A Breakdown of *Brassica* Self-Incompatibility in ARC1 Antisense Transgenic Plants

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Self-incompatibility, the rejection of self pollen, is the most widespread mechanism by which flowering plants prevent inbreeding. In *Brassica*, the S receptor kinase (SRK) has been implicated in the self-incompatibility response, but the molecular mechanisms involving SRK are unknown. One putative downstream effector for SRK is ARC1, a protein that binds to the SRK kinase domain. Here it is shown that suppression of ARC1 messenger RNA levels in the self-incompatible *Brassica napus* W1 line is correlated with a partial breakdown of self-incompatibility, resulting in seed production. This provides strong evidence that ARC1 is a positive effector of the *Brassica* self-incompatibility response.

Because the close proximity of their male and female reproductive organs can promote self-fertilization, many flowering plants have evolved mechanisms to prevent inbreeding. One such mechanism is self-incompatibility, which allows the recognition and rejection of self pollen (*1*). In *Brassica*, compatible pollination results in pollen adhering to the stigmatic papillae of the pistil, followed by pollen hydration and germination to form pollen tubes. The pollen tubes then penetrate the stigma and grow down the pistil to fertilize the ovary. During the self-incompatibility response, self pollen can be rejected at many stages, including the inhibition of pollen hydration and germination and the prevention of pollen tube growth through the stigma (*2*). The *S* locus glycoprotein (SLG) present in the stigmatic papilla cell wall and the S receptor kinase (SRK) localized to the stigmatic papilla plasma membrane have been implicated in the recognition of self pollen, although more recent evidence has questioned the importance of SLG in this process (*3*). During pollen-pistil interactions, SRK is thought to be activated upon binding of a pollen component from self pollen (*4*). An activity in the pollen coat corresponding to a protein fraction of low molecular weight has been detected but not identified (*5*). It is predicted that SRK activation triggers a signaling cascade in the stigmatic papillae, leading to pollen rejection (*4*).

In plants, a number of putative receptor kinases have been identified, all belonging to the receptor serine-threonine kinase family. However, the function of many of these receptor kinases is unknown, and downstream signaling proteins have not been identified (*6*). The KAPP protein phosphatase is the only substrate that has been identified for plant receptor kinases and shown to have a biological role, specifically with the CLV1 receptor kinase (*7*). Previously, we identified three proteins that specifically interact with SRK kinase domains in vitro (*8*, *9*). One of these, ARC1, shows a phosphorylation-dependent interaction that is mediated through arm repeats that are present in the COOH-terminal region. In addition, ARC1 expression is localized to the stigma, which is the region of the pistil contacting the pollen (*9*). Thus, although the in vitro results suggest that ARC1 is a substrate for SRK, evidence for a biological role for ARC1 during pollen-pistil interactions, particularly in self incompatibility, is lacking. To address this, transgenic plants were generated in the self-incompatible *Brassica napus* W1 cultivar (*10*) using the full-length ARC1 cDNA in the sense or antisense orientation (*11*).

Primary transformants were identified as kanamycin-resistant and were self-pollinated to produce the next generation of plants for analysis. Three primary transformants were generated for each of the ARC1 sense and antisense vectors, of which two each showed inheritance of the transgene in the next generation. Of the two ARC1-sense transgenic W1 lines, only the S1 line showed increased levels of ARC1 mRNA; however, it did not appear to have any detectable change in phenotype. Both of the ARC1-antisense transgenic W1 lines (A1 and A2) had decreased levels of ARC1 mRNA and also showed a partial breakdown of the self-incompatibility phenotype. In addition, sibling W1 plants that did not inherit the transgene [nontransgenic (nt) sibs] remained self-incompatible. The analysis of one ARC1-sense transgenic W1 plant (S1-1) and of the two independent ARC1-antisense transgenic W1 lines are described in detail.

