomic region is highly conserved and present in all nine Brassicaceae species surveyed. Interestingly, the *PUB17* genomic region also shares some synteny with the *ARC1* genomic region. For example, *DUF1296-related* genes flank both *ARC1* and *PUB17* on the right side, and a *SecY-related* gene appears in the same orientation relative to *ARC1* and *PUB17*. The *WD40-repeat* genes in the two genomic regions are paralogs, but in different positions relative to *ARC1* and *PUB17*. The high degree of similarity of these two genomic regions likely originated from an ancient angiosperm whole-genome duplication (Jiao et al., 2011), followed by *ARC1* and *PUB17* acquiring divergent functions in the Brassicaceae (*ARC1* in self-incompatibility; *PUB17* in plant defense responses; Stone et al., 1999; Yang et al., 2006).

To confirm that the predicted *ARC1* orthologs are in fact most closely related to the originally identified *B. napus* ARC1 (Gu et al., 1998), a phylogenetic analysis was conducted with a subset of eight most closely related U-box proteins, PUB12-19, that share the same domain organization as ARC1: a conserved UND domain followed by the U-box domain and the ARM repeat domain (Mudgil et al., 2004; Samuel et al., 2006). The full-length *A. thaliana* PUB12-PUB19 and *B. napus* ARC1 sequences were used to conduct BLAST analyses of sequenced genomes from *A. lyrata* (Hu et al., 2011), *B. rapa* (Wang et al., 2011), *C. rubella* (www.phytozome.net/capsella), and *T. halophila* (www.phytozome.net/thellungiella). All of the sequences that were identified in the BLAST search were full-length protein sequences that were then used for the phylogenetic analysis. Most species appeared to contain a single-copy gene for each of these PUB proteins (Figure 2, Table 3). The one exception was *B. rapa*, which appeared to have two copies of PUB11, PUB13, PUB16, and PUB17, likely resulting from the triplication of the genome (Wang et al., 2011). Predicted ARC1 proteins from two other self-incompatible plants, *B. oleracea* and *C. grandiflora*, were also included in this analysis. The phylogenetic tree generated by this analysis clearly resolves that the predicted PUB17 proteins are different from the predicted ARC1 proteins. The tree also shows that the ARC1 orthologs form a monophyletic clade, and the *A. lyrata* and *Capsella* ARC1 proteins cluster with the *Brassica* ARC1 proteins. As predicted from the synteny analysis in Figure 1, an ARC1 ortholog was not found in the self-compatible species, *A. thaliana* (Kitashiba et al., 2011) and *T. halophila*. Also, self-compatible *C. rubella* contains a copy of ARC1 that is very closely related to the ARC1 ortholog from the very closely related self-incompatible *C. grandiflora*.

Loss of the *ARC1* Region in *A. thaliana* Is Conserved across All Ecotypes Surveyed

The source of self-compatibility in *A. thaliana* has been attributed to the loss of functional *SRK* and *SCR* genes (Kusaba et al., 2001; Tang et al., 2007; Tsuchimatsu et al., 2010). While *A. lyrata SRK* and *SCR* genes can be transformed into *A. thaliana* to confer self-incompatibility, the strength of the response can be quite variable, with some ecotypes showing a good self-incompatibility response to other ecotypes being completely self-compatible with a wild-type acceptance of pollen grains (Nasrallah et al., 2004; Boggs et al., 2009; Tsuchimatsu et al., 2010). Also, in an extensive survey of different *A. thaliana* ecotypes, several ecotypes were found to contain a functional copy of SRK_a, and varying self-incompatibility phenotypes were observed when these *A. thaliana* pistils were pollinated with *A. halleri* SCR_a pollen (Tsuchimatsu et al., 2010). With *ARC1* being deleted from the *A. thaliana* Col-0 and C24 ecotypes, we were interested to determine if the *ARC1* deletion is more widespread than previously reported (Kitashiba et al., 2011). A genomic PCR screen was conducted on two different natural accession sets: the Nordborg collection (96 ecotypes; Nordborg et al., 2005) and the Beck collection (261 ecotypes; Beck et al., 2008), which cover a wide geographic range of *A. thaliana* ecotypes and include ecotypes carrying a functional copy of SRK_a (Tsuchimatsu et al., 2010). The PCR screen was designed such that if the *ARC1* deletion region is present in an *A. thaliana* ecotype, a PCR product of 1.4 kb in size would be obtained (Figure 3). Thus, this screen served as a quick and straightforward method to examine the genetic diversity of the *ARC1* deletion region across the *A. thaliana* ecotypes. The PCR screen revealed that all of the *A. thaliana* ecotypes surveyed contained the deletion region and therefore lacked a functional copy of *ARC1* (Figure 3; see Supplemental Data Set 1 online). To verify that a similar deletion was present, PCR bands from 10 different *A. thaliana* ecotypes were selected for sequencing: C24, Kro-0, Ler-0, and Mrk-0 from Germany, Ra-0 from France, Br-0 from the Czech Republic, Wei-1 from Switzerland, LL-0 from Spain, Bur-0 from Ireland, Col-0 from the US, and Cvi-0 from Cape Verde Islands. The sequences from the PCR products indicated that the 10 ecotypes shared identical sequences for the ARC1 5′ coding region and the deletion breakpoint (Figure 4). A few single nucleotide polymorphisms were detected between the 10 different ecotypes in the region upstream of the *A. thaliana* ARC1 pseudogene. Thus, *ARC1* has undergone a widespread deletion in

