RNA Silencing of Exocyst Genes in the Stigma Impairs the Acceptance of Compatible Pollen in Arabidopsis[1][OPEN]

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Initial pollen-pistil interactions in the Brassicaceae are regulated by rapid communication between pollen grains and stigmatic papillae and are fundamentally important, as they are the first step toward successful fertilization. The goal of this study was to examine the requirement of exocyst subunits, which function in docking secretory vesicles to sites of polarized secretion, in the context of pollen-pistil interactions. One of the exocyst subunit genes, *EXO70A1*, was previously identified as an essential factor in the stigma for the acceptance of compatible pollen in Arabidopsis (*Arabidopsis thaliana*) and *Brassica napus*. We hypothesized that EXO70A1, along with other exocyst subunits, functions in the Brassicaceae dry stigma to deliver cargo-bearing secretory vesicles to the stigmatic papillar plasma membrane, under the pollen attachment site, for pollen hydration and pollen tube entry. Here, we investigated the functions of exocyst complex genes encoding the remaining seven subunits, *SECRETORY3* (*SEC3*), *SEC5*, *SEC6*, *SEC8*, *SEC10*, *SEC15*, and *EXO84*, in Arabidopsis stigmas following compatible pollinations. Stigma-specific RNA-silencing constructs were used to suppress the expression of each exocyst subunit individually. The early postpollination stages of pollen grain adhesion, pollen hydration, pollen tube penetration, seed set, and overall fertility were analyzed in the transgenic lines to evaluate the requirement of each exocyst subunit. Our findings provide comprehensive evidence that all eight exocyst subunits are necessary in the stigma for the acceptance of compatible pollen. Thus, this work implicates a fully functional exocyst complex as a component of the compatible pollen response pathway to promote pollen acceptance.

In flowering plants, sexual reproduction occurs as a result of constant communication between the male gametophyte and the female reproductive organ, from the initial acceptance of compatible pollen to final step of successful fertilization (for review, see Beale and Johnson, 2013; Dresselhaus and Franklin-Tong, 2013; Higashiyama and Takeuchi, 2015). In the Brassicaceae, the stigmas that present a receptive surface for pollen are categorized as dry and covered with unicellular papillae (Heslop-Harrison and Shivanna, 1977). Communication is initiated rapidly following contact of a pollen grain with a stigmatic papilla, as the role of the papillae is to regulate the early cellular responses leading to compatible pollen germination. The basal compatible pollen recognition response also presents a barrier to foreign pollen or is inhibited with self-incompatible pollen (for review, see Dickinson, 1995; Hiscock and Allen, 2008; Chapman and Goring, 2010; Indriolo et al., 2014b).

The initial adhesive interaction between the pollen grain and the papilla cell in the Brassicaceae is mediated by the exine of the pollen grain and the surface of the stigmatic papilla (Preuss et al., 1993; Zinkl et al., 1999). A stronger connection results between the adhered pollen grain and the stigmatic papilla with the formation of a lipid-protein interface (foot) derived from the pollen coat and the stigmatic papillar surface (Mattson et al., 1974; Stead et al., 1980; Gaude and Dumas, 1986; Elleman and Dickinson, 1990; Elleman et al., 1992; Preuss et al., 1993; Mayfield et al., 2001). It is at this point that a Brassicaceae-specific recognition of compatible pollen is proposed to occur (Hülskamp et al., 1995; Pruitt, 1999), though the nature of this recognition system is not clearly defined. Two stigma-specific *Brassica oleracea* glycoproteins, the S-Locus Glycoprotein and S-Locus Related1 (SLR1) protein, play a role in compatible pollen adhesion (Luu et al., 1997, 1999), potentially through interactions with the pollen coat proteins, PCP-A1 and SLR1-BP, respectively (Doughty et al., 1998; Takayama et al., 2000). The simultaneous recognition of self-incompatible pollen would also take place at this stage

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(for review, see Dresselhaus and Franklin-Tong, 2013; Indriolo et al., 2014b; Sawada et al., 2014). Thus, this interface not only provides a strengthened bond between the pollen grain and stigmatic papilla, but likely facilitates the interaction of signaling proteins from both partners to promote specific cellular responses in the stigmatic papilla toward the pollen grain.

One response regulated by these interactions is the release of water from the stigmatic papilla to the adhered compatible pollen grain to enable the pollen grain to rehydrate, germinate, and produce a pollen tube (Zuberi and Dickinson, 1985; Preuss et al., 1993). Upon hydration, the pollen tube emerges at the site of pollen-papilla contact and penetrates the stigma surface between the plasma membrane and the overlaying cell wall (Elleman et al., 1992; Kandasamy et al., 1994). Pollen tube entry into the stigmatic surface represents a second barrier, selecting compatible pollen tubes. Subsequently, the compatible pollen tubes traverse down to the base of the stigma, enter the transmitting tract, and grow intracellularly toward ovules for fertilization. Pollen-pistil interactions at these later stages are also highly regulated (for review, see Beale and Johnson, 2013; Dresselhaus and Franklin-Tong, 2013; Higashiyama and Takeuchi, 2015).

EXO70A1, a subunit of the exocyst, was identified as a factor involved in early pollen-stigma interactions, where it is required in the stigma for the acceptance of compatible pollen and inhibited by the self-incompatibility response (Samuel et al., 2009). Stigmas from the Arabidopsis (*Arabidopsis thaliana*) *exo70A1* mutant display constitutive rejection of wild-type-compatible pollen (Samuel et al., 2009; Safavian et al., 2014). This stigmatic defect was rescued by the stigma-specific expression of an Red Fluorescent Protein (RFP):EXO70A1 transgene (Samuel et al., 2009) or partially rescued by providing a high relative humidity environment (Safavian et al., 2014). In addition, the stigma-specific expression of an EXO70A1 RNA interference construct in *Brassica napus* 'Westar' resulted in impaired compatible pollen acceptance and a corresponding reduction in seed production compared with compatible pollinations with wild-type *B. napus* 'Westar' pistils (Samuel et al., 2009). From these studies, EXO70A1 was found to be a critical component in stigmatic papillae to promote compatible pollen hydration and pollen tube entry through the stigma surface. One of the functions of the exocyst is to mediate polar secretion (for review, see Heider and Munson, 2012; Zárský et al., 2013; Synek et al., 2014). Consistent with this, previous studies have observed vesicle-like structures in proximity to the stigmatic papillar plasma membrane in response to compatible pollen in both *Brassica* spp. and Arabidopsis species (Elleman and Dickinson, 1990, 1996; Dickinson, 1995; Safavian and Goring, 2013; Indriolo et al., 2014a). The secretory activity is predicted to promote pollen hydration and pollen tube entry. As well, consistent with the proposed inhibition of EXO70A1 by the self-incompatibility pathway (Samuel et al., 2009), a complete absence or a significant reduction of vesicle-like structures at the stigmatic papillar plasma membrane was observed in the *exo70A1* mutant and with self-incompatible pollen (Safavian and Goring, 2013; Indriolo et al., 2014a).

The exocyst is a well-defined complex in yeast (*Saccharomyces cerevisiae*) and animal systems, consisting of eight subunits, SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70, and EXO84 (TerBush et al., 1996; Guo et al., 1999). Exocyst subunit mutants were first identified in yeast as secretory mutants displaying a cytosolic accumulation of secretory vesicles (Novick et al., 1980). Subsequent work defined roles for the exocyst in vesicle docking at target membranes in processes such as regulated secretion, polarized exocytosis, and cytokinesis to facilitate membrane fusion by Soluble NSF Attachment protein Receptor (SNARE) complexes (for review, see Heider and Munson, 2012; Liu and Guo, 2012). In plants, genes encoding all eight exocyst subunits have been identified, and many of these genes exist as multiple copies. For example, the Arabidopsis genome contains single copy genes for *SEC6* and *SEC8*, two copies each for *SECRETORY3* (*SEC3*), *SEC5*, *SEC10*, and *SEC15*, three *EXO84* genes, and 23 *EXO70* genes (Chong et al., 2010; Cvrčková et al., 2012; Vukašinović et al., 2014). Ultrastructural studies using electron tomography uncovered the existence of a structure resembling the exocyst in Arabidopsis (Otegui and Staehelin, 2004; Seguí-Simarro et al., 2004). Localization studies of specific Arabidopsis exocyst subunits also supported conserved roles in polarized exocytosis and cytokinesis in plants. Localization studies have shown EXO70, SEC6, and SEC8 at the growing tip of pollen tubes (Hála et al., 2008), EXO70A1 at the stigmatic papillar plasma membrane (Samuel et al., 2009), SEC3a, SEC6, SEC8, SEC15b, EXO70A1, and EXO84b at the root epidermal cell plasma membrane and developing cell plate (Fendrych et al., 2010, 2013; Wu et al., 2013; Zhang et al., 2013; Rybak et al., 2014), and SEC3a at the plasma membrane in the embryo and root hair (Zhang et al., 2013). Similar to the yeast exocyst mutants, vesicle accumulation has also been observed in the *exo70A1* and *exo84b* mutants (Fendrych et al., 2010; Safavian and Goring, 2013). Taken together, these findings strongly support that plant exocyst subunits function in vivo in vesicle docking at sites of polarized secretion and cytokinesis (for review, see Zárský et al., 2013). In support of this, a recent study investigating Transport Protein Particle (TRAPP)II and exocyst complexes during cytokinesis in Arabidopsis has identified all eight exocyst components in immunoprecipitated complexes (SEC3a/SEC3b, SEC5a, SEC6, SEC8, SEC10, SEC15b, EXO70A1, EXO70H2, and EXO84b; Rybak et al., 2014).

