It is well established that specific pollen recognition in the self-incompatibility (SI) response of the Brassicaceae is determined by allele-specific interactions that occur at the stigma surface between two highly polymorphic proteins encoded in the S locus: the S-locus receptor kinase SRK and its ligand, the S-locus cysteine-rich protein SCR. Arabidopsis thaliana lacks a functional SI system and harbors nonfunctional S-locus variants that contain defective alleles of the SRK and/or SCR genes (Kusaba et al., 2001; Sherman-Broyles et al., 2007; Tang et al., 2007; Shimizu et al., 2008; Boggs et al., 2009a; Tsuchimatsu et al., 2010; Dwyer et al., 2013). Despite being highly self-fertile, A. thaliana can be made to express SI upon transformation with functional SRK-SCR gene pairs isolated from its self-incompatible close relatives (Nasrallah et al., 2002, 2004; Boggs et al., 2009a, 2009b). The first transfer of the SI trait into A. thaliana was achieved using the SRKb-SCRb gene pair isolated from the Sb locus of Arabidopsis lyrata (Kusaba et al., 2001; Nasrallah et al., 2002, 2004). Many of the subsequent studies that have been performed in the transgenic A. thaliana SRK-SCR system have used plants transformed with p548, a plasmid that we constructed by inserting the A. lyrata SRKb and SCRb genes with their 5’ and 3’ regulatory sequences into the pBIN+ binary vector (Nasrallah et al., 2004).

Indriolo et al. (2014) recently used the p548 plasmid to generate SRKb-SCRb transformants and test the role of the ARM Repeat Containing 1 (ARC1) gene in SI. ARC1 was originally identified as a Brassica napus protein that interacts with the SRK kinase domain in yeast (Gu et al., 1998), and it was subsequently inferred to be required for SI because downregulation of the ARC1 gene in B. napus (Stone et al., 1999) and A. lyrata (Indriolo et al., 2012), as well as overexpression of ARC1’s target, Exo70A1, in B. napus (Samuel et al., 2009), caused partial breakdown of the SI response. However, the involvement of the proposed SRK-ARC1-Exo70A1 pathway in SI has been questioned because the ARC1 gene was found to be deleted in all A. thaliana accessions analyzed to date (Kitashiba et al., 2011; Indriolo et al., 2012), including those in which the SRKb-SCRb transgenes confer a strong SI phenotype (Kitashiba et al., 2011). Additionally, overexpression of Exo70A1 did not cause weakening of the SI response in A. thaliana SRKb-SCRb plants (Kitashiba et al., 2011).

Indriolo et al. (2014) reported on their characterization of the SI response in plants of the Sha and Columbia-0 (Col-0) accessions, which either transformed with the p548 plasmid alone or cotransformed with p548 and a plasmid containing an ARC1 gene isolated from A. lyrata or B. napus. They concluded that, along with SRK and SCR, “ARC1 is the third component that is required to return A. thaliana to its ancestral self-incompatibility state.” However, this conclusion is inconsistent with results of previous studies of SI in transgenic A. thaliana SRK-SCR transformants, which have shown that several A. thaliana accessions are rendered fully self-incompatible by transformation with the p548 plasmid without the addition of a functional ARC1 gene. Contrary to Indriolo et al.’s assertion that in previous studies of A. thaliana SRK-SCR transformants, “the self-pollen rejection response was incomplete,” we reported that among 11 A. thaliana accessions tested by transformation with the p548 plasmid, five accessions (C24, Cvi-0, Hodja, Kas-2, and Sha) were converted to full SI by expression of the SRKb and SCRb genes alone (Nasrallah et al., 2004; Boggs et al., 2009a). Importantly, the SI phenotype of these self-incompatible A. thaliana SRK-SCRb transformants faithfully recapitulates the SI phenotype of naturally self-incompatible Brassicaceae with respect to the four defining features of SI in this family: (1) site of pollen inhibition at the stigma surface, (2) intensity of the response, (3) developmental regulation over the course of stigma maturation, and (4) heritability. These features suggest that the inhibition of self pollen in self-incompatible A. thaliana SRK-SCRb transformants is achieved via the same
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signaling pathway as that utilized by other self-incompatible Brassicaceae species.