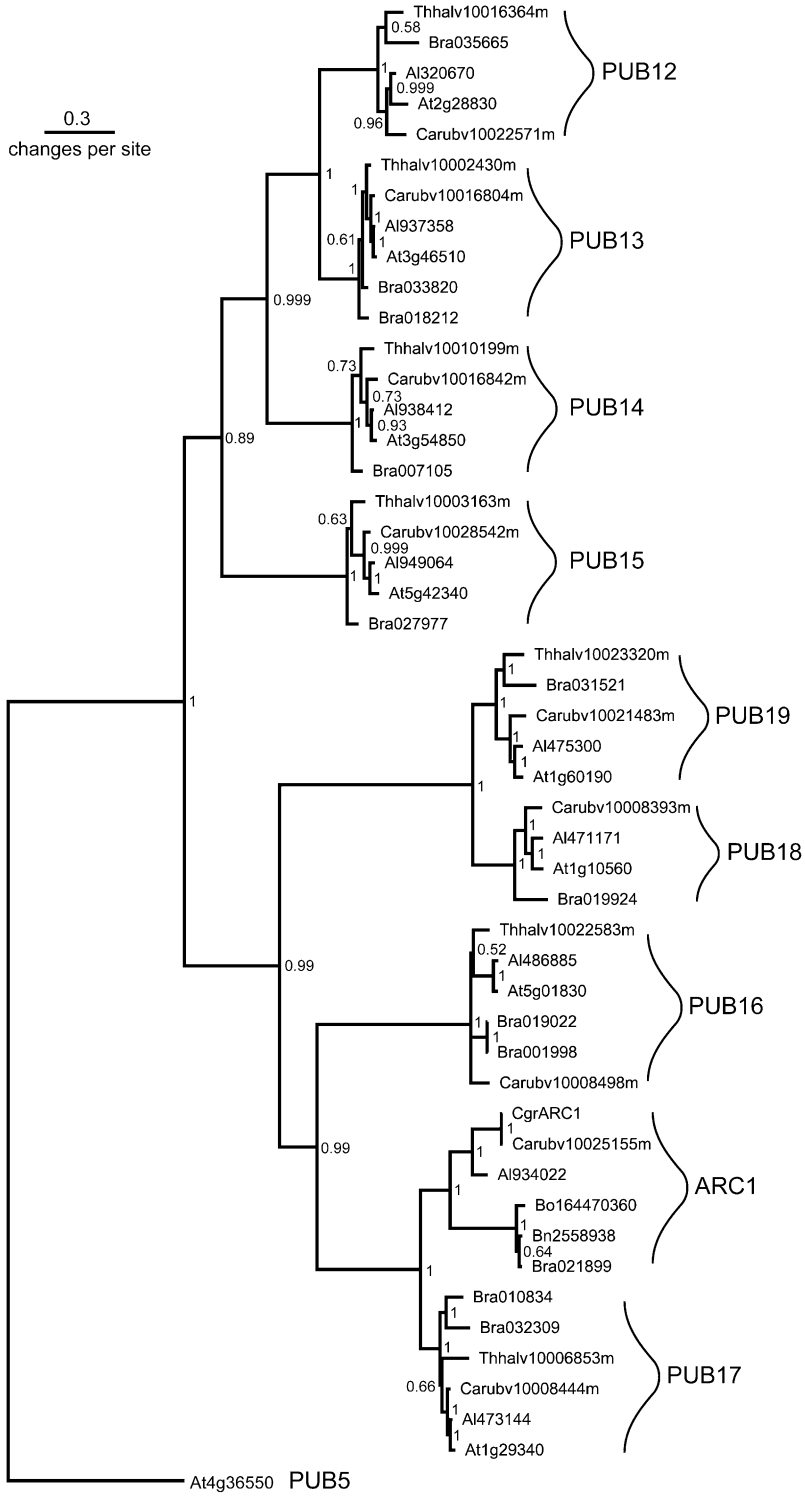

Figure 2. Bayesian Phylogeny Showing the Relationships between the ARC1 Proteins and the PUB5 and PUB12-19 Proteins from Different Brassicaceae Species.

Predicted amino acid sequences were identified from the *A. lyrata* (Al), *C. rubella* (Carub), *T. halophila* (Thhalv), and *B. rapa* (Bra) genomes by BLAST searches with the *A. thaliana* (At) PUB protein sequences. Predicted ARC1 sequences from *B. oleracea* (Bo), *B. napus* (Bn), and *C. grandiflora* (Cgr) were also included. ARC1 proteins are found in a clade sister to the most closely related PUB17 proteins, and PUB16 forms a single clade sister to the closely related ARC1 and PUB17 proteins. Node values = posterior probabilities.

Table 2. Gene Identifiers and Accession Numbers for the *PUB17* Genomic Region

Species	Gene Identifiers and Accession Numbers							
A. thaliana	At1g29310; NP_174225	At1g29320; NP_174226	At1g29330; NP_564326	At1g29340; NP_174228	At1g29350; NP_174229	At1g29370; NP_174230	At1g29380; NP_174231	At1g29390; NP_973936
A. lyrata	473141; XP_002890804	921968; XP_002890807	473143; XP_002893557	473144; XP_002890808	473146; XP_002893558	–	912742; XP_002890809	912743; XP_002893559
C. rubella	10009028	10009063	10010280	10008444	11008305	–	10011350	10010957
B. rapa LF	Bra032303	Bra032305	Bra032306	Bra032309		Bra032310	Bra032314	Bra032315
B. rapa MF2	–	Bra010836	Bra010835	Bra010834	Bra010832	Bra010833	Bra010831	–
T. halophila	10007527	10007651	10008757	10006858	10006794	–	10008139	10008700

Dashes represent the absence of a corresponding orthologue in the *PUB17* genomic region.

A. thaliana, and we did not identify any ecotypes that carried an intact copy of *ARC1*. Furthermore, the deletion of the *ARC1* region appears to have been a single event, given the conservation observed for the sequence and deletion breakpoint in the 10 diverse ecotypes.

Knockdown of *ARC1* Expression in Transgenic *A. lyrata* Plants Leads to the Breakdown of Self-Incompatibility

With our observation that the *ARC1* genomic region has undergone distinct deletions in several self-compatible species, we were interested in testing *ARC1*'s role in a naturally occurring self-incompatible *Arabidopsis* species, *A. lyrata.* Previously, we found that when *ARC1* expression was knocked down in transgenic *B. napus* plants, the self-incompatibility response was attenuated resulting in successful self-fertilization (Stone et al., 1999). Quantitative RT-PCR (qRT-PCR) was first used to examine the expression patterns of *A. lyrata ARC1* (Al-*ARC1*), and it was found to be expressed at highest levels in the pistil and buds, and lower levels of expression could also be detected in the anthers and leaves (Figure 5A; see Supplemental Figure 1 online). The closely related Al-*PUB17* was also tested, and it was expressed in all tissues with the highest level of expression detected in flower bud. Thus, Al-*ARC1* was found to be expressed in the pistil where it could function in the self-incompatibility response, and the expression pattern is consistent with self-incompatibility genes often showing increased or specific expression in the pistil (e.g., *SRK* and *B. napus ARC1*; Goring et al., 1992; Gu et al., 1998).