Several plant exocyst subunit genes have been implicated in biological processes that rely on regulated vesicle trafficking, where corresponding mutants have displayed a range of growth defects. At the cellular level, these phenotypes have been associated with decreased cell elongation and polar growth (Cole et al., 2005, 2014; Wen et al., 2005; Synek et al., 2006), defects in cytokinesis and cell plate formation (Fendrych et al., 2010; Wu et al., 2013; Rybak et al., 2014), and disrupted Pin-Formed (PIN) auxin efflux carrier recycling and

polar auxin transport (Drdová et al., 2013). Several Arabidopsis subunit mutants display strong growth defects such as the *sec3a* mutant with an embryo-lethal phenotype (Zhang et al., 2013), *sec6*, *sec8*, and *exo84b* mutants with severely dwarfed phenotypes and defects in root growth (Fendrych et al., 2010; Wu et al., 2013; Cole et al., 2014), and *exo70A1* with a milder dwarf phenotype (Synek et al., 2006). The Arabidopsis *exo70A1* mutant has also been reported to have defects in root hair elongation, hypocotyl elongation, compatible pollen acceptance, seed coat deposition, and tracheary element differentiation (Synek et al., 2006; Samuel et al., 2009; Kulich et al., 2010; Li et al., 2013). Essential roles for other exocyst subunits include Arabidopsis SEC5a/SEC5b, SEC6, SEC8, and SEC15a/SEC15b in male gametophyte development and pollen tube growth (Cole et al., 2005; Hála et al., 2008; Wu et al., 2013), SEC8 in seed coat deposition (Kulich et al., 2010), SEC5a, SEC8, EXO70A1, and EXO84b in root meristem size and root cell elongation (Cole et al., 2014), and a maize (*Zea mays*) SEC3 homolog in root hair elongation (Wen et al., 2005). Finally, the Arabidopsis EXO70B1, EXO70B2, and EXO70H1 subunits have been implicated in plant defense responses (Pecenková et al., 2011; Stegmann et al., 2012; Kulich et al., 2013; Stegmann et al., 2013).

Even with these detailed studies on the functions of exocyst subunits in plants, a systematic demonstration of the requirement of all eight exocyst subunits in a specific plant biological process is currently lacking. EXO70A1 was previously identified as an essential factor in the stigma for compatible pollen-pistil interactions in Arabidopsis and *B. napus* (Samuel et al., 2009), and we hypothesized that this protein functions as part of the exocyst complex to tether post-Golgi secretory vesicles to stigmatic papillar plasma membrane (Safavian and Goring, 2013). To provide support for the proposed biological role of the exocyst in the stigma for compatible pollen acceptance, we investigated the roles of the remaining seven subunits, SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, and EXO84, in Arabidopsis stigmatic papillae. Given that some Arabidopsis exocyst subunits were previously determined to be essential at earlier growth stages, stigma-specific RNA-silencing constructs were used for each exocyst subunit, and the early post-pollination stages were analyzed for these transgenic lines. Our collective data demonstrates that all eight exocyst subunits are required in the stigma for the early stages of compatible pollen-pistil interactions.

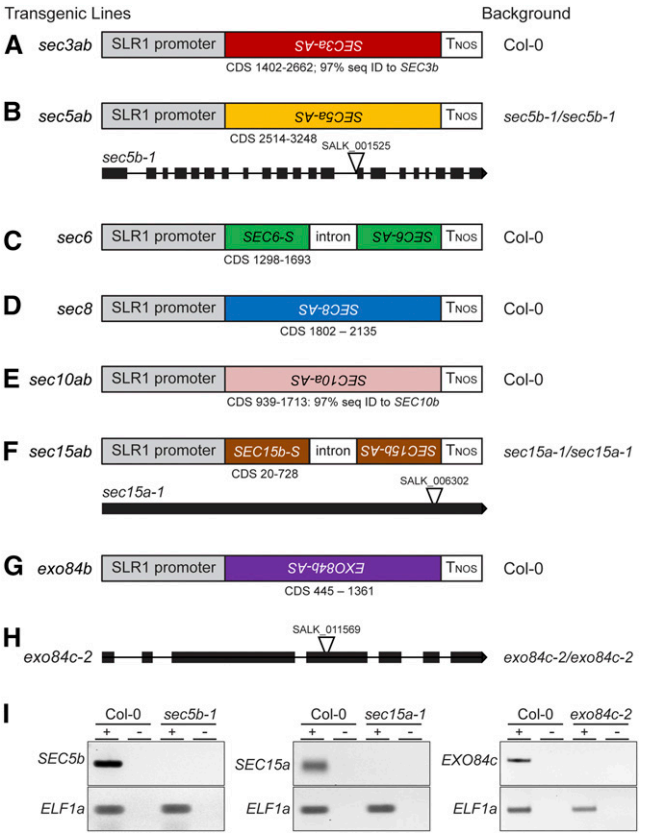

Figure 1. RNA-silencing constructs and exocyst mutants used in this study. A to G, RNA-silencing constructs and their corresponding wild-type or mutant backgrounds. All constructs are driven by the stigma-specific *SLR1* promoter. A, The *SLR1*:*SEC3a* AS construct contains *SEC3a* $CDS_{1402-2662}$, with 97% identity to *SEC3b*, and was transformed into wild-type Col-0. B, The *SLR1*:*SEC5a* AS construct contains *SEC5a* $CDS_{2514-3248}$ and was transformed into the *sec5b-1* mutant. C, The *SLR1*:*SEC6* hpRNAi construct contains *SEC6* $CDS_{1298-1693}$ and was transformed into wild-type Col-0. D, The *SLR1*:*SEC8* AS construct contains *SEC8* $CDS_{1802-2135}$ and was transformed into wild-type Col-0. E, The *SLR1*:*SEC10a* AS construct contains *SEC10a* $CDS_{939-1713}$, with 97% identity to *SEC10b*, and was transformed into wild-type Col-0. F, The *SLR1*:*SEC15b* hpRNAi construct contains *SEC15b* CDS_{20-728} and was transformed into the *sec15a-1* mutant. G, The *SLR1*:*EXO84b* AS construct contains *EXO84b* $CDS_{445-1361}$ and was transformed into wild-type Col-0. H, For the *EXO84c* gene, the *exo84c-2* T-DNA insertion mutant was analyzed. I, RT-PCR analyses confirming that the *sec5b-1*, *sec15a-1*, and *exo84c-2* homozygous mutants are knockout lines. *Elf1α* was included as a positive control. +, Reverse transcriptase was added; –, reverse transcriptase was not added to the cDNA reaction. The RT– reactions verified the removal of any contaminating genomic DNA. % Seq ID, Percentage nucleotide sequence identity; CDS, coding DNA sequence; TNOS, Nopaline Synthase terminator.

