Received: 24 October 2023 | Revised: 23 January 2024 | Accepted: 3 July 2024

DOI: 10.1002/pld3.622

RESEARCH ARTICLE

Investigating a role for PUB17 and PUB16 in the self-incompatibility signaling pathway in transgenic *Arabidopsis thaliana*

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Funding information
Natural Sciences and Engineering Research Council of Canada, Grant/Award Number: 5010470

Abstract

In Brassicaceae self-incompatibility (SI), self-pollen rejection is initiated by the *S*-haplotype specific interactions between the pollen S cysteine-rich/S-locus protein 11 (SCR/SP11) ligands and the stigma S receptor kinases (SRK). In *Brassica* SI, a member of the Plant U-Box (PUB) E3 ubiquitin ligases, ARM-repeat containing 1 (ARC1), is then activated by SRK in this stigma and cellular events downstream of this cause SI pollen rejection by inhibiting pollen hydration and pollen tube growth. During the transition to selfing, *Arabidopsis thaliana* lost the SI components, *SCR*, *SRK*, and *ARC1*. However, this trait can be reintroduced into *A. thaliana* by adding back functional copies of these genes from closely related SI species. Both SCR and SRK are required for this, though the degree of SI pollen rejection varies between *A. thaliana* accessions, and ARC1 is not always needed to produce a strong SI response. For the *A. thaliana* C24 accession, only transforming with *Arabidopsis lyrata SCR* and *SRK* confers a strong SI trait (SI-C24), and so here, we investigated if ARC1-related PUBs were involved in the SI pathway in the transgenic *A. thaliana* SI-C24 line. Two close ARC1 homologs, PUB17 and PUB16, were selected, and (Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology was used to generate *pub17* and *pub16* mutations in the C24 accession. These mutants were then crossed into the transgenic *A. thaliana* SI-C24 line and their potential impact on SI pollen rejection was investigated. Overall, we did not observe any significant differences in SI responses to implicate PUB17 and PUB16 functioning in the transgenic *A. thaliana* SI-C24 stigma to reject SI pollen.

KEYWORDS
Arabidopsis, ARC1, *Brassica*, PUB16, PUB17, self-incompatibility, SRK

1 | INTRODUCTION

In the Brassicaceae, the dry stigma employs a strong selectivity for pollen as it determines the fate of the pollen in contact through the activation of intracellular events to facilitate pollen hydration (Dickinson, 1995). This is followed by the germination of the pollen grain and the emergence of a pollen tube which penetrates the stigmatic papilla cell wall and travels down the reproductive tract until it reaches an unfertilized ovule for fertilization (Hafidh & Honys, 2021; Robichaux & Wallace, 2021). In self-incompatible (SI) Brassicaceae

species, the stigma blocks self-incompatible pollen by preventing pollen hydration and germination (Abhinandan et al., 2022; Goring et al., 2023). Brassicaceae SI is genetically controlled by two *S*-locus linked genes encoding the pollen S cysteine-rich/S-locus protein 11 (SCR/SP11) peptide (Schopfer et al., 1999; Shiba et al., 2001; Takayama et al., 2000) and the stigma S receptor kinase (SRK) (Goring & Rothstein, 1992; Silva et al., 2001; Stein et al., 1991; Takasaki et al., 2000). Both genes are highly polymorphic and have specific allelic combinations (*S*-haplotypes) that determine SI pollen recognition. Following *S*-haplotype-specific receptor–ligand interactions between the stigma SRK and the pollen SCR, SRK activates the SI signaling pathway in the stigmatic papillae to reject the SI pollen. The functions of SCR and SRK have been primarily studied in *Brassica* and *Arabidopsis* species, but the signaling events and mechanisms underlying the SI pathway have been best characterized in *Brassica* species (Abhinandan et al., 2022; Goring et al., 2023).

There are three *Brassica* SRK interacting proteins that have been shown to function in the *Brassica* SI pathway: ARM-repeat containing 1 (ARC1; Gu et al., 1998, Stone et al., 1999), M-locus protein kinase (MLPK; Murase et al., 2004, Kakita et al., 2007), and the FERONIA (FER) receptor kinase (Huang et al., 2023; Zhang et al., 2021). *Brassica napus* ARC1 is a plant U-box (PUB) E3 ubiquitin ligase (Azevedo et al., 2001; Gu et al., 1998; Stone et al., 2003) and was shown to be a key player in the stigma SI pathway through the partial and complete breakdown of SI pollen rejection with RNAi knockdowns and CRISPR/Cas9 mutations, respectively (Abhinandan et al., 2023; Huang et al., 2023; Stone et al., 1999). In the SI pathway, *B. napus* ARC1 confers pollen rejection by targeting compatibility factors in the stigma for ubiquitination and proteasomal degradation. The identified targets include glyoxylase 1 (GLO1; Sankaranarayanan et al., 2015), the EXO70A1 exocyst subunit (Safavian & Goring, 2013; Samuel et al., 2009), and phospholipase D α1 (PLD1; Scandola & Samuel, 2019). The degradation of these factors through ARC1 leads to SI pollen rejection by disrupting cellular responses in the stigmatic papillae that are needed for compatible pollen (cellular homeostasis, exocytosis, and membrane remodeling; Abhinandan et al., 2022). Recently, *Brassica rapa* FER was implicated as another signaling branch downstream of SRK (Huang et al., 2023; Zhang et al., 2021). It is proposed that SRK interacts with FER which then activates NADPH oxidases causing the accumulation of reactive oxygen species (ROS) to inhibitory levels (Huang et al., 2023; Zhang et al., 2021). The SRK-activated ARC1 and FER pathways are proposed to function in parallel as disrupting either causes a breakdown in SI pollen rejection.