The presence and the number of transgenes integrated in each line were determined by Southern (DNA) blot analysis, and all of the transgenic lines appeared to possess a single copy (*12*). In the A1 line, A1-1 and A1-2 carried the ARC1 antisense transgene, whereas A1-3(nt) did not. Similarly in the A2 line, A2-1, A2-2, and A2-3 carried the ARC1 antisense transgene, whereas A2-4(nt) did not. The ARC1 mRNA levels were examined by RNA blot analysis (Fig. 1). Similar ARC1 mRNA levels were detected in nt sibs [A1-3(nt) and A2-4(nt); Fig. 1, A and B] and in the W1 control (Fig. 1, A and B). In the ARC1-sense transgenic W1 plant, ARC1 mRNA levels were increased approximately threefold (S1-1; Fig. 1, A and B). In the two indepen-

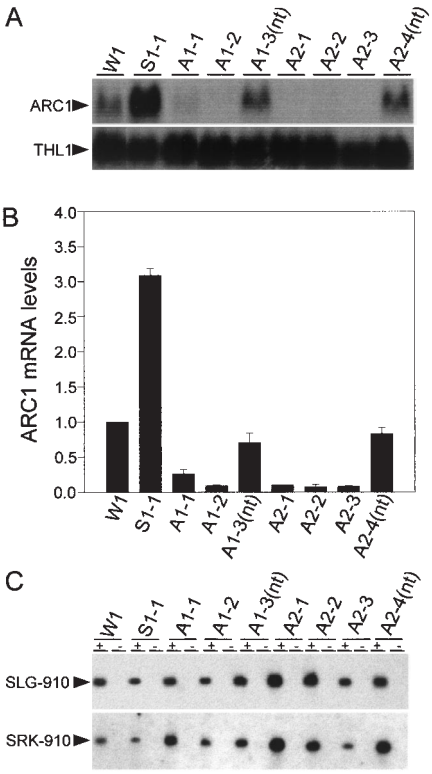

Fig. 1. Analysis of ARC1, SLG-910, and SRK-910 mRNA levels in ARC1 transgenic W1 plants. (**A**) ARC1 RNA blot analysis. Each lane contained 20 μg of total RNA extracted from pistils. The membrane was first hybridized to the ^{32}P-labeled ARC1 cDNA and then hybridized to the ^{32}P-labeled THL1 cDNA as a control for even loading. (**B**) Instant Imager quantification of ARC1 mRNA levels. The levels of ARC1 mRNA were normalized to the level of THL1 mRNA. The data represent the mean of two independent experiments. Error bars indicate ±SE. (**C**) RT-PCR analysis for SLG-910 and SRK-910. To specifically detect SLG-910 and SRK-910 transcripts, RT-PCR was carried out for 20 cycles with gene-specific primers, followed by Southern blotting (*13*). The RT-PCR analysis was repeated several times with different RNA preparations and varying PCR cycle numbers. No overall decrease in either transcript was observed for any transgenic plant.

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dent ARC1-antisense transgenic W1 lines, ARC1 mRNA levels were drastically reduced by up to 90% (A1-1, A1-2, A2-1, A2-2, and A2-3; Fig. 1, A and B). To ensure that any potential effects seen in these plants were not due to a loss of SLG or SRK, the plants were analyzed for the presence and expression of the W1 self-incompatibility genes *SLG-910* and *SRK-910* (*10*, *13*). All the plants carried and expressed the *SLG-910* and *SRK-910* genes, as determined by Southern blot analysis (*12*) and reverse transcriptase–polymerase chain reaction (RT-PCR) analysis (Fig. 1C).

To determine whether alteration of ARC1 mRNA levels affected self-incompatibility, plants were analyzed for their ability to produce seed by self-pollination as well as by reciprocal crosses to W1 (*14*) (Fig. 2). In addition, reciprocal crosses were performed with the self-compatible *Brassica napus* Westar cultivar to examine any effects of the transgene on compatible pollinations. The W1 cultivar has a strong, early-acting self-incompatibility phenotype and typically shows no seed production with selfing (Fig. 2, A and B) while showing ample seed production when crossed with the compatible Westar cultivar (Fig. 2, D and E). The ARC1-sense S1-1 plant showed a similar profile when selfed and crossed to W1 and Westar (Fig. 2, A, B, and D). Thus, the overexpression of ARC1 did not alter the pollination behavior of this transgenic W1 plant.

