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Binding of an arm repeat protein to the kinase domain of the *S*-locus receptor kinase

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ABSTRACT **Screening of a yeast two-hybrid library for proteins that interact with the kinase domain of an *S*-locus receptor kinase (SRK) resulted in the isolation of a plant protein called ARC1 (Arm Repeat Containing). This interaction was mediated by the C-terminal region of ARC1 in which five arm repeat units were identified. Using the yeast two-hybrid system and *in vitro* binding assays, ARC1 was found to interact specifically with the kinase domains from SRK-910 and SRK-A14 but failed to interact with kinase domains from two different *Arabidopsis* receptor-like kinases. In addition, treatment with a protein phosphatase or the use of a kinase-inactive mutant reduced or abolished the binding of ARC1 to the SRK-910 kinase domain, indicating that the interaction was phosphorylation dependent. Lastly, RNA blot analysis revealed that the expression of ARC1 is restricted to the stigma, the site of the self-incompatibility response.**

Many flowering plants employ self-incompatibility systems to prevent inbreeding and promote outcrossing (for review, see refs. 1–3). In the *Brassica* family, the sporophytic self-incompatibility system is controlled by the multi-allelic *S* locus (4). When the pollen parent and the pistil share the same S allele, pollen germination or pollen tube growth is inhibited, thereby leading to failure of fertilization. Molecular and biochemical characterization of the *S* locus has led to the isolation of two genes, the *S*-locus glycoprotein (SLG) and *S*-locus receptor kinase (SRK) genes, both of which are highly expressed in the pistil (5, 6). These two genes are tightly linked, and for one S-allele, the SLG and SRK were found within a region of 30 kb (7). Within the pistil, the SLG has been localized to the cell wall of the stigma papillae, whereas the SRK is present as a transmembrane protein in the stigma (8–10). Based on DNA sequence comparisons, the N-terminal structure of the SRK gene resembles the SLG coding region, and its C-terminal portion encodes a putative serine/threonine kinase (6). Further characterization of the kinase domain has confirmed that the SRK encoded a functional serine/threonine kinase (11, 12).

Both SLG and SRK have been shown to be determinants of self-incompatibility in *Brassica*, as either loss of SLG gene expression (13–15) or mutations in the SRK gene (16, 17) were found to be associated with self-compatibility. As the *S* locus determines the specific interaction between the pollen and stigma, it has been postulated that the pollen component of the self-incompatibility system may be encoded by a functional gene linked to the SLG and SRK genes. One candidate for this pollen component is the anther-specific SLA gene (18). The pollen component on the incompatible pollen may serve as a ligand, which is recruited likely by the SLG to interact with the SRK. The specific interaction between the ligand and SRK would in turn activate the SRK and trigger a signaling cascade, leading to pollen rejection by the stigma papillae cells (11, 19, 20). Little is understood about the molecular mechanisms in the SRK-mediated signal transduction pathway. In an attempt to dissect this pathway, we have employed the yeast two-hybrid system to isolate proteins that may function as downstream targets of the SRK protein. Using this approach, we have isolated a plant gene called *ARC1* (Arm Repeat Containing), which encodes a protein that interacts specifically with the kinase domains from two different *S*-locus receptor kinases.

MATERIALS AND METHODS

Screening of the Yeast Two-Hybrid Library. The lexA-VP16 system was used in the yeast two-hybrid screening (21). The construction of the W1 pistil cDNA library in the pVP16 vector and the screening of the library was conducted as previously described (22). Essentially, the L40 yeast strain (21) was first transformed with the lexAkinase^{910} plasmid and then transformed with the VP16–pistil cDNA library. Transformants were plated on synthetic dextrose minimal medium (SD) plates supplemented with 100 mg/liter adenine sulfate (SD_{Ade}) to select for activation of the *HIS3* reporter gene. β-Galactosidase assays were then performed on filter lifts of the colonies to detect activation of the *lacZ* reporter gene (21).

β-Galactosidase activity levels were determined by using the permeabilized cell assay as described by Rose *et al.* (23). Overnight cultures were diluted to 5×10^6 cells per ml and grown for 3–4 h; 5×10^6 cells were pelleted and resuspended in 750 μl of Z buffer. Three drops of chloroform and two drops of 0.1% SDS were added and vortexed for 10 s. The samples were placed at 28°C for 5 min, and then 150 μl of a 4 mg/ml stock of *o*-nitrophenyl β-D-galactoside was added to each sample. The samples were timed for the length of time required to develop a pale yellow color, at which point 375 μl of 1 M Na_2CO_3 was added to stop the reaction. After 1 h, the remainder of the samples were stopped regardless of color development. Samples were centrifuged for 10 min to pellet cell debris and quantitated by measuring the OD_{420}. Three separate colonies were assayed for each construct. β-Galactosidase activity was determined as the OD_{420} per hour.