In *Arabidopsis* species, identifying SRK interacting proteins has proven to be more elusive, but some downstream cellular responses following SI pollen perception have been uncovered (Indriolo et al., 2014; Iwano et al., 2015; Macgregor et al., 2022; Macgregor & Goring, 2022; Safavian & Goring, 2013). While *Arabidopsis thaliana* carries pseudogenized *SCR*, *SRK*, and *ARC1* genes, the SI trait can be reinstated using transgenes from SI *Arabidopsis* species such as *Arabidopsis lyrata* and *Arabidopsis halleri* (Boggs, Nasrallah, & Nasrallah, 2009; Indriolo et al., 2014; Iwano et al., 2015; Nasrallah et al., 2004; Rozier et al., 2020; Zhang et al., 2019). However, accession differences were uncovered in these studies where, for example, the Al*SCR* and Al*SRK* transgenes successfully conferred a strong SI phenotype in C24 resulting in about 100 seeds produced per transgenic plant, which is significantly less compared with seeds produced by untransformed *A. thaliana* C24 (Indriolo et al., 2014; Iwano et al., 2015). In the Col-0 accession, the Al*SCR* and Al*SRK* transgenes did not confer a strong SI phenotype as about 10,000 seeds were produced per transgenic plant which is equivalent to the number of seeds produced by untransformed *A. thaliana* Col-0 (Boggs, Nasrallah, & Nasrallah, 2009; Nasrallah et al., 2004). This variation was found to be caused by RNA-induced silencing of the Al*SRK* transgene by small RNAs originating from inverted repeats in the expressed *A. thaliana* Col-0 *SRKA* pseudogene (Fujii et al., 2020). We previously found that ARC1 is required for the SI trait in *A. lyrata* and adding Al*ARC1* along with the Al*SCR* and Al*SRK* transgenes into *A. thaliana* Col-0 also restored the SI phenotype (Indriolo et al., 2012, 2014). The interaction of AlARC1 with SRK was found to be conserved, and interestingly a close AlARC1 homolog, AlPUB17, interacted with AlSRK as well (Indriolo & Goring, 2016). This raised the question of whether closely related ARC1 homologs could also be functioning in *Arabidopsis* SI. Here, using a transgenic *A. thaliana* Al*SCR* Al*SRK* C24 line that displays a strong SI phenotype (Iwano et al., 2015), we investigated the involvement of two close ARC1 homologs, PUB16 and PUB17, in the transgenic *A. thaliana* SI pathway.

2 | RESULTS

2.1 | Generation of *pub16* and *pub17* mutations in the C24 accession

ARC1 belongs to a subgroup of PUB E3 ligases that have the domain organization of a U-box N-terminal domain (UND), followed by the U-box domain and C-terminal ARM domain (Mudgil et al., 2004; Samuel et al., 2008; Trenner et al., 2022). A phylogeny of AlARC1 with UND-PUBs from *A. lyrata* and *A. thaliana* showed that the closest homologs to AlARC1 are PUB17 and PUB16 (Figure 1; Indriolo et al., 2012). Because AlPUB17 was also previously found to interact with $AlSRK_1$ (Indriolo & Goring, 2016), PUB17 and PUB16 were selected to investigate if they functioned in the transgenic *A. thaliana* SI pathway. The transgenic *A. thaliana* Al*SCR* Al*SRK* C24 line (SI-C24 #15-1) was selected for this study as it displays a strong and stable SI phenotype (Iwano et al., 2015; Macgregor et al., 2022). *PUB17* and *PUB16* are expressed across a broad range of tissues in *A. thaliana* including the stigma (Klepikova et al., 2016), and the expression of *PUB17* and *PUB16* was confirmed by RT-PCR analyses using RNA extracted from the top halves of pistils (including the stigma) (Figure 2a).

Because *pub17* and *pub16* mutants were not available in the C24 accession, we generated individual mutants using CRISPR/Cas9 vectors targeting each gene with two sgRNAs designed to generate deletion mutations (Figure 2b). Wildtype *A. thaliana* C24 was transformed with these vectors, and transformed T1 plants carrying deletion

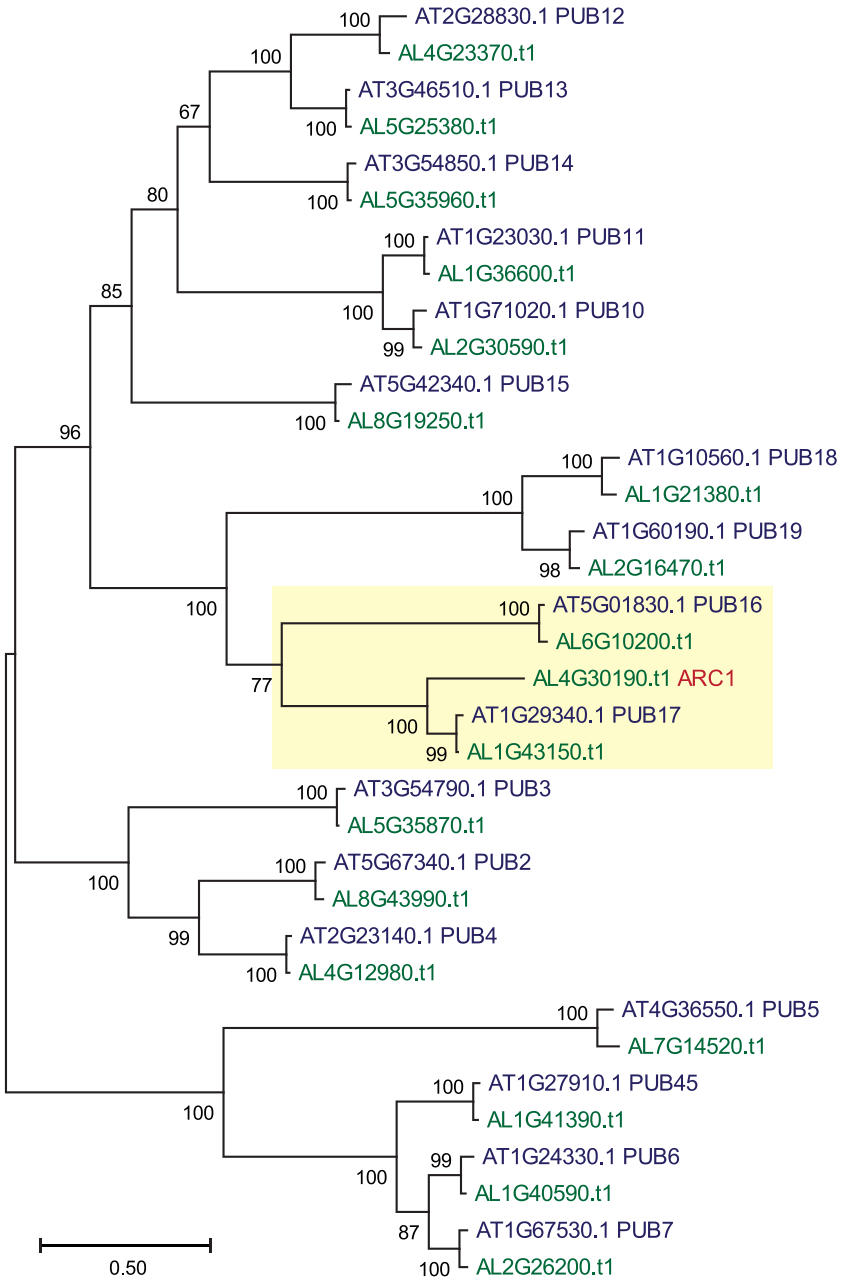

FIGURE 1 Phylogenetic analysis of predicted U-box N-terminal domain (UND)-plant U-Box (PUB) proteins in *Arabidopsis thaliana* and *Arabidopsis lyrata*. The amino acid sequences for *A. thaliana* UND-PUBs are shown in blue and *A. lyrata* UND-PUBs are in green (the *A. lyrata* ARM-repeat containing 1 [ARC1] name is in red). The yellow box highlights the clustering of *A. lyrata* ARC1 with PUB17 and PUB16. The maximum likelihood tree was built in Molecular Evolutionary Genetics Analysis Version 11 (MEGA11) using default parameters and 1000 bootstrap replications.

