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COMMENTARY

The ARC1 E3 Ligase Promotes a Strong and Stable Self-Incompatibility Response in *Arabidopsis* Species: Response to the Nasrallah and Nasrallah Commentary[OPEN]

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Following the identification of the male (S-locus Cysteine Rich/S-locus Protein 11) and female (*S* Receptor kinase [SRK]) factors controlling self-incompatibility in the Brassicaceae, research in this field has focused on understanding the nature of the cellular responses activated by these regulators. We previously identified the ARM Repeat Containing1 (ARC1) E3 ligase as a component of the SRK signaling pathway and demonstrated ARC1's requirement in the stigma for self-incompatible pollen rejection in *Brassica napus*, *Arabidopsis lyrata*, and *Arabidopsis thaliana*. Here, we discuss our findings on the role of ARC1 in reconstructing a strong and stable *A. thaliana* self-incompatibility phenotype, in the context of the putative issues outlined in a commentary by Nasrallah and Nasrallah. Additionally, with their proposed standardized strategy for studying self-incompatibility in *A. thaliana*, we offer our perspective on what constitutes a strong and stable self-incompatibility phenotype in *A. thaliana* and how this should be investigated and reported to the greater community.

With many angiosperms possessing hermaphroditic flowers, self-incompatibility (SI) systems have evolved to avoid the deleterious effects of inbreeding (Figures 1A and 1B). As defined by Charlesworth et al. (2005), "plant SI systems all prevent self-fertilization through recognition and rejection of pollen by pistils expressing 'cognate' allelic specificity." In Brassicaceae species, the allele specificity is conferred by two well-characterized polymorphic genes encoding the female *S* Receptor kinase (SRK) and the male S-locus Protein 11/S-locus Cysteine Rich (SP11/SCR; hereby referred to as SCR) (reviewed in Iwano and Takayama, 2012). The major outstanding area in this field is identifying the signaling proteins activated by SRK, determining their function at the cellular level, and investigating whether these signaling proteins have conserved functions across the self-incompatible species in the Brassicaceae. Despite strong interest in finding these potential factors by us and other groups, only the *Brassica rapa M* Locus Protein Kinase (Murase et al., 2004; Kakita et al., 2007a, 2007b) and the ARM Repeat Containing1 (ARC1) E3 ligase have emerged as direct downstream signaling proteins. We demonstrated a conserved role for ARC1 in self-incompatible *Brassica napus* (Gu et al., 1998; Stone et al., 1999, 2003), self-incompatible *Arabidopsis lyrata* (Indriolo et al., 2012), and self-incompatible *Arabidopsis thaliana* expressing *A. lyrata SCRb*, *SRKb*, and *ARC1* transgenes (Indriolo et al., 2014). The commentary by Nasrallah and Nasrallah (2014) focuses on our proposed role for ARC1 in reconstituting self-incompatibility in transgenic *A. thaliana*.

ARC1'S FUNCTION IN THE SI RESPONSE

There is a widespread loss of the *ARC1* gene from the *A. thaliana* genome across 357 ecotypes tested, including the Columbia-0 (Col-0) and Sha ecotypes discussed here, and other ecotypes that still carry an intact *SCR* or *SRK* gene (Indriolo et al., 2012 and references therein). Therefore, we proposed that loss of the *ARC1* gene likely predated the loss of *SCR* and *SRK* genes in *A. thaliana*, during the transition to selfing from the ancestral self-incompatible state (reviewed in Castric et al., 2014). The requirement of *ARC1* for self-pollen rejection in naturally occurring self-incompatible *A. lyrata* was validated through transgenic *ARC1* RNA interference knockdown plants (Indriolo et al., 2012). In the follow-up study, we examined how transforming *ARC1* combined with *A. lyrata SCRb* and *SRKb* genes into *A. thaliana* contributed to recapitulating the *A. lyrata* SI response and demonstrated that the presence of all three transgenes (*SCRb-SRKb+ARC1*) was correlated with the presence of a strong and stable SI response in *A. thaliana* (Indriolo et al., 2014).

