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The Search for Components of the Self-incompatibility Signalling Pathway(s) in *Brassica napus*

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The *Brassica* self-incompatibility system is controlled by a multi-allelic *S* receptor kinase (SRK) gene. Upon contact between self-incompatible pollen and the stigmatic papillae at the surface of the pistil, this receptor kinase is predicted to initiate a signalling pathway in the stigmatic papilla leading to the rejection of the self-incompatible pollen. Several different approaches have been taken to determine the molecular events occurring in the stigmatic papillae upon activation of the self-incompatible response. Examination of cytosolic calcium levels, electrical responses, cytoskeletal organization, and callose deposition in the stigmatic papillae during compatible and incompatible pollinations fail to reveal any responses specific to self-incompatibility. However, the identification of stigmatic proteins which interact with the SRK kinase domain has led to the characterization of a novel protein called ARC1 which appears to be a substrate for the *S* receptor kinase and is a positive effector of the self-incompatibility response.

Key words: *Brassica napus*, self-incompatibility, signal transduction, calcium, electrophysiology, cytoskeleton, callose, SRK, ARC1, THL1.

In *Brassica*, as in many other flowering plants, pollination is followed by complex pollen–pistil interactions leading to successful fertilization by compatible pollen. The initial step involves adhesion of the pollen to stigmatic papillae and is mediated through the pollen coat (reviewed in Dickinson, 1995). Both the *S* locus glycoprotein (SLG) and *S* locus related-1 (SLR1) proteins, present in the cell walls of the stigmatic papillae, are proposed to be involved in pollen adhesion, and have been found to interact with pollen coat proteins (Doughty *et al*., 1993; Hiscock *et al*., 1995; Luu *et al*., 1999). Pollen adhesion is then followed by pollen hydration and germination. Due to the dry nature of the stigma, the pollen is dependent on the pistil for providing water for hydration (Stead *et al*., 1980; Elleman and Dickinson, 1996). Pollen–pistil interactions continue during pollen tube growth, and the stigmatic papillae also play an essential role at this stage. Kandasamy *et al*. (1993) showed that ablation of the stigmatic papillae in *B. napus*, by the targeted expression of the diphtheria toxin A chain, resulted in essentially sterile plants which were unable to support pollen tube growth. In addition, treatment of pistils with phosphatase inhibitors also had a similar effect suggesting that signalling through phosphorylation or dephosphorylation in the pistil is essential for pollen tube growth (Kandasamy *et al*., 1993; Rundle *et al*., 1993; Scutt *et al*., 1993).

Successful fertilization in *Brassica* can also be influenced by a sporophytic self-incompatibility system which is controlled by a single genetic locus called the *S* locus (Bateman, 1955). There are multiple alleles at this *S* locus, and pollen rejection occurs when the pollen originates from a pollen parent sharing the same *S* allele as the pistil. Therefore, the phenotype of the haploid pollen grain is derived from the diploid parent. This has been hypothesized to occur either by the deposition of an *S* factor in the exine of the pollen grain upon dissolution of the surrounding diploid anther tapetum (Heslop-Harrison *et al*., 1974) or by the expression of the pollen *S* gene prior to meiosis during pollen development (Pandey, 1970). More recently, Doughty *et al*. (1998) have proposed a third model involving gametophytic expression of the pollen *S* gene late in pollen development, followed by secretion of the *S* factor into the surrounding region and mixing prior to deposition on the pollen surface.

Molecular analysis of the *S* locus has led to the isolation of more than one gene tightly linked to the *S* locus, and consequently a change in the nomenclature to *S* haplotype was proposed (Nasrallah and Nasrallah, 1993). The first

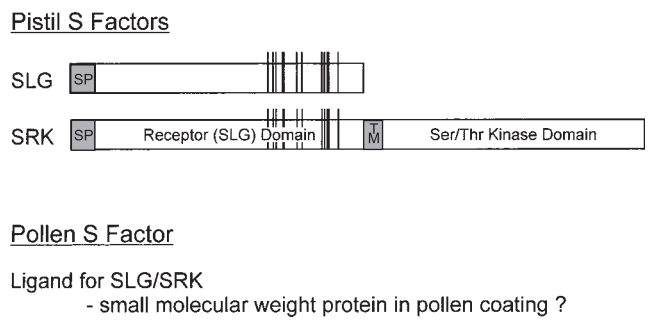

FIG. 1. Self-incompatibility genes which have been shown (SLG, SRK) or predicted (pollen *S* factor) to be linked to the *Brassica S* locus. The structure of the predicted proteins for the SLG (*S* locus glycoprotein) and SRK (*S* receptor kinase) genes are shown. SP, Signal peptide; TM, transmembrane domain. See text for explanation of the putative pollen *S* factor.