mutations were identified by PCR. Following the selfing of these T1 plants, the T2 generation was screened to identify homozygous *pub* mutants (*pub17-4*, *pub17-5*, *pub16-1*, and *pub16-2*). While there is still expression of these mutant alleles (Figure S1), the deletion mutations remove a large portion of the predicted PUB17 and PUB16 protein sequences and would not be functional (Figure 2b, Figure S2). These *pub* mutants were then crossed individually into the SI-C24 line. To generate the SI-C24 *pub16-1 pub17-5* double homozygous mutant line, the *pub16-1* mutant was crossed into the SI-C24 *pub17-5* mutant line. Progeny from these crosses was left to set seed (some seeds are produced with selfing in the SI-C24 line), and subsequent generations were screened for SI-C24 plants that were homozygous for the respective *pub* mutations for further analysis. The plants and flowers from the *pub16* and *pub17* single mutants as well as the SI-C24 *pub16-1 pub17-5* double mutant all displayed normal phenotypes with no visible developmental or morphological defects.

2.2 | SI pollen hydration on SI-C24 *pub* mutant stigmas

Pollen hydration is dependent on water transferred from the stigmatic papilla to the contacted pollen grain, and SI pollen is rejected by blocking this pollen hydration step. Thus, we tested the impact of the *pub* mutations on the transgenic *A. thaliana* SI pathway by conducting pollen hydration assays (Figure 3). For this assay, SI-C24 pollen or C24 pollen was used to pollinate wildtype and mutant stigmas in the C24 and SI-C24 backgrounds. As the pollen grain hydrates, it evolves

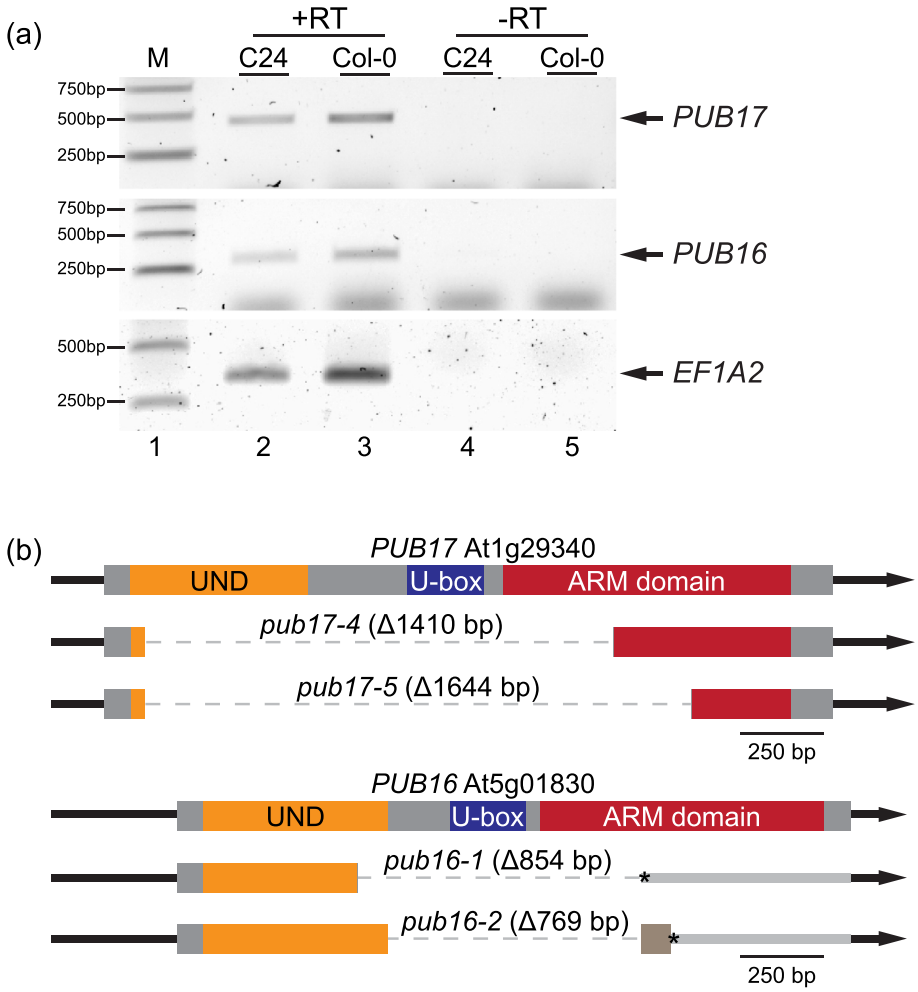

FIGURE 2 Gene expression of *PUB17* and *PUB16* in *Arabidopsis thaliana*. (a) RT-PCR analysis of *PUB17* and *PUB16* expression in pistil tissues from *A. thaliana* Col-0 and C24. cDNA was synthesized from RNA extracted from the top half of pistils (including the stigmas), and RT-PCR was conducted to confirm the expression of *PUB17* and *PUB16* in both the Col-0 and C24 accessions. Elongation factor 1α2 (*EF1A2*) was used as a positive control. *PUB17* = 467 bp, *PUB16* = 305 bp, *EF1A2* = 355 bp. (b) Schematic representation of CRISPR/Cas9 deletion mutants for *PUB17* and *PUB16*. Both genes are encoded by a single exon (gray rectangle), and dashed lines represent deletions in the mutant alleles. Overlaid on the coding regions are the three predicted protein domains (U-box N-terminal domain [UND], U-box, ARM domain) from Mudgil et al. (2004) for PUB17 and PUB16. Both of the *pub17* mutations are predicted to have in-frame deletions and the predicted partial remaining UND and ARM domains are shown. Both of the *pub16* mutations are predicted to have frameshifts with premature stop codons (*) following the deletions and only contain predicted UND domains as shown. Scale bars = 250 bp.