In our experimental setup, the Nasrallah group's *A. lyrata SCRb-SRKb* construct alone or in combination with the *A. lyrata ARC1* or *B. napus ARC1* transgenes were transformed into *A. thaliana* Col-0 and Sha ecotypes, and 20 independent primary transformants were selected for each transgene combination. This approach was selected to avoid differences and biases due to expression variation, transgene position effects, or any potential phenotypic plasticity in the ecotypes tested. An initial survey was conducted on these randomly selected primary transformants, and then detailed analyses were conducted on T2 generation plants for three independent lines from each transgenic combination in the Col-0 and Sha ecotypes (Indriolo et al., 2014). All self-incompatible T2 plants were found to carry both the *SCRb-SRKb* and *ARC1* T-DNAs (Indriolo et al., 2014). Transgene expression levels were quantified by quantitative RT-PCR (qRT-PCR) using RNA samples extracted from anther or stigma tissue (harvested from T2 flowers)

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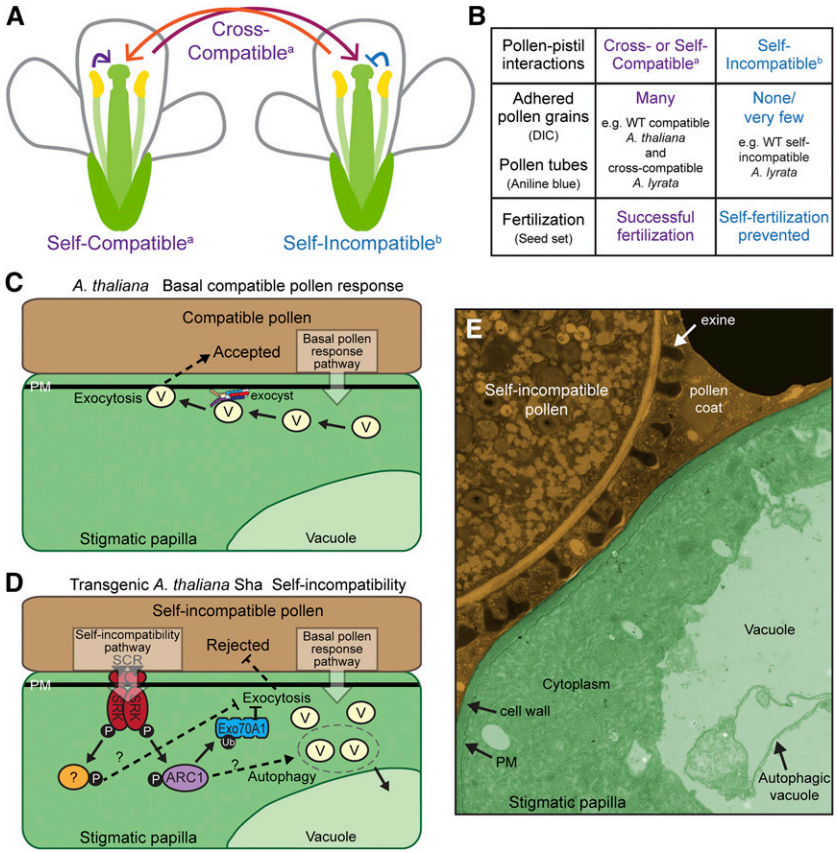

Figure 1. Pathways for Compatible and Self-Incompatible Pollen Responses in *A. thaliana*.

(A) Compatible (arrow) and self-incompatible (bar) pollen-pistil interactions.

(B) Criteria for assessing compatible versus self-incompatible pollinations.

(C) Model for the basal compatible pollen response. An unknown basal pollen response pathway is activated in the stigmatic papilla under the compatible pollen grain leading to the activation of vesicle secretion. Our research on *Brassica* and *Arabidopsis* Exo70A1 revealed a putative role for the exocyst complex in docking secretory vesicles at the stigmatic papillae plasma membrane (Samuel et al., 2009; Safavian and Goring, 2013; Safavian et al., 2014). Exo70A1 is proposed to assemble with the remaining subunits of the exocyst complex to dock secretory vesicles (reviewed in Zárský et al., 2013). SNARE proteins mediate vesicle fusion to the plasma membrane, and unknown cargo (ACA13 as one candidate; Iwano et al., 2014) are released to enable pollen hydration pollen tube entry through the stigmatic papillar cell wall (compatible pollen is accepted).