To test the requirement of Al-*ARC1* for the self-incompatibility response, transgenic Al-*ARC1* RNAi *A. lyrata* lines were generated to suppress Al-*ARC1* expression. An Al-*ARC1*–specific hairpin RNAi driven by either the stigma-specific *SLR1* promoter or the cauliflower mosaic virus 35S promoter (Franklin et al., 1996; Helliwell and Waterhouse, 2003) was transformed into self-incompatible perennial *A. lyrata* spp *petraea* (Kivimäki et al., 2007) using a modified *Agrobacterium tumefaciens*–

Table 3. Gene Identifiers and Accession Numbers for the Predicted PUB Proteins Used in the Phylogenetic Analysis

Species	PUB Proteins				
	PUB5	PUB12	PUB13	PUB14	PUB15
A. thaliana	At4g36550 NP_195373	At2g28830 NP_565676	At3g46510 NP_190235	At3g54850 NP_191045	At5g42340 NP_199049
A. lyrata		320670; XP_002881013	937358; XP_002877490	938412; XP_002876275	949064; XP_002863798
C. rubella		1002257	10016804	10016842	10028542
B. rapa		Bra035665	Bra033820; Bra018212	Bra007105	Bra027977
T. halophila		10016364	10002430	10010199	10003163
	PUB16	PUB17	PUB18	PUB19	ARC1
A. thaliana	At5g01830; NP_195803	At1g29340; NP_174228	At1g10560; NP_172526	At1g60190; NP_176225	–
A. lyrata	486885; XP_002872992	473144; XP_002890808	471171; XP_002889817	475300; XP_002888147	934022; XP_002879489
C. rubella	10008498	10008444	10008393	10021483	10025155
B. rapa	Bra019022; Bra001998	Bra010834; Bra032309	Bra019924	Bra031521	Bra021899
T. halophila	10022583	10008444	10008393	10023320	–
B. napus					2558938; AAB97738
B. oleracea					164470360; ABY58019

Dashes represent the absence of a corresponding ARC1 orthologue. For empty cells, the corresponding orthologue was not identified for the phylogenetic analysis.

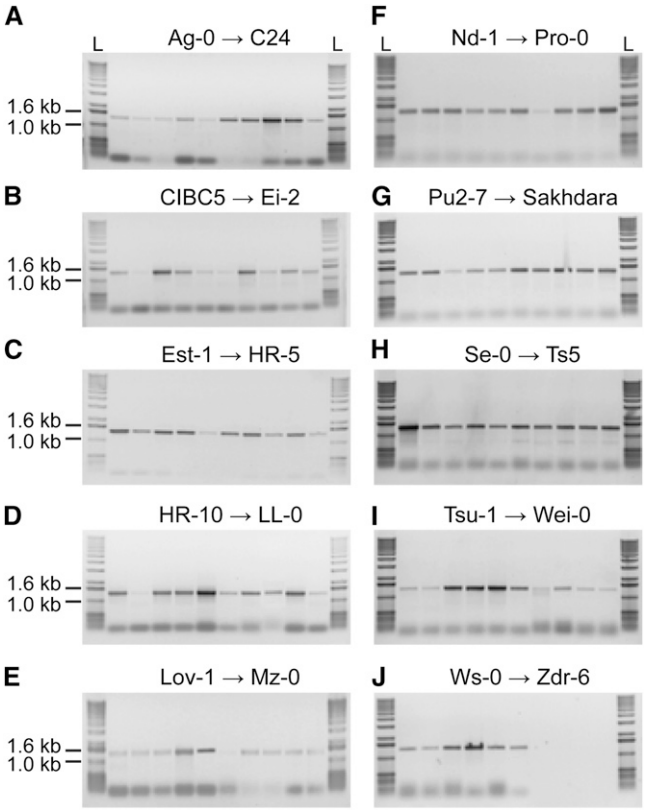

Figure 3. PCR Screen to Survey for the *ARC1* Deletion Region in 96 *A. thaliana* Ecotypes.

The PCR results from the 96 Nordborg ecotype set are shown in alphabetical order with 10 samples per gel. The PCR product for the deleted *ARC1* region is 1.4 kb, and all ecotypes were found to have the deletion, based on the presence of this PCR product. The different intensities in the PCR bands reflect only experimental variation. L, Gibco BRL 1-kb ladder.

(A) Ag-0, An-1, Bay-0, Bil-5, Bil-7, Bor-4, Br-0, Bur-0, and C24.

(B) CIBC-5, CIBC-17, Col-0, CS22491, Ct-1, Cvi-0, Eden-1, Eden-2, Edi-0, and Ei-2.

(C) Est-1, Fab-2, Fab-4, Fei-1, Ga-0, Got-7, Got-22, Gu-0, Gy-0, and HR-5.

(D) HR-10, Kas-2, Kin-0, Knox-10, Knox-18, Kondara, Kz-1, Kz-9, Ler-1, and LL-0.

(E) Lov-1, Lov-5, Lp2-2, Lp2-6, Lz-0, Mr-0, Mrk-0, Ms-0, Mt-0, and Mz-0.

(F) Nd-1, NFA-8, NFA-10, Nok-3, Omo2-1, Omo2-3, Oy-0, Pna-10, Pna-17, and Pro-0.

(G) Pu2-7, Pu2-23, Ra-0, Ren-1, Ren-11, Rmx-A02, Rmx-A180, RRS-7, RRS-10, and Sakhadara.

(H) Se-0, Sorbo, Spr1-2, Spr1-6, Sq-1, Sq-8, Tamm-2, Tamm-27, Ts-1, and Ts-5.

(I) Tsu-1, Ull2-3, Ull2-5, Uod-1, Uod-7, Van-0, Var2-1, Var2-6, Wa-1, and Wei-0.

(J) Ws-0, Ws-2, Wt-5, Yo-0, Zdr-1, and Zdr-6.

mediated floral dip transformation method (Clough and Bent, 1998; Lu and Kang, 2008). As the *A. lyrata* plants were self-incompatible, flowers were manually pollinated with cross-compatible pollen following transformation. Eleven independent transgenic *A. lyrata* plants were generated, and stigma RNA from each line was analyzed by qRT-PCR to determine the degree of the *ARC1* RNAi knockdown. The *ARC1* mRNA levels were found to be reduced to varying degrees in all 11 independent transgenic lines (Figure 5B). While the 489-bp *ARC1* fragment used in the hairpin RNAi vector was chosen for its specificity, this region does share 72% sequence identify to the closely related *PUB17* gene and, as a result, could affect *PUB17* expression as well. No other significant target genes were identified in BLAST searches with the 489-bp Al-*ARC1* sequence. The qRT-PCR analysis indicated that *PUB17* mRNA levels were also reduced in all lines, except the 35SRNAi 6-8 where *PUB17* mRNA levels were increased (Figure 5B). These results were also verified for the transgenic *A. lyrata* 35SRNAi 6-8 line using a standard RT-PCR analysis (see Supplemental Figure 1 online).