from an oval shape to a rounder shape, and this shape change is quantified by measuring the pollen diameter as a proxy for pollen hydration at 0 and 10-min post-pollination (Lee et al., 2020). First, we verified that the *pub* mutations did not impact compatible pollen hydration. As controls for compatible pollen hydration, SI-C24 or C24 pollen was used to pollinate C24 stigmas, and the hydrated pollen grains displayed large increases in pollen diameter as expected (Figure 3a–d). This level of hydration is sufficient for the next step of compatible pollen germination (Rozier et al., 2020). Large increases in SI-C24 pollen diameter were also observed on the compatible single *pub17* and *pub16* mutant stigmas. Similarly, when compatible C24 pollen was used to pollinate the SI-C24 *pub16-1 pub17-5* mutant stigmas, the compatible C24 pollen grains hydrated normally (Figure 3d). The average pollen diameters at 10-min post-pollination were not significantly different from that seen for the compatible controls (SI-C24 or C24 pollen on C24 stigmas) (Figure 3a–d). These results indicate that with compatible pollinations, the single *pub17* and *pub16* mutants and the double *pub16-1 pub17-5* mutant have normal compatible pollen hydration responses in the stigma.

Next, we tested the potential impact of the *pub17* and *pub16* mutations on the SI response in the transgenic *A. thaliana* stigma. As a control for SI pollen hydration, SI-C24 pollen was placed on SI-C24 stigmas. These pollen grains showed very little hydration which was detected by the very small increase in pollen diameter (Figure 3a–c). This level of pollen hydration is not sufficient for the next step of pollen germination and thus causes SI pollen rejection (Rozier et al., 2020). Similarly, when SI-C24 pollen was used to pollinate stigmas from the SI-C24 *pub17* and SI-C24 *pub16* lines, there were only small increases in pollen diameter, and the average pollen diameters at 10-min post-pollination were not significantly different from that seen for the SI control (SI-C24 pollen on SI-C24 stigmas) (Figure 3a,b). These results indicate that the individual *pub17* and *pub16* mutations in the SI-C24 background did not have any noticeable impact on the SI pollen rejection response of blocking SI pollen hydration. Finally, to address whether *PUB17* and *PUB16* may have redundant functions in the rejection of SI pollen, pollen hydration assays were conducted on SI-C24 *pub16-1 pub17-5* double mutant stigmas (Figure 3c). SI-C24 pollen placed on the SI-C24 *pub16-1 pub17-5* mutant stigmas exhibited a similar small increase in SI pollen hydration which was equivalent to that observed for the SI control of SI-C24 pollen on SI-C24 stigmas. This indicates that the SI pollen hydration block was unaffected by the loss of both PUB17 and PUB16 function in the SI-C24 stigma. The collective results from the pollen hydration assay indicate that PUB17 and PUB16 are not required in the transgenic *A. thaliana* SI-C24 stigma for the rejection of SI-C24 pollen at the pollen hydration stage.