(D) Model for the reconstituted self-incompatibility signaling pathway in the Sha ecotype. Following self-pollination in transgenic *SCR-SRK+ARC1* Sha ecotype flowers, the pollen SCR ligand binds to SRK at the stigmatic papillar plasma membrane, resulting in the activation of the downstream signaling pathway. The ARC1 E3 ligase is recruited by SRK and targets Exo70A1 for ubiquitination. Even though the basal compatible pollen response pathway has been also activated, ubiquitinated Exo70A1 is somehow inhibited so that exocyst-mediated vesicle secretion to the self-incompatible pollen grain is blocked. In addition, secretory vesicles are degraded in the vacuole through autophagy. An unknown signaling protein (yellow) also has activity in the Sha ecotype in blocking exocytosis (see Samuel et al. [2009], Safavian and Goring [2013], and Indriolo et al. [2014] for further details and references therein).

(E) Transmission electron microscopy image of a self-incompatible pollen-stigmatic papillar interaction at 10 min postpollination from the transgenic *SCRb-SRKb+ARC1* Sha ecotype. Pseudocoloring has been added to distinguish the pollen (brown) from the stigmatic papilla (green). Autophagy is detected with the autophagic vacuole in the vacuole (see Rose et al. [2006] and Indriolo et al. [2014] for details). (*Figures 1C to 1E adapted from Indriolo et al. [2014], Figures 9 and 10.*)

at the correct developmental stage for all samples (flower buds just starting to open; before anther dehiscence). The transgene qRT-PCR expression analyses included an *A. lyrata* control for comparison, and the overall conclusion was that ARC1's impact on the SI response was not due to variable transgene expression levels (see Indriolo et al. [2014] for a detailed discussion).

We found *ARC1* to be absolutely required, along with *SCRb* and *SRKb*, for generating a stable and robust self-incompatible response in Col-0 plants, but the presence of *ARC1* had less of an additive effect in the Sha ecotype (Indriolo et al., 2014). To make this conclusion, we use the widely accepted definition of self-incompatibility: the rejection of self-pollen to prevent

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self-fertilization (Figures 1A and 1B). This is required to avoid inbreeding and to promote outcrossing: the end goal of self-incompatibility systems (reviewed in Charlesworth et al., 2005; Iwano and Takayama, 2012; Castric et al., 2014). For Col-0, Figure 2 from Nasrallah and Nasrallah (2014) presents data illustrating a "transient self-incompatibility" response for Col-0:*AlSCRb-SRKb* transformants, following a 2-h pollination at stage 14E [described as "young flowers (early stage 14), before anthers had extended above the stigma and deposited their pollen on the stigma epidermis"; Nasrallah et al., 2002]. These Col-0:*AlSCRb-SRKb* transformants were described as follows: "Unlike *Brassica*, however, older SRKb/SCRb flowers regained a measure of receptivity to self pollen" (Nasrallah et al., 2002); are "compatible to self-pollination in mature flowers," and positive for "open-pollinated seed set" (Table 1 in Nasrallah et al., 2004); and "set as much seed as untransformed plants" (Table 1 in Boggs et al., 2009). The Col-0:*AlSCRb-SRKb* transformants in Kitashiba et al. (2011) and Yamamoto and Nasrallah (2013) are also fully self-fertile. Based on these published data, the Col-0:*AlSCRb-SRKb* transformants fail to meet the criteria of preventing self-fertilization and therefore are not fully self-incompatible. In our study, the Col-0:*AlSCRb-SRKb* transformants produced seed levels (49.1 seeds/silique) comparable to wild-type Col-0 (50.3 seeds/silique). With the addition of *ARC1* (Col-0:*AlSCRb-SRKb*+*ARC1*), however, these transformants displayed a statistically significant decrease in seed set following self-pollination (5.6 seeds/silique in the strongest line). As expected for an SI response, self-pollinated pistils from these transformants displayed reduced self-pollen attachment (differential interference contrast) and pollen tube growth (aniline blue). Following cross-compatible pollinations between the transformants and wild-type Col-0 flowers, many attached pollen grains and growing pollen tubes were observed as predicted (Figures 1A and 1B). Thus, ARC1 is required for the Col-0:*AlSCRb-SRKb*+*ARC1* transformants to meet the criteria of a self-incompatible plant (Figures 1A and 1B). The effect of ARC1 was not due to changes in *A. lyrata SRKb* expression levels as shown by qRT-PCR analyses (Indriolo et al., 2014). In addition, the Indriolo et al. (2014) study was conducted in a true wild-type Col-0 background and not in the *RNA-dependent RNA polymerase6* mutant background described as a "modifier" by Nasrallah and Nasrallah (2014) and Tantikanjana et al. (2009).