RESULTS

Strategies for the RNA Silencing of Exocyst Subunit Genes in the Stigma

A number of Arabidopsis exocyst subunit loss-of-function mutants display severe growth defects; thus, flowering is compromised in these mutants (Fendrych et al., 2010; Wu et al., 2013; Zhang et al., 2013). As we were interested in investigating if the exocyst subunits are required in the stigmatic papilla to accept compatible pollen, a focused strategy was adopted to knockdown the expression of individual exocyst subunit genes. We have previously used the *Brassica oleracea* SLR1 promoter in a number of studies (Samuel et al., 2009; Indriolo et al., 2012, 2014a), as it is a well-characterized promoter that drives strong expression in the stigma as the flower reaches maturity (Franklin et al., 1996; Foster et al., 2005; Fobis-Loisy

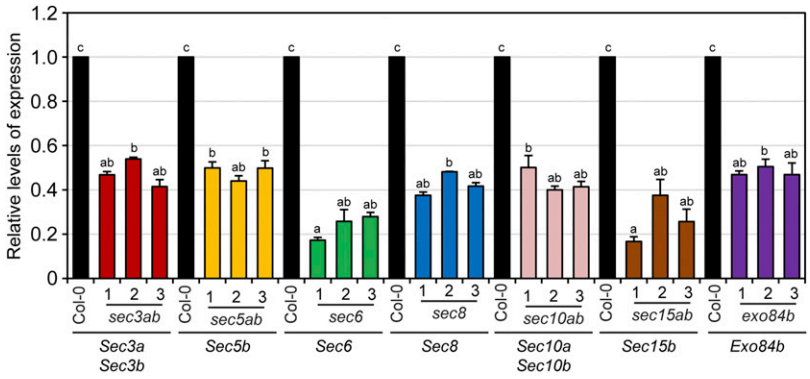

Figure 2. qRT-PCR analyses of exocyst subunit gene expression levels in wild-type and transgenic RNA-silencing lines. The qRT-PCR analyses were conducted on RNA samples extracted from half pistils (with stigmas and styles) for three independent transgenic lines per RNA-silencing construct. The exocyst subunit gene expression levels were normalized across the samples with the *Elf1α* and *TUB4* controls, and the data for the transgenic RNA-silencing lines are shown relative to wild-type Col-0 (set to value of 1). Error bars = SE. The different letters represent means that are significantly different at $P < 0.05$ (one-way ANOVA with Scheffe post hoc tests).

et al., 2007). Thus the stigma-specific SLR1 promoter was used to drive the expression of RNA-silencing constructs for each of the exocyst subunit genes (Fig. 1). Initially, hairpin RNA interference (hpRNAi) constructs were attempted for all the target genes, but some clones were unsuccessful due to technical issues, and antisense (AS) constructs were assembled for some genes instead.

The number of genes encoding each of the seven exocyst subunits under investigation ranged from a single copy (*SEC6* and *SEC8*), two genes (*SEC3a* and *SEC3b*, *SEC5a* and *SEC5b*, *SEC10a* and *SEC10b*, and *SEC15a* and SEC15*b*), and three genes (*EXO84a*, *EXO84b*, and *EXO84c*; Chong et al., 2010; Cvrčková et al., 2012; Vukašinović et al., 2014). As all of these exocyst subunit genes showed some level of expression in Arabidopsis stigmas (except *EXO84a*; Supplemental Fig. S1; Safavian et al., 2014), these constructs were designed to target either more than one gene (*SEC3* and *SEC10*; Fig. 1, A and E) or the constructs were combined with homozygous transfer DNA (T-DNA) insertion mutants (*SEC5* and *SEC15*; Fig. 1, B and F). The RNA-silencing constructs were designed to knockdown the expression of the more highly expressed gene paralogs, and the T-DNA insertion line was used to knockout the expression of the other gene paralogs with lower expression levels. Thus, based on the stigma microarray expression data obtained from the Bio-Array Resource (Swanson et al., 2005; Toufighi et al., 2005), the *SEC5a-AS* construct was transformed into homozygous *sec5b-1* plants (SALK_001525; Fig. 1B), and the *SEC15b-hpRNAi* was transformed into homozygous *sec15a-1* plants (SALK_006302; Fig. 1F). The need for this approach was discovered early on in this work, where the RNA silencing of *SEC15b* by the *SLR1*:*SEC15b-hpRNAi* transgene alone did not reduce the acceptance of compatible pollen (Supplemental Fig. S2). The exception to this strategy was the *EXO84* genes, as *EXO84a* appears to be pollen specific (Supplemental Fig. S1), and for some unknown reason, we were unable to recover any *exo84c-2* mutants transformed with the *EXO84b-AS* construct (Fig. 1, G and H). Thus, the transgenic *exo84b* lines and homozygous *exo84c-2* mutant (SALK_011569; Cole, 2008) were characterized separately. The *sec5b-1*, *sec15a-1*, and *exo84c-2* homozygous mutants were confirmed by reverse transcription (RT)-PCR to be knockout lines (Fig. 1I).

Following transformation of the RNA-silencing constructs, T1 transgenic plants were screened for reduced seed set phenotypes, and three independent transformants for each of the seven exocyst subunits were selected from this screen for detailed characterization in the T2 and T3 generations. The mRNA levels of the target exocyst subunit genes in the transgenic RNA-silencing lines were analyzed by quantitative reverse transcription (qRT)-PCR to determine the degree of RNA silencing in these lines. As a result of the small stigma size, half-pistils (with stigmas) were collected for RNA extraction and qRT-PCR analysis. When exocyst subunit gene expression levels were compared with wild-type ecotype Columbia (Col-0), all of the RNA-silencing lines showed a significant decrease in the respective exocyst subunit gene (Fig. 2). The levels of reduced expression ranged from 26% (*sec6* line 1 and *sec15ab* line 1) to 53% (*sec3ab* line 1 and *sec5ab* line 1) compared with wild-type Col-0. As well, the level of RNA silencing in the stigmas is likely lower than our reported values because the half-pistils collected for this analysis included style tissues that would not express the stigma-specific SLR1 promoter-driven RNA-silencing constructs.

RNA Silencing of Exocyst Subunit Genes in the Stigma Causes Reduced Acceptance of Wild-Type-Compatible Pollen

The early stages of compatible pollen acceptance were investigated by hand pollinating transgenic pistils and staining with aniline blue to visualize pollen tube growth. Wild-type Col-0 pollen was used for all pollinations of transgenic stigmas. The differential interference contrast (DIC) images of these transgenic pistils showed a large reduction in the number of Col-0 pollen grains adhered to the stigmas at 2 h postpollination. This indicated that most of the pollen grains had failed to adhere and germinate on the stigma surface, and they would have been washed away during the staining process (Fig. 3, A–H; Supplemental Fig. S2). The number of pollen grains per stigma was scored for each of the three transgenic lines per exocyst subunit and the *exo84c-2* mutant. All transgenic and mutant stigmas showed a statistically significant decrease in the number of adhered pollen grains