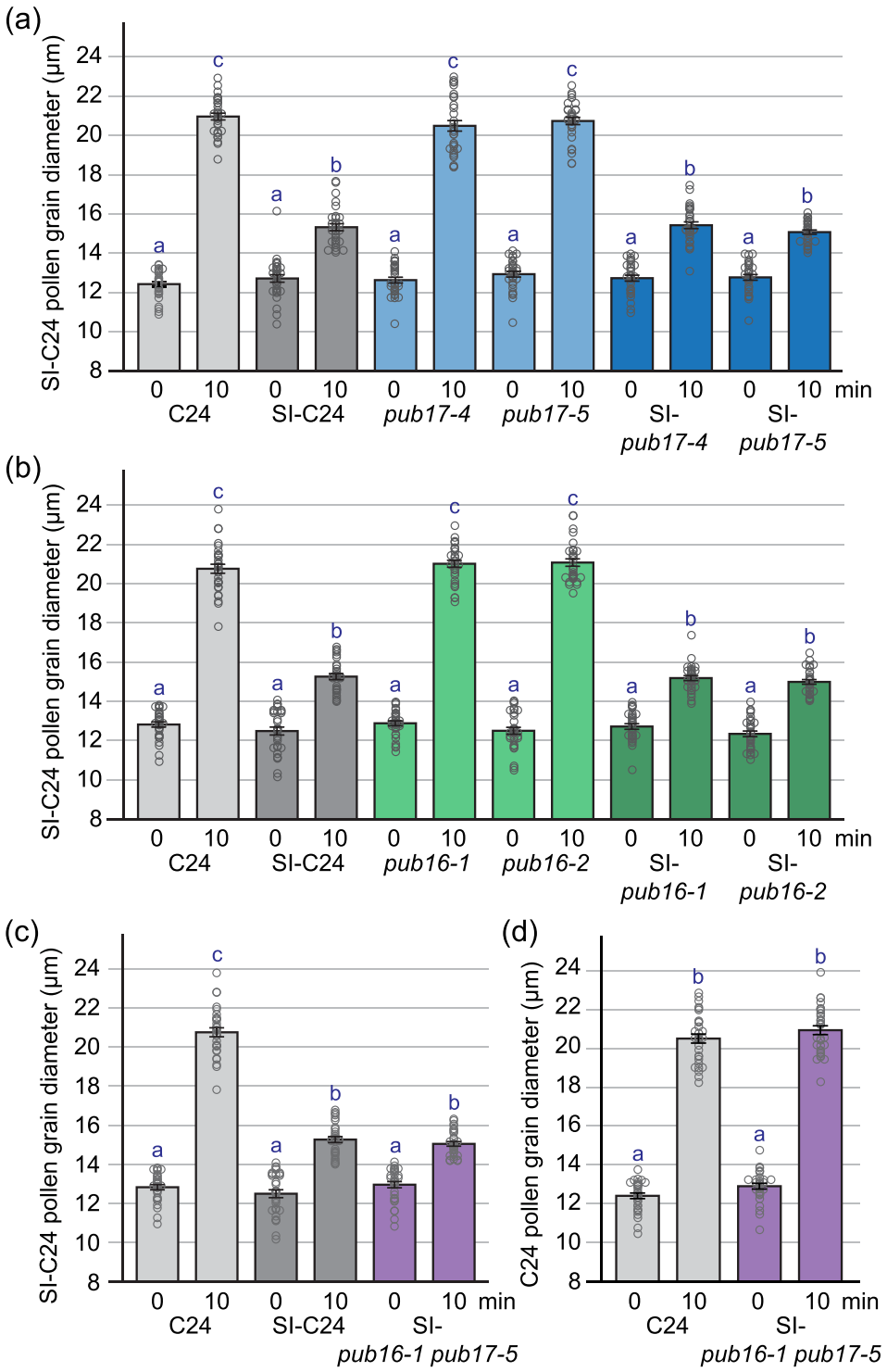

FIGURE 3 Pollen hydration assays on SI-C24 and SI-C24 *pub* mutant stigmas. (a–c) SI-C24 pollen hydration assays on stigmas from (a) C24, SI-C24, *pub17*, and SI-C24 *pub17* mutants; (b) C24, SI-C24, *pub16*, and SI-C24 *pub16* mutants; and (c) C24, SI-C24, and the SI-C24 *pub16-1 pub17-5* mutant. (d) C24 pollen hydration assays on stigmas from C24 and the SI-C24 *pub16-1 pub17-5* mutant. Pollen diameters were measured at 0- and 10-min post-pollination. Data are shown as bar graphs of the means ± SE with all the data points displayed. $n = 30$ pollen grains per line. Letters represent statistically significant groupings of $p < .05$ based on a one-way analysis of variance (ANOVA) with a Tukey's honest significance difference (HSD) post-hoc test. Pollen hydration assays on stigmas from C24 and the individual *pub* mutants (a, b) and C24 pollen on stigmas from SI-C24 *pub16-1 pub17-5* (d) represent control pollinations to confirm the single and double *pub* mutations did not impact compatible pollen hydration. The single and double *pub* mutations also did not impact the stigma SI response at the level of pollen hydration (a–c).

2.3 | SI pollen tube growth and seed set in the SI-C24 *pub* mutants

The majority of SI pollen grains are rejected by blocking pollen hydration, but some do successfully hydrate and produce a pollen tube that grows into the pistil. As a result, we wanted to further investigate if the loss of *PUB17* and *PUB16* had any impact on the number of pollen tubes that successfully overcame the SI stigma barrier. Control and mutant pistils were pollinated with SI-C24 pollen and collected at 24-h post-pollination for aniline blue staining to visualize the pollen tubes (Figure 4). A control compatible pollination of C24 pistils with SI-C24 pollen showed abundant pollen tubes growing through the C24 pistils signifying pollen acceptance (Figure 4a,b). Similarly, compatible pollinations of the *pub17* and *pub16* mutants with SI-C24 pollen resulted in abundant pollen tube growth through the pistils (Figure 4e,f,i,j). As well, compatible pollinations of the SI-C24

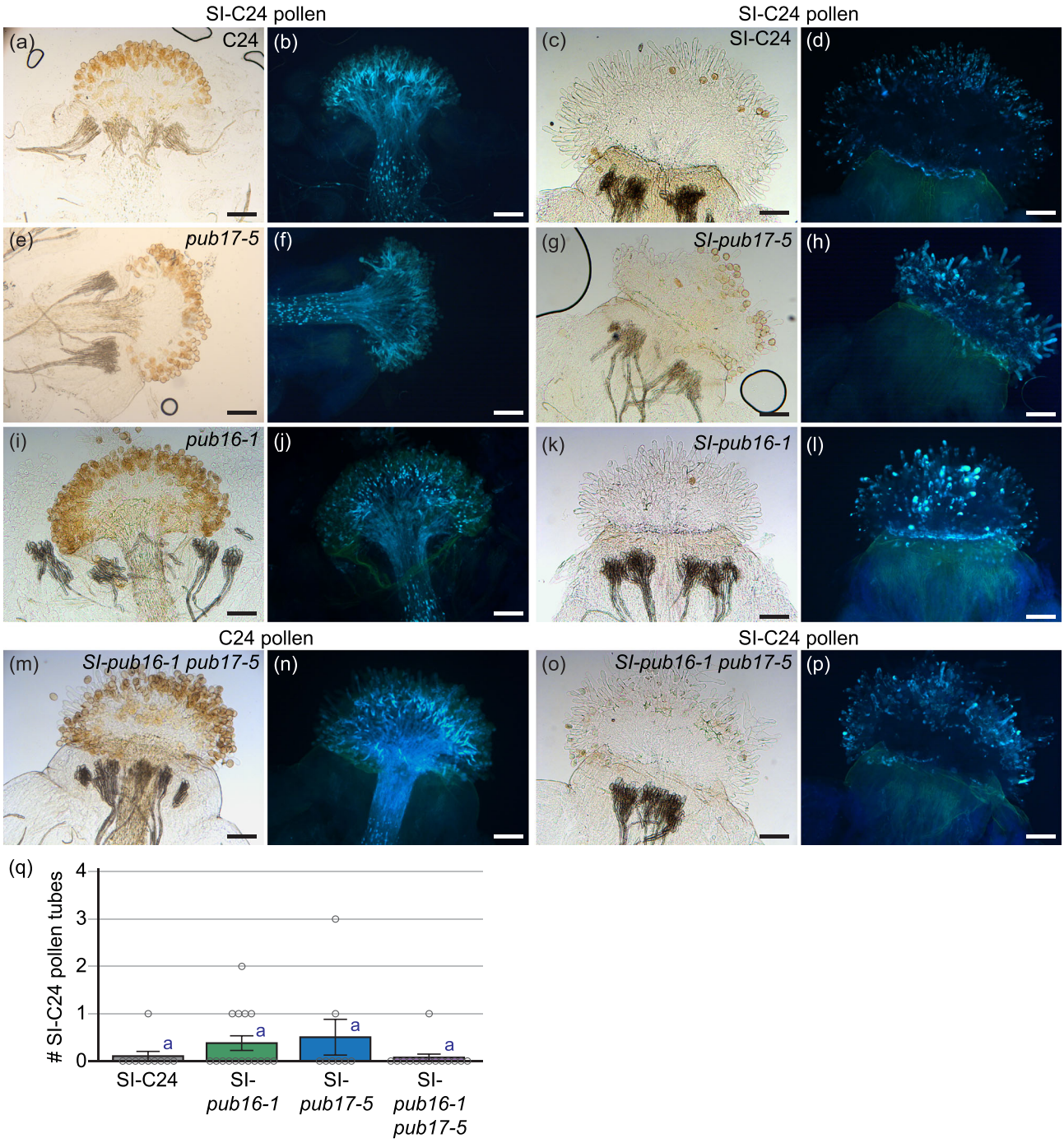

FIGURE 4 Aniline blue images of SI-C24 pollinated SI-C24 and SI-C24 *pub* mutant pistils. (a–p) Representative images of pistils collected at 24-h post-pollination for aniline blue staining. The pistils were pollinated with SI-C24 pollen, except for (m, n) which were pollinated with C24 pollen. The genotypes of the pistils are indicated above, and brightfield (left) and aniline blue images (right) are shown for each sample. Pollen tubes growing through the pistil are indicative of pollen acceptance as shown in panels (b, f, j, n) which represent compatible pollination controls. The *pub* mutations also had no impact on the stigma SI response as observed by the lack of SI-C24 pollen tube growth (panels d, h, l, p). Scale bars = 100 µm. (q) Bar graph showing the mean number of pollen tubes/pistil at 24-h post-pollination. Data are shown as mean ± SE with all the data points displayed. $n = 8–16$ pistils per line. Letters represent statistically significant groupings of $p < .05$ based on a one-way analysis of variance (ANOVA) with a Tukey's honest significance difference (HSD) post-hoc test.