Because *A. thaliana* Sha:*AlSCRb-SRKb* transformants were previously reported by the Nasrallah group to have a self-incompatibility phenotype in open flowers with reduced seed production (~219 seeds/plant; Table 1 in Boggs et al., 2009), we conducted the same transgenic analyses in the Sha ecotype as a comparison. Consistent with Boggs et al. (2009), Indriolo et al. (2014) identified *A. thaliana* Sha:*AlSCRb-SRKb* transformants that met the definition of self-incompatibility with statistically significant reductions in seed set in the absence of *ARC1* (16.6 seeds/silique in the strongest line). Therefore, both studies show that Sha:*AlSCRb-SRKb* transformants have self-incompatibility responses preventing self-fertilization. However, we found that adding the *ARC1* transgene with *SCRb* and *SRKb* significantly enhanced the self-incompatibility phenotype in the Sha ecotype to cause a stronger pollen rejection response resulting in an even greater decrease in seed set (0.7 seeds/silique in the strongest line). All of these transformants also displayed a corresponding decrease in attached pollen grains and pollen tube growth on self-pollinated pistils. Nasrallah and Nasrallah (2014) argue that ARC1 is not needed as the reduced amount of seed set that they observed is lower than what we reported. In our qRT-PCR analyses, equivalent *SCRb* and *SRKb* expression levels were observed in the self-incompatible Sha:*AlSCRb-SRKb* transformants versus the Sha:*AlSCRb-SRKb*+*ARC1* transformants (Indriolo et al., 2014). Since the Nasrallah group publications did not provide quantitative *SCRb* and *SRKb* transgene expression data (Nasrallah et al., 2002, 2004; Boggs et al., 2009; Kitashiba et al., 2011; Yamamoto and Nasrallah, 2013), any potential expression level differences could not be determined. Environmental conditions can also influence the self-incompatibility trait (Horisaki and Niikura, 2008), so this will also need to be explored further.

The second question that we investigated was whether ARC1 was required for reconstituting a self-incompatibility response in *A. thaliana* that produced similar cellular responses to wild-type self-incompatible *A. lyrata* (Safavian and Goring, 2013). Based on our work and others (see Samuel et al., 2009; Safavian and Goring, 2013; Indriolo et al., 2014 and references therein), our working models for the acceptance of compatible pollen versus the rejection of self-incompatible pollen by the stigmatic papillae are shown in Figures 1C and 1D. Compatible pollen is accepted through a basal signaling pathway that leads to vesicle secretion at the pollen contact site in the stigmatic papilla, which in turn promotes pollen hydration and pollen tube entry into the stigma (Figure 1C). Self-incompatible pollen is rejected by the activation of allele-specific SCR-SRK interactions, and the downstream SRK signaling pathway disrupts vesicle secretion from the simultaneously activated basal pollen response pathway (Figure 1D). ARC1 functions downstream of activated SRK to inhibit Exo70A1 and prevent exocyst-mediated vesicle secretion, resulting in rejection of self-pollen (Figure 1D). In the *A. lyrata* SI response, we discovered that autophagy is induced by 10 min postpollination, sending vesicles to the vacuole for degradation (Figure 1D; Safavian and Goring, 2013). Similar to *A. lyrata*, autophagic organelles were detected in the vacuoles of *A. thaliana SCRb-SRKb*+*ARC1* transgenic plants but not in the *AlSCRb-SRKb* transformants at 10 min postpollination. In addition, the Sha:*AlSCRb-SRKb*+*ARC1* transformants were most similar to *A. lyrata* with the highest frequency of autophagic organelles in the vacuole at 10 min postpollination (Figure 1E; Indriolo et al., 2014). This suggested that ARC1 is directly or indirectly linked to the autophagy pathway during the *Arabidopsis* sp self-incompatibility response. Therefore, we concluded that *ARC1* is required (along with *A. lyrata SCR* and *SRK* genes) to reconstitute an *A. lyrata*-type self-incompatibility cellular response in *A. thaliana.*