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gene isolated corresponds to the SLG (Fig. 1), a secreted glycoprotein found in the cell wall of the stigmatic papillae (Nasrallah *et al.*, 1985; Kandasamy *et al.*, 1989). The second gene isolated encodes the *S* receptor kinase (SRK, Fig. 1), a membrane localized protein with an SLG-related extracellular domain, followed by a transmembrane domain and a ser/thr kinase domain (Stein *et al.*, 1991; Goring and Rothstein, 1992; Delorme *et al.*, 1995). Several studies leading to a reduction in SLG expression have shown a correlation with a loss of pistil self-incompatibility, suggesting that SLG is required in the pistil for the self-incompatibility response (Toriyama *et al.*, 1991; Nasrallah *et al.*, 1992; Shiba *et al.*, 1995). Similarly, several studies have also implicated the SRK as a requirement for the pistil side of the self-incompatibility response. Reduced SRK expression or mutations in the SRK gene have been correlated with self-compatibility (Goring *et al.*, 1993; Nasrallah *et al.*, 1994; Conner *et al.*, 1997; Watanabe *et al.*, 1997; Stahl *et al.*, 1998). In addition, the expression of a dominant-negative SRK has led to a breakdown in self-incompatibility (Stahl *et al.*, 1998). Since both SLG and SRK function is the pistil, there is probably a third gene linked to the *S* haplotype which encodes the pollen *S* factor and represents a ligand for SRK. While the pollen gene has not been identified, a biological activity corresponding to a small molecular weight protein fraction has been detected in pollen coat extracts (Stephenson *et al.*, 1997).

Upon binding of the pollen *S* factor, SLG and SRK are proposed to initiate a signalling pathway(s) in the pistil leading to the rejection of incompatible pollen (Stein *et al.*, 1991; Goring and Rothstein, 1992). Since SLG is found throughout the papillar cell wall, it may help the pollen *S* factor cross the cell wall and/or form a complex with the extracellular domain of the membrane bound SRK. Ligand binding would then be predicted to lead to activation of the SRK kinase domain which would in turn initiate a signalling cascade(s) in the papilla leading to pollen rejection. Very little is known about this signalling pathway(s). Treatment of pistils with okadaic acid has led to a breakdown in *Brassica* self-incompatibility suggesting that type 1 or type 2A phosphatases are involved (Scutt *et al.*, 1993). In addition, the kinase associated protein phosphatase (KAPP) which interacts with a number of plant receptor kinases, was found to bind *in vitro* to the SRK-A14 kinase domain, again implicating protein phosphatases (Braun *et al.*, 1997). An aquaporin like protein, MOD, has also been implicated in this response where a mutation in the MOD gene has been correlated with a breakdown in self-incompatibility. MOD has been proposed to be activated by SRK and draw water away from the incompatible pollen to prevent pollen hydration (Ikeda *et al.*, 1997).

BRASSICA NAPUS

B. napus is an amphidiploid species which is generally self-compatible while its two progenitor species, *B. oleracea* and *B. campestris*, are both self-incompatible (Downey and Rakow, 1987). A few naturally occurring self-incompatible *B. napus* cultivars do exist (Olsson, 1960; Gowers, 1982), and self-incompatible lines have been generated by introgressing an *S* haplotype from *B. campestris* (Mackay, 1977). We have been working primarily with two different *B. napus* ssp. *oleifera* cultivars. The first is Westar which is a self-compatible cultivar, and the second is W1 which has an early-acting self-incompatibility system. W1 was produced by introgressing a *B. campestris S* haplotype into the self-compatible Westar cultivar (Goring *et al.*, 1992). The SLG and SRK cDNAs, SLG-910 and SRK-910, have been cloned from the functional *S* haplotype in W1 (Goring *et al.*, 1992; Goring and Rothstein, 1992) and the chromosomal region surrounding these two genes has been analysed (Yu *et al.*, 1996).

