Plant Molecular Biology **45:** 365–376, 2001.

Further analysis of the interactions between the *Brassica S* receptor kinase and three interacting proteins (ARC1, THL1 and THL2) in the yeast two-hybrid system

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Received 24 July 2000; accepted in revised form 30 November 2000

Key words: ARC1, *Brassica* self-incompatibility, RLK5, *S* receptor kinase, thioredoxin h

Abstract

The yeast two-hybrid system was used to further characterize the interactions between the *Brassica S* receptor kinase (SRK) and three putative substrates, ARC1 and the two thioredoxin h proteins, THL1 and THL2. Interactions were generally detectable with kinase domains of both Class I and Class II SRKs. Chimeric constructs were made between the SRK_{910} kinase domain and the non-interacting *Arabidopsis* RLK5 kinase domain. Only one chimeric construct, SRR2, interacted with THL1 and THL2, while none of the chimeras were able to interact with ARC1. SRR2 is largely made up of RLK5 kinase domain with the N-terminal end being derived from the SRK_{910} kinase domain and was the only chimeric construct that retained kinase activity. Deletion or substitution of a conserved cysteine at the N-terminal end of the SRK_{910} kinase domain resulted in loss of interaction with THL1 and THL2, while the addition of this cysteine to a related receptor kinase, SFR1, conferred the ability to interact with the thioredoxin h proteins. In addition, substitution of the cysteines in the THL1 active site abolished the interaction. Lastly, the two *Arabidopsis* thioredoxin h clones most closely related to THL1 and THL2 were found to interact with the SRK kinase domains. Thus, the nature of the interaction of the thioredoxin h clones with SRK involves the reducing activity of these proteins and is restricted to the class of thioredoxin h proteins which have the variant CPPC active site.

Introduction

In self-incompatible *Brassica*, pollination is regulated by the self-incompatibility system which acts to prevent self-pollination, as well as pollination between two plants carrying the same *S* allele (for review, see De Nettancourt, 1997; Suzuki *et al.*, 1997; Cock, 2000). While classically the self-incompatibility system has been defined to be controlled by a single genetic *S* locus, molecular studies have led to the characterization of a number of genes tightly linked to the *S* locus (Cui *et al.*, 1999; Suzuki *et al.*, 1999; Casselman *et al.*, 2000). Of these, the *S* receptor kinase (SRK) gene was found to encode the female determinant of self-incompatibility (Nasrallah *et al.*, 1991; Takasaki *et al.*, 2000) while the *S* locus cysteine-rich protein (SCR) was found to encode the male determinant (Schopfer *et al.*, 1999; Takayama *et al.*, 2000). The *S* locus glycoprotein (SLG), long thought to be involved in *Brassica* self-incompatibility, is not essential and while Takasaki *et al.* (2000) found that SLG enhanced the self-incompatibility response in the pistil

(Nunes *et al.*, 2000) did not see any effect with the addition of SLG.

SRK, located in the pistil, is thought to recognize the pollen-borne SCR protein and activate a signalling pathway in the pistil leading to self-pollen rejection. The pathway appears to act at several stages such as blocking pollen hydration and germination and preventing the germinated pollen from penetrating the stigma cell wall (Stead *et al.*, 1997). As a first step towards understanding the signalling pathway activated by SRK and leading to pollen rejection, we had previously carried out a yeast two-hybrid screen of a *Brassica* pistil cDNA library with the SRK_{910} kinase domain. Three proteins, THL1, THL2 and ARC1, were found to interact with the kinase domain of SRK_{910} and thus represented potential downstream targets of the kinase (Bower *et al.*, 1996; Gu *et al.*, 1998). ARC1 shows a specific and phosphorylation-dependent interaction with SRK, and binds to the SRK kinase domain through an arm repeat region present in the C-terminal half of the protein (Gu *et al.*, 1998). We have recently shown through the analysis of ARC1 antisense transgenic plants that ARC1 is required for the *Brassica* self-incompatibility response (Stone *et al.*, 1999). The remaining two clones, THL1 and THL2, encode thioredoxin h proteins which specifically interact with SRK_{910} and not with two *Arabidopsis* receptor kinases, RLK4 and RLK5 (Bower *et al.*, 1996). In this paper, we have used the yeast two-hybrid system to further characterize the nature of the SRK interactions with its three putative substrates and have identified a cysteine that is required for the THL1 and THL2 interactions.

Materials and methods

Plasmid constructs

The original SRK_{910} construct was made using a naturally occurring *Bcl*I site at amino acid 462, which includes six amino acids at the end of the predicted transmembrane domain and the kinase domain (Bower *et al.*, 1996). For $SRK_{910ser465}$, the cysteine at amino acid 465 was changed to a serine using the QuikChange™ site-directed mutagenesis kit (Stratagene) on the original SRK_{910} kinase construct. For the remaining kinase constructs, PCR was used to introduce a *Bam*HI site at the end of the transmembrane domain. *Bam*HI sites were introduced into $SRK_{910\text{-}cys}$ at amino acid 466, SRK_{A14} at amino acid 459, $SRK_{A14\text{-}cys}$ at amino acid 462, SRK_9 at amino acid 432, SRK_{15} at amino acid 468, SRK_{29} at amino acid 470, SFR1 at amino acid 464, and SFR2 at amino acid 455. $SFR1_{cys466}$ is the same as SFR1 with the exception that Arg^{466} is substituted with a cysteine. In the mutant form of SFR2, the invariant lysine in the kinase domain (Lys^{545}) was substituted with an arginine.

For the chimeric SRK_{910}/RLK5 kinases, the starting constructs were the RLK5 kinase domain starting at the end of the transmembrane domain, and the SRK_{910} kinase domain starting seven amino acids from the end of the transmembrane domain at the *Bcl*I site. The N-terminal and C-terminal regions were swapped using restriction enzyme sites present in the cDNAs or created by PCR mutagenesis. For SRK_{910}, the sites used were the *Mfe*I site at nucleotide 1590, the *Nsi*I site at nucleotide 2343, and an *Acc*I site created at nucleotide 2357 (GTCTCT → GTCTAC). For RLK5, the sites used were an *Eco*RI site created at nucleotide 2052 (TGAAAA → GAATTC), an *Nsi*I site created at nucleotide 2813 (AGTCAT → ATGCAT), and the *Acc*I site at nucleotide 2827. All the kinase constructs were cloned in frame into the pBTM116 yeast vector and the pGEX vector.

For the *Arabidopsis* TRX clones, *Bam*HI sites were introduced 3 bp upstream of the start codons using PCR and cloned in frame into the pVP16 yeast vector. The cysteines in the active site of THL1 were mutated to serines using the QuikChange™ site-directed mutagenesis kit.

Yeast transformations and β-galactosidase assays

Yeast transformations were carried out according to Gietz and Woods (1994) and β-galactosidase assays were conducted as previously described (Bower *et al.*, 1996; Bu *et al.*, 1998). Essentially, for each β-galactosidase assay, 0.5 OD of cells were spun down from 5 ml overnight cultures. After the addition of ONPG, the samples were timed for the length of time required to develop a pale yellow colour and the reaction was stopped with the addition of Na_2CO_3. Those samples which did not show the development of a pale yellow colour within a few hours were left to incubate overnight and then stopped regardless of colour development. All constructs were tested several times to confirm the positive interactions. For the data shown in Figures 1, 3B, 4B, and 5B, β-galactosidase levels were measured from at least four different colonies for

each construct. β-galactosidase activity was measured as the OD_{420} per hour per 10^6 cells.