For the two ARC1-antisense W1 lines, selfing of the transgenic plants resulted in some seed production, with the highest mean being 6.5 seeds per pod for the A1-2 plant (approximately one-third of a fully compatible seed set), whereas the nontransgenic sibs had zero seed production as expected (Fig. 2A). The pods produced by the transgenic plants were approximately half the size of pods resulting from a compatible pollination (Fig. 3). The morphology of the pods produced by the nontransgenic sibs was identical to that of pods produced by a self-incompatible W1 pollination (Fig. 3). Similar seed set and pod sizes were also obtained when the plants were pollinated with W1 pollen (Figs. 2B and 3). Statistical analysis of the seed set by the ARC1-antisense transgenic W1 plants showed that the seeds per pod were significantly different from both the self-incompatible pollinations and the fully compatible pollinations (Fig. 2). Thus, the ARC1-antisense transgenic W1 lines displayed a phenotype that was intermediate between self-incompatible and compatible, and the change in phenotype was due to the presence of the transgene, because the nt sibs were completely self-incompatible.

RNA blot analysis has previously shown that ARC1 mRNA is only detected in the pistil (*9*). Thus, it is predicted that any effects seen in the transgenic plants will be confined to the pistil, and the self-incompatibility system should be intact for the pollen. When W1 pistils were pollinated with transgenic pollen, there was essentially no seed production (Fig. 2C), and this was not due to a loss of pollen viability because crosses onto Westar pistils produced mean seed sets similar to those of the W1-Westar compatible pollinations (Fig. 2E).