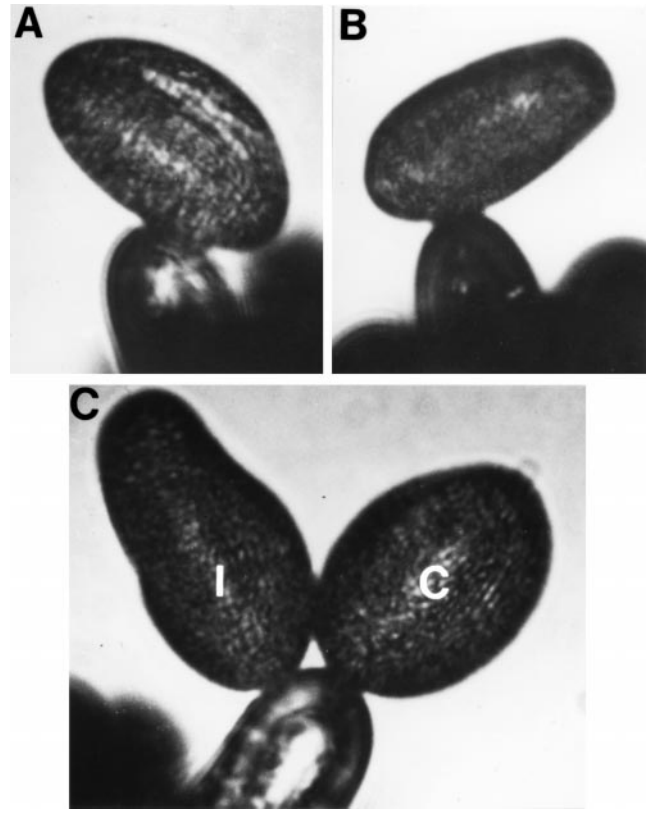

FIG. 2. Pollinations in self-compatible Westar and self-incompatible W1. A, Compatible Westar pollen placed on a stigmatic papilla; B, incompatible W1 pollen placed on a W1 stigmatic papilla; C, both incompatible W1 pollen (I) and compatible Westar pollen (C) have been placed on a W1 stigmatic papilla. Photographs courtesy of Dr John Dearnaley, University of Southern Queensland, Australia.

Typically, in a compatible Westar pollination, the pollen lands on the stigmatic surface and goes through the stages of pollen adhesion, hydration, and germination (Figs 2A and 3A). The pollen tube then grows through the cell wall of the stigmatic papilla cell and down the style to eventually fertilize the ovule (Fig. 3A). In a self-incompatible W1 pollination, the pollen is rejected at an early stage and does not adhere to the stigmatic papillae and hydration is prevented even when placed next to a compatible Westar pollen (Fig. 2B and C). Very rarely is a pollen grain able to adhere, hydrate and germinate to form a pollen tube which is unable to penetrate the stigmatic surface (Fig. 3B).

Our main objective over the last few years has been to gain a better understanding of the molecular events

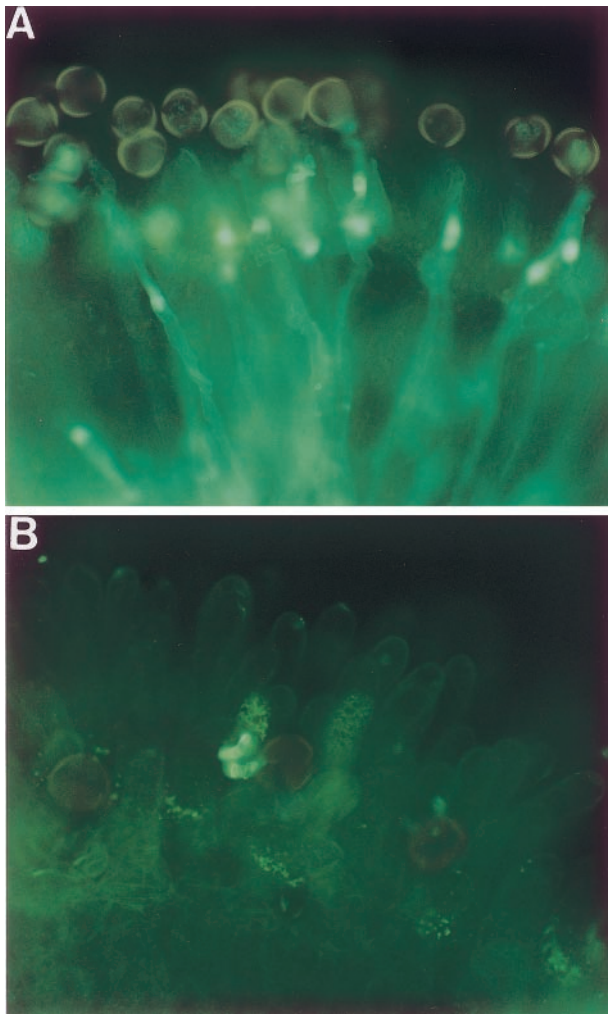

FIG. 3. Pollen tube growth in self-compatible Westar and self-incompatible W1. A, Compatible Westar pollination resulting in large numbers of pollen tubes growing down the style; B, incompatible W1 pollination resulting in very few pollen grains adhering. One pollen grain has germinated, but the pollen tube has curled around and is unable to penetrate the stigma. Pollinated pistils are stained with aniline blue.

occurring during the *Brassica* self-incompatibility response. To this end, we have investigated the following during pollen–pistil interactions in *B. napus*: (1) stigmatic cytosolic calcium levels during self-incompatible and self-compatible pollinations; (2) electrical responses in the stigma during self-incompatible and self-compatible pollinations; (3) stigmatic cytoskeletal organization during self-incompatible and self-compatible pollinations; (4) the role of callose deposition in self-incompatible pollinations; and (5) identification of stigmatic proteins which interact with the SRK kinase domain.