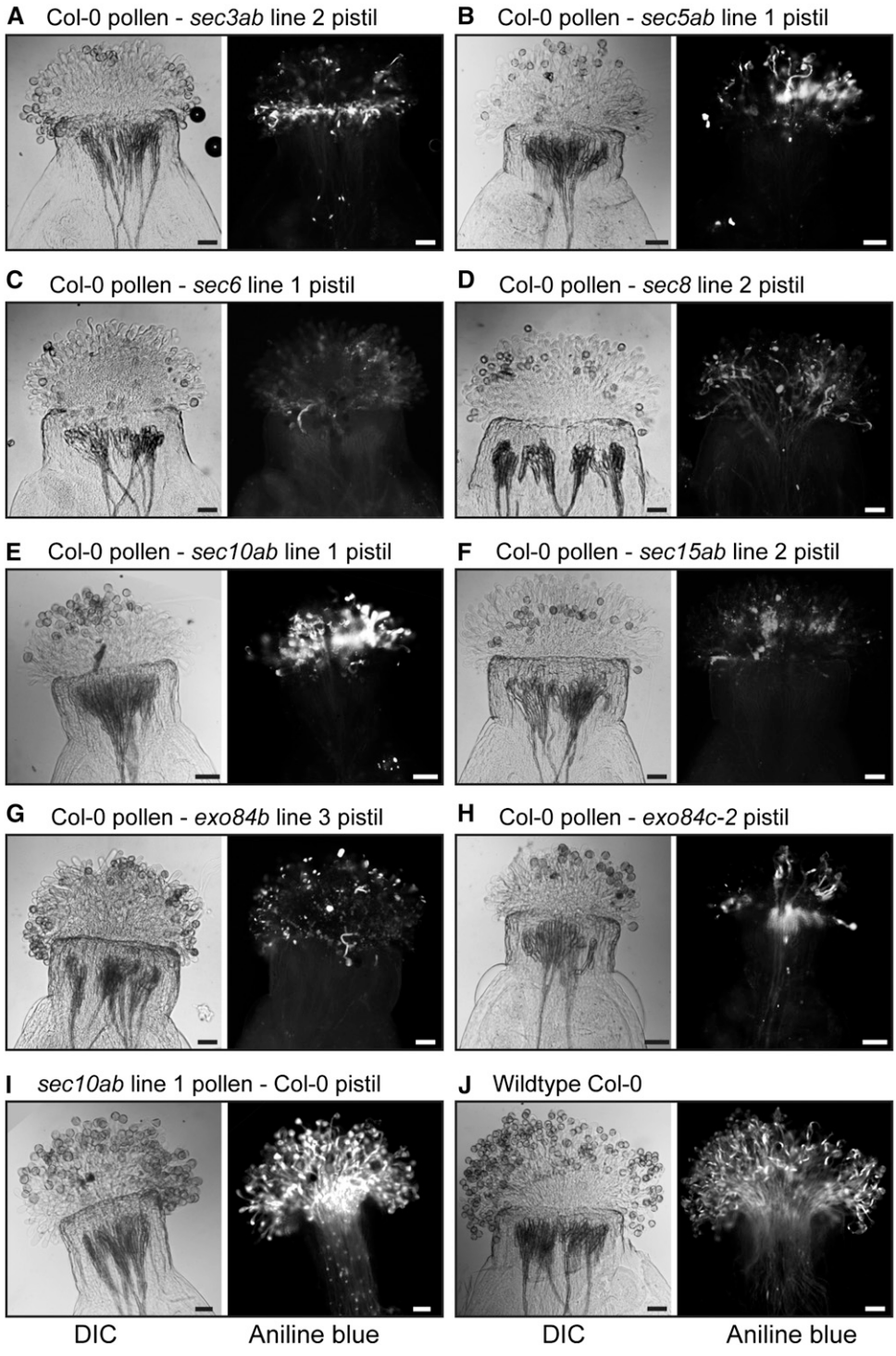

Figure 3. Pollen grain attachment and pollen tube growth following manual pollination of pistils from the exocyst subunit RNA-silencing/knockout lines with wild-type Col-0 pollen. A to H, Col-0 pollen on transgenic stigmas at 2 h postpollination. Three independent transgenic lines tested for each RNA-silencing construct (Supplemental Fig. S2), and one representative image for each is shown here. All transgenic pistils showed reduced compatible pollen responses with reduced pollen grain adhesion (DIC images) and pollen tube growth (aniline blue images). I, Control reciprocal pollination of *sec10ab* line1 pollen on Col-0 stigma. These control pollinations were conducted for the three independent transgenic lines tested per RNA-silencing construct (Supplemental Fig. S3), and one representation line is shown here, where abundant adhered pollen grains (DIC) and pollen tubes (aniline blue image) were observed. J, Control wild-type Arabidopsis Col-0 self-pollination showing abundant adhered pollen grains (DIC image) and numerous pollen tubes as expected (aniline blue image). Bars = 50 μm.

relative to wild-type Col-0 stigmas (Fig. 4A). The mean numbers of adhered pollen grains for the transgenic plants ranged from 9 pollen per stigma (*sec15ab* line 1) to 49 pollen per stigma (*exo84b* line 2) compared with Col-0 at 133 pollen per stigma (Fig. 4A). The aniline blue-stained images of the transgenic pistils pollinated with Col-0 pollen also showed corresponding decreases in the number of pollen tubes growing through the pistils (Fig. 3, A–H; Supplemental Fig. S2) compared with the control wild-type Col-0 pistils (Fig. 3J). By contrast, reciprocal control pollinations of the various transgenic pollen on wild-type Col-0 pistils produced abundant pollen tubes with aniline blue staining and numerous pollen grains present on the stigmas in the DIC images (Fig. 3I; Supplemental Fig. S3). Thus, the transgenic pollen grains were unaffected, and only the transgenic stigmas

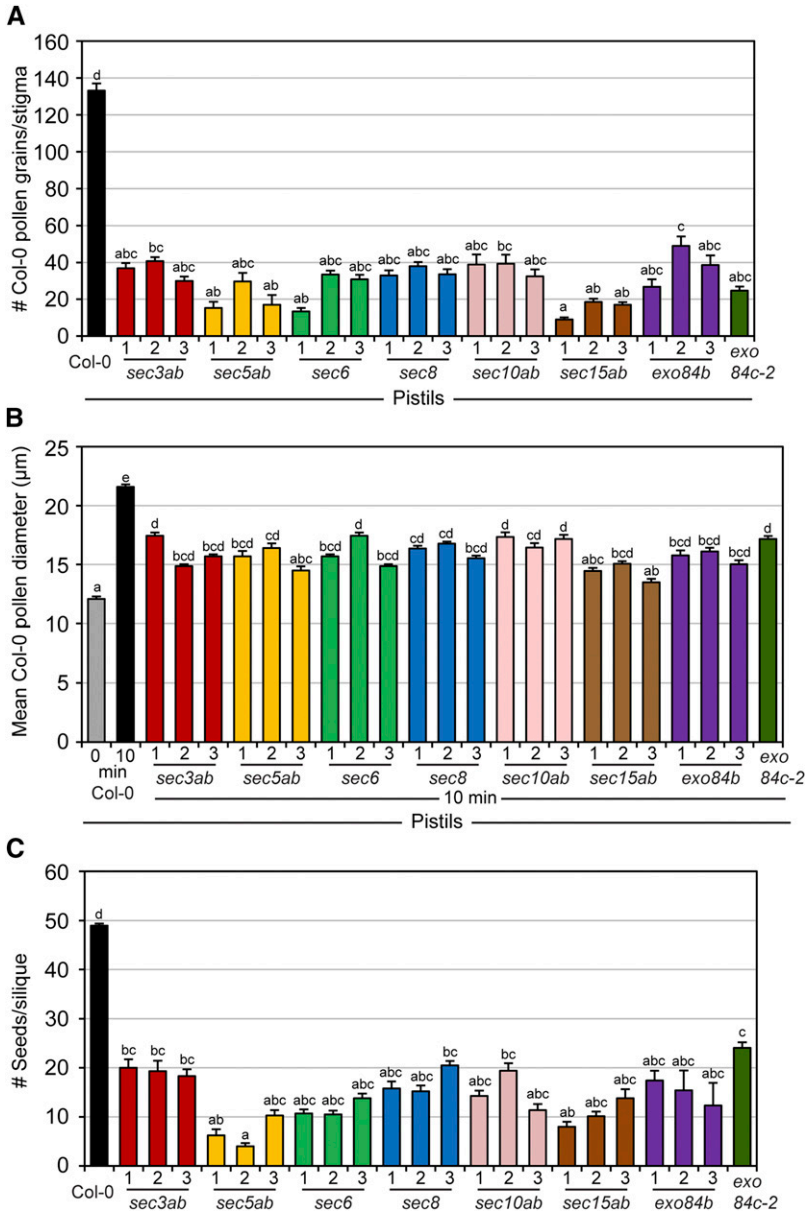

Figure 4. Graphs showing adhered pollen grains, pollen grain hydration, and seed set for wild-type Col-0, the three independent transgenic lines per exocyst subunit RNA-silencing construct, and the *exo84c-2* mutant. A, Mean number of adhered pollen grains per stigma at 2 h postpollination following aniline blue staining. All pistils were pollinated with wild-type Col-0 pollen ($n \geq 10$ stigmas for each plant line). B, Pollen hydration as measured by mean pollen diameter at 10 min postpollination. All pistils were pollinated with Col-0 pollen ($n = 30$ pollen grains [10 pollen grains $\times$ 3 pistils] for each plant line). C, Mean number of seeds per silique for average silique sizes in each plant line, following natural self-pollination. Error bars = SE. The different letters represent means that are significantly different at $P < 0.05$ (one-way ANOVA with Scheffe post hoc tests).

displayed a reduced pollen acceptance phenotype. This confirmed that the strategy of using a stigma-specific promoter to drive the expression of the RNA-silencing constructs allowed us to successfully investigate exocyst subunit functions in the stigma following pollination.