Yeast protein extractions and western blot analysis

Protein extracts were prepared according to Adams *et al.* (1997) using 2 OD_{600} units of cells from a 5 ml overnight culture of synthetic complete media minus trp and leu inoculated with a single colony.

For the western blot analysis for the VP16 fusion proteins, 5 μl of each protein extract was separated on a 12% SDS-PAGE gel and transferred to nitrocellulose. The membrane was incubated with a 1:200 dilution of the anti-VP16 antibody (Santa Cruz Biotechnology Inc.) according to the manufacturer's instructions. For western blot analysis to detect the lexA fusion proteins, 15 μl of each protein extract was separated on a 9% SDS-PAGE gel and transferred to nitrocellulose. The membrane was incubated with a 1:100 dilution of the anti-lexA antibody (Santa Cruz Biotechnology Inc.) according to the manufacturer's instructions. The secondary antibody was a 1:5000 dilution of goat anti-mouse antibody conjugated with horseradish peroxidase (Kirkegaard & Perry Laboratories). The secondary antibody was detected using the LumiGLO chemiluminescent substrate (Kirkegaard & Perry Laboratories).

Kinase assays

GST fusion proteins were purified as previously described (Gu *et al.*, 1998) except that glycerol was added to a final concentration of 10% (v/v) to the G-lysis buffer for the washes, and to the elution buffer. The purified fusion proteins were stored in the elution buffer at -20 °C. For the kinase assay, approximately 0.5 μg of each protein was mixed in a 10 μl reaction with 20 mM Pipes pH 7, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μg/ml aprotinin, 25 μM cold ATP, and 10 μCi [^{32}P]γ-ATP and incubated for 20 min at room temperature. The proteins were separated on a 8% SDS-PAGE gel, stained with Coomassie Blue, dried, and subjected to autoradiography to detect the phosphoproteins. Protein markers were from New England Biolabs.

Results

Using the yeast two-hybrid system, we had previously isolated three putative substrates, THL1, THL2 and ARC1, which interact specifically with the SRK kinase domain. The interactions were confirmed through *in vitro* binding studies indicating that the yeast two-hybrid system was a valid method to assess interactions between kinase domains and THL1, THL2 and ARC1 (Bower *et al.*, 1996; Gu *et al.*, 1988). Therefore, a more detailed analysis was carried out using this system. All kinase domains were cloned into the lexA DNA binding domain vector, while THL1, THL2 and ARC1 were the original VP16 activation domain plasmids isolated from the yeast two-hybrid library. In the case of ARC1, the library clone encodes the C-terminal half of ARC1 containing the arm repeats. Interactions were tested by co-transforming the plasmids into the yeast L40 strain and testing for activation of the *lacZ* reporter gene by measuring β-galactosidase activity. Protein extracts prepared from the transformed yeast were incubated with the β-galactosidase substrate, ONPG, and the timed reactions were stopped upon the detection of a pale yellow colour. The remaining reactions were stopped after an overnight incubation. Positive interactions resulted in the detection of the yellow colour anywhere from thirty seconds to a few hours, depending on the kinase domain being tested. The VP16 vector co-transformed with the kinase domains served as a control for background levels.

Interactions between different members of the S receptor kinase superfamily and THL1, THL2 and ARC1

The *Brassica S* locus is highly polymorphic and consequently, a large number of alleles of SRK have been identified. These alleles can be divided into two classes, Class I and Class II, based on sequence identity and the strength of the self-incompatibility response (Nasrallah *et al.*, 1991). The yeast two-hybrid library was originally screened with the SRK_{910} kinase domain which is a Class I SRK. Here we have tested interactions with two other Class I SRKs, SRK_{A14} and SRK_9, and two Class II SRKs, SRK_{15} and SRK_{29} (Figure 1A). There are also a number of receptor kinases that are not involved in self-incompatibility, but share sequence identity in the extracellular *S* domain of SRK, and together with SRK, these genes constitute the S receptor kinase superfamily (Hardie, 1999). Two *Brassica* receptor kinases belonging to this group, SFR1 and SFR2, were also tested in the yeast two-hybrid system (Figure 1A). All of the active kinases tested were found to interact with