The 11 transgenic *ARC1* RNAi *A. lyrata* lines with reduced *ARC1* RNA levels were then examined for the robustness of their self-incompatibility response phenotypes. Similar to the *Brassica* species, self-pollen rejection in *A. lyrata* occurs very early as rejected pollen grains are unable to germinate and form pollen tubes. Therefore, the ability of the *ARC1* RNAi plants to reject or accept self-pollen was measured by examining pollen grains and pollen tube penetration on the stigmatic surface. For the *A. lyrata ARC1* RNAi lines, the pistils were stained with aniline blue to visualize the pollen tubes 24 h after pollination. Typically in the aniline blue staining process, only pollen grains that have been accepted remain on the surface. All rejected pollen grains would no longer remain on the stigmatic papillae as they would have been removed by the washes inherent in the aniline blue staining protocol. Therefore, in a successful self-incompatibility response, pollen grains are absent from the stigmatic surface (Figure 6A) and no pollen tubes can be observed (Figure 6B). By contrast, the cross-compatible pollinated stigma shows pollen grains on the stigmatic surface (Figure 6C) and numerous pollen tubes growing into the stigma (Figure 6D) and into the style and ovary (Figure 6G). For all 11 transgenic *ARC1* RNAi *A. lyrata* lines, the self-pollen rejection response was impaired following self-pollination, and both pollen grains (Figure 6E; see Supplemental Figure 2 online) and pollen tubes could be observed growing into the stigmas (Figure 6F; see Supplemental Figure 2 online). Also, pollen tubes were observed growing into the style and ovary (Figure 6H). Thus, the reduction in Al-*ARC1* mRNA levels was associated with a breakdown in the self-incompatibility response.

To determine the extent of self-pollen acceptance in the Al-*ARC1* RNAi lines, seed production, as a measure of successful pollen tube growth and fertilization, was examined. While cross-compatible pollinations led to well-developed siliques with ~13 seeds/silique, the siliques from self-incompatible pollinations were very small and empty (Figures 6I to 6K). Following self-pollinations, transgenic *ARC1* RNAi *A. lyrata* lines displayed a range of silique sizes, and some examples of larger siliques are shown for the SLR1RNAi 6-3 line in Figure 6I. The average silique sizes from the RNAi lines ranged from 6 mm in the 35SRNAi line 6-8 to10 mm in 35SRNAi line 1-1 and fell between the average 4-mm siliques from self-incompatible pollinations and 22.4-mm siliques from cross-compatible crosses (Figure 6K). Additionally, the number of seeds present in the siliques from the

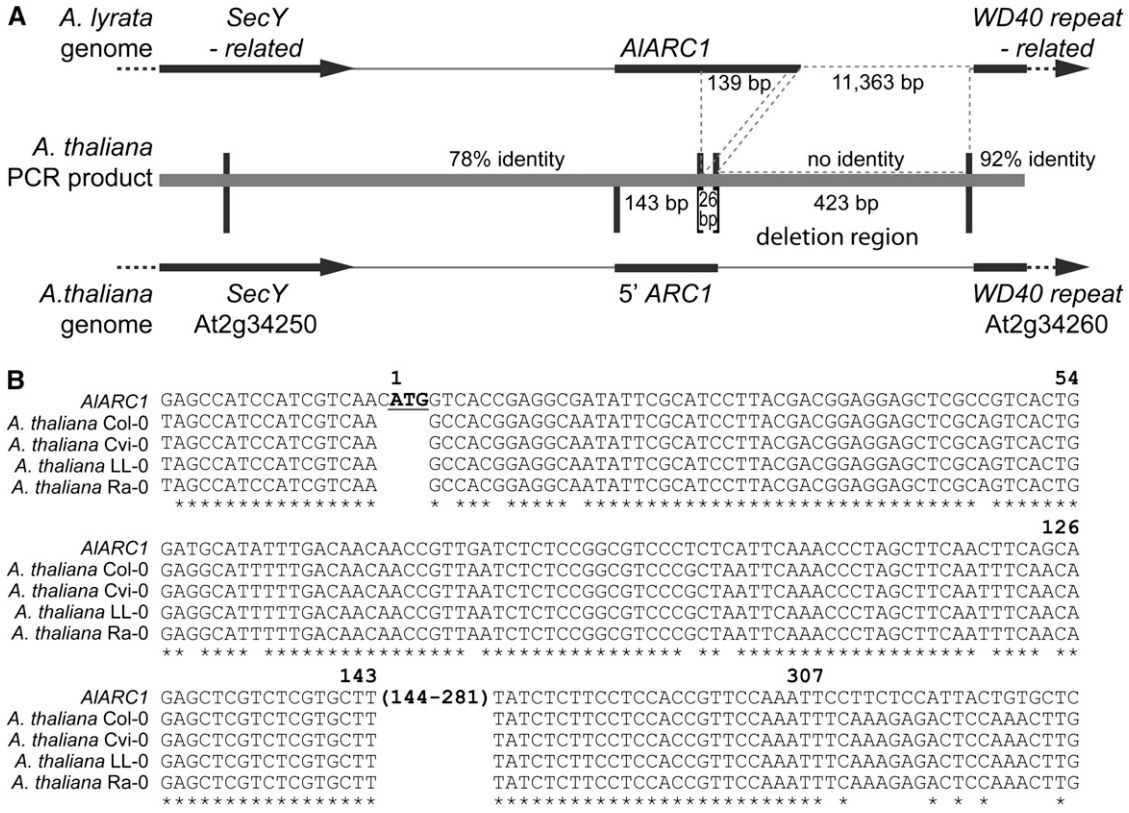

Figure 4. *ARC1* Deletion Region in the *A. thaliana* Genome.

(A) Schematic of the ARC1 region in *A. lyrata* and *A. thaliana*. The *A. lyrata* genome contains an intact *ARC1* gene and when compared with *A. thaliana* shares sequences identity until the deletion break point in the *A. thaliana ARC1* pseudogene. The At2g34250 3′ untranslated region and the ARC1 5′ untranslated region/start of the coding region are 78% identical at the DNA sequence level. This is followed by a 139-bp deletion in *A. thaliana* ARC1 and a small 26-bp sequence of *ARC1* that is 100% identical between the two species genomes. The next 423 bp from *A. thaliana* has no sequence identity to the 11,363-bp region from *A. lyrata*. The sequence identity returns at 9 bp before the start of At2g34260, and the region is 92% similar up until the end of the sequencing read. The sequence identities were determined using the *A. lyrata* genome sequence and the sequenced PCR products from 10 different *A. thaliana* ecotypes.