Cloning and Sequencing of the ARC1 cDNA. The original ARC1 clone isolated from the two-hybrid screen was a 1-kb partial cDNA encoding the C-terminal region. This partial cDNA was used to screen pistil libraries leading to the identification of two additional clones. The cDNAs were then subcloned into pBluescript KS+ (Stratagene) and sequenced

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Abbreviations: SLG, *S*-locus glycoprotein; SRK, *S*-locus receptor kinase; RLK, receptor-like kinase; MBP, maltose-binding protein; GST, glutathione *S*-transferase; PMSF, phenylmethylsulfonyl fluoride; KAPP, kinase-associated protein phosphatase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF024625).

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by cycle sequencing on an Applied Biosystems 373 DNA sequencer (Molecular Biology Core Facility, York University, Canada). These clones, together with the original C-terminal clone, spanned the entire cDNA based on the estimated size of the ARC1 transcript. To clear up sequence ambiguities, additional cDNAs were isolated by PCR amplification by using primers close to the 5′ and 3′ regions of ARC1 and sequenced. Sequence comparisons were done by using DNASIS and PROSIS software (Hitachi, Tokyo) or by FASTA searches of the GenBank database.

RNA Blot Analysis. Extraction of total RNA from vegetative and floral tissues of *Brassica napus* W1 was conducted by the procedure of Jones *et al.* (24). Approximately 20 μg of total RNA was size-fractionated on a 1.2% agarose–formaldehyde gel. Blotting, hybridization, and washing were carried out as previously described (22). The ARC1 mRNA was detected by using the 1.0-kb C-terminal ARC1 clone, and a 1.5-kb 18S ribosomal cDNA from *Brassica rapa* (25) was used as a control probe.

***In Vitro* Binding and Phosphorylation of ARC1.** The kinase domains used to construct the maltose-binding protein (MBP) or glutathione *S*-transferase (GST) fusions consisted of the entire catalytic region starting just at the 3′ end of the putative transmembrane domain. The GSTARC1 fusions were constructed with either the original C-terminal region isolated in the two-hybrid screen or the entire ARC1 coding region. All MBP and GST protein fusions were purified by the method of Horn and Walker (26) with modifications. Fifty to 200 ml of 2× YT containing 100 μg/ml ampicillin was inoculated with 1/100th volume of an overnight culture and grown to an OD_{600} of 0.8 at 37°C. Isopropyl β-D-thiogalactoside was added to a final concentration of 0.1 mM and induced overnight at 20°C.

For the GST fusion proteins, the cells were pelleted, resuspended in 10–20 ml of G-lysis buffer [50 mM Hepes, pH 7.4/150 mM NaCl/10 mM EDTA/1 mM DTT/200 μM phenylmethylsulfonyl fluoride (PMSF)] and frozen overnight at −20°C. The samples were thawed, PMSF was added again to a final concentration of 200 μM, and the samples were sonicated in 15-s bursts by using a probe sonicator. Triton X-100 was added to a final concentration of 1%, and the samples were spun at 17,000 rpm for 10 min at 4°C. To the supernatant, 2 ml of 10% (vol/vol) glutathione-agarose was added and mixed for 30 min at 4°C. The beads were washed three times each with 2 ml of lysis buffer, resuspended in 6 ml of lysis buffer, and poured into a column. The GST fusions were eluted with elution buffer (50 mM Hepes, pH 8.0/15 mM glutathione), glycerol was added to a final concentration of 20%, and the samples were stored at −20°C.

To purify the MBP fusion proteins, the method used was similar to above except that the cells were resuspended in M-lysis buffer (50 mM Hepes/200 mM NaCl/1 mM EDTA/1 mM DTT/200 μM PMSF); Triton X-100 was omitted, and 2 ml of 10% amylose resin (New England Biolabs) was added to the supernatant. The MBP fusion proteins bound to the amylose resin were washed four times each with M-lysis buffer containing 10% glycerol, resuspended in 200 μl of M-lysis buffer with 10% glycerol, and stored at 4°C.

For the *in vitro* binding assay, 5–10 μl of the resin-bound MBP fusion proteins were separated by SDS-PAGE and stained with Coomassie blue to determine approximate concentrations. Approximately 2 μg each of the resin-bound MBP fusion proteins were washed twice with binding buffer (20 mM Hepes, pH 7.4/1 mM EDTA/5 mM $MgCl_2$/1 mM DTT/0.1% Triton X-100) and resuspended in 300 μl of binding buffer. Approximately 2 μg of the eluted GSTARC1 (C-terminal) fusion protein was added to each sample and mixed for 1.5–2 h at 4°C. The samples were then washed three times with binding buffer and resuspended in 20 μl of sample buffer. Phosphatase treatment of the MBPkinase^{910} was performed by washing approximately 2 μg of the resin-bound MBPkinase^{910} once with the λ protein phosphatase buffer (New England Biolabs) with or without phosphatase inhibitors (2 mM Na_3VO_4/5 mM NaF/5 mM β-glycerophosphate/5 mM *p*-nitrophenyl phosphate) and then resuspended in 50 μl of the same buffer. To each sample, 800 units of the λ protein phosphatase (New England Biolabs) was added and incubated at 30°C for 30 min. The samples were washed twice with binding buffer containing 2 mM Na_3VO_4 and 1 mM *p*-nitrophenyl phosphate, resuspended in 300 μl of binding buffer, and processed as above. To detect the bound GSTARC1 (C-terminal) fusion protein, 10 μl of each sample were separated on a 12% SDS-PAGE gel and transferred to nitrocellulose membrane. The GSTARC1 (C-terminal) fusion protein was detected by using a GST-Western blotting kit (Boehringer). A duplicate gel was also stained with Coomassie Blue to visualize the MBP fusion proteins bound on the beads.