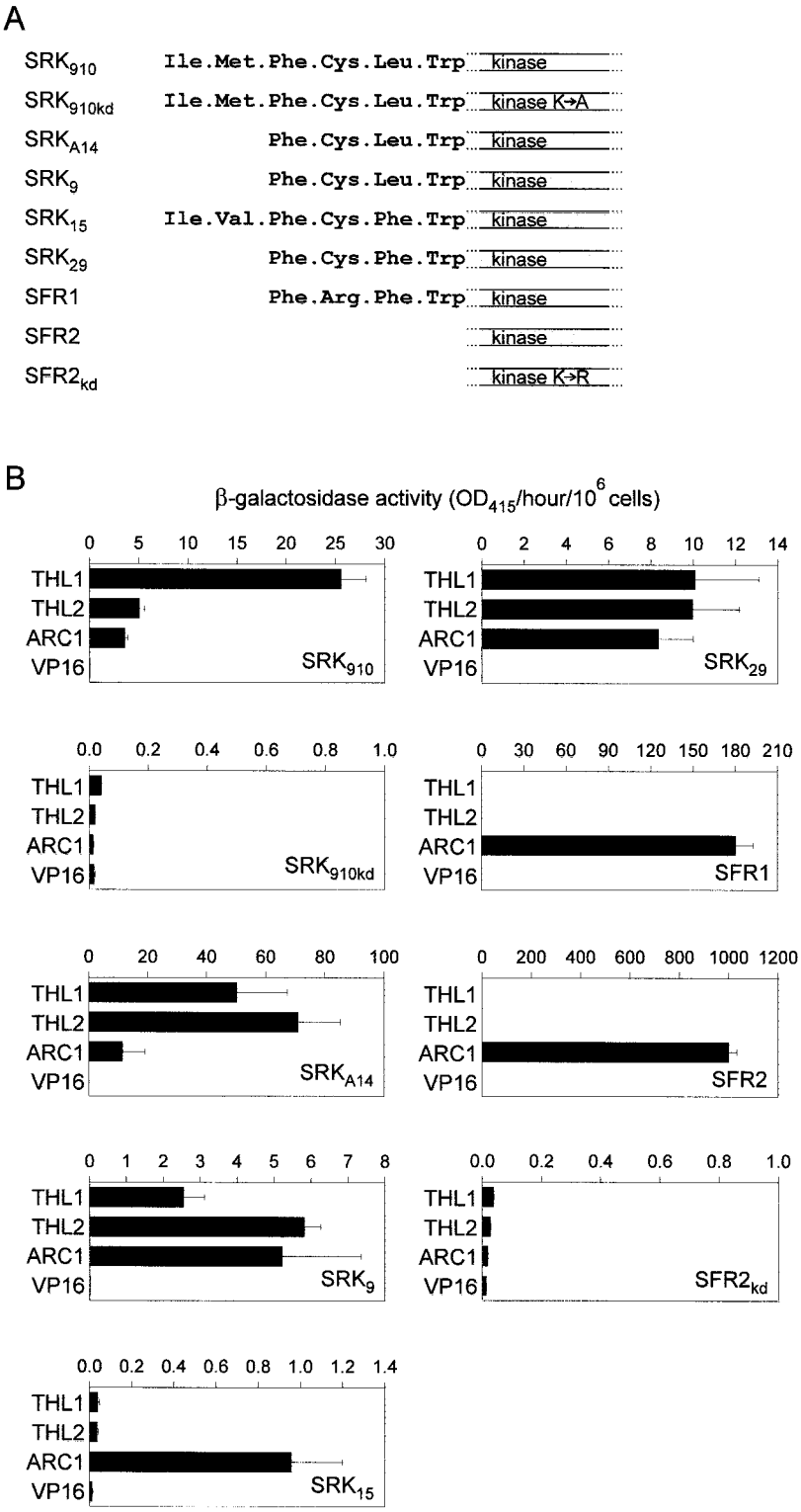

Figure 1. Interactions in the yeast two-hybrid system. A. Schematic diagram of the SRK and SRK kinase constructs showing the transmembrane domain amino acids at the N-terminal end. The kinase constructs contain the last few amino acids of the predicted transmembrane domains as shown. kd = kinase dead. B. Plasmids encoding lexA (DNA binding domain) and VP16 (activation domain) fusions were transformed into the L40 yeast strain which carries a *lacZ* reporter gene with lexA DNA binding sites in the promoter. Interactions between the lexA and VP16 fusions resulting in the expression of the *lacZ* reporter gene were determined by measuring the β-galactosidase activity. Transformation of the kinase domains with the VP16 vector serves as a control for background. The lexA-kinase fusion with SRK_{910}, SRK_{910kd}, SRK_{A14}, SRK_9, SRK_{15}, SRK_{29}, SFR1, SFR2 or $SFR2_{kd}$ was co-transformed with VP16-THL1, VP16-THL2 VP16-ARC1 or VP16 alone.

ARC1; however, only a subset interacted with THL1 and THL2 (Figure 1B). All of the SRKs interacted with THL1 and THL2 with the exception of SRK_{15} which also showed only a very weak interaction with ARC1 (Figure 1B). Neither of the SFR kinases interacted with THL1 and THL2, although they showed the highest levels of β-galactosidase activity with ARC1 (Figure 1B). SRK_{910kd} and $SFR2_{kd}$ represent kinase-inactive forms where a conserved lysine in the kinase domain was replaced with alanine (SRK_{910}) or arginine (SFR2). No interactions were detected for either of these kinase constructs (Figure 1B).

The kinase domains were tested for activity by producing GST-kinase fusion proteins in *E. coli* and testing for autophosphorylation in the presence of $[^{32}P]\gamma$-ATP. We confirmed that the 'kinase-active' SRK and SFR kinase domain constructs autophosphorylated *in vitro* (Figure 2A). SRK_{910kd} showed no detectable kinase activity (not shown, Goring and Rothstein, 1992) while $SFR2_{kd}$ showed extremely low levels of activity (Figure 2A) only detectable after longer exposures (data not shown). Immunoblot analysis with an anti-lexA antibody showed that all the lexA-kinase fusion proteins were expressed in the yeast cells (Figure 2B). We also confirmed that THL1, THL2 and ARC1 were expressed in the yeast cells using an anti-VP16 antibody (Figure 2C).

When comparing the level of β-galactosidase activity resulting from the interactions, there was quite a large range with SRK_{15} showing the lowest levels in its interaction with ARC1 and SFR2 showing the highest levels in its interaction with ARC1. This is likely to be due to several different factors. For example, SFR2 is present at higher levels in the yeast cells (Figure 2B), and it also has the highest autophosphorylation activity *in vitro*. Generally, the kinases that showed stronger autophosphorylation activity *in vitro* (SRK_{A14}, SFR1, SFR2) tended to show higher levels of β-galactosidase activity with interactions.

Interactions between chimeric SRK_{910}/RLK5 kinases and THL1, THL2 and ARC1

Previously, we have shown that the kinase domain from the *Arabidopsis* RLK5 receptor-like kinase does not interact with THL1, THL2 nor ARC1 (Bower *et al.*, 1996; Gu *et al.*, 1998). To identify regions in the SRK kinase domain that are important for interaction with these proteins, chimeras were constructed between the SRK_{910} and RLK5 kinase domains (Figure 3A). Sequences encoding domains that were N-terminal and C-terminal to the catalytic domain were exchanged either singly or together as shown in Figure 3A.

Of the six different SRK_{910}/RLK5 chimeric kinase constructs, only SRR2 interacted with both THL1 and THL2. In both cases, the interaction was stronger than with the original SRK_{910} kinase domain as determined by the level of the β-galactosidase activity compared with the VP16 background (Figure 3B). SRR2 contains the N-terminal end of the SRK_{910} and the catalytic and C-terminal domains are derived from RLK5 (Figure 3A). Two other chimeric constructs containing this region from SRK_{910}, SSR3 and SRS5 did not show any interaction with THL1 and THL2 (Figure 3B).

To determine if the chimeras had retained their kinase activity, GST-kinase fusion proteins were tested for autophosphorylation in the presence of $[^{32}P]\gamma$-ATP. Of the six SRK_{910}/RLK5 chimeras, only SRR2 has retained its catalytic activity (Figure 2A). The remaining chimeric kinases did not have any detectable activity (data not shown). The fact that SRR2 is active while SSR3 and SRS5 are not may explain why THL1 and THL2 only interacted with SRR2. Western blot analysis with the anti-lexA antibody showed that all the kinases were present in the yeast extracts at the expected sizes (Figure 2B).

When the SRK_{910}/RLK5 chimeric kinases were tested with ARC1, none had detectable levels of β-galactosidase activity and, thus, were all negative for ARC1 interactions (Figure 3B). The inability of the five kinase-inactive chimeras to interact with ARC1 is consistent with its requirement for phosphorylation (Gu *et al.*, 1998). While SRR2 is an active kinase, a lack of interaction with ARC1 suggests that the N-terminal region of SRK_{910} is not sufficient or is not being phosphorylated at the required site.