(B) Sequence alignment of *A. lyrata ARC1* to the corresponding region in *A. thaliana*. PCR products covering this region (shown in **[A]**) were sequenced for 10 ecotypes (Br-0, Bur-0, C24, Col-0, Cvi-0, Kro-0, Ler-0, LL-0, Mrk-0, Ra-0, and Wei-1). The 10 ecotypes shared identical sequences for the ARC1 5′ coding region and the deletion breakpoint, and sequences from four of these ecotypes are shown aligned to Al-*ARC1*. The start codon for Al-*ARC1* is marked in bold and underlined and is absent in corresponding *A. thaliana* sequences.

transgenic plants was variable and was usually less than the cross-compatible control (Figure 6J). Importantly though, all of the transgenic Al-*ARC1* RNAi lines produced some seeds when self-pollinated while the wild-type self-incompatible pollinations failed to produce any seeds (Figure 6J). Thus, the transgenic *ARC1* RNAi *A. lyrata* lines show clear evidence of breakdown in the self-incompatibility response following self-pollination. Also, the observed pseudo-self-compatibility phenotype was similar to that seen for *B. napus ARC1* antisense lines (Stone et al., 1999).

DISCUSSION

ARC1 is an E3 ubiquitin ligase that functions in the pistil as part of the *Brassica* self-incompatibility pathway (Gu et al., 1998; Stone et al., 1999, 2003; Samuel et al., 2009), and this study demonstrates that ARC1 is also required for the *A. lyrata* self-incompatibility response. We found that the RNAi suppression of Al-*ARC1* resulted in the acceptance of self-incompatible pollen, despite the presence of the upstream SCR/SP11 and SRK components. While *PUB17*, the most closely related paralog to *ARC1*, was also suppressed in many of these lines, *PUB17* is unlikely to be responsible for this phenotype as one line, 35SRNAi 6-8, had increased *PUB17* mRNA levels and still showed an attenuated self-incompatibility phenotype. Also, Rea et al. (2010) reported that when the *A. lyrata SRK* and *SCR/SP11* genes were transformed into the *A. thaliana pub17* mutant, the self-incompatible phenotype was unaffected, implying that PUB17 is not involved in the SRK signaling pathway. Finally, PUB17 has been previously reported to have a very different function where it is required for disease resistance

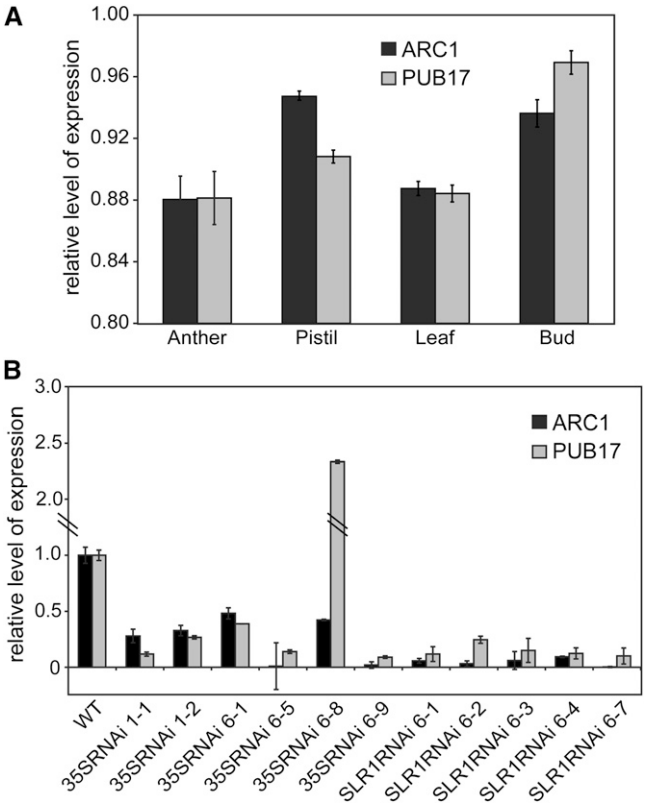

Figure 5. qRT-PCR Analysis of *ARC1* and *PUB17* Expression in Wild-Type and Transgenic *ARC1* RNAi *A. lyrata* Lines.

(A) qRT-PCR analysis of *ARC1* and *PUB17* mRNA levels in anthers, pistil, leaves, and flower buds. *ARC1* and *PUB17* expression levels were normalized across tissue samples with *TUB4* (Bassel et al., 2008), and data from two biological replicates are shown. Error bars indicate ± SE.

(B) Quantitative PCR analysis of *ARC1* and *PUB17* expression levels in stigmas from the transgenic *ARC1* RNAi *A. lyrata* lines. The expression levels of *ARC1* and *PUB17* are shown relative to a wild-type (WT) sample (set to a value of 1). *ARC1* and *PUB17* expression levels were normalized across the samples with *Elf1*α, and data from two biological replicates are shown. Error bars indicate ± SE.

and the hypersensitive response in tomato (*Solanum lycopersicum*) and *A. thaliana* (Yang et al., 2006).

With ARC1 being required for the self-incompatibility response in both *B. napus* and *A. lyrata*, ARC1 may play a more widespread signaling role in self-pollen rejection in the Brassicaceae than previously suggested by Kitashiba et al. (2011). *Brassica* and *Arabidopsis* belong to separate lineages in the Brassicaceae with *Brassica* in lineage II and *Arabidopsis* in lineage I (Franzke et al., 2011). As part of this study, we also investigated the occurrence of the *ARC1* gene in other species in the Brassicaceae. This includes lineage I species (*A. thaliana*, *A. lyrata*, *C. grandiflora*, *C. rubella*, and *L. alabamica*), lineage II species (*B. rapa*, *S. irio*, *T. halophila*, and *T. parvula*), and a species outside of the core Brassicaceae (*A. arabicum*) (Franzke et al., 2011). Previously, *ARC1* was reported to be present in the genome of self-incompatible *A. lyrata* but deleted from two self-compatible *A. thaliana* ecotypes, Col-0 and C24 (Kitashiba et al., 2011). A genome-wide survey of these sequenced genomes led to the intriguing observation that there were independent deletions in the *ARC1* genomic region for most of the self-compatible species tested (*A. thaliana*, *L. alabamica*, *T. halophila*, *T. parvula*, and *A. arabicum*). In contrast with *ARC1*, the genomic region including *PUB17* was highly conserved across all the genomes examined. The *PUB17* gene was present in all the genomes and the structure and gene order in the *PUB17* region was quite conserved.