To examine an earlier time point in the compatible pollen acceptance response of the transgenic stigmas, pollen hydration was examined. As the pollen grain hydrates, its shape changes from elongated to round, and the increased pollen diameter is a well-established proxy for pollen hydration (Zuberi and Dickinson, 1985). Col-0 pollen was used to pollinate stigmas from three transgenic lines per exocyst subunit and the *exo84c-2* mutant, and pollen diameters were measured at 10 min postpollination (Fig. 4B). All pollen hydration experiments were conducted under relative humidity levels ranging from 20% to 35% to reduce any potential interference from ambient humidity (for further discussion, see Safavian et al., 2014). Col-0 pollen diameters on all the transgenic stigmas were significantly reduced compared with those on wild-type Col-0 stigmas, indicating that pollen hydration was also impaired with the RNA silencing/knockout of each of the exocyst subunits. The mean Col-0 pollen grain diameters on transgenic stigmas ranged from 13.5 (*sec15ab* line 3) to 17.4 μm (*sec3ab* line 1 and *sec6* line 2) at 10 min postpollination compared with Col-0 stigmas with 12.1 μm at 0 min and 21.6 μm at 10 min postpollination. Thus, these data extend our earlier study showing that EXO70A1 is required in the stigma for the early postpollination stage of pollen hydration (Samuel et al., 2009).

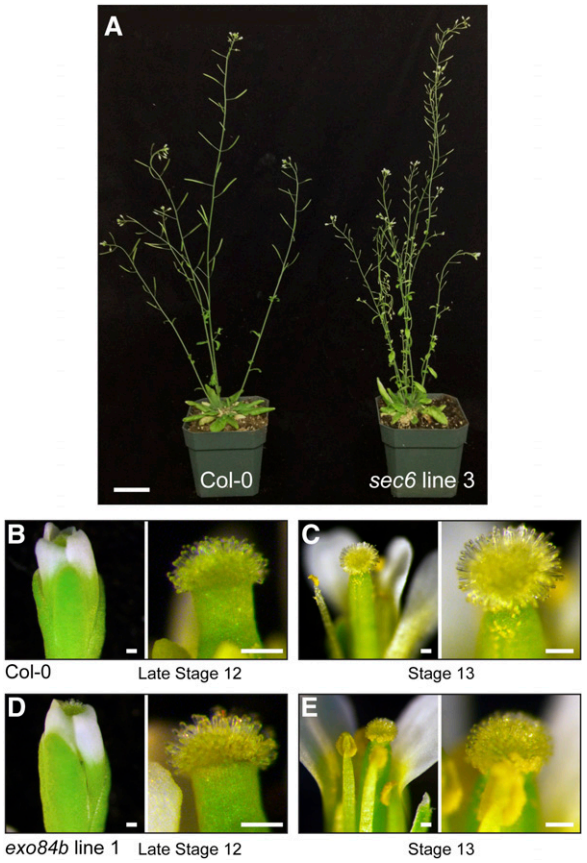

Figure 5. Phenotypes of flowering plants and stigmatic papillae. A, Images of wild-type Col-0 (left) and *sec6* line 3 (right) whole plants. While the overall morphology is similar to wild-type Col-0, *sec6* line 3 shows smaller siliques relative to Col-0. Smaller siliques were also seen for the other transgenic lines (Supplemental Fig. S4). B to E, Images of wild-type Col-0 (B and C) and *exo84b* line 1 (D and E) stigmas. Elongated stigmatic papillae are present in late-stage 12 flower buds (just prior to anthesis and anther dehiscence; Smyth et al., 1990; Sanders et al., 1999) for both wild-type Col-0 (B) and *exo84b* line 1 (D). The flower bud is shown in the left section, while a close-up of the stigma is shown in the right section. The other transgenic lines were also wild type for stigmatic papillar elongation ($n > 30$ for each line; Supplemental Fig. S5). A freshly opened flower (stage 13) and close-up of the stigma are shown for comparison (C and E). Bars = 3 cm (A) and 150 μm (B–E).

With reduced pollen hydration and pollen adhesion, we investigated whether this resulted in reduced seed set. The number of seeds per silique was counted for the most common silique size from the three transgenic lines per exocyst subunit and the *exo84c-2* mutant. All siliques carried some seeds, which would be expected because a few pollen tubes were seen in the aniline blue-stained images. However, all transgenic siliques had significant reductions in seed set compared with wild-type Col-0 (Fig. 4C). The mean numbers for the transgenic lines ranged from 4 seeds per silique (*sec5ab* line 2) to 24 seeds per silique (*exo84c-2*) compared with Col-0 at 49 seeds per silique.

RNA Silencing of Exocyst Subunit Genes in the Stigma Does Not Affect Plant Growth and Morphology

As several Arabidopsis exocyst subunits play essential roles in growth and development (Cole et al., 2005, 2014; Synek et al., 2006; Fendrych et al., 2010; Wu et al., 2013; Zhang et al., 2013), we examined the transgenic lines carefully for any other defects resulting from the RNA-silencing constructs. The use of the stigma-specific SLR1 promoter in the RNA-silencing constructs would be predicted to drive expression of these constructs late in the life cycle (as flower buds approach maturity). All the transgenic lines were wild type in appearance and flowered similarly to wild-type Col-0 (Fig. 5A; Supplemental Fig. S4). A clearly noticeable altered phenotype was the reduced silique sizes resulting from the reduced seed set (described above; Fig. 4C). The *exo70A1* mutant pistils have also been previously described to have variable stigmatic papillar elongation and defects in tracheary element differentiation (Synek et al., 2006; Samuel et al., 2009; Li et al., 2013; Safavian et al., 2014). Late-stage 12 transgenic flower buds, just prior to anthesis, were carefully examined for stigmatic papillar elongation. At this stage, the flower buds are starting to open, but anther dehiscence has not occurred yet (Smyth et al., 1990; Sanders et al., 1999). All transgenic stigmas revealed fully elongated stigmatic papillae that were wild type in appearance (Fig. 5, B–E; Supplemental Fig. S5). Finally, mature tracheary elements could be seen in cleared pistils from the transgenic plants (Supplemental Fig. S6). Thus, the effects of the RNA-silencing constructs appear to be specific to responses of the stigmatic papillae to compatible pollen.

High Relative Humidity Can Increase the Number of Compatible Pollen Grains on Transgenic Stigmas

We have previously shown that the defects in the acceptance of wild-type Col-0 pollen by the *exo70A1* mutant can be partially overcome by exposing plants to high relative humidity growth conditions (Safavian et al., 2014). Pollinations were carried out, using wild-type Col-0 pollen, under high relative humidity conditions (high humidity [HH], approximately 90%) for one representative transgenic line per exocyst subunit and the *exo84c-2* mutant, followed by aniline blue staining of the pollinated pistils, and then compared with results from low relative humidity conditions (low humidity [LH], approximately 35%; Fig. 6). Consistent with our previous observations on the effect of high relative humidity on pollen-pistil interactions in *exo70A1* mutants, there were increased numbers of adhered pollen grains and pollen tubes per transgenic stigma under HH conditions (Fig. 6, A and B). When the number of adhered Col-0 pollen grains per stigma were counted, each transgenic line showed a significant increase under high relative humidity compared with LH conditions (Fig. 6C). Thus, these results confirm that high relative humidity can partially restore stigmatic fertility, by circumventing pollen hydration control by the