Identification of a cysteine required for the interaction with THL1 and THL2

In the original SRK_{910} kinase domain construct, the N-terminus includes six amino acids from the end of the predicted transmembrane domain due to the use of a convenient *Bcl*I site (Figure 1A). In this region, there is a cysteine at position 465 (Figure 1A) which is the only cysteine present in the SRK_{910} region of SRR2. This cysteine is conserved in all the SRKs tested, but not present in the SFR1 or SFR2 constructs (Figure 1A). When N-terminal amino acids from either the SRK_{910} or the SRK_{A14} constructs,

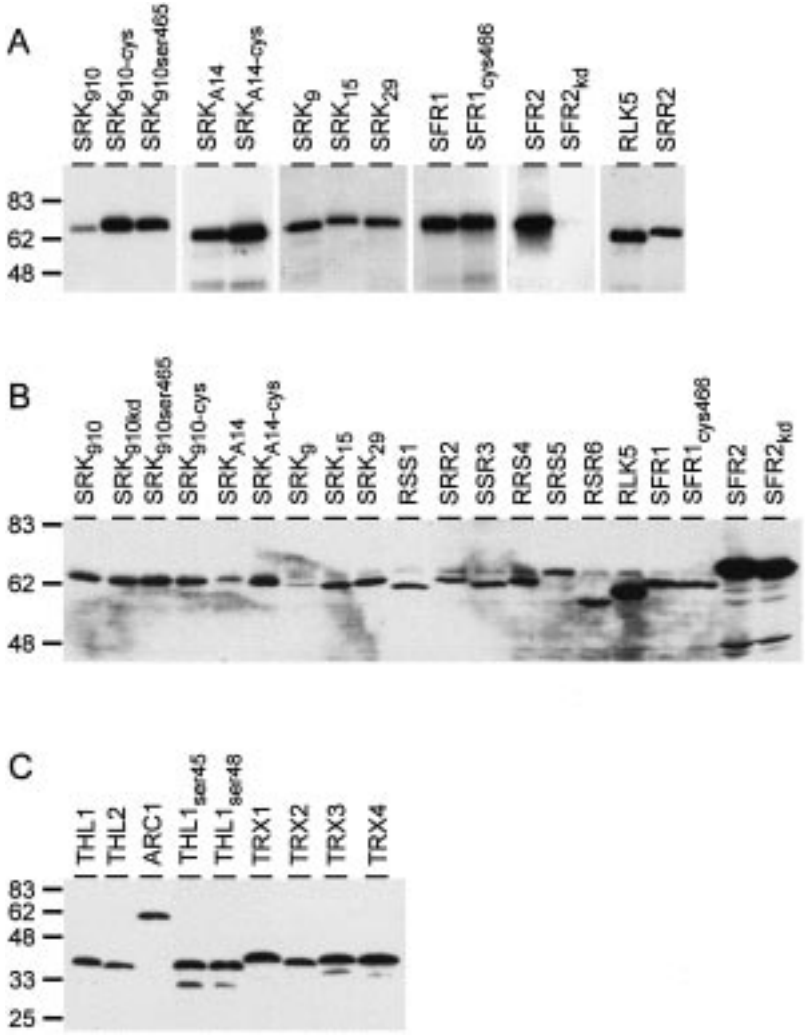

Figure 2. A. Autoradiograph of GST-kinase assays. Approximately 0.5 μg of each purified GST fusion protein was incubated with [^{32}P]γ-ATP, and separated on an 8% SDS-PAGE gel. The gel was then stained, dried and exposed to film to detect the phosphoproteins as shown. Different exposure times were used to detect the phosphoproteins with SRK_{14}, SFR1 and SFR2 requiring shorter exposures compared to the remaining kinases. The positions of the molecular weight markers are shown on the left. B. Immunoblot analysis of yeast extracts containing the lexA-kinase fusion proteins. Protein extracts were separated on a 9% SDS-PAGE gel, transferred to nitrocellulose and incubated with an anti-lexA antibody. The positions of the molecular weight markers are shown on the left. C. Immunoblot analysis of yeast extracts containing the VP16 fusion proteins. Protein extracts were separated on a 12% SDS-PAGE gel, transferred to nitrocellulose and incubated with an anti-VP16 antibody. The positions of the molecular weight markers are shown on the left.

including the cysteine, were deleted ($SRK_{910\text{-}cys}$, $SRK_{A14\text{-}cys}$, Figure 4A), neither THL1 nor THL2 interacted with the kinase domains (Figure 4B). Similarly, when the cysteine was replaced by serine in SRK_{910} ($SRK_{910ser465}$, Figure 4A), interaction with THL1 and THL2 was abolished (Figure 4B). When a cysteine was introduced into the same position in SFR1 ($SFR1_{cys466}$, Figure 4A), SFR1 gained the ability to interact with THL1 and THL2 (Figure 4B). All four kinase constructs with modified N-termini ($SRK_{910\text{-}cys}$, $SRK_{A14\text{-}cys}$, $SRK_{910ser465}$, $SFR1_{cys466}$) retained their kinase activity when tested as GST-kinase fusions (Figure 2A), and were present at detectable levels in the yeast cells (Figure 2B). Thus, the loss of interaction with THL1 and THL2 is not due to the loss of phosphorylation, but rather due to the loss of Cys^{465}. In keeping with this, all four constructs were still able to interact with ARC1 (Figure 4B). The $SRK_{A14\text{-}cys}$ construct gave a higher level of background than the other three constructs tested