While most of the self-compatible species did not carry a functional copy of the *ARC1* gene, *C. rubella* was one self-compatible species that retained ARC1, and this may reflect the recent the transition to self-pollination that occurred for this species. The breakdown of self-incompatibility in *C. rubella* occurred ~30,000 to 50,000 years ago (Foxe et al., 2009; Guo et al., 2009), while the breakdown of self-incompatibility of *A. thaliana* has been estimated to be much more distant, between ~400,000 and 1 million years ago (Bechsgaard et al., 2006; Tang et al., 2007; Bomblies and Weigel, 2010). The speciation of *C. rubella* and the transition to a self-pollinating species was also a direct result of a population bottleneck, and the inactivation of the *SCR/SP11-SRK* genes was likely the initial event that allowed self-pollination to occur (Foxe et al., 2009; Guo et al., 2009).

Previously, it has been hypothesized that *A. thaliana* become a self-compatible species due to the pseudogenization of the *SCR/SP11* and *SRK* genes (Kusaba et al., 2001). While the majority of the *A. thaliana* ecotypes surveyed to date carry pseudogenes for *SCR/SP11* and *SRK*, some ecotypes were discovered to have intact copies of *SRK* or *SCR/SP11* (Kusaba et al., 2001; Bechsgaard et al., 2006; Tang et al., 2007; Shimizu et al., 2008; Boggs et al., 2009; Tsuchimatsu et al., 2010; Guo et al., 2011). This indicated that the transition from self-incompatibility to self-compatibility in *A. thaliana* has occurred multiple times with independent *SCR/SP11-SRK* mutations. With the previous report that the *ARC1* gene was deleted in the *A. thaliana* Col-0 and C24 ecotypes (Kitashiba et al., 2011), we also investigated how widespread this specific *ARC1* deletion was in 357 *A. thaliana* ecotypes. The complete loss of the *ARC1* gene in all 357 ecotypes tested, including those with a functional *SCR/SP11* or *SRK* gene, suggests that *ARC1* became a pseudogene before the inactivation of *SCR/SP11* or *SRK* in *A. thaliana*. Tsuchimatsu et al. (2010) observed variations in the manifestation of the reconstituted self-incompatibility responses in different *A. thaliana* ecotypes, with the self-incompatibility response weakening in older flowers (pseudo-self-compatibility). They suggested that mutations conferring pseudo-self-compatibility could have occurred prior to the loss of the *SCR/SP11* or *SRK* self-incompatibility genes and account for the pseudo-self-compatibility in *A. thaliana*. The widespread loss of *A. thaliana ARC1* could be one such pseudo-self-compatibility mutation. In both *B. napus* and *A. lyrata*, we observed that the suppression of *ARC1* expression is correlated with an incomplete breakdown of self-incompatibility. While this could be due to inefficient *ARC1* suppression, it may also indicate that there are other elements functioning downstream of SRK (e.g., MLPK) and that loss of these elements, in addition to ARC1, would be required to produce a full breakdown of the self-incompatibility response. Thus, perhaps in *A. thaliana*, the weakening of the self-incompatibility response with the loss of

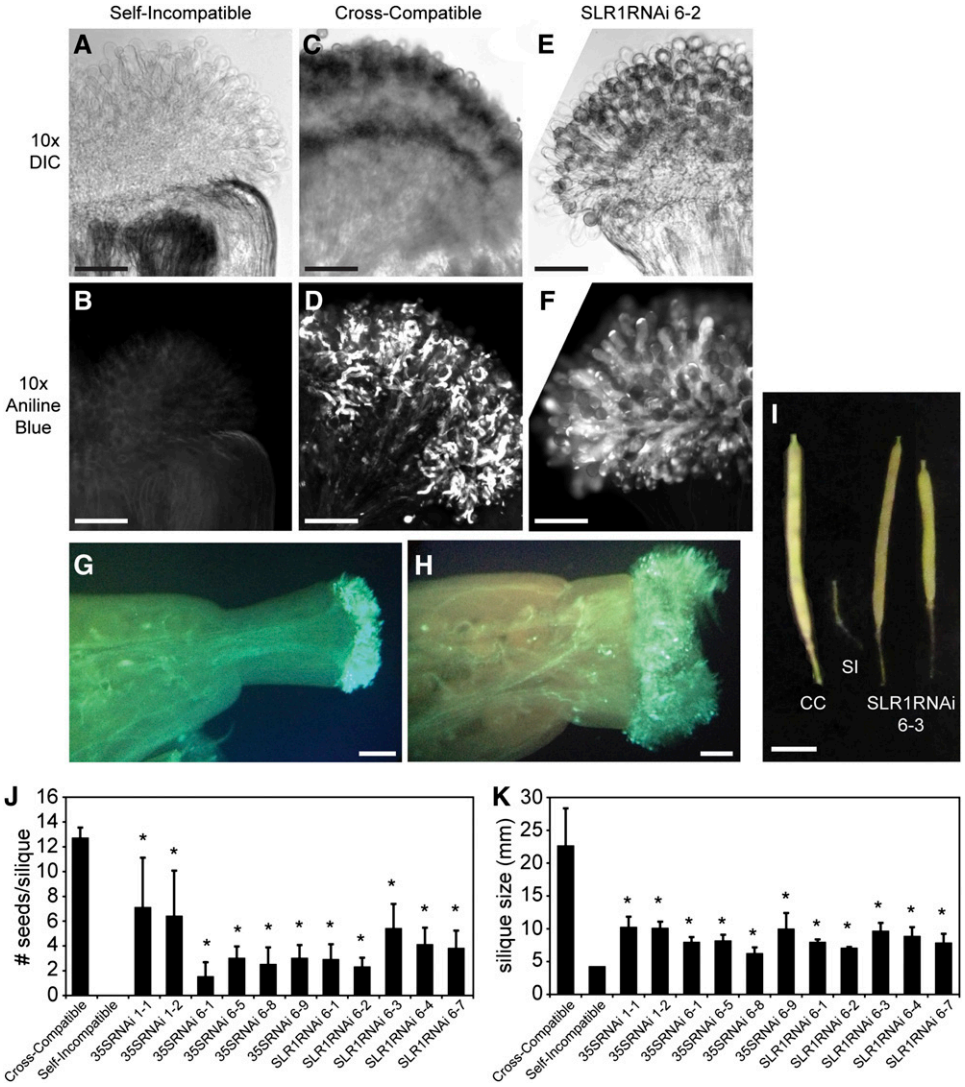

Figure 6. Pollen Tube Growth and Seed Set in the Transgenic *ARC1* RNAi *A. lyrata* Lines.