For the kinase assay, approximately 0.5 μg of each protein was mixed in a 10-μl reaction with 20 mM Pipes, pH 7.0, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μg/ml aprotinin, and 20 μCi [γ-^{32}P]ATP and incubated for 30 min at room temperature. The proteins were separated on 13% SDS-PAGE gel. The gel was then stained with Coomassie blue, dried, and subjected to autoradiography to detect the phosphoproteins. The protein markers used were from New England Biolabs.

RESULTS

ARC1 Interactions in the Yeast Two-Hybrid System. The two-hybrid pistil cDNA library was screened by cotransforming the yeast L40 strain with the DNA binding domain plasmid carrying the lexAkinase^{910} fusion and the activation domain plasmid with the VP16–pistil cDNA library (22). Interactions between the lexAkinase^{910} and a VP16–cDNA fusion protein resulted in the activation of two reporter genes, *HIS3* and *lacZ*, and were identified by histidine prototrophy and β-galactosidase activity. Initial screening lead to the identification of two different thioredoxin-h clones, which interacted with the lexAkinase^{910} (22), and further screening led to the isolation of ARC1. To test the specificity of the ARC1 interactions in the yeast two-hybrid system, several different lexA fusions were used: (*i*) lexAkinase^{910} used to screen the library; (*ii*) the kinase inactive lexAmukinase^{910} with a lysine-to-alanine substitution at position 557; (*iii*) the protein kinase domains from two *Arabidopsis* receptor-like kinases (RLK), lexAkinase^{RLK4} and lexAkinase^{RLK5} (27); (*iv*) lexAlamin to detect nonspecific interactions; and (*v*) lexAkinase^{A14}, which contains an SRK kinase domain from another *Brassica S* locus (28).

The VP16:ARC1 fusion showed interactions with the kinase domains from both of the SRK genes, lexAkinase^{910} and lexAkinase^{A14}, but not with either of the *Arabidopsis* kinases, lexAkinase^{RLK4} and lexAkinase^{RLK5} (Fig. 1*A*). Cotransformation of lexAkinase^{910} with the VP16 vector alone does result in very weak transcriptional activation of the reporter genes, but the levels are much lower than that seen with the VP16:ARC1 fusion (Fig. 1*B*). The lexAkinase^{A14} has much higher background levels leading to growth in the absence of histidine when cotransformed with either VP16 or VP16:ARC1 (Fig. 1 *A* and *B*). However, when β-galactosidase levels were measured, the lexAkinase^{A14} fusion in the presence of VP16:ARC1 had 17 times more activity than in the presence of VP16 alone (Fig. 1 *A* and *B*). Thus, the interaction between the SRK-A14 kinase domain and VP16:ARC1 results in much higher levels of reporter gene activation. The lack of interaction between the mutant lexAkinase^{910} and VP16:ARC1 suggests a requirement of phosphorylation for this interaction. No interaction was seen for the nonspecific control lexAlamin.

Sequence Analysis of ARC1. The original clone isolated in the two-hybrid system was approximately 1 kb in length and encoded the C-terminal region of the ARC1 protein. The

A

	VP16:ARC1		
	Growth on His$^+$ plates	Growth on His$^-$ plates	β-gal activity
LexAkinase^{910}			0.48±0.08
LexAmukinase^{910}			0
LexAkinase^{RLK4}			0
LexAkinase^{RLK5}			0
LexAlamin			0
LexAkinase^{A14}			24.68±5.57

B

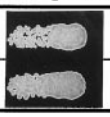
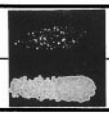
	VP16		
	Growth on His$^+$ plates	Growth on His$^-$ plates	β-gal activity
LexAkinase^{910}			0
LexAkinase^{A14}			1.41±0.19

FIG. 1. Interaction of ARC1 with kinase domains from two different *S*-locus receptor kinases. lexA fusions were tested for their ability to interact with either VP16:ARC1 (*A*) or VP16 alone (*B*). Positive interactions resulting in the activation of the *HIS3* and *lacZ* genes were detected by growth in the absence of histidine and β-galactosidase (β-gal) activity. β-Galactosidase activity was measured by using an *o*-nitrophenyl β-D-galactoside assay and is reported in units of OD_{420}/h. (*A*) Only lexAkinase^{910} and lexAkinase^{A14} interact with the VP16:ARC1 fusion, resulting in growth in the absence of histidine and detectable levels of β-galactosidase activity. (*B*) lexAkinase^{910} in the presence of VP16 alone shows slight growth in the absence of histidine but no measurable levels of β-galactosidase activity. lexAkinase^{A14} in the presence of VP16 alone has higher background activity, resulting in growth in the absence of histidine and measurable levels of β-galactosidase activity. However, the β-galactosidase levels are only 1/17th of that seen for the interaction with VP16:ARC1 in *A*.