pub16-1 pub17-5 mutant pistils with C24 pollen resulted in many pollen tubes growing through the pistils (Figure 4m,n). Thus, there were no observable differences in the abundance of compatible pollen tubes growing through the *pub* mutant pistils in comparison to C24 signifying that compatible pollen tube growth was not affected in these *pub* mutants.

In contrast, the control SI pollination of SI-C24 pistils with SI-C24 pollen resulted in no observable pollen tube growth in the majority of

these pistils, indicating SI pollen rejection (Figure 4c,d,q). Similarly, when SI-C24 *pub17-5* and SI-C24 *pub16-1* mutants were pollinated with SI-C24 pollen, little to no pollen tubes were observed in the pistils (Figure 4g,h,k,l,q). Correspondingly, this phenotype was observed in the SI-C24 *pub16-1 pub17-5* mutant pistils pollinated with SI-C24 pollen (Figure 4o–q). To verify that there was no impact on SI-C24 pollen rejection at the level of pollen tube growth in the SI-C24 *pub* mutants, the number of SI-C24 pollen tubes in the style were counted in the SI-C24 and SI-C24 *pub* mutant pistils (Figure 4q). While there was a slight trend of more pollen tubes growing through the SI-C24 *pub17-5* and SI-C24 *pub16-1* mutant pistils, it was not significantly different from that observed for the SI-C24 pistils. Thus, these data indicate that the loss of *PUB17* and *PUB16* function in the transgenic *A. thaliana* SI-C24 pistil did not impact the rejection of SI-C24 pollen tube growth.

While the *pub* mutations did not disrupt SI-C24 pollen rejection at the hydration and pollen tube growth stages, we wanted to determine if there would be any impact on the final stage of seed production. The SI-C24 *pub* mutants were pollinated with C24 and SI-C24 pollen, and the number of seeds/siliques was counted (Figure 5). With compatible C24 pollen, abundant seeds were produced for all samples and SI-C24 *pub* mutants did not show any significant differences from the SI-C24 control (Figure 5). Interestingly, with SI-C24 pollen, there was the same trend as seen with the number of pollen tubes (Figure 4q), where the SI-C24 *pub17-5* and SI-C24 *pub16-1* mutant siliques had a slight increase in the number of seeds per silique, but they were not significantly different from the SI-C24 siliques (Figure 5). Overall, these results indicate that the loss of *PUB17* and *PUB16* in the transgenic *A. thaliana* SI-C24 pistil did not impact seed yield with either compatible C24 or SI-C24 pollinations.

3 | DISCUSSION

The bulk of our understanding of the mechanisms underlying the Brassicaceae SI pathway originates from findings in *Brassica* species, and here we investigated if similar SI pathway components are regulating *Arabidopsis* SI. The factors for initiating SI pollen rejection, pollen SCR and stigma SRK, are conserved between *Brassica* and *Arabidopsis*, but investigations on the downstream players of this pathway suggest there are genus-specific differences (Abhinandan et al., 2022; Goring et al., 2023). For example, *A. thaliana* lost functional copies of the *SCR*, *SRK*, and *ARC1* genes during the transition to selfing, and while functional copies of *SCR* and *SRK* are required to restore the SI trait in *A. thaliana*, ARC1 is not always required (Boggs, Nasrallah, & Nasrallah, 2009; Fujii et al., 2020; Indriolo et al., 2014; Indriolo & Goring, 2014; Nasrallah et al., 2004; Rozier et al., 2020). As well, introducing *SCR-SRK* pairs from closely-related *Arabidopsis* and *Capsella* SI species can reinstate the SI trait in transgenic *A. thaliana*, but *Brassica SCR-SRK* pairs fail to do so (Bi et al., 2000; Boggs, Dwyer, et al., 2009; Yamamoto et al., 2022; Zhang et al., 2019). These studies infer that at least some components of the downstream SI pathway have diverged between *Brassica* and *Arabidopsis*.

In this study, we investigated if the closely related ARC1 homologs, PUB17 and PUB16, were functioning as an alternative to ARC1 in the transgenic *A. thaliana* SI-C24 line. A number of PUBs have been implicated in plant immunity, often working together with redundant functions (Trenner et al., 2022; Trujillo, 2018). PUB17 was shown to be a positive regulator of plant immunity through the activation of programmed cell death (Yang et al., 2006). A putative K-homology RNA-binding protein, KH17, which acts as a negative regulator of immunity was proposed to be targeted by PUB17 for degradation (McLellan et al., 2020). For PUB16, little is known other than its increased expression observed in flowers with Gibberellic acid (GA) treatment (Acosta et al., 2012). Previously we found a positive interaction between $AlSRK_1$ and AlPUB17 (Indriolo & Goring, 2016), and our phylogenetic analysis showed that PUB17 and PUB16 are the two most closely related UND-PUBs to AlARC1 (Figure 1; Indriolo et al., 2012). The CRISPR/Cas9 system was used to generate *pub17* and *pub16* mutations in the C24 accession (Figure 2), and these mutations were then crossed into the transgenic *A. thaliana* SI-C24 line which carries the $AlSCR_b$ and $AlSRK_b$ transgenes and displays a strong SI phenotype (Iwano et al., 2015; Macgregor et al., 2022). Our experiments provided a thorough examination of the requirements of *PUB17* and *PUB16* at three different stages that are impacted by SI pollen rejection: pollen hydration, pollen tube growth, and seed yield. In response to SI-C24 pollen, the transgenic *A. thaliana* SI-C24 *pub*