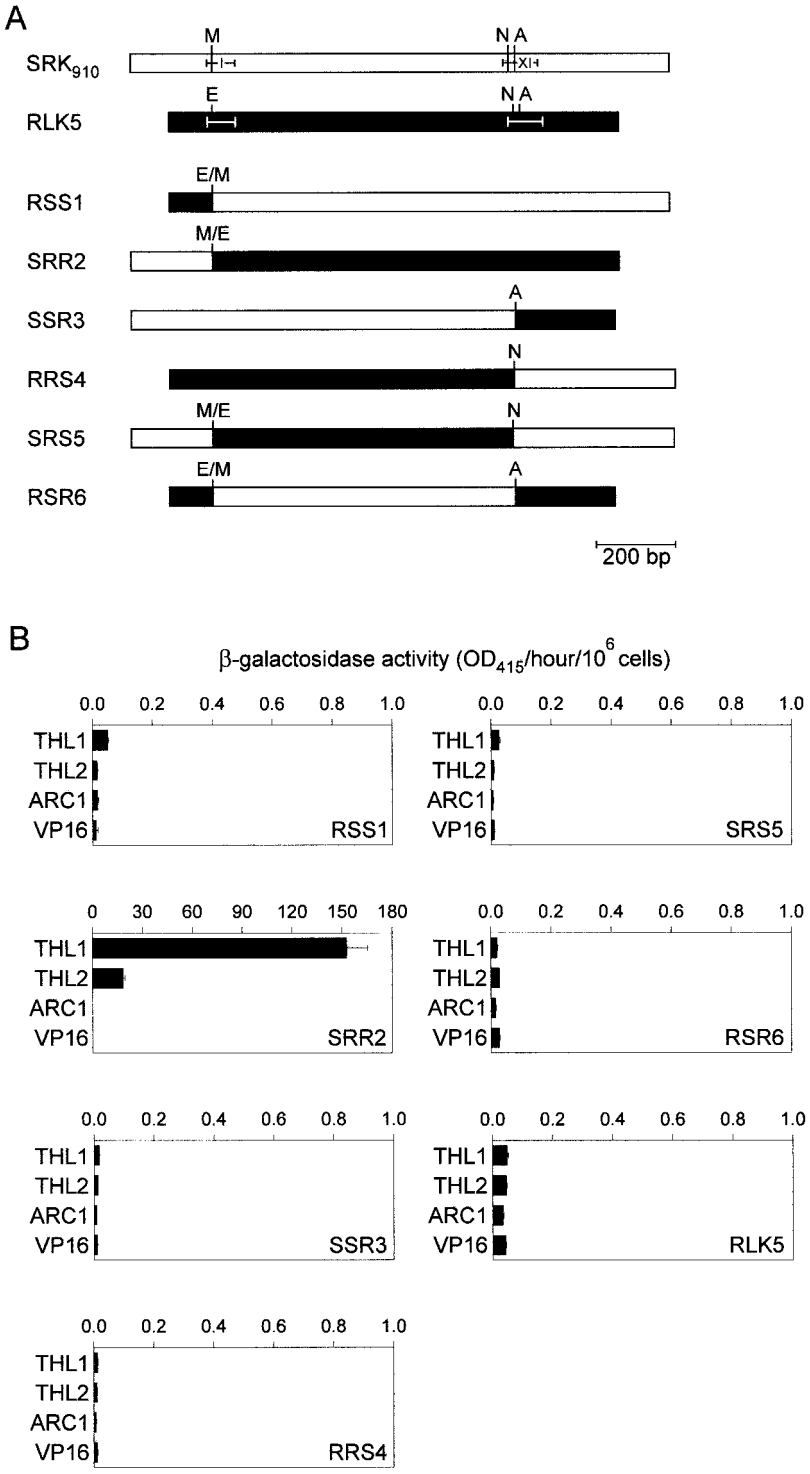

Figure 3. A. Schematic diagram of the chimeric SRK_{910} and RLK5 kinase constructs used in the yeast two-hybrid system. The coding regions of the SRK_{910} and RLK5 kinase constructs starting near or at the end of the predicted transmembrane domains are shown. For the chimeric kinases, the N- and/or C-terminal ends were exchanged between SRK_{910} and RLK5 using the restriction sites shown in domain I or domain XI (I and XI are the first and last conserved kinase domains as defined by Hanks *et al.*, 1988). For SRK_{910}, the *Mfe*I (M) and *Nsi*I (N) sites were already present, and the *Acc*I (A) site was created using PCR. For RLK5, the *Eco*RI (E) and *Nsi*I (N) sites were created using PCR, and the *Acc*I (A) site was already present. See Materials and methods for more details. B. Yeast two-hybrid interactions with the chimeric SRK_{910} and RLK5 kinase domains. The lexA:kinase fusions with SRK_{910}, RLK5, or SRK_{910}/RLK5 chimeric kinases were co-transformed with VP16-THL1, VP16-THL2, VP16-ARC1, or VP16 alone.

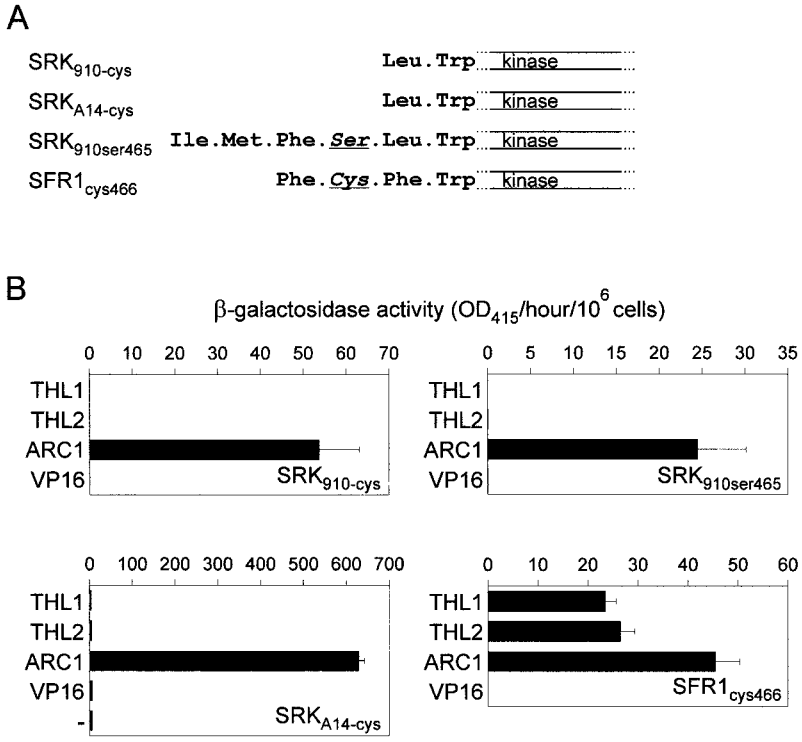

Figure 4. A. Schematic diagram of the SRK and SFR1 kinase constructs with alterations in the transmembrane domain amino acids at the N-termini. The amino acids which have been substituted in SRK_{910} and SFR1 are underlined. B. Yeast two-hybrid interactions with the altered SRK and SFR1 kinase constructs. The lexA:kinase fusions with $SRK_{910\text{-}cys}$, $SRK_{A14\text{-}cys}$, $SRK_{910ser465}$, or $SRK_{A14\text{-}cys}$ were co-transformed with VP16-THL1, VP16-THL2, VP16-ARC 1, or VP16 alone. $SRK_{A14-cys}$ was also transformed alone (represented by −).

(Figure 4B). Transformation of yeast with $SRK_{A14\text{-}cys}$ alone showed that the background was due to this construct (Figure 4B).

Mutagenesis of the thioredoxin active site and interactions with the Arabidopsis *thioredoxin h family*

In thioredoxins, the typical active site consists of CGPC, where the two cysteines have essential roles in the redox activity. In plants, there is a subset of thoiredoxin h members who have a variant active site consisting of CPPC, and this active site is found in THL1 and THL2. To test the requirement of the two cysteines for the interaction with the SRKs, the cysteines were individually replaced by serines in THL1 (Figure 5A). Both $THL1_{ser45}$ and $THL1_{ser48}$ were unable to interact with either SRK_{910} or SRK_{29}, thus, demonstrating that the redox activity is needed for this interaction (Figure 5B).

In *Arabidopsis*, there are several members of the thioredoxin h family (Rivera-Madrid *et al.*, 1995). To determine if the *Arabidopsis* thioredoxin h clones could similarly interact with the SRK kinase domains, four of the *Arabidopsis* thioredoxin h clones, TRX1, TRX2, TRX3 and TRX4, were tested. While immunoblot analysis with an anti-VP16 antibody detected all four TRX proteins (Figure 2B), TRX1 and TRX2 did not show any interactions with the kinase constructs (Figure 5B). However, TRX3 and TRX4 did interact with the two SRKs tested, SRK_{910} and SRK_{29}. When TRX3 and TRX4 were tested with $SRK_{910ser465}$, the interaction was abolished (Figure 5B). Therefore, TRX3 and TRX4 displayed exactly the same pattern of interactions as seen with THL1 and THL2. Interestingly, both TRX3 and TRX4 have the variant CPPC active site, while TRX1 and TRX2 have the typical CGPC active site. In addition, THL1 is most similar to TRX3 (75% sequence identity at the amino acid level), and THL2 is most similar to TRX4 (85% sequence identity at the amino acid level).