entire cDNA was subsequently characterized and was found to be 2186 bp in length. Based on RNA blot analysis (see below), the cDNA was considered to be approximately full length. The predicted amino acid sequence shown in Fig. 2 corresponds to

```
1    MATDSAMFASSRRRQSPSLEAFLSPVDLSDVPLLQTLSSISSEIVSCFSNARFSF  55
56   QRRNTRSLIRKVQVFAVLLQHLAPESSLDPTAVLCFKELYLLLHHSKFLLRYCAH  110
111  SSKLWLLLQSPSLSSFFHDLSKDYSTLLDVFLPVDSLCLNDDVREQVQLLHMQHY  165
166  VDDNNDETLRNRLYSFLDEFENGSVPNSEELRFFFFEKLAIKDPTSYREEIEFLE  220
221  EQIKSHGCDLDPTRSVINGFIDITRYVMFLLFKIEDGNEIKKQRKRLISEEIENT  275
276  FTTTLPKDFICSISLNLMNDPVIISTGQTYDRTSIARWIHQEGRSTCPKTGQKLV  330
331  DLSFVSNLALRHLTTLWCEVTGLSHDSPKESLPKVFQTRASTEANKATLSILVQN  385
386  LAHGSELAAGEIRVLTRTVTETRTLIVETGAIPYLRSLLKSQNAVAQENAVASIF  440
441  NLSIDEANRSLIVEEHDCLEPIMSVLVSGLTMRAKEIAAATLYTLSSVHDYKKAI  495
496  ANADGCIEALALVLRNGTVRGKKDAVYALHSLWLHPDNCSLMVKRGGVSALVGAL  550
551  GEEAVAEKVAWVLGVMATESLGAESIGREETVVTGLMELMRCGRPRGKEKAIATL  605
606  LQLCTAGGAVVTEKVVKTPALAVLTRKLLLTGTDRAKRKAVSLSKVCKGCDQNTQR 661
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FIG. 2. Predicted amino acid sequence of ARC1. The longest ORF, which also corresponds to the frame fused to VP16 in the original two-hybrid clone, is shown. The arrow marks the start of the two-hybrid clone isolated. The five putative arm repeats are underlined and separated by triangles.

the reading frame found in the two-hybrid clone following the VP16 reading frame. The other two reading frames were found to contain multiple stop codons (data not shown). The initiation methionine at nucleotide 114 was identified as the first methionine following a potential stop codon at position 84. The full-length ARC1 protein is predicted to consist of 661 amino acids, and the C-terminal two-hybrid clone begins at amino acid 362 (Fig. 2).

Database searches with both the DNA and amino acid sequences revealed that ARC1 was a novel plant protein. For the ARC1 amino acid sequence, the N-terminal region did not show any significant sequence similarities to proteins in the database. The C-terminal region, which was present in the two-hybrid clone, showed approximately 25% sequence identity to the arm repeat regions in the mouse pendulin protein and the β-catenin/armadillo family of proteins. From the defined arm repeats in the armadillo gene, five potential arm repeats were identified in ARC1 (Figs. 2 and 3). In Fig. 3, each ARC1 arm repeat is aligned to the closest armadillo arm repeat. The ARC1 arm repeats are fairly degenerate showing 29% (R1), 19% (R2), 33% (R3), 29% (R4), and 24% (R5) sequence identity to the closest armadillo arm repeat (Fig. 3).

Southern blot analysis of genomic DNA from two *Brassica napus* lines, the self-incompatible W1 line and the self-compatible Westar line, indicated that *ARC1* is a single copy gene found in both lines. However, when the blot was washed under lower stringency conditions, some faint cross-hybridizing bands could be detected (data not shown).

ARC1 RNA Expression Patterns. RNA blot analysis of ARC1 was performed to determine its expression patterns and the approximate size of the transcript. Total RNA from W1 leaf, roots, stems, anthers, and pistils were hybridized with the ARC1 cDNA. High levels of steady-state ARC1 mRNA were only detected in the pistils from developing flower buds, and the size of the ARC1 transcript was approximately 2.3 kb (Fig. 4*A*, lanes 8–10). Slightly higher levels of ARC1 transcripts were detected in the larger buds approaching anthesis (Fig. 4*A*, lane 10). Similar results were also observed with tissues from the self-compatible Westar line (data not shown). To determine if ARC1 expression was restricted within the pistil, W1 pistils were separated into the stigma, style, and ovary, and total RNA was extracted from each section. High levels of ARC1 mRNA were only detected in the stigma (Fig. 4*B*, lane 2). This is the region at the top of the pistil that comes in