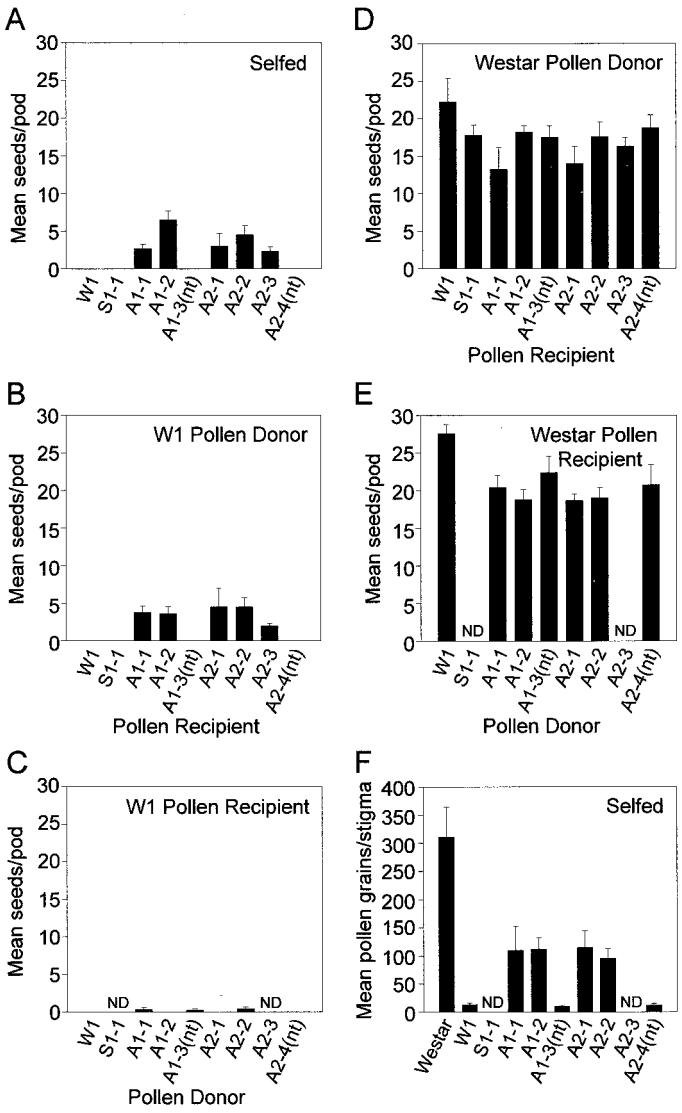

Fig. 2. Seed set and pollination analysis. [(A) through (E)] Mean number of seeds per pod after controlled pollinations. (**A**) Self pollinations. (**B**) Transgenic and nontransgenic W1 pistils pollinated with W1 pollen. (**C**) W1 pistils pollinated with transgenic and nontransgenic W1 pollen. (**D**) Transgenic and nontransgenic W1 pistils pollinated with Westar pollen. (**E**) Westar pistils pollinated with transgenic and nontransgenic W1 pollen. (**F**) Mean number of pollen grains adhering to the stigmatic surface after self-pollination. W1 self-pollinations were self-incompatible and showed no seed production, whereas W1-Westar pollinations were compatible and showed full seed production. Means represent no less than five values. Error bars indicate ±SE. ND, not determined. For the statistical analysis, the data were pooled into the following three groups: (i) self-incompatible, which includes the data from (A) and (B) for W1 and from the two nontransgenic sibs to give a mean of 0 ± 0 seeds per pod; (ii) intermediate compatible, which includes the data from (A) and (B) for the five ARC1-antisense transgenic W1 plants to give a mean of 3.7 ± 0.37 seeds per pod; and (iii) fully compatible, which includes all the data from (D) to give a mean of 17.2 ± 0.75 seeds per pod. Mean seed set differed significantly among the three groups of plants when analyzed by a nested analysis of variance followed by Scheffe's a posteriori test ($F_{2,13} = 137.3$, $P < 0.0001$). Thus, the ARC1-antisense transgenic W1 plants showed an intermediate level of seed set.

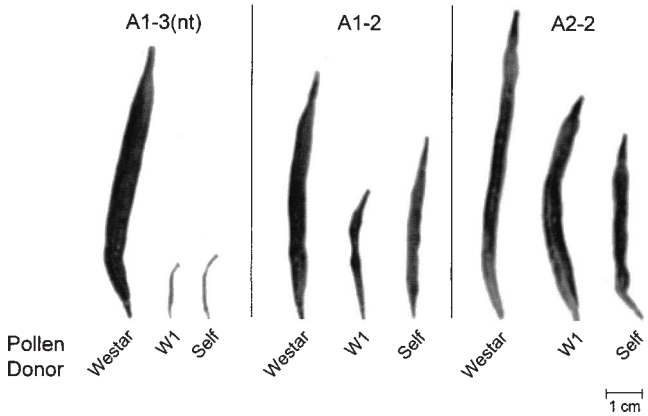

Fig. 3. Comparison of mature seed pods from the A1-3(nt) nontransgenic sib and the A1-2 and A2-2 ARC1-antisense transgenic W1 plants. Pistils from each plant were pollinated with Westar, W1, and self pollen. Pods were allowed to develop for an equal length of time.

Thus, as predicted, the transgenic pollen did not show an altered phenotype. To rule out the possibility that the partial seed set in the ARC1-antisense W1 plants was from reduced pistil fertility, transgenic pistils were pollinated with Westar pollen. Both the transgenic and nontransgenic W1 plants produced ample seed, indicating that pistil fertility was not affected in any of these plants (Fig. 2D). The pods produced were fully developed and were similar in size to those of a compatible Westar pollination (Fig. 3). Similar results occurred when the ARC1 sense and antisense constructs were introduced into the self-compatible Westar background (*12*). This indicates that ARC1 does not have an essential function in compatible pollinations and its effects are confined to the self-incompatibility system.

Pollen germination and pollen tube growth were examined more closely in the transgenic plants by means of fluorescence microscopy of aniline blue–stained pistils to determine whether a particular stage of self-incompatibility was most affected (*15*). The transgenic and nontransgenic sibs were either self-pollinated or pollinated with incompatible W1 pollen. As controls, W1 pistils were either self-pollinated or pollinated with compatible Westar pollen. Typically, during a compatible pollination, a large number of pollen grains adhere to the stigmatic surface (approximately 300 pollen grains; Fig. 2F), hydrate, and germinate to produce pollen tubes that penetrate the stigmatic surface (Fig. 4A). In the W1 cultivar, a self-incompatible pollination results in few pollen grains adhering to the stigmatic surface (approximately 13; Fig. 2F), little or no pollen germination, and no pollen tube penetration of the stigmatic surface (Fig. 4B). The stigmas of the self-pollinated nontransgenic sibs were similar to those of a typical self-incompatible W1 pollination (Fig. 2F). However, self-pollination of the ARC1-antisense transgenic W1 plants resulted in a large number of pollen grains adhering to the stigmatic surface (approximately 100 pollen grains; Fig. 2F) and germinating to form pollen tubes (Fig. 4C). However, many of the pollen tubes were curled up on the stigmatic surface and only a few penetrated the surface to grow down the style (Fig. 4C). This probably accounts for the partial seed set.