Discussion

While the *Brassica* self-incompatibility response is known to require the *S* receptor kinase, not much

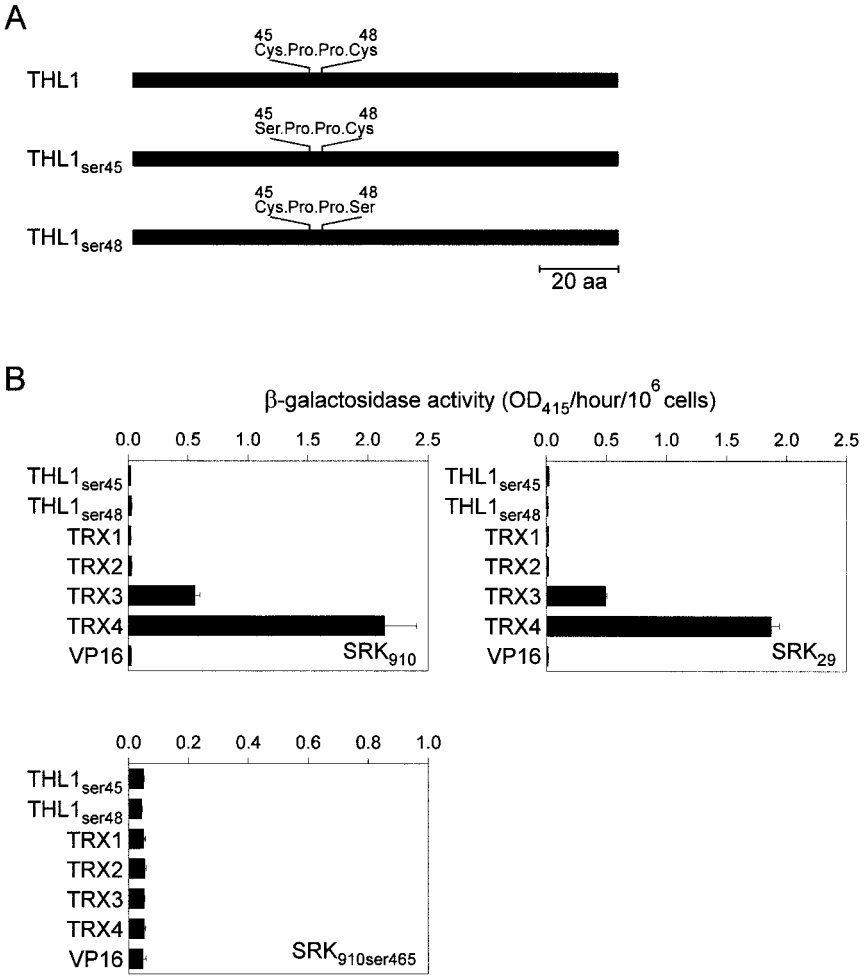

Figure 5. A. Schematic diagram of the mutagenized THL1 constructs used in the yeast two-hybrid system. The cysteines in the active site of THL1 were substituted with serines. B. Yeast two-hybrid interactions with the mutagenized THL1 and with the *Arabidopsis* thioredoxin h members. The lexA:kinase constructs with SRK_{910}, $SRK_{910ser465}$ or SRK_{29} were co-transformed with VP16-$THL1_{ser45}$, VP16-$THL1_{ser48}$, the *Arabidopsis* thioredoxin clones represented by VP16-TRX1, VP16-TRX2, VP16-TRX3 and VP16-TRX4, or VP16 alone. The VP16 data are the same as shown in Figures 1 and 4B.

is known about the signalling pathways that lead to pollen rejection. Using the yeast two-hybrid system, we have previously isolated three putative substrates, THL1, THL2 and ARC1, which interact specifically with the SRK kinase domain (Bower *et al.*, 1996; Gu *et al.*, 1998). ARC1 was found to bind to the SRK kinase domain in a phosphorylation-dependent manner. In addition, ARC1 has a very tissue-specific pattern of expression. ARC1 mRNA is only detected in the top portion of the pistil, the stigma, where pollen grains germinate (Gu *et al.*, 1998). Finally, we have shown that ARC1 is essential for the self-incompatibility response and have proposed that ARC1 represents a downstream effector of signal transduction via SRK (Stone *et al.*, 1999). Therefore, it was somewhat surprising to detect interactions between ARC1 and the SFR kinases. One interpretation of this data is that ARC1 belongs to a family of downstream effector molecules for the *S* receptor kinase superfamily. ARC1 may not necessarily be involved in signalling through the SFR receptor kinases *in vivo*, but related proteins may do so by binding to the SFR kinase domain through similar arm repeat domains. This hypothesis was supported by searches of the *Arabidopsis* genome sequence which revealed several predicted genes showing sequence identity to ARC1 (data not shown). *Arabidopsis thaliana* does not possess a self-

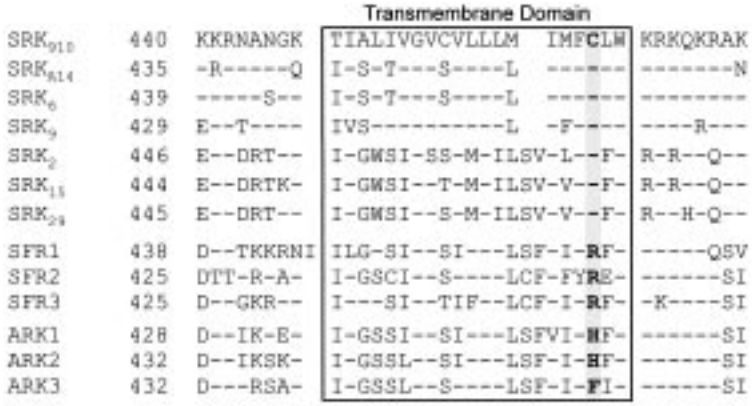

Figure 6. Alignment of the predicted transmembrane regions from SRKs and other closely related receptor kinases belonging to the *S* receptor kinase superfamily. SRK_{910} (Goring and Rothstein, 1992) and SRK_{A14} (Glavin *et al.*, 1994) are from *Brassica napus*. SRK_2, SRK_6 (Stein *et al.*, 1991), SRK_{15} (Cabrillac *et al.*, 1999), SFR1 (accession number Y14285), SFR2 (Pastuglia *et al.*, 1997), and SFR3 (accession number Y14286) are from *Brassica oleracea*. SRK_9 (Watanabe *et al.*, 1994) and SRK_{29} (Hatakeyama *et al.*, 1998) are from *Brassica rapa*. ARK1, ARK2 and ARK3 (Dwyer *et al.*, 1994) are from *Arabidopsis*. Dashes represent amino acids that are identical to the SRK_{910} sequence, gaps were introduced to maximize the alignment, the predicted transmembrane domain region is boxed, and the conserved cysteine in the SRKs is highlighted.

incompatibility system, but does have receptor kinases belonging to the *S* receptor kinase superfamily.