```
Armadillo R12   631  LCEL AADKEGAEIIEQEGATGPLTDLLHSRNEGVATYAAAV  671
                     .  :    :   .:   :: . :  :: : : ..   :.:
ARC1 R1         397  IRVLTRTVTETRTLIVETGAIPYLRSLLKSQNAVAQENAVAS 438

Armadillo R2    183  VHQLSKKEASRHAIMNSPQMVAALVRAISNSNDLESTKAAVGT 225
                     . .::  :::.: .:.    .. ... .. .    .     :.:
ARC1 R2         439  IFNLSIDEANRSLIVEEHDCLEPIMSVLVSGLTMRAKEIAAAT 481

Armadillo R6    352  LKVLSVCSSNKPAIVDAGG-MQALAMHLGNMSPRLVQNCLWT 391
                     :  :: .  .: ::. : : . :::. : :....:   ..
ARC1 R3         482  LYTLSSVHDYKKAIANADGCIEALALVLRNGTVRGKKDAVYA 523

Armadillo R6    352  LKVLSVCSSNKPAIVDAGGMQALAMHLGNMSP RLVQNCLWT 391
                     :. : .   :  ..:  ::..::.. ::   .   :  :.
ARC1 R4         524  LHSLWLHPDNCSLMVKRGGVSALVGALGEEAVAEKVAWVLGV 565