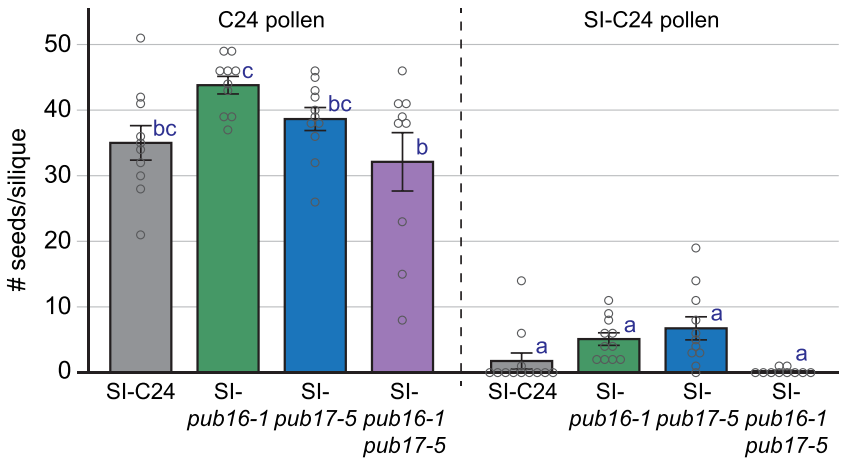

FIGURE 5 Seed yield for SI-C24 and SI-C24 *pub* mutant siliques. Pistils were pollinated with C24 pollen (left) or SI-C24 pollen (right), and siliques were harvested at 2-weeks post-pollination for counting. Data are shown as a bar graph of the means ± SE with all data points displayed. $n = 10–11$ siliques per line. Letters represent statistically significant groupings of $p < .05$ based on a one-way analysis of variance (ANOVA) with a Tukey's honest significance difference (HSD) post-hoc test. The *pub* mutations do not impact seed set upon pollinations with compatible C24 pollen and self-incompatible SI-C24 pollen.

mutant pistils display strong SI pollen rejection responses with very low increases in pollen hydration, the absence of pollen tube growth in the pistil, and low seed counts (Figures 3–5). In all of these assays, there were no significant differences to the control SI pollen rejection responses displayed by wild-type transgenic *A. thaliana* SI-C24 pistils. As well, the *pub17* and *pub16* mutant pistils did not have any impact on compatible pollen responses (Figures 3–5). Collectively, these findings highlight that PUB17 and PUB16 are not compensating for the loss of ARC1 in the transgenic *A. thaliana* SI-C24 line, and do not influence the ability of the SI-C24 line to reject SI pollen. Thus, based on this work, PUB17 and PUB16 are not required for self-pollen rejection in the transgenic *A. thaliana* SI-C24 line. In two other studies, *PUB17* and *PUB2*, which is more distantly related to *ARC1* (Figure 1), were tested in a transgenic *A. thaliana* A*lSCR*-A*lSRK* Col-0 line that has a weak SI trait and no roles were found as well (Rea et al., 2010; Zhang et al., 2014). However, this A*lSCR*-A*lSRK* Col-0 line was reported to be essentially self-compatible, setting ~10,000 seeds per plant (Boggs, Dwyer, et al., 2009). In comparison, the *A. thaliana* SI-C24 line used in this study displays a very strong SI phenotype setting only ~100 seeds per plant (Iwano et al., 2015), and this allowed us to more fully assess if any subtle changes had occurred to the SI phenotype with the loss of *PUB17* and *PUB16*.

In terms of cellular responses identified using transgenic *A. thaliana* SI lines, Iwano et al. (2015) discovered a rapid increase in cytosolic Ca^{2+} levels in the SI-C24 stigmatic papillae upon SI pollination. While a cytosolic Ca^{2+} flux was also observed with compatible pollen (Iwano et al., 2004), the size of the Ca^{2+} flux was much greater with SI pollen (Iwano et al., 2015) and would be predicted to disrupt actin cytoskeleton and vesicle secretion (Goring, 2017). Furthermore, Iwano et al. (2015) implicated glutamate-like receptors (GLRs) to be responsible for the Ca^{2+} influx following SI pollination. Other cellular responses that have been identified in the transgenic *A. thaliana* SI lines include changes to actin filaments (Rozier et al., 2020) and the activation of autophagy (Indriolo et al., 2014; Macgregor et al., 2022; Safavian & Goring, 2013). We found that autophagy was necessary for the SI pathway as crossing autophagy mutants into transgenic *A. thaliana* SI-C24 and SI-Col-0 lines led to a partial breakdown of SI pollen rejection by the SI *atg* mutant pistils (Macgregor et al., 2022; Macgregor & Goring, 2022). Nevertheless, the mechanisms underlying the activation of autophagy and GLRs remain unknown. Thus, further investigations are required to uncover the signaling proteins directly activated by SRK and to determine how they are connected to the cellular events activated in the stigmatic papillae with SI pollen contact. Furthermore, these investigations could also reveal differences in the signaling compounds that act downstream of SRK in *Arabidopsis* SI compared with *Brassica* SI.

4 | METHODS

4.1 | Plant materials and growth conditions

All *A. thaliana* seeds (C24, SI-C24, and CRISPR/Cas9 deletion mutants) were sterilized and stratified at 4°C for 5 days in the dark. Stratified seeds were placed directly on Sunshine #1 soil supplemented with Plant Prod All Purpose 20-20-20 fertilizer for germination. Plants were grown in chambers under a 16-h light/8-h dark growth cycle at 22°C, with humidity kept under 50%. The *pub* mutants (*pub17-4*, *pub17-5*, *pub16-1*, and *pub16-2*) generated through CRISPR/Cas9 (see below) were crossed with the transgenic *A. thaliana* SI-C24 line #15-1 (Iwano et al., 2015) to establish the single *pub* mutants in the SI-C24 background. The *pub16*-mutant was then crossed with SI-C24 *pub17-5* to generate the SI-C24 *pub16-1 pub17-5* double mutant line. The SI-C24 line was genotyped for the presence of the *SCRb* and *SRKb* transgenes using the *SCRb FP* and *RP* primers and the *SRKb FP* and *RP* primers. The *pub* mutants were genotyped by using primers that flanked the deletion sites (*PUB17 KO FP* and *RP*; *PUB16 KO FP* and *RP*) and internal primers to the deleted regions (*PUB17 INT FP* and *RP*; *PUB16 INT FP* and *RP*). All primer sequences are listed in Table S1, and PCR reactions used the 2X Taq Mix (FroggaBio) and ran for 30 cycles.