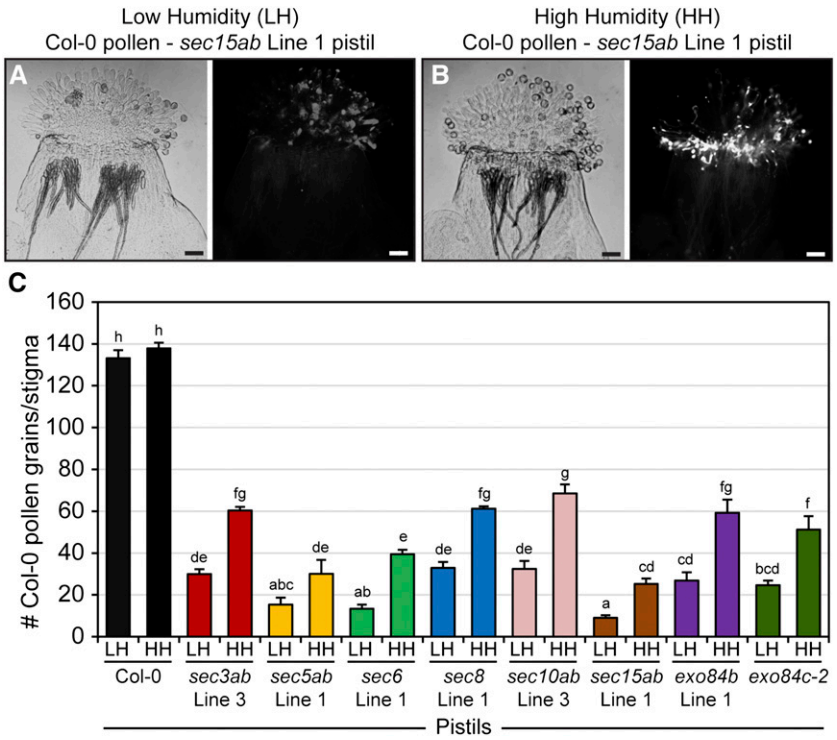

Figure 6. Pollen grain adhesion under low and high relative humidity for representative exocyst subunit RNA-silencing lines and the *exo84c-2* mutant pollinated with wild-type Col-0 pollen. A and B, Col-0 pollen grain attachment and pollen tube growth on *sec15ab* line 1 pistils under low (LH, approximately 35%) and high (HH, approximately 90%) humidity. Pistils were harvested at 2 h postpollination followed by aniline blue staining. C, Mean number of adhered wild-type Col-0 pollen grains per stigma at LH and HH at 2 h postpollination following aniline blue staining ($n \geq 10$ stigmas for each plant line). Error bars = SE. The different letters represent means that are significantly different at $P < 0.05$ (one-way ANOVA with Duncan post hoc test).

dry Arabidopsis stigma and resulting in higher germination of wild-type Col-0 pollen on transgenic stigmas.

DISCUSSION

In Arabidopsis, pollen-pistil interactions trigger a cascade of complex events in stigmatic papillae that mediate the acceptance of compatible pollen and the rejection of self-incompatible pollen (for review, see Indriolo et al., 2014b). While considerable progress has been made in deciphering the cellular signaling regulating the rejection of self-incompatible pollen in the Brassicaceae (for review, see Iwano and Takayama, 2012; Dresselhaus and Franklin-Tong, 2013; Indriolo et al., 2014b), comparatively little is known about the cellular responses of stigmatic papillae to compatible pollen. Previously, we have demonstrated that EXO70A1 is an essential factor in the stigma for compatible pollen-pistil interactions in Arabidopsis and *B. napus* promoting pollen hydration and pollen tube penetration into the stigma surface (Samuel et al., 2009; Safavian et al., 2014). In this study, we have demonstrated the involvement of the remaining seven Arabidopsis exocyst subunits, SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, and EXO84, in compatible pollen acceptance. The RNA silencing of these subunit genes in the stigma was correlated with impaired compatible pollen recognition, where the transgenic plants displayed reduced pollen hydration, pollen attachment, and pollen tube growth and decreased seed set compared with wild-type Col-0.

There are two significant implications of our collective data demonstrating that genes corresponding to all eight exocyst subunits (SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70A1, and EXO84) are required in the stigma for accepting compatible pollen acceptance. First, this work supports our hypothesis that the exocyst is regulating polarized secretion in the stigmatic papilla toward the attachment site of the compatible pollen grain (see model in Fig. 7). Distinct roles are beginning to be identified for subsets of exocyst subunits such as EXO84 and SEC5 in autophagy and innate immunity signaling in mammalian cells (Bodemann et al., 2011; Simicek et al., 2013). SEC5 and EXO84, along with EXO70B1, have also been linked to autophagy in Arabidopsis (Kulich et al., 2013). Thus, our work presented here supports the more widely known function of the eight-subunit exocyst in vesicle trafficking. Second, our work is, to our knowledge, the first demonstration of the requirement of all eight exocyst subunits for a specific biological process in plants. This includes the plant SEC10 subunit for which no prior biological function was known, as Vukašinović et al. (2014) showed that single Arabidopsis knockout mutants for SEC10a or SEC10b displayed wild-type phenotypes.

There are several plant functions where more than one exocyst subunit has been implicated, though not the entire set of eight subunits. For example, SEC5a, SEC8, EXO70A1, and EXO84B are involved in primary root growth (Cole et al., 2014) and the maize SEC3 homolog and Arabidopsis Exo70A1 in polar growth of root hairs (Wen et al., 2005; Synek et al., 2006). Arabidopsis SEC5a/SEC5b, SEC6, SEC8, and SEC15a/SEC15b are required for pollen development and polar growth of the pollen tube (Cole et al., 2005; Hála et al., 2008; Wu et al., 2013). Arabidopsis knockout mutants for SEC3a, SEC6, SEC8, EXO70A1, and EXO84b

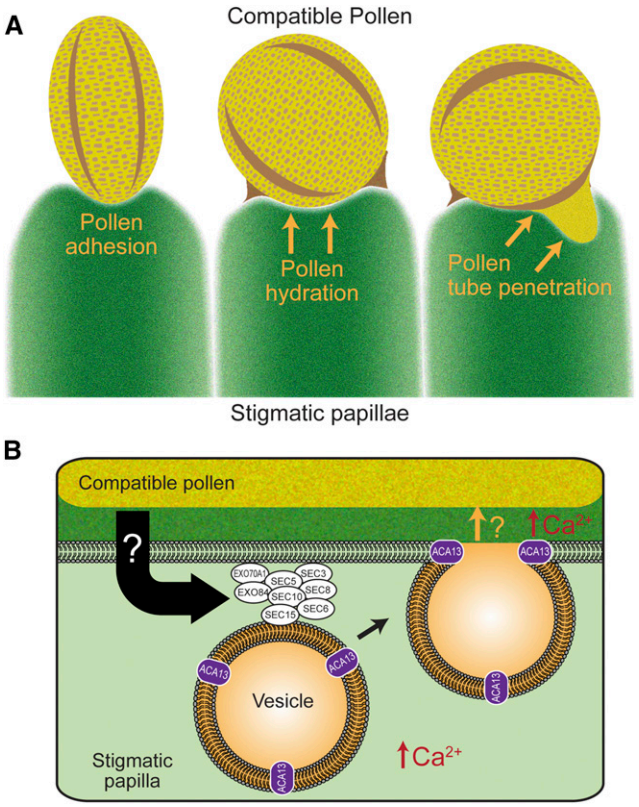

Figure 7. Proposed model of exocyst function during compatible pollen-stigma interactions in Arabidopsis. A, Early stages of the Arabidopsis compatible pollen response: (1) the pollen exine and pollen coat mediate pollen adhesion to stigmatic papilla; (2) the pollen grain hydrates at 5 to 10 min postpollination; and (3) the pollen tube penetrates and grows through papillar cell wall at 15 to 20 min postpollination (Preuss et al., 1993; Zinkl et al., 1999; Iwano et al., 2004, 2014; Safavian and Goring, 2013). B, Following compatible pollination, an unknown signaling event (black arrow) is proposed to initiate exocyst complex assembly and docking of secretory vesicles to the papillar plasma membrane for delivery of cargo to the compatible pollen grain. The ACA13 Ca^{2+} pump is one possible cargo candidate to deliver extracellular Ca^{2+} to the growing pollen tube (Iwano et al., 2014). Other unknown cargo (orange arrow) is proposed to facilitate water transfer for pollen hydration and papillar cell wall expansion for pollen tube penetration. Three different events have rapidly been observed in the stigmatic papilla underneath the pollen grains: (1) vesicle-like structures at the papillar plasma membrane at 5 to 10 min; (2) three successive local cytoplasmic increases in $[Ca^{2+}]$ levels at 6, 10, and 12 min; and (3) the relocalization of the ACA13 Ca^{2+} pump from internal Golgi-associated vesicles to the plasma membrane at 16 to 20 min (Iwano et al., 2004, 2014; Safavian and Goring, 2013).

display plant growth defects ranging from embryo-lethal to dwarf phenotypes; and related to this, SEC6, EXO84b, and EXO70A1 have been directly linked to cytokinesis and cell plate formation (Synek et al., 2006; Fendrych et al., 2010; Wu et al., 2013; Zhang et al., 2013; Rybak et al., 2014). Finally, EXO70A1 and SEC8 are required for seed coat deposition (Kulich et al., 2010). Overall, these studies have implicated plant exocyst subunits in very diverse biological functions, but there are common themes of cellular processes typically associated with the exocyst in other systems such as regulated secretion, cell polarity, and cytokinesis (for review, see Zárský et al., 2013).