Through the analysis of the various kinase constructs in the yeast two-hybrid system, we have identified a cysteine (cys^{465}) which is required for the interaction between the SRKs and the thioredoxin h clones, THL1 and THL2. Cys^{465} is the only cysteine that is present in the SRK_{910} region of the SRR2 chimeric kinase. In addition, deletion or substitution of this cysteine in either SRK_{910} or SRK_{A14} resulted in loss of this interaction. In addition, we found that the same pattern of interaction and requirement for Cys^{465} is conserved with the *Arabidopsis* TRX3 and TRX4 thioredoxin h proteins and appears to be limited to the class of thioredoxin h proteins which contain the variant CPPC active site. Since the role of thioredoxins is to reduce disulfide bonds, the requirement for a particular cysteine is in keeping with this role and suggests that Cys^{465} is involved in a disulfide bond. In support of this, mutagenesis of the cysteines in the active site for THL1 abolished the interactions with the SRKs. The data in this paper also suggest that the thioredoxin h proteins only interact with active kinases. All kinase inactive constructs failed to interact with the thioredoxins even if they possessed Cys^{465}.

An examination of the predicted SRK amino acid sequences has shown that Cys^{465} is present in all SRK sequences present in the GenBank database (some examples are shown in Figure 6). The SRKs generally fall into two classes where the Class I sequences including SRK_{910}, SRK_{A14}, SRK_6 and SRK_9 show roughly 75% to 80% DNA sequence identity to each other, while the Class II SRKs represented by SRK_2, SRK_{15} and SRK_{29} only show approximately 65% DNA sequence identity to the Class I SRKs. Class I alleles tend to mount stronger self-incompatibility responses compared to Class II alleles (Nasrallah *et al.*, 1991). It is not known why these differences occur and it may be related to the signalling pathways activated by the two classes of SRKs. The one discrepancy in this study was with the Class II SRK_{15} which contains this conserved cysteine, but failed to interact with THL1 and THL2. However, it was also very difficult to detect the interaction between SRK_{15} and ARC1 suggesting that there were some problems with this particular kinase. It is unclear whether this is related to SRK_{15} being a weaker Class II allele or just the effect of expressing this particular kinase in yeast.

A number of other receptor kinases have also been characterized which are similar in sequence to the SRKs, but are not involved in self-incompatibility such as the *Brassica* SFR receptor kinases and the *Arabidopsis* ARK receptor kinases (Pastuglia *et al.*, 1997; Tobias *et al.*, 1992; Dwyer *et al.*, 1994; Tobias and Nasrallah, 1996). These non-SRK receptor kinases have similar overall levels of sequence identity to SRK_{910} as to the Class II SRKs, but they do not possess Cys^{465} (Figure 6). Neither SFR1 nor SRF2 interacted with THL1 and THL2; however, the addition of this Cys^{465} to SFR1 allowed it to interact with THL1 and THL2 confirming the importance of this cysteine. Searches of the *Arabidopsis* genome

sequence identified a number of predicted proteins belonging to the *S* receptor kinase superfamily; however, the large majority of these predicted proteins did not possess Cys^{465} (data not shown). Since this Cys^{465} is for the most part specific to the SRKs, any potential regulatory roles by thioredoxin h may be limited to this class of receptor kinases.

In plants, thioredoxins have been classically divided into three types, thioredoxin h, f and m. Thioredoxin f and m play a role in the regulation of metabolic enzymes in the chloroplast (Buchanan, 1991). Thioredoxin h proteins, which are encoded by a gene family and are generally cytoplasmic, are most closely related to the single animal thioredoxin protein (Buchanan, 1991; Rivera-Madrid *et al.*, 1995). There are also some unusual thioredoxin forms such as two membrane bound thioredoxins found in soybean (Shi and Bhattacharyya, 1996) and a maize nuclear protein with thioredoxin domains (Laughner *et al.*, 1998). There is growing evidence, particularly in animal systems, that thioredoxin plays an integral role in several signalling pathways (Sen, 2000). For example, redox regulation by thioredoxin occurs for transcription factors such as the glucocorticoid receptor and NF-κB (Makino *et al.*, 1999; Hirota *et al.*, 1999). Thioredoxin has also been found to be an inhibitor of kinases such as PKC and ASK1 (Watson *et al.*, 1999; Liu *et al.*, 2000). In the example of the ASK1 kinase, thioredoxin is proposed to bind and inhibit ASK1 in the absence of stimulus (Liu *et al.*, 2000). Following oxidative stress, thioredoxin becomes dissociated from ASK1. This allows another protein, TRAF2, to interact with ASK1 and mediate ASK1 oligomerization which in turn leads to ASK1 activation (Liu *et al.*, 2000). Thus, one model for the function of THL1 and THL2 in self-incompatibility is that they are negative regulators of SRK in the absence of stimulus. This negative regulation may be required to reduce basal SRK activity resulting from spontaneous SRK dimerization or autophosphorylation. Activation of SRK by the pollen-borne SCR protein would then be predicted to overcome this putative negative regulation and lead to SRK signalling through proteins such as ARC1.

Acknowledgements

We wish to thank Dr K. Hinata for providing the SRK_{29} cDNA, Dr M. Watanabe for providing the SRK_9 cDNA, Dr J. Giraudat and the *Arabidopsis* Biological Resource Center for providing the *Arabidopsis* thioredoxin h clones, and Dr J. Walker for providing the *Arabidopsis* RLK5 cDNA. We are also very grateful to Dr P. Bartel and Dr S. Fields for providing the pBTM116 plasmid, Dr S. Hollenberg for the pVP16 plasmid and L40 yeast strain. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to D.R.G.

References

Adams, A., Gotlschling, D.E., Kaiser, C.A. and Stearns T. 1997. Techniques and protocols #4 – Yeast protein extracts. In: Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, Cold Spring Harbor Laboratory Press, Plainview, NY, pp. 115–116.

Bower, M.S., Matias, D.D., Fernandes-Carvalho, E., Mazzurco, M., Gu, T., Rothstein, S. and Goring, D.R. 1996. Two members of the thioredoxin-h family interact with the kinase domain of a *Brassica S* locus receptor kinase. Plant Cell 8: 1641–1650.

Buchanan, B.B. 1991. Regulation of CO_2 assimilation in oxygenic photosynthesis: the ferredoxin/thioredoxin system. Arch. Biochem. Biophys. 288: 1–9.

Cabrillac, D., Delorme, V., Garin, J., Ruffio-Chable, V., Giranton, J.L., Dumas, C., Gaude, T. and Cock, J.M. 1999. The S15 self-incompatibility haplotype in *Brassica oleracea* includes three S gene family members expressed in stigmas. Plant Cell 11: 971–986.

Casselman, A.L., Vrebalov, J., Conner, J.A., Singhal, A., Giovannoni, J., Nasrallah, M.E. and Nasrallah, J.B. 2000. Determining the physical limits of the *Brassica* S locus by recombinational analysis. Plant Cell 12: 23–33.

Cock, J.M. 2000. A receptor kinase and the self-incompatibility response in *Brassica*. In M. Kreis and J. Walker (Eds.) Advances in Botanical Research, vol. 32, Academic Press, New York, pp. 270–298.

Cui, Y., Brugiere, N., Jackman, L., Bi, Y.M. and Rothstein, S.J. 1999. Structural and transcriptional comparative analysis of the S locus regions in two self-incompatible *Brassica napus* lines. Plant Cell 11: 2217–2231.