Armadillo R10   521  LIRNLALCPANHAPLREHGAIHHLVRLLMRAFQDTERQRSSI 562
                     ..  : :: :  :.. :...   .. .: : .  :   :
ARC1 R5         602  IATLLQLCTAGGAVVTEKVVKTPALAVLTRKLLLTGTDRAKR 643
                          609       619       629       639
```

FIG. 3. Alignment of the five ARC1 arm repeats to the armadillo arm repeats. Each of the five ARC1 arm repeats is aligned to the most similar arm repeat found in the armadillo protein. The sequence identities range from 19 to 33% sequence identity. Double dots indicate identical amino acids, and single dots indicate similar amino acids. The following groups were used to define similar amino acids: nonpolar side chains (G, A, V, L, I, P, F, M, W, C), uncharged polar side chains (N, Q, S, T, Y), acidic side chains (D, E), and basic side chains (K, R, H).

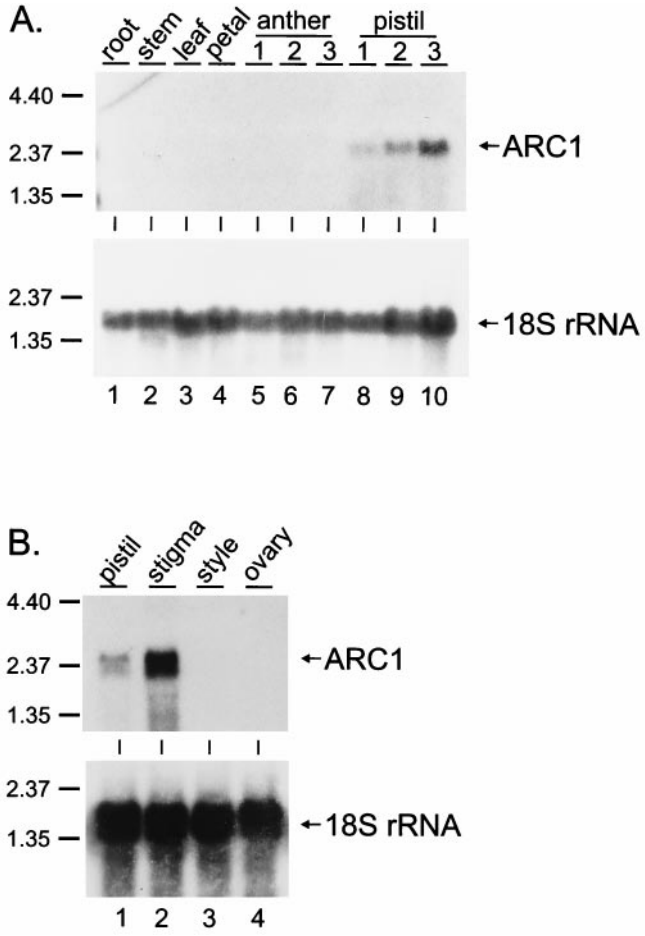

FIG. 4. Tissue-specific expression of ARC1. Total RNA from (*A*) root, stem, leaf, petal, anther, and pistil or (*B*) pistil, stigma, style, and ovary were hybridized to the ARC1 cDNA. The numbers 1 to 3 represent different bud sizes with 1 = 1–2-mm buds, 2 = 3–4-mm buds, and 3 = 5–7-mm buds. ARC1 transcripts are only detected in the pistil, and within the pistil, the ARC1 mRNA is restricted to the stigma. Hybridization of the filters with the thioredoxin-h clone, THL-1 (22), indicated that intact mRNA was present in all lanes (not shown). The filters were also hybridized with the 18S cDNA probe to show the relative amount of total RNA present in each lane.

contact with the pollen and corresponds to where the SLG and SRK genes are expressed (9, 29). Thus, the pattern of ARC1 expression is tissue-specific and corresponds to the site of the self-incompatibility reaction.

***In Vitro* Binding and Phosphorylation of ARC1.** To confirm that a direct interaction was occurring between ARC1 and the SRK kinase domains, binding assays were performed using MBPkinase fusion proteins bound to the amylose resin. A GSTARC1 (C-terminal) fusion protein, which contained the same region isolated from the yeast two-hybrid library, was mixed with the MBP fusion proteins. After washing, the samples were then separated by SDS-PAGE, and the presence of bound GSTARC1 (C-terminal) fusion protein was detected by a GST antibody. As shown in Fig. 5, the GSTARC1 (C-terminal) fusion protein showed preferential binding to the two SRK kinases, MBPkinase^{910} and MBPkinase^{A14} (Fig. 5*A*, lanes 5 and 8). Binding to MBP and the two *Arabidopsis* kinases, MBPkinase^{RLK4} and MBPkinase^{RLK5}, was not detected (Fig. 5*A*, lanes 2–4). With longer exposures, some nonspecific binding was detected to all the MBP samples under these conditions. Treatment of the MBPkinase^{910} with the λ protein phosphatase resulted in very little binding of GSTARC1 (Fig. 5*A*, lane 6), and inhibition of the phosphatase restored GSTARC1 binding to MBPkinase^{910} (Fig. 5*A*, lane 7).

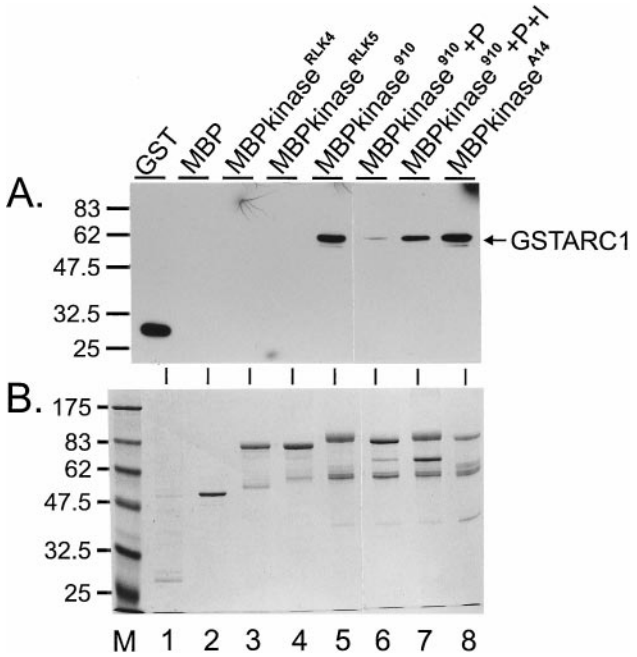

FIG. 5. In Vitro Binding of ARC1 to the SRK kinase domains. (*A*) The GSTARC1 (C-terminal) fusion protein was mixed with various resin-bound MBP fusion proteins. The presence of the GSTARC1 (C-terminal) fusion protein was detected by using a GST Western blotting kit. Lane 1, 5 μl of the GST positive control provided with the GST Western blotting kit; lanes 2–8, *in vitro* binding assays. (*B*) Coomassie blue-stained gel of the *in vitro* binding assays. Lane M, marker lane; lane 1, 5 μl of the GST positive control provided with the GST Western blotting kit; lanes 2–8, the various MBP fusion proteins in the *in vitro* binding assays. For the MBP fusion proteins, the top band in each lane represents the full-length fusion protein. The bands below represent degradation products.

The GST protein by itself did not bind to any of the MBP fusion proteins (data not shown). Fig. 5*B* is a Coomassie-stained gel showing the relative levels of the MBP fusions present in the binding reactions. Thus, ARC1 binds directly to the SRK kinase domains in a phosphorylation-dependent interaction.