ROBUST SI IN SRKb-SCRb TRANSFORMANTS OF THE Sha ACCESSION IN THE ABSENCE OF FUNCTIONAL ARC1

In the particular case of the Sha accession, Boggs et al. (2009a) reported that 80% (8 of 10) of the independent primary SRKb-SCRb transformants analyzed expressed an intense SI phenotype, which was faithfully transmitted over ten generations. Accordingly, instead of the thousands of seeds produced by untransformed Sha plants, Sha[SRKb-SCRb] transformants were found to produce on average 219 ± 25 seeds over their lifetime (i.e., ~1 seed per silique on average, with the majority of siliques containing no seed). While somewhat higher than the amount of seed produced by SRKb-SCRb transformants of the C24 and Cvi-0 accessions, which set only ~50 to 60 seeds (or ~0.25 to 0.3 seeds per silique on average) over their lifetime (Boggs et al., 2009a), the low amount of seed produced by Sha[SRKb-SCRb] plants still underscores the stability of the SI phenotype throughout the life of these plants. Moreover, as for SRKb-SCRb transformants of the C24, Cvi-0, Hodja, and Kas-2 accessions, the strength and developmental stability of SI in Sha[SRKb-SCRb] transformants is demonstrated by the observation that manual self-pollination of siliques from stage 13 floral buds and older flowers results in severe inhibition of pollen at the surface of the self-pollinated stigma and the profuse pollen tube growth on the cross-pollinated stigma. The images were generated by UV fluorescence microscopy observation of stigmas that were stained with aniline blue two hours after manual self-pollination as previously described (Nasrallah et al., 2004). Bars = 10 μm.

Figure 1. Intense SI Exhibited by Sha[SRKb-SCRb] Transformants in the Absence of a Functional ARC1 Gene.

The figure shows typical pollination results obtained by manual self-pollination of open flower stigmas in transgenic self-incompatible Sha[SRKb-SCRb] plants. A T12 plant homozygous for a single integration of the SRKb-SCRb transgenes was used for self-pollination (left) and for cross-pollination with pollen from an untransformed plant (right). Note the severe inhibition of pollen at the surface of the self-pollinated stigma and the profuse pollen tube growth on the cross-pollinated stigma. The images were generated by UV fluorescence microscopy observation of stigmas that were stained with aniline blue two hours after manual self-pollination as previously described (Nasrallah et al., 2004). Bars = 10 μm.

Figure 2. Developmentally Stable SI in Sha[SRKb-SCRb] Transformants and Transient SI in Col-0 [SRKb-SCRb] Transformants Lacking a Functional ARC1 Gene.

The graph shows the developmental regulation of SI in the stigmas of Sha[SRKb-SCRb] and Col-0[SRKb-SCRb] plants carrying a single integration of the SRKb-SCRb transgenes. Manual pollinations were performed on the stigmas of floral buds and flowers at stage 12, stage 13, early stage 14 (14E), and late stage 14 (14L) of development. Representative images of Col-0 floral buds and flowers at these developmental stages are shown below the graph. The results of replicate pollinations are expressed as the mean number (±se) of pollen tubes that form upon manual self-pollination of stigmas (filled columns) or manual cross-pollination with pollen from untransformed plants (adjoining white columns). For Sha[SRKb-SCRb] (T2 generation, homozygous for the transgenes), each column represents the results of 16 replicate pollinations. The Col-0[SRKb-SCRb] pollination data were previously reported by Nasrallah et al. (2002).
In Brassica oleracea, instead of 20 to 25 seeds per pod produced in compatible pollinations, manual self-pollination of self-incompatible stigmas produces, on average, 0.15 to 0.25 seeds per pod (Nasrallah and Wallace, 1968; Nakanishi and Hinata, 1975) or as much as one to two seeds per pod (Thompson and Taylor, 1971).

It is puzzling that none of the Sha[SRKb-SCRb] plants generated by Indriolo et al. exhibited as strong an SI phenotype as that observed in the Sha[SRKb-SCRb] transformants reported by Boggs et al. (2009a). Among 17 transformants that they analyzed by manual self-pollination of open flower stigmas, none exhibited strong SI (defined by the growth of <10 pollen tubes per stigma) and three exhibited only a moderate level of SI. The level of SRK transcripts and protein that accumulate in stigma epidermal cells is the primary determinant of the strength of the SI response and its stability over the course of stigma development in A. thaliana SRK-SCR transformants, as in naturally self-incompatible members of the Brassicaceae. Therefore, it is possible that the Indriolo et al. transformants might have had relatively low expression levels of the SRKb transgene.

**ROBUST SI SIGNALING IN SRKb-SCRb TRANSFORMANTS OF THE COL-0 ACCESSION IN THE ABSENCE OF FUNCTIONAL ARC1**

We previously reported that SRKb-SCRb transformants of the Col-0 accession produce amounts of seed equivalent to those produced by untransformed plants (Nasrallah et al., 2002, 2004; Tantikanjana et al., 2009; Tantikanjana and Nasrallah, 2012), and this phenotype was observed in the Indriolo et al. (2014) study. However, this high seed set is not due to the inability of Col-0[SRKb-SCRb] plants to effect SI signaling, as implied by Indriolo et al.’s description of these plants as “remaining self-compatible.” Rather, as previously reported (Nasrallah et al., 2002; Tantikanjana et al., 2009; Tantikanjana and Nasrallah, 2012) and shown in Figure 2, Col-0[SRKb-SCRb] plants (unlike untransformed Col-0 plants) express an intense SI response in the stigmas of mature floral buds (stage 13) and young flowers (early stage 14), and the high number of seeds produced by these plants is due to subsequent weakening of SI at later stages of flower development. This transient SI phenotype, which is often observed in natural and cultivated populations of various species, was also observed in SRKb-SCRb transformants of the RLD and Ws-0 accessions (Nasrallah et al., 2004; Liu et al., 2007) and independently by Tsuchimatsu et al. (2010) in plants of the Wei accession that were transformed with another S-locus transgene. It should also be noted that the transient SI phenotype of Col-0[SRKb-SCRb] plants, which is caused by modifier loci harbored in the Col-0 genome (Boggs et al., 2009a), has been converted to full SI in the absence of functional ARC1: Loss-of-function mutations in the RDR6 gene (Tantikanjana et al., 2009) and overexpression of the auxin response factor ARF3 both resulted in expression of a strong self-pollen rejection phenotype that persisted into late stages of flower development, causing lack of seed set (Tantikanjana and Nasrallah, 2012).