The exocyst belongs to the group of multisubunit tethering complexes that function in directing transport vesicles to different subcompartments in intracellular trafficking pathways (for review, see Yu and Hughson, 2010; Hong and Lev, 2014). The function of the exocyst has been extensively studied in yeast and animal systems (for review, see Heider and Munson, 2012; Liu and Guo, 2012). Well-defined cellular roles in yeast include localization of the exocyst to sites of active secretion in yeast budding (TerBush et al., 1996) and in animal systems include neurite outgrowth (Vega and Hsu, 2001) and regulated trafficking of the Glucose transporter type4 (GLUT4) in adipocytes (Inoue et al., 2003). In these various systems, the exocyst function is in turn regulated by members of the Ras homolous (Rho), Ras-like (Ral), and Ras-related in brain (Rab) families of small Guanosinetriphosphatases (GTPases; for review, see Mukherjee et al., 2014). Following a compatible pollination on the Brassicaceae dry stigma, unknown factors are proposed to be released towards the pollen grain, and this is likely accomplished by the exocyst functioning in its conserved role as a multisubunit tethering complex to dock secretory vesicles under the pollen attachment site (see model in Fig. 7; for review, see Chapman and Goring, 2010). However, still unknown are the signaling events that regulate the initiation of polarized secretion following compatible pollination as well as the cargo being delivered to the compatible pollen.

In the context of vesicle secretion, earlier studies had provided some evidence for vesicle-like structures in *B. oleracea* stigmatic papillae in response to compatible pollen (Elleman and Dickinson, 1990, 1996; Dickinson, 1995). More recently, we investigated this in more detail in three Brassicaceae species: Arabidopsis, *Arabidopsis lyrata*, and *B. napus* (Safavian and Goring, 2013; Indriolo et al., 2014a). Detailed examinations of post-pollination events in the pollen-papillar contact area were conducted using transmission electron microscopy. As expected, vesicle-like structures were observed fusing to the papillar plasma membrane underneath the pollen contact site at 5- to 10-min postcompatible pollinations in Arabidopsis and *A. lyrata* (Safavian and Goring, 2013). Interestingly, *B. napus* stigmatic papillae appeared instead to use multivesicular bodies (MVBs) to release extracellular vesicles at the plasma membrane (Safavian and Goring, 2013), thus explaining the observation by Elleman and Dickinson (1996) of vesicle-like structures within the stigmatic papillar cell wall. The exocyst may very well be mediating both vesicle and MVB trafficking to the stigmatic papillar plasma membrane, following compatible pollinations, as EXO70A1 was found to be required in both Arabidopsis and *B. napus* for compatible pollinations (Samuel et al., 2009; Safavian et al., 2014). As well, vesicle-like structures were observed to accumulate in the cytoplasm of Arabidopsis *exo70A1* stigmatic papillae, and the stigma-specific RNA

silencing of EXO70A1 in *B. napus* also resulted in the accumulation of MVBs in the papillar cytoplasm (Safavian and Goring, 2013). Accumulation of secretory vesicles in the cytoplasm is a distinct trait that was originally observed for yeast exocyst mutants (Finger and Novick, 1997).

The polarized secretion at the pollen-papilla interface is proposed to facilitate pollen hydration and pollen tube entry into the papillar cell wall (see model in Fig. 7). Although the cargo of the vesicles has yet to be identified, it likely includes factors to promote water movement from the stigma and cell wall-loosening enzymes (Dickinson, 1995; Elleman and Dickinson, 1996; Safavian and Goring, 2013). As well, one very strong cargo candidate is the Autoinhibited Ca^{2+} Adenosine Triphosphatase13 (ACA13) that is required in the stigma for compatible pollinations and proposed to pump extracellular Ca^{2+} to the growing pollen tube (see model in Fig. 7; Iwano et al., 2014). ACA13 has Ca^{2+} transporter activity and was found to rapidly localize from Golgi-associated vesicles to the papillar plasma membrane under the compatible pollen grain (Iwano et al., 2014). Ca^{2+} gradients have been well documented in growing pollen tubes (for review, see Hepler et al., 2012), and Iwano et al. (2004) had previously shown that following compatible pollinations, there were local cytoplasmic increases in stigmatic papillar [Ca^{2+}] levels at 6 to 12 min under the pollen contact site. ACA13 is proposed to pump Ca^{2+} out of the stigmatic papilla for the growing pollen tube (Iwano et al., 2014).

In the Brassicaceae, high relative humidity can partially rescue pollen germination arising from pollen coat defects. For example, Arabidopsis pollen coat mutants have been reported to show higher pollen germination rates under high relative humidity (Preuss et al., 1993; Hülskamp et al., 1995; Fiebig et al., 2000). We previously showed that high relative humidity can partially rescue the Arabidopsis *exo70A1* stigmatic defect for accepting compatible pollen, essentially bypassing the control of pollen hydration by the dry stigma (Safavian et al., 2014). Similarly in this study, when representative exocyst subunit RNA-silencing transgenic lines and the *exo84c-2* mutant were pollinated under high relative humidity, the ability of wild-type Col-0 pollen to adhere on the stigmas and produce pollen tubes was partially restored, whereas pollinations under low relative humidity resulted in a reduced ability of transgenic stigmas to support pollen adhesion and pollen tube growth.

In summary, we present conclusive evidence in this study that the remaining seven exocyst subunits are involved in the stigma for pollen hydration/germination and pollen tube entry. Our strategy of using the stigma-specific SLR1 promoter to drive the expression of RNA-silencing constructs, along with select T-DNA knockout lines, was successful in allowing the transgenic lines to produce phenotypically wild-type flowers so that compatible pollen acceptance and reproduction could be assessed. Our data supporting that all exocyst subunits are required in the stigma for accepting compatible pollen suggests that the absence or knockdown of any one subunit leads to a dysfunctional exocyst and disruption of polarized secretion.

MATERIALS AND METHODS

Plant Material and Growth Conditions

All Arabidopsis (*Arabidopsis thaliana*) seeds (wild-type Col-0, transgenic lines, and T-DNA insertion mutants) were sterilized and cold treated for 3 d at 4°C. Afterward, they were either transferred directly to soil or germinated on one-half-strength Murashige and Skoog medium plates with 0.4% (w/v) phytoagar at pH 5.8 in 16-h light at 22°C. Seven-day-old seedlings were transferred to soil with Plant Prod All Purpose Fertilizer 20-20-20 (1 g L^{-1} water) and placed in growth chambers under a 16-h-light/8-h-dark cycle at 22°C. The Arabidopsis T-DNA insertion mutants were obtained from the Arabidopsis Biological Resource Center, and homozygous mutants were identified by PCR (primers listed in Supplemental Table S1). The *exo84c-2* (SALK_011569; Cole, 2008) was a newly characterized mutant, and the *sec5b-1* (SALK_001525) and *sec15a-1* (SALK_006302) mutants were previously published (Hála et al., 2008). To confirm that all three T-DNA insertion lines were null mutants, RNA was extracted from whole seedlings from *sec5ab* line 1, *sec15ab* line 1, and *exo84c-2* and subjected to RT-PCR analyses (Fig. 1I). Primers for the *exo84c-2* RT-PCR analysis are listed in Supplemental Table S1, and primers from Hála et al. (2008) were used for the *sec5b-1* and *sec15a-1* RT-PCR analyses.