To determine if the interaction between ARC1 and the SRK kinase domains led to the phosphorylation of ARC1, the GSTARC1 (full length) fusion protein was mixed with the GSTkinase^{910}, GSTmukinase^{910}, GSTkinase^{RLK5}, or GSTkinase^{A14} in a kinase assay with [γ-^{32}P]ATP. GST alone was also included as a control to show that ARC1 was being specifically phosphorylated. ARC1 was found to be phosphorylated by both of the SRK kinases, GSTkinase^{910} and GSTkinase^{A14} (Fig. 6, lanes 2 and 10). GSTkinase^{RLK5} only showed very weak phosphorylation of ARC1 (Fig. 6, lane 7), and GST was not phosphorylated by any of the kinases (Fig. 6, lanes 3, 8, and 11). In addition, no ARC1 phosphorylation was detected in the presence of the inactive GSTmukinase^{910} (Fig. 6, lane 5). Thus, the interaction between the GSTARC1 (full length) fusion protein and the GSTkinase^{910} and GSTkinase^{A14} fusion proteins does lead to some phosphorylation of ARC1.

DISCUSSION

In a search for components of the *Brassica* self-incompatibility signaling cascade, we have identified a plant protein, ARC1, which can interact with the kinase domain of two different *S*-locus receptor kinases. Although there is allele specificity in the *Brassica* self-incompatibility system, it is expected that this specificity would occur at the level of pollen recognition such as a "pollen ligand" binding to the extracellular S-domain. Once the SRK is activated, the downstream events would be

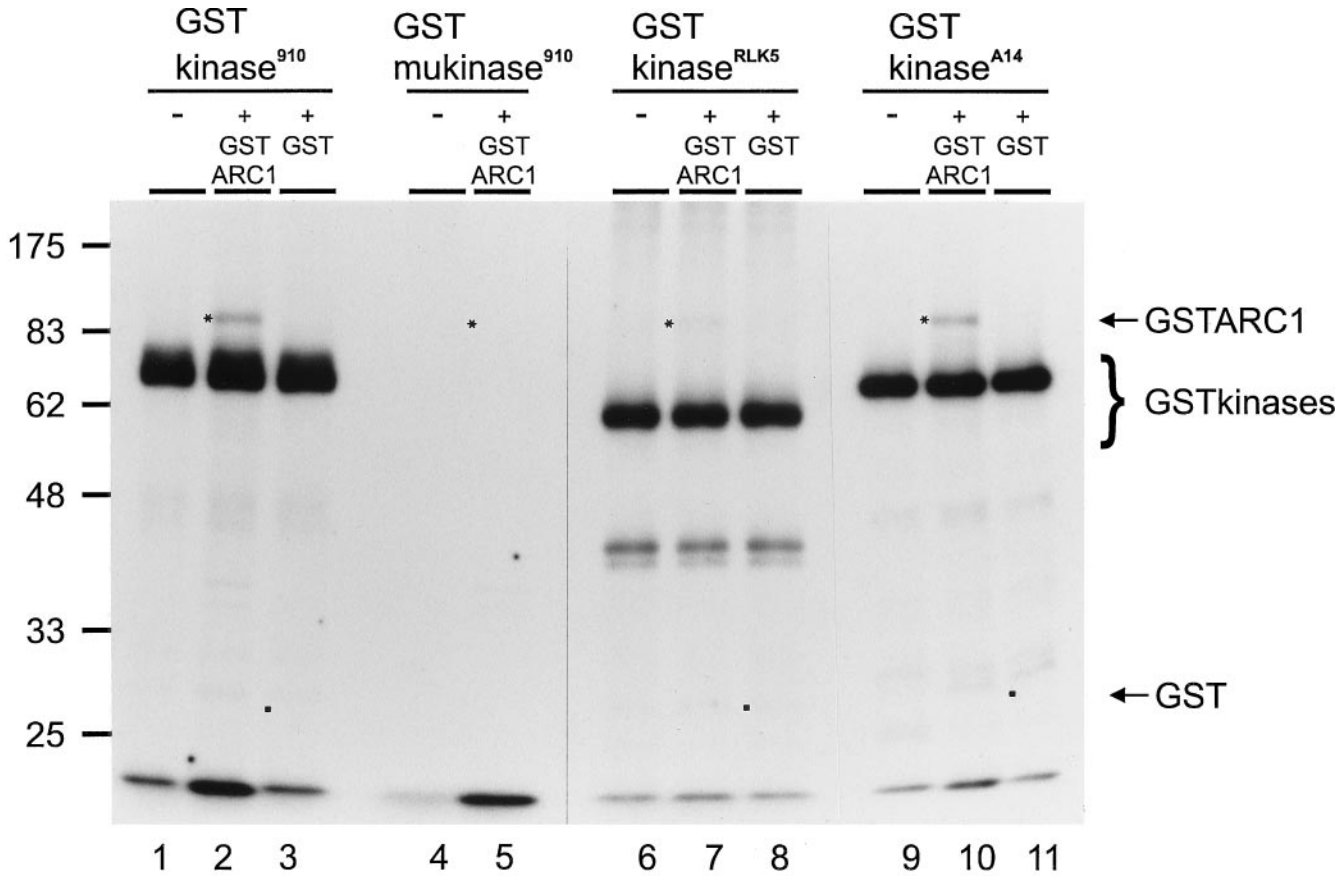

FIG. 6. Phosphorylation of ARC1. The functional SRK-910 kinase domain (GSTkinase^{910}, lanes 1–3), the inactive form (GSTmukinase^{910}, lanes 4 and 5), the *Arabidopsis* RLK5 kinase domain (GSTkinase^{RLK5}, lanes 6–8), or the SRK-A14 kinase domain (GSTkinase^{A14}, lanes 9–11) were mixed with either GST or GSTARC1 to examine phosphorylation. Lanes 1, 4, 6, and 9 contain the kinases alone. GSTARC1 is present in lanes 2, 5, 7, and 10. GST alone is in lanes 3, 8, and 9. No phosphorylation of GST is seen, but a clear phosphorylation of GSTARC1 is detected in the presence of GSTkinase^{910} and GSTkinase^{A14}. The asterisks mark the position of the GSTARC1 protein, and the dots mark the position of the GST protein detected by Coomassie Blue staining. The predicted sizes of the proteins are GSTARC1 (full length) fusion protein, 99 kDa; GSTkinase^{910}, 71 kDa; GSTmukinase^{910}, 71 kDa; GSTkinase^{RLK5}, 65 kDa; and GSTkinase^{A14}, 70 kDa.

expected to be conserved for different SRKs. Thus, binding of ARC1 to both the SRK-910 and SRK-A14 kinase domains is consistent with these expectations and what is known for receptor tyrosine kinase signaling systems (30). The lack of interaction between ARC1 and the *Arabidopsis* RLK5 is not surprising, as RLK5 belongs to a different family of receptor-like kinases, the leucine-rich repeat family (27). On the other hand, the lack of interaction with the *Arabidopsis* RLK4, which belongs to the *S*-locus superfamily, indicates that this interaction is only specific to a subset of these receptors, the SRKs. RLK4 is primarily expressed in root tissue and is thus thought to play quite a different role from the SRKs (27).

In previous work, we have shown that two members of the thioredoxin-h family also interact specifically with the SRK-910 kinase domain; however, the interaction did not seem to be phosphorylation dependent (22). Although we have isolated two classes of proteins, ARC1 and the thioredoxin-h proteins, which interact with the SRK kinase domain, both may play a role in the self-incompatibility response. With receptor tyrosine kinases, one receptor kinase can interact with more than one substrate leading to the activation of multiple pathways (31). Another protein that has been found to bind to the SRK-A14 kinase domain is the kinase-associated protein phosphatase (KAPP) (32). KAPP was first isolated through an interaction screen with RLK5 (33). Subsequent analysis revealed that KAPP binds to a number of different plant receptor kinases, suggesting that it may play a more general regulatory role (32).