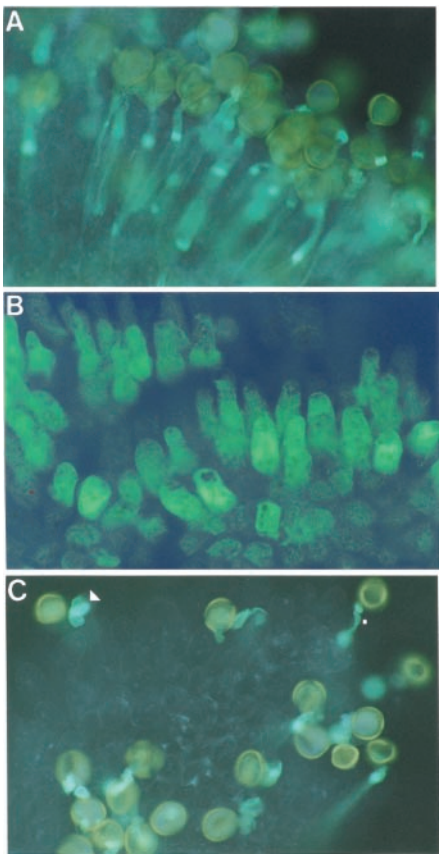

Fig. 4. Analysis of pollen grain germination and pollen tube growth by fluorescence microscopy. (**A**) Stigma of a self-compatible Westar pollination. (**B**) Stigma of a self-incompatible W1 pollination. There are no pollen grains adhering to the stigma papillae. (**C**) Stigma of the A2-2 ARC1-antisense transgenic W1 plant, self-pollinated. A pollen tube growing down the stigmatic papilla is indicated by a white square. A curled pollen tube unable to penetrate the stigma is indicated by a white triangle.

The intermediate phenotype observed in the ARC1-antisense W1 plants may result from some residual ARC1 mRNA levels that are sufficient to maintain some level of self-incompatibility. Alternatively, because self-incompatible *Brassica* pollen can be blocked at several stages (*2*), and the ARC1-antisense W1 plants are most affected at the early stages of the self-incompatibility response, ARC1 may function in these early stages to inhibit pollen adhesion, hydration, or germination. Another unknown component of the signaling pathway may act later, perhaps to block the infiltration of the pollen tube into the stigmatic papillar cell wall; this component may be compensating for the loss of ARC1 expression, but is not capable of fully restoring the self-incompatibility phenotype.

The cellular function of ARC1 is unknown, and sequence similarities to other proteins only exist in the arm repeat region, which is required for binding to the SRK kinase domain (*9*). In animals, arm repeat proteins have diverse functions, with the arm repeats constituting protein-protein interaction domains (*16*). One particularly interesting class is the β-catenin/Armadillo family involved in cadherin-mediated cell adhesion and the Wnt/Wingless signaling pathway (*16*), though ARC1 does not appear to be more closely related to this family in relation to the other classes. One possible function for ARC1 is that of an adaptor protein. Upon binding to the activated SRK through its COOH-terminal arm repeats, the NH_2-terminal region of ARC1 may bind and recruit the next putative protein in the pathway. Thus, based on the analysis of the ARC1 transgenic W1 plants and coupled with the in vitro interaction studies (*9*), we propose that ARC1 is involved in the SRK-mediated signaling pathway as a positive downstream effector of SRK, leading to the rejection of self-incompatible pollen.

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14. Pollination bags were applied 1 day before anthesis, and self-pollinations were carried out on opened flowers. Otherwise, buds were stripped of all male tissue before anthesis and were covered. Pistils were pollinated 1 day later and were covered for no less than 1 week, after which the resulting pods were left uncovered to mature.
15. For fluorescence microscopy, 18 to 24 hours after pollination (*14*) pistils were collected and fixed in 3:1 (v/v) ethanol:glacial acetic solution for at least 15 min, after which whole or hand-cut pistils were treated with 1N NaOH for 1 hour at 60°C, rinsed, and stained in 0.1% aniline blue in 0.1 M K_3PO_4 (pH 7) buffer for 1 hour at 60°C.
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