De Nettancourt, D. 1997. Incompatibility in angiosperms. Sex. Plant Reprod. 10: 185–199.

Dwyer, K.G., Kandasamy, M.K., Mahosky, D.I., Acciai, J., Kudish, B.I., Miller, J.E., Nasrallah, M.E. and Nasrallah, J.B. 1994. A superfamily of S locus-related sequences in *Arabidopsis*: diverse structures and expression patterns. Plant Cell 6: 1829–43.

Gietz, R.D. and Woods, R.A. 1994. High efficiency transformation with lithium acetate. In: John R. Johnston (Ed.) Molecular Genetics of Yeast: A Practical Approach, Oxford University Press, Oxford, pp. 121–134.

Glavin, T.L., Goring, D.R., Shafer, U. and Rothstein, S.J. 1994. Features of the extracellular domain of the *S*-locus receptor kinase from *Brassica*. Mol. Gen. Genet. 244: 630–637.

Goring, D.R. and Rothstein, S.J. 1992. The *S*-locus receptor kinase gene in a self-incompatible *Brassica napus* line encodes a functional serine/threonine kinase. Plant Cell 4: 1273–1281.

Gu, T., Mazzurco, M., Matias, D.D., Sulaman, W. and Goring, D.R. 1998. Binding of a novel arm repeat protein to the kinase domain of the *S* locus receptor kinase. Proc. Natl. Acad. Sci. USA 95: 382–387.

Hanks, S., Quinn, A.M. and Hunter, T. 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 241: 42–52.

Hardie, D.G. 1999. Plant protein serine/threonine kinases: Classifications and functions. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50: 97–131.

Hatakeyama, K., Takasaki, T., Watanabe, M. and Hinata, K. 1998. Molecular characterization of S locus genes, SLG and SRK, in a pollen-recessive self-incompatibility haplotype of *Brassica rapa* L. Genetics 149: 1587–1597.

Hirota, K., Murata, M., Sachi, Y., Nakamura, H., Takeuchi, J., Mori, K. and Yodoi, J. 1999. Distinct roles of thioredoxin in the cytoplasm and in the nucleus. A two-step mechanism of redox regulation of transcription factor NF-κB. J. Biol. Chem. 274: 27891–27897.

Laughner, B.J., Sehnke, P.C. and Ferl, R.J. 1998. A novel nuclear member of the thioredoxin superfamily. Plant Physiol. 118: 987–996.

Liu, H., Nishitoh, H., Ichijo, H. and Kyriakis, J.M. 2000. Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. Mol. Cell. Biol. 20: 2198–2208.

Makino, Y., Yoshikawa, N., Okamoto, K., Hirota, K., Yodoi, J., Makino, I. and Tanaka, H. 1999. Direct association with thioredoxin allows redox regulation of glucocorticoid receptor function. J. Biol. Chem. 274: 3182–3188.

Nasrallah, J.B., Nishio, T. and Nasrallah, M.E. 1991. The self-incompatibility genes of *Brassica*: expression and use in genetic ablation of floral tissues. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42: 393–422.

Nunes, L.A., Silva, N.F., Stone, S.L., Sulaman, W., Nazarian, K.A.P., Burnett, L.A., Arnoldo, M., Rothstein, S. J. and Goring, D.R. 2000. Expression of the *S* receptor kinase in self-compatible *Brassica napus cv.* Westar leads to the allele-specific rejection of self-incompatible *Brassica napus* pollen. Mol. Gen. Genet. accepted.

Pastuglia, M., Roby, D., Dumas, C. and Cock, J.M. 1997. Rapid induction by wounding and bacterial infection of an S gene family receptor-like kinase gene in *Brassica oleracea*. Plant Cell 9: 49–60.

Rivera-Madrid, R., Mestres, D., Marinho, P., Jacquot, J.-P., Decottignies, P., Miginiac-Maslow, M. and Meyer, Y. 1995. Evidence for five divergent thioredoxin h sequences in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA 92: 6520–5624.

Sen, C.K. 2000. Cellular thiols and redox-regulated signal transduction. Curr. Top. Cell. Regul. 36: 1–31.

Schopfer, C.R., Nasrallah, M.E. and Nasrallah, J.B. 1999. The male determinant of self-incompatibility in *Brassica*. Science 286: 1697–1700.

Shi, J. and Bhattacharyya, M.K. 1996. A novel plasma membrane-bound thioredoxin from soybean. Plant Mol. Biol. 32: 653–662.

Stead, A.D., Roberts, I.N. and Dickinson, H.G. 1997. Pollen-pistil interaction in *Brassica oleracea*. Planta 146: 211–216.

Stein, J.C., Howlett, B., Boyes, D.C., Nasrallah, M.E. and Nasrallah, J.B. 1991. Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. Proc. Natl. Acad. Sci. USA 88: 8816–8820.

Stone, S.L., Arnoldo, M. and Goring D.R. 1999. A breakdown of *Brassica* self-incompatibility in ARC1 antisense transgenic plants. Science 286: 1729–1731.

Suzuki, G., Watanabe, M. and Hinata, K. 1997. Molecular genetics of self-incompatibility in *Brassica*. Rec. Res. Dev. Agric. Biol. Chem. 1: 235–242.

Suzuki, G., Kai, N., Hirose, T., Fukui, K., Nishio, T., Takayama, S., Isogai, A., Watanabe, M. and Hinata, K. 1999. Genomic organization of the S locus: identification and characterization of genes in SLG/SRK region of S(9) haplotype of *Brassica campestris* (syn. *rapa*). Genetics 153: 391–400.

Takasaki, T., Hatakeyama, K., Suzuki, G., Watanabe, M., Isogai, A. and Hinata, K. 2000. The S receptor kinase determines self-incompatibility in *Brassica* stigma. Nature 403: 913–916.

Takayama, S., Shiba, H., Iwano, M., Shimosato, H., Che, F.S., Kai, N., Watanabe, M., Suzuki, G., Hinata, K. and Isogai, A. 2000. The pollen determinant of self-incompatibility in *Brassica campestris*. Proc. Natl. Acad. Sci. USA 97: 1920–1925.

Tobias, C.M., Howlett, B. and Nasrallah, J.B. 1992. An *Arabidopsis thaliana* gene with sequence similarity to the S locus receptor kinase of *Brassica oleracea*. Plant Physiol. 99: 282–290.

Tobias, C.M. and Nasrallah, J.B. 1996. An S locus related gene in *Arabidopsis* encodes a functional kinase and produces two classes of transcripts. Plant J. 10: 523–531.

Watanabe, M., Takasaki, T., Toriyama, K., Yamakawa, S., Isogai, A., Suzuki, A., and Hinata, K. 1994. A high degree of homology exists between the protein encoded by *SLG* and the *S* receptor domain encoded by SRK in self-incompatible *Brassica campestris* L. Plant Cell Physiol. 35: 1221–1229.

Watson, J.A., Rumsby, M.G. and Wolowacz, R.G. 1999. Phage display identifies thioredoxin and superoxide dismutase as novel protein kinase C-interacting proteins: thioredoxin inhibits protein kinase C-mediated phosphorylation of histone. Biochem. J. 343: 301–305.