ARC1 represents a particularly interesting interacting protein for several reasons. First, ARC1 mRNA is detected only in the stigma, where the self-incompatibility pathway would be occurring. Second, a homology search of the amino acid sequence revealed the presence of arm repeats in the C-terminal region of ARC1. Arm repeats are short 42 amino acid hydrophobic sequences originally found in the *Drosophila* armadillo protein. They have been found in other proteins of diverse function such as the β-catenin/armadillo family, pendulin, yeast SRP1, human APC tumor suppressor, and the guanine nucleotide exchanger, smgGDS (34). For some of these proteins, the arm repeats have been found to participate in protein–protein interaction (35, 36). As only the C-terminal region of ARC1 (which contains the arm repeats) was originally isolated from the two-hybrid system, it is likely that the arm repeats are mediating the phosphorylation-dependent binding to the SRK kinase domain.

The two proteins to which ARC1 showed the highest levels of sequence identity were the mouse pendulin and armadillo/β-catenin proteins. Pendulin is required for normal cell proliferation and is translocated from the cytoplasm to the nucleus in a cell cycle-dependent manner. Most of pendulin consists of ten arm repeats, and presumably the protein functions as an adaptor molecule that may be involved in nuclear protein import (37). β-Catenins have two different roles. First, these proteins participate in cadherin-mediated cell adhesion where β-catenins link the cadherins to the cytoskeleton and are thought to have a role in regulating adhesion (38). Second, they are involved in the Wnt signaling pathway during *Drosophila*

and *Xenopus* development. For the *Drosophila* pathway, it has been established that serine/threonine phosphorylation plays a role in regulating armadillo accumulation in the cytosol (38). Recently, β-catenin has been found to associate with the transcription factor LEF-1, and this interaction is thought to be part of its role in regulating *Xenopus* development (39, 40). Due to limited sequence identity, it is unclear whether ARC1 represents a plant homologue of either pendulin or β-catenin or whether it represents a protein with a novel function. One discrepancy is the lower number of arm repeats found in ARC1 compared with pendulin and β-catenin. However, it is possible that more degenerate repeats are present that have not been identified.

Other than the presence of arm repeats in the C-terminal region, there are no other sequence similarities to indicate a possible function for ARC1. The N-terminal half of ARC1 may represent another binding domain to interact with the next step of the signaling pathway. One possible candidate for this step is the aquaporin-like MOD protein. The characterization of the *mod* mutation, which results in the loss of self-incompatibility, has led to the isolation of an aquaporin-like gene, suggesting that self-incompatibility involves the activation of a water channel in the stigma (41). Like the β-catenins, a role for ARC1 regulating cell adhesion during pollination is also plausible, although the nature of the adhesion in plants would be quite different from that in animals. Although plants do not have an extracellular matrix as seen in animals, there is some suggestion that the plant cell wall fulfills a similar function (42). In this role, the primary function of ARC1 would be in the basic pollination process. That is, it may be involved in *Brassica* pollen recognition leading to pollen adhesion, hydration, and pollen tube penetration. Following this, the self-incompatibility pathway may act as a negative regulator of ARC1 to block incompatible *Brassica* pollinations. Further work will be required to determine the function of ARC1 and its role in the self-incompatibility pathway.

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