**A STANDARDIZED STRATEGY FOR FUNCTIONAL STUDIES OF SI IN TRANSGENIC A. THALIANA**

The results reviewed here indicate that all factors required for self-pollen rejection have been retained in several A. thaliana accessions and that a functional ARC1 gene is not required for SRK-mediated signaling or for conversion of these accessions to full SI. The contradictory results reported for A. thaliana SRKb-SCRb transformants in previous studies and by Indriolo et al. (2014) cannot be easily reconciled. However, we note that proper interpretation of pollination phenotypes in the A. thaliana SRK-SCR system requires consideration of the following issues.

(1) The major source of phenotypic variability among independent SRK-SCR transformants is differential SRK expression levels that inevitably result from different sites of transgene integration. Consequently, careful comparison of SRK transcript levels in independent SRK-SCR transformants is critical. In view of the well-documented developmental regulation of SI and of SRK transcript levels in stigmas, accurate quantification of these transcripts requires the use of stigmas or pistils harvested from floral buds or flowers at the same stage of development rather than from a mixture of buds or flowers at different stages of development, as may have been done by Indriolo et al. (2014). Indeed, the levels of SRK transcripts will be overestimated in samples containing an excess of stigmas from stage 13 or stage 14 buds and underestimated in samples containing an excess of stigmas from immature buds or older flowers.

(2) In view of the variable levels of transgene expression in independent transformants, it is imperative that experiments aimed at testing the role of a gene of interest in SI use a true-breeding SRK-SCR transgenic line having a well-defined, stable, and heritable SI phenotype and ideally harboring a single integration of the SRK-SCR transgenes (to minimize the possibility of transgene silencing in subsequent generations and to facilitate progeny analysis). This homozygous SRK-SCR line would then be transformed with a construct designed to test the role of a gene of interest (GOI), either by root transformation in cases where the SRK-SCR line is fully self-incompatible and sets little or no seed, or by floral dipping in cases where the SRK-SCR line expresses transient SI. This strategy leads to unambiguous evaluation of the role of the GOI in SI because it allows direct comparison of plants having or lacking the GOI construct in the same genetic background (i.e., in plants having the same integration site and expression levels of the SRK and SCR transgenes) as well as a straightforward determination of whether a pollination phenotype cosegregates with the GOI transgene in subsequent generations. By contrast, a strategy of cotransformation with the SRK-SCR genes and the GOI construct, as used by Indriolo et al. (2014) for testing the role of ARC1, will inevitably produce transformants having a range of expression levels, not only of the GOI transgene, but also of SRK and SCR, thus complicating interpretation of observed phenotypes. This issue is compounded in transgenic progenies due to the segregation of plants that are homozygous or hemizygous for the SRK-SCR and/or GOI transgene integrations and is even more problematic when the primary transformants contain more than one integration of the transgenes.
To avoid the pitfalls associated with variable SRK expression in independent A. thaliana SRK-SCR transformants and to facilitate comparison of results obtained in different laboratories, we suggest the adoption of a standardized strategy for functional studies of SI based on three elements: (1) the use of well-defined true-breeding SRK-SCR lines; (2) analysis of SRK transcript levels in carefully staged stigmas; and (3) evaluation of SI phenotypes by microscopic examination of pollen tube growth in manually self-pollinated stigmas over the course of their development, which allows detection of transient SI phenotypes. The microscopic pollination assay is preferred over the seed counting method because it is a rapid assay that samples large numbers of pollen grains (~1000 pollen grains may be sampled by microscopy observation of just 10 self-pollinated stigmas). By contrast, the seed count method can produce ambiguous results, as previously noted in naturally self-incompatible Brassicaceae (Sampson, 1964), because it reports only on the fate of the few pollen grains that produce seed and because factors other than pollen inhibition at the stigma surface can affect seed production.

ACKNOWLEDGMENTS

Work in the Nasrallah laboratory is supported by National Science Foundation Grant IOS-1146725. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the article.

AUTHOR CONTRIBUTIONS

J.B.N. and M.E.N. wrote the commentary.

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Robust Self-Incompatibility in the Absence of a Functional ARC1 Gene in Arabidopsis thaliana
June B. Nasrallah and Mikhail E. Nasrallah
Plant Cell; originally published online October 21, 2014;
DOI 10.1105/tpc.114.129387

This information is current as of January 8, 2018

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