(A) to **(F)** Pollen grain attachment and pollen tube growth in *A. lyrata* stigmas from control self-incompatible cross (**[A]** and **[B]**), control cross-compatible cross (**[C]** and **[D]**), and SLR1RNAi 6-2 self-pollination (**[E]** and **[F]**). After pollination, pistils were stained with aniline blue to visualize the pollen tubes. DIC, differential interference contrast. Bars = 0.1 mm.

(G) and **(H)** Pollen tube growth in *A. lyrata* pistils from control cross-compatible cross **(G)** and SLR1RNAi 6-2 self-pollination **(H)**. After pollination, pistils were stained with aniline blue to visualize the pollen tubes. Bars = 2 mm.

(I) Siliques from cross- and self-pollinated pistils. Wild-type cross-compatible (CC) pollinations result in a large silique while self-incompatible (SI) crosses result in a small undeveloped silique. Siliques from a self-pollinated SLR1RNAi 6-3 plant were much larger and developed. Bar = 5 mm

(J) Average number of seeds per silique following cross- and self-pollinations. All the transgenic *ARC1* RNAi *A. lyrata* lines produced significant increases in the number of seeds/silique when compared with wild-type self-incompatible crosses (*t* test, $P < 0.05$). $n = 10$. Error bars indicate ± SE.

(K) Average silique sizes following cross- and self-pollinations. All the transgenic *ARC1* RNAi *A. lyrata* lines produced significant increases in silique lengths when compared with siliques from wild-type self-incompatible crosses (*t* test, $P < 0.05$). $n = 10$. Error bars indicate ± SE.

ARC1 could have allowed for some self-pollination to occur, leading to conditions for further breakdown by the pseudogenization of the *SCR/SP11* and *SRK* genes.

While we have described a role for ARC1 in a simple linear SRK signaling pathway (Samuel et al., 2009; Chapman, 2010), receptor kinase signaling pathways in animal systems are typically much more complex with multiple branches (Seet et al., 2006; Lemmon and Schlessinger, 2010). Thus, it would not be unexpected for SRK to signal through other unknown branches to cause self-pollen rejection, and the loss of one branch involving ARC1 may lead only to a pseudo-self-compatibility phenotype. This may also offer an explanation for the large ecotype-dependent range of self-incompatibility phenotypes observed in transgenic *A. thaliana* expressing the *A. lyrata SCR/SP11* and *SRK* genes (Nasrallah

et al., 2004; Boggs et al., 2009; Tsuchimatsu et al., 2010). The range of phenotypes from self-compatibility to stronger levels of self-incompatibility may reflect activity levels of other elements or pathways functioning downstream of SRK in these different ecotypes. In the future, it would be of interest to test whether the transformation of Al-*ARC1* into transgenic *A. thaliana* displaying a pseudo-self-compatibility phenotype would strengthen the self-incompatibility response. In conclusion, we have shown that *ARC1* has been independently deleted in the genomes of a number of self-compatible species, and its role in the self-incompatibility response is conserved between *Arabidopsis* and *Brassica* species.

METHODS

Synteny and Phylogenetic Analyses

For the nine-species whole-genome orthologous alignment assembled by the VEGI consortium (available on their genome browser at http://grandiflora.eeb.utoronto.ca:8086), the genomes of six species were obtained from the genome assemblers or from public databases (www.phytozome.org). Three additional species, *Sisymbrium irio*, *Leavenworthia alabamica* race a4 (Busch et al., 2011), and *Aethionema arabicum* were sequenced by the VEGI consortium using exclusively Illumina paired-end and mate-pair approaches and were assembled using a combination of Ray (Boisvert et al., 2010) for contiging and SOAPdenovo (Li et al., 2010) for scaffolding. A whole-genome multiple alignment of the nine genomes was produced using a progressive alignment pipeline similar to that previously published for the alignment of vertebrate genomes (Miller et al., 2007). Briefly, the procedure starts by computing pairwise alignments between each species and the *Arabidopsis lyrata* genome, which is used as a reference, using the lastZ program (Schwartz et al., 2003). A progressive multiple alignment procedure is then applied to assemble a multiple alignment, using the Mutliz program (Blanchette et al., 2004). In the case of the *Brassica rapa* and *L. alabamica* genomes, which have both undergone whole-genome triplications, only the best conserved, up to three remaining paralogous regions, was retained for the alignment.

The nine-species whole-genome orthologous alignment was accessed using the Synteny viewer in the University of California, Santa Cruz Genome Browser (Fujita et al., 2011) to construct the gene syntenies for the *ARC1* and *PUB17* genomic regions from the *Arabidopsis thaliana*, *A. lyrata*, *S. irio*, *L. alabamica*, *Thellungiella halophila*, *Thellungiella parvula*, *B. rapa*, and *A. arabicum* genomes. The synteny viewers in The Arabidopsis Information Resource (TAIR; Rhee et al., 2003) and the *Brassica* database (BRAD; Cheng et al., 2011) were also used in these analyses. Finally, *A. lyrata*, *Capsella rubella*, *B. rapa*, and *T. halophila* genomic sequences were extracted and protein BLAST searches were conducted using Phytozome v8.0 (Altschul et al., 1990; Goodstein et al., 2012). Gene identifiers are listed in Tables 1 and 2.

For the phylogenetic analysis, the *A. thaliana* PUB12-19 and *A. lyrata* ARC1 amino acid sequences were used in TBLASTN searches to identify orthologs from the *A. lyrata*, *C. rubella*, *T. halophila*, and *B. rapa* genomes. Default TBLASTN parameters were used in the Phytozome and BRAD databases (expect threshold −1, comparison matrix BLOSUM62, word length 3, allowed for gaps). The synteny viewers from TAIR (Rhee et al., 2003) and BRAD (Cheng et al., 2011) were also used to identify the genes encoding *A. lyrata* and *B. rapa* PUB10-19 and ARC1 proteins. Gene identifiers are listed in Table 3. The ARC1 sequences from *B. napus* (2,558,938) and *B. oleracea* (164,470,360) were obtained from GenBank, and the *Capsella grandiflora* ARC1 sequence was extracted from an RNASeq analysis (K. Hazzouri and S.I. Wright, unpublished data). Full-length PUB5, PUB12-19, and ARC1 amino acid sequences were aligned using MAFFT (Katoh et al., 2005). The alignment is available as Supplemental Data Set 2 online. The protein FASTA alignment file was used in MrBayes for a Bayesian phylogenetic analysis, with the mixed model option and 300,000 generations, and two runs used to check for convergence (Ronquist et al., 2012).