4.2 | Phylogenetic analysis

The amino acid sequences for *A. thaliana* PUB proteins with UND domains (Mudgil et al., 2004; Trenner et al., 2022) were retrieved fromThe Arabidopsis Information Resource (TAIR, Araport11) and used in Basic Local Alignment Search Tool (BLAST) searches of the *A. lyrata* genome in Phytozome to retrieve the *A. lyrata* UND-PUBs. In Molecular Evolutionary Genetics Analysis Version 11 (MEGA11), muscle set at default parameters was used to align the amino acid sequences, and then using default parameters, a maximum likelihood tree was built with 1000 bootstrap replications (Edgar, 2004; Jones et al., 1992; Tamura et al., 2021). Gene identifiers for the genes used for this study are shown in Figure 1.

4.3 | RT-PCR analysis of *PUB17* and *PUB16* expression

To confirm expression of *PUB17* and *PUB16* in C24 pistils, the top half of approximately 30 *A. thaliana* pistils (including the stigmas) were collected from stage 12 flower buds and flash frozen in liquid nitrogen. Total RNA was extracted using the standard protocol for the RNeasy Plant Mini kit (Qiagen). Extracted RNA was treated using RQ1 RNase-Free DNase (Promega) to eliminate genomic DNA contamination. cDNA was synthesized using Superscipt™ IV reverse transcriptase (ThermoFisher) with oligo (dT)20 primers and then used for PCR amplification (+RT). As a negative control, the cDNA synthesis reaction was set up with all components except the Superscipt™ IV reverse transcriptase and used for PCR amplification (−RT). Elongation factor 1α2 (EF1A2; At1g07930) was used as a positive control for the RT-PCR reactions. PCR reactions were performed at 30 cycles for *PUB16* and *PUB17* and at 25 cycles for *EF1A2* using 2X Taq Mix (FroggaBio), and the following primers: *PUB17 LP* and *PUB17 RP*; *PUB16 LP* and *PUB16 RP*; and *EF1A2 LP* and *EF1A2 RP*. To test for expression of the *pub* deletion mutations, RNA was extracted from seedling tissues, and RT-PCR reactions were performed as described

above using primers that flanked the deletion sites (*PUB17 KO FP* and *RP*; *PUB16 KO FP* and *RP*). See Table S1 for primer sequences.

4.4 | Plasmid construction and plant transformation

The CRISPR/Cas9 vectors for creating deletion mutations in the C24 *PUB17* and *PUB16* genes were generated as previously described (Doucet, Lee, et al., 2019, Doucet, Truong, et al., 2019; Wang et al., 2015). The final pBEE401E vectors each contained two guide RNAs targeting the single exons of *PUB17* and *PUB16*. Wildtype *A. thaliana* C24 plants were transformed through *Agrobacterium*-mediated floral dip and to maximize transformation efficiency, plants were dipped twice, with a 7-day interval (Clough & Bent, 1998). Seeds from transformed plants were collected, stratified, and germinated on soil. Seven-day-old seedlings were sprayed with BASTA herbicide every 2 days, for a total of three times to select for transformants. Transformants were confirmed through the detection of the BASTA resistance gene by PCR, and deletion mutations were identified through PCR genotyping. Plants in the T1 generation were self-fertilized, and in the T2 generation, two homozygous mutant alleles were detected for each gene *pub17-4*, *pub17-5*, *pub16-1*, and *pub16-2*. See Table S1 for primers used.

4.5 | Pollen hydration, aniline blue staining, and seed set

All post-pollination assays used manual pollinations and were conducted as described in Lee et al. (2020). All assays began with the emasculation of stage 12 flower buds from wild-type and mutant lines. Emasculated flower buds were wrapped with plastic wrap and stored in the growth chambers. To conduct the pollen hydration assay, the emasculated pistils were removed from the plant at 24-h post-emasculation and mounted on ½ MS plates. A single anther from SI-C24 open flowers was used to lightly pollinate pistils. Images were captured at 0- and 10-min post-pollination using a Nikon sMz800 microscope, and 10 pollen grains per pistil were randomly measured using the NIS-elements imaging software. This assay was repeated on three pistils per line, measuring a total of 30 pollen grains per group ($n = 30$). To conduct the aniline blue staining assay, emasculated pistils were pollinated with a single anther from SI-C24 open flowers at 24-h post-emasculation and were returned to the growth chambers. At 24-h post-pollination, the pistils were collected and stained as described in (Lee et al., 2020). Aniline blue-stained pistils were mounted on a microscope slide with sterile water, flattened with a coverslip, and imaged at 10× magnification on a Zeiss Axioskop2 Plus fluorescent microscope. Aniline blue images were captured using the UV laser, and brightfield images were captured using the built-in halogen lamp. The number of pollen tubes/style was quantified for 8–16 pistils per line. To quantify seed yield, emasculated pistils were pollinated at 24-h post-emasculation using anthers from opened C24 or SI-C24 flowers and left for 10–14 days. Developed and matured siliques were collected and submerged in 70% (v/v) ethanol for 3–5 days or until siliques were cleared and seeds were visible (Beuder et al., 2020). Cleared siliques were mounted on a dry microscope slide with the septum facing upwards and imaged using a Nikon sMz800 microscope, and seeds were counted using the NIS-elements imaging software. For each line, the number of seeds/silique from 10 siliques were counted. For all statistical analyses, one-way analysis of variance (ANOVA) with Tukey's honest significance difference (HSD) post-hoc tests were performed using SPSS (IBM, Armonk, NY, USA). The cut-off value was specified as $p < .05$.

AUTHOR CONTRIBUTIONS

Paula K.S. Beronilla and Daphne R. Goring designed the research; Paula K.S. Beronilla performed the research and wrote the paper; Paula K.S. Beronilla and Daphne R. Goring analyzed data, edited the paper, and approved the final version.

ACKNOWLEDGMENTS

We thank Samantha Lee, Erin Navaratnam, Cecilia Widjaja, and Hamna Ammar for technical assistance, and members of the Goring lab for critically reading this article. We are also very grateful to Seiji Takayama (Nara Institute of Science and Technology; University of Tokyo) for providing the SI-C24 #15-1 seeds (*A. thaliana* C24). PKSB was supported by a Aiken-Woods Memorial scholarship and this research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to DRG.

CONFLICT OF INTEREST STATEMENT

The authors did not report any conflict of interest.

DATA AVAILABILITY STATEMENT

The data is available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Beronilla, P. K. S., & Goring, D. R. (2024). Investigating a role for PUB17 and PUB16 in the self-incompatibility signaling pathway in transgenic *Arabidopsis thaliana*. *Plant Direct*, *8*(7), e622. https://doi.org/10.1002/pld3.622