Exocyst RNA-Silencing Constructs and Plant Transformations

RNA-silencing constructs for *SEC3ab*, *SEC5a*, *SEC6*, *SEC8*, *SEC10ab*, *SEC15b*, and *EXO84b* were all cloned into the pORE3:SLR1 promoter binary vector (Chapman, 2010; Indriolo et al., 2012, 2014a). PCR amplification steps were carried out with the Advantage 2 DNA Polymerase (Clontech), and the PCR primers used in these reactions are listed in Supplemental Table S1. For *SEC3a-AS*, a full-length complementary DNA (cDNA) was synthesized (Integrated DNA Technologies), and a cDNA fragment with nucleotides 1402 to 2662 from the coding sequence ($CDS_{1402-2662}$) was cloned as an *Eco*RI and *Xma*I antisense fragment into pORE3:SLR1. For *SEC5a-AS*, $CDS_{2514-3248}$ was PCR amplified from the *SEC5a* cDNA in pRTL2ΔNS (Chong et al., 2010) and cloned as a *Sac*I and *Cla*I antisense fragment into pORE3:SLR1. For *SEC6* hpRNAi, $CDS_{1298-1693}$ was PCR amplified from the *SEC6* cDNA in pRTL2ΔNS (Chong et al., 2010) and ligated into pGEM-T-Easy (Promega). Both the sense (*Eco*RI and *Kpn*I) and antisense (*Hin*dIII and *Cla*I) orientations were transferred to pKANNIBAL (Wesley et al., 2001), and the hpRNAi fragment was then cloned as a *Not*I and *Sac*I fragment into pORE3:SLR1. For *SEC8-AS*, $CDS_{1802-2135}$ was PCR amplified from the *SEC8* cDNA in pRTL2ΔNS (Chong et al., 2010) and cloned as a *Not*I and *Sac*I antisense fragment into pORE3:SLR1. For *SEC10a-AS*, a full-length cDNA was synthesized (Integrated DNA Technologies), and $CDS_{939-1713}$ was cloned as an *Sma*I and *Eco*RI antisense fragment into pORE3:SLR1. For *EXO84b-AS*, $CDS_{445-1361}$ was PCR amplified from Col-0 genomic DNA and cloned as an *Sac*I and *Cla*I antisense fragment into pORE3:SLR1. For *SEC15b* hpRNAi, CDS_{20-728} was PCR amplified from the *SEC15b* cDNA in pRTL2ΔNS (Chong et al., 2010), and the sense or antisense fragments were ligated into the TOPO cloning vector (Life Technologies). The *Sec15b* reverse primer for the sense PCR fragment also included an intron sequence. The *Bam*HI-*Eco*RI sense fragment and the *Eco*RI-*Sal*I antisense fragment were then subcloned into pBluescript KSII+, and finally *Xma*I was used to clone this hpRNAi fragment into pORE3:SLR1.

The RNA-silencing constructs were transformed into Arabidopsis by *Agrobacterium tumefaciens*-mediated transformation with the floral dip method (Clough and Bent, 1998). T1 seeds were germinated on soil, and transformants were selected by spraying seedlings with 1g L^{-1} Basta herbicide. T1 plants surviving this screen were then verified to carry the RNA-silencing constructs by PCR screening with primers to the $Basta^R$ gene. Following flowering, transformants were assessed for reduced seed set, and three independent transgenic lines were selected for each RNA-silencing construct (*SEC3ab*, *SEC5a*, *SEC6*, *SEC8*, *SEC10ab*, *SEC15b*, and *EXO84b*) for detailed assessments in the T2 and T3 generations. The *exo84c-2* mutant was analyzed along with these transgenic lines for altered compatible pollen responses. Images of whole plants were captured using a Canon digital camera, and images of whole flowers were taken with a Nikon sMz800 microscope and NIS-Elements imaging software at magnifications of 2× to 5× to view the stigmatic papillae. For observations of tracheary elements, freshly opened flowers from wild-type Col-0 and

transgenic lines were incubated in chloral hydrate in 30% (v/v) glycerol for several hours to clear the tissue. Once cleared, the samples were washed three times with distilled water and viewed using a Zeiss Axiovert 100M microscope.

Pollen Adhesion, Pollen Tube Growth, Pollen Hydration, and Seed Set

To visualize pollen grain adhesion and pollen tube growth, stage 12 flower buds (final stage prior to bud opening; Smyth et al., 1990) from wild-type Col-0 and transgenic plants were emasculated and covered in plastic wrap to maintain tissue hydration. After 24 h, three anthers from an Arabidopsis Col-0 flower were used to apply pollen grains to emasculated stigmas with elongated papillae. At 2 h postpollination, whole pistils were collected and placed in the fixative (300 μL of ethanol:glacial acetic acid [3:1]) at room temperature for 30 min. The fixative was removed, and pistils were washed three times with sterile water and incubated in 500 μL of 1 M NaOH at 60°C for 1 h. Pistils were then stained with 500 μL of 0.1% (w/v) aniline blue at room temperature for 30 min or overnight at 4°C. Subsequently, pistils were mounted on slides in Vectashield (Vector Laboratories, H-1400) to prevent photobleaching, and pollen tubes were visualized with a Zeiss Axioskop2Plus fluorescence microscope. A minimum of 10 pistils were examined for the number of adhered pollen grains (DIC) and pollen tube growth (UV light). Reciprocal crosses were conducted by pollinating wild-type Col-0 pistils with pollen from transgenic plants to confirm pollen viability in the transgenic lines. The pollination assays were performed under relative humidity levels of 35% or lower and monitored with a digital hygrometer. For the high relative humidity experiments, pollination assays were performed at relative humidity levels of approximately 90%. For seed set analysis, seeds per silique were counted for Col-0 and each independent transgenic line (n = 10).

For pollen hydration assays, 24 h after emasculation, pistils with fully elongated papillae were removed from flowers and embedded vertically in one-half-strength Murashige and Skoog plates to view the top surface area of the stigma. A single Col-0 anther was used to lightly pollinate the stigma. Pictures were taken at 0 min (immediately after pollination) and 10 min postpollination using a Nikon sMz800 microscope at 6.0×. The pollen diameter was measured for 10 pollen grains per stigma from three stigmas for each transgenic line and Col-0 using the NIS-Elements imaging software. The pollen hydration assays were conducted under conditions with humidity levels of less than 35% (monitored with a digital hygrometer).

qRT-PCR Assays

For the three independent transgenic lines selected for each RNA-silencing construct (*SEC3ab*, *SEC5a*, *SEC6*, *SEC8*, *SEC10ab*, *SEC15b*, and *EXO84b*), half pistils, including the stigma and style, were collected for RNA extraction and qRT-PCR analyses to determine the degree of RNA silencing of the target exocyst subunit genes in each line. RNA was extracted from the half pistils using the SV Total RNA Isolation Kit (Promega). The extracted RNA was quantified with a Nanodrop spectrophotometer (GE Nanovue Plus), and 1 μg of RNA was used to synthesize cDNA using the Super Script III reverse transcriptase kit (Invitrogen). The cDNA was tested by PCR with Arabidopsis Elongation Factor-1α (EIF-1α)-specific primers, using RT-minus reactions to verify the removal of any contaminating genomic DNA in the cDNA synthesis process. The qRT-PCR was performed for two biological replicates using the half-pistil cDNA with a 2× KAPA SYBR FAST Master Mix (KAPA) and a Real-Time PCR Detection System (Bio-Rad). Following the qRT-PCR reactions, the target exocyst subunit gene expression was normalized to two controls, *Elf1α* and Tubulin β4 (TUB4) as previously published (Indriolo et al., 2014a). All primers used for the qRT-PCR analyses are listed in Supplemental Table S2.

Gene identifiers are as follows: *SEC3a*, At1g47550; *SEC3b*, At1g47560; *SEC5a*, At1g76850; *SEC5b*, At1g21170; *SEC6*, At1g71820; *SEC8*, At3g10380; *SEC10a*, At5g12370; *SEC10b*, At5g12365; *SEC15a*, At3g56640; *SEC15b*, At4g02350; *EXO84a*, At1g10385; *EXO84b*, At5g49830; *EXO84c*, At1g10180; *EXO70A1*, At5g03540; *EXO70A2*, At5g52340; and *EXO70A3*, At5g52350. Arabidopsis T-DNA mutants are as follows: *sec5b-1*, SALK_001525; *sec15a-1*, SALK_006302; and *exo84c-2*, SALK_011569.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Abundance of each Arabidopsis exocyst subunit in different tissues from the Protein Abundance Across Organisms database (Wang et al., 2012).

Supplemental Figure S2. Pollen grain attachment and pollen tube growth following pollination of transgenic pistils with wild-type Col-0 pollen.

Supplemental Figure S3. Pollen grain attachment and pollen tube growth following pollination of wild-type Col-0 pistils with transgenic pollen.

Supplemental Figure S4. Phenotypes of flowering plants from the exocyst subunit RNA-silencing/knockout lines.

Supplemental Figure S5. Phenotypes of stigmatic papillae from the exocyst subunit RNA-silencing/knockout lines.

Supplemental Figure S6. Phenotypes of tracheary elements in pistils from the exocyst subunit RNA-silencing/knockout lines.

Supplemental Table S1. Primers used for PCR cloning and genotyping transgenic lines.

Supplemental Table S2. Primers used for qRT-PCR analyses.

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