Plant Material

The *A. lyrata* plants used in this study were from the self-incompatible perennial *A. lyrata* spp *petraea* P6 and P7 populations collected by Kivimäki et al. (2007) in Northern Sweden. Plants from the P6 population were cross-compatible with the P7 population. The *A. thaliana* ecotypes screened for the *ARC1* deletion were obtained from the ABRC and were from the Nordborg collection (96 ecotype set; Nordborg et al., 2005) and Beck collection (261 ecotype set; Beck et al., 2008). All plants were grown under long-day growth chamber conditions consisting of a 16-h-light/8-h-dark photoperiod at 22°C. Flowering was induced in the *A. lyrata* plants by vernalizing rosettes of ~12 cm in size for 30 d on a short-day cycle of an 8-h-light/16-h-dark photoperiod at 5°C (Kuittinen et al., 2008).

Nucleic Acid Extractions and PCR Assays

For PCR genotyping of the transgenic *A. lyrata* plants and screening for the presence of the *ARC1* deletion in the *A. thaliana* ecotypes, genomic DNA was extracted from young leaves using the Plant DNeasy kit (Qiagen). The *A. thaliana* ARC1 deletion region was PCR amplified using primers to the last exon of At2g34250 and the first exon of At2g34260. For the RT-PCR assays, RNA was extracted from *A. lyrata* anthers, leaves, buds, pistils, and stigmas tissues using of the Plant RNeasy kit (Qiagen). The RNA was quantified with a Nanodrop (GE Nanovue Plus) and then treated with DNase I (amplification grade; Invitrogen) to remove any contaminating genomic DNA. The DNase I was removed from the RNA samples by acid-phenol:chloroform (Ambion) extraction, followed by a phenol-chloroform extraction and nucleic acid precipitation (Sambrook and Russel, 2001). cDNA synthesis was performed on 50 ng/μL of RNA per tissue using the Super Script III reverse transcriptase (Invitrogen). The cDNA was tested by PCR reaction with *A. lyrata Elf1*α-specific primers. RT-PCR and qRT-PCR assays were then conducted on the cDNA samples. The qRT-PCR on the transgenic *ARC1* RNAi *A. lyrata* lines was performed as previously described (Bassel et al., 2008; Indriolo et al., 2010) using stigma cDNA and 2× Power SYBR green (Applied Biosystems). All primers were optimized before use, and the *ARC1* and *PUB17* expression levels were normalized to *Elf1*α (for the different RNAi lines) or *TUB4* (for the different tissues). The standard PCR conditions used for all reactions were a 2-min denaturation at 94°C, followed by a three-step cycle of 30 s 94°C denaturation, 30 s at the appropriate annealing temperature for each primer pair, and then extension at 72°C with a time of 1 kb/1 min for 25 to 35 cycles, followed by a final extension of 10 min at 72°C. Tsg polymerase (Biobasics) was used for all PCR reactions. See Supplemental Table 1 online for all primers used in this study.

A. lyrata ARC1 RNAi Constructs and Transformation

The Al-*ARC1* hairpin RNAi construct was designed using an Al-*ARC1* fragment covering nucleotides 1260 to 1748 of the coding sequence. The cauliflower mosaic virus 35S promoter:Al-*ARC1* RNAi construct was made by amplifying the 489-bp Al-*ARC1* fragment with Advantage 2 polymerase (Clontech) using a forward primer with *Bam*HI and *Xho*I sites and a reverse primer with *Cla*I and *Kpn*I sites. The PCR product was

subcloned into pGEMT-easy (Promega) and was verified by sequencing. The Al-*ARC1* hairpin was assembled in the pKannibal vector (*Xho*I and *Kpn*I for the sense fragment, and *Bam*HI and *Cla*I for the antisense fragment) and then transferred into the pART27 plant transformation vector using *Not*I (Helliwell and Waterhouse, 2003). To generate the *SLR1* promoter:Al-*ARC1* RNAi construct, the Al-*ARC1* hairpin was amplified from pKannibal using a forward primer with an *Xma*I site and a reverse primer with an *Eco*RI site. The PCR product was then digested with *Xma*I and *Eco*RI and ligated into the pORE3 vector (Coutu et al., 2007) containing the *SLR1* promoter (Chapman, 2010). For the transformation of the Al-*ARC1* hairpin RNAi plant transformation vectors into *A. lyrata*, the *Agrobacterium tumefaciens*–mediated floral dip transformation method for *A. thaliana* (Clough and Bent, 1998) was modified with a *Camilina sativa* protocol that included placing the dipped plants under vacuum for 5 min (Lu and Kang, 2008). As the *A. lyrata* plants were self-incompatible, flowers were manually pollinated with cross-compatible pollen for 1 week after dipping.

Crosses and Pollination Assays

For the *A. lyrata* crosses, pistils from 1- to 2-d-old flowers were pollinated with pollen from 2- to 3-d-old flowers as this was determined to be the optimal timing for pistil receptivity and pollen viability using flower development in *A. thaliana* as a guide (Smyth et al., 1990). Aniline blue staining for pollen tubes was conducted on pistils that had been pollinated for 24 h as previously described (Samuel et al., 2009). Siliques from self-incompatible and cross-compatible crosses were characterized by measuring length and the number of seeds present in each silique for 10 randomly selected siliques from each control and transgenic *ARC1* RNAi *A. lyrata* line.

Accession Numbers

Sequence data from this article can be found in the TAIR, BRAD, and Phytozome data libraries under the gene identifiers listed in Tables 1–3.

Supplemental Data

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

Supplemental Figure 1. RT-PCR Analysis of *ARC1* Expression in Wild-Type and Transgenic *ARC1* RNAi *A. lyrata* Lines.

Supplemental Figure 2. Pollen Grain Attachment and Pollen Tube Growth in Pistils from the Transgenic *ARC1* RNAi *A. lyrata* Lines.

Supplemental Table 1. Primers Used in This Study.

Supplemental Data Set 1. List of *A. thaliana* Ecotypes Identified by PCR to Contain the ARC1 Deletion Region.

Supplemental Data Set 2. Text File of the Alignment Used for the Phylogenetic Analysis in Figure 2.

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AUTHOR CONTRIBUTIONS

E.I. designed the research, performed research, analyzed data, and wrote the article. P.T. performed research and analyzed data. S.I.W. designed the research, contributed new computational tools, and analyzed data. D.R.G. designed the research, analyzed data, and wrote the article.

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