Finally, the fact that the Sha:*AlSCRb-SRKb* transformants (in the absence of ARC1) can produce a self-incompatibility response preventing self-fertilization indicates the presence of another downstream signaling protein in

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the Sha ecotype (Figure 1D). In our *B. napus* and *A. lyrata ARC1* knockdown studies (Stone et al., 1999; Indriolo et al., 2012), we typically observed a partial breakdown of self-incompatibility, and while this phenotype could be due to residual ARC1 activity, it is equally likely to result from the activity of other unknown downstream signaling proteins. Plant receptor kinase signaling pathways are complex (reviewed in Osakabe et al., 2013; Macho and Zipfel, 2014), and so it would be reasonable to expect multiple signaling proteins functioning downstream of SRK. The activity of this putative unknown signaling protein(s) appears to be attenuated in Col-0 (see Indriolo et al. [2014] for an in-depth discussion), and the differences between the Sha and Col-0 ecotypes for this activity will only be fully explained once it has been identified. Whether this unknown signaling protein(s) is another member of the PUB E3 ligase family that ARC1 belongs to (Mudgil et al., 2004; Samuel et al., 2008), and whether it targets Exo70A1, another exocyst subunit, or acts elsewhere in the secretory system to disrupt exocytosis will need to be investigated (Figure 1D).

ISSUES TO CONSIDER IN A STANDARDIZED STRATEGY FOR STUDYING SI IN *A. THALIANA*

In terms of the recommendations for the adoption of a standardized strategy for functional studies of self-incompatibility in *A. thaliana*, Nasrallah and Nasrallah (2014) recommended the use of "well-defined true breeding *SCR-SRK* lines." We feel that in presenting new data, multiple independently transformed plants should be studied in both the T1 and T2 generations to ensure that the reported phenotypes are reproducible and segregate with the introduced transgenes in progeny of the primary transformants. After this has been established, then working in more detail with well-defined homozygous transgenic self-incompatible line is reasonable. It is also important to work with transgenic lines showing similar transgene expression levels to *A. lyrata* self-incompatibility genes (i.e., qRT-PCR analyses with *A. lyrata* controls for comparison). Relative humidity levels should also be tracked during these analyses as pollinations on Brassicaceae dry stigmas can be affected (Safavian et al., 2014 and references therein).

We agree with Nasrallah and Nasrallah (2014) that the pollination analyses should include aniline blue staining for pollen tubes (Figure 1B). Given that self-pollen rejection occurs rapidly at the stigmatic surface, we additionally would like to see corresponding differential interference contrast images of these aniline blue-stained pollinated pistils to view the elongated stigmatic papillae for correct staging and the number of adhered pollen grains. However, this cannot be a stand-alone assay and should be done in conjunction with seed counts. Seed set data are an important measure since self-incompatibility systems exist to prevent self-fertilization (reviewed in Charlesworth et al., 2005; Iwano and Takayama, 2012; Castric et al., 2014). This would certainly help to clear the extensive confusion in the field by identifying transgenic *A. thaliana* lines that are genuinely self-incompatible. This type of analysis would certainly be expected for any practical application of this breeding system. A simple experiment to confirm that the lack of seed set is not due to other factors is to simultaneously perform reciprocal hand pollinations with a cross-compatible plant (Figure 1A) and quantify seed set (full seed set would be expected in both directions). In our experience, seed set is a very reliable measure in *A. thaliana* plants grown in growth chambers under standard growth conditions. Finally, it will be important to examine the stigmatic papillar cellular responses and extend these studies to other self-incompatible Brassicaceae species to fully understand the contributions of a candidate signaling protein in the SRK-activated signaling pathway.

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AUTHOR CONTRIBUTIONS

D.R.G., E.I., and M.A.S. discussed the ideas and wrote the article.

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