STIGMATIC CYTOSOLIC CALCIUM LEVELS DURING SELF-INCOMPATIBLE AND SELF-COMPATIBLE POLLINATIONS

There were several reasons for examining calcium as a second messenger in the self-incompatibility reaction. First of all, calcium has been implicated as a second messenger for signal transduction pathways in plants (Sanders *et al.*, 1999), and is, in fact, part of the gametophytic self-incompatibility response in the pollen tubes of *Papaver rhoeas* (Franklin-Tong, 1999). Secondly, calcium also plays a prominent role in animal signal transduction pathways (Berridge *et al.*, 1999). Lastly, in response incompatible pollen, a rapid deposition of 1,3-β-D-glucan (callose) at the base of the stigmatic papillae cells has been observed within a few minutes of exposure to the pollen (Dumas and Knox, 1983; Kerhoas *et al.*, 1983). The enzyme responsible for callose synthesis, 1,3,-β-D-glucan synthase, has been shown in soybean cells to be a calcium-dependent enzyme and is thought to be activated by increases in calcium (Kauss, 1985). Thus, free calcium levels were analysed in the stigmatic papillae, on a single cell basis, during the application of compatible or incompatible pollen (Dearnaley *et al.*, 1997).

The cytoplasm of a stigmatic papilla was first micro-injected with calcium green-1 dextran, a pollen grain was then placed on the injected papilla, and the cytoplasmic fluorescence was monitored. Low intensity, transient papillar calcium peaks were detected in both incompatible W1 pollinations and compatible Westar pollinations within several minutes of pollination. This was not a species-specific response since pollen from other *Brassica* species and *Arabidopsis* could also elicit a calcium peak. At a lower level, calcium peaks were also observed with latex spheres (Dearnaley *et al.*, 1997).

In comparing the incompatible W1 and compatible Westar pollinations, the only difference was that a higher percentage of compatible Westar pollinations (54%) produced a calcium peak compared with the incompatible W1 pollinations (23%). The calcium peaks also seemed to correlate with pollen hydration where the incompatible W1 pollinations had a lower rate of hydration compared with the compatible Westar pollinations. Thus, while there was no association of calcium fluxes with the self-incompatibility response, calcium may be playing a role in pollen hydration during compatible pollinations. Perhaps, low level calcium peaks in self-incompatible W1 occur before the self-incompatibility response blocks the incompatible pollen (Dearnaley *et al.*, 1997).

ELECTRICAL RESPONSES IN THE STIGMA DURING SELF-INCOMPATIBLE AND SELF-COMPATIBLE POLLINATIONS

The signalling events that occur during pollen–pistil interactions with either incompatible pollen or compatible pollen may involve ion movement across the plasma membrane, through the activation of ion channels in the plasma membrane. Two possible roles for ion channels are (1) activation of inward calcium fluxes resulting in increases in cytoplasmic calcium; and (2) in compatible pollinations, the pollen is allowed to hydrate and the mechanism behind this may involve the activation of outward movements of both K^+ and Cl^- which would result in water efflux from the papillae cells (Zeuthen and Stein, 1994). The prevention

of hydration in incompatible pollinations may result from a repression of the water efflux due to ion efflux.

In the case of stigmatic papillae, the ability to determine the involvement of ion channels, and to look for differences between incompatible and compatible pollinations, requires an electrophysiological approach utilizing a measurement technique called voltage clamp. This technique, common in animal studies but seldom used in higher plants, makes it possible to determine the voltage dependence of ionic currents across the plasma membrane and to identify the ionic species (Smith *et al*., 1980; Lew, 1991). Voltage clamping was used to measure ionic conductance and membrane potentials across the stigmatic papilla membrane. Measurements were taken on papillae before and after pollinations for both incompatible W1 and compatible Westar pollen (Dearnaley *et al*., 1997).

In general, there were considerable intercellular variations in the membrane potentials, clamping currents, and conductances. Consequently, significant differences were not seen. However, a general trend for a decrease in the membrane potentials (increasing membrane permeability) before and after pollination with both self-incompatible W1 and self-compatible Westar was observed. In addition, there was a slight increase in the membrane conductance (increase in ion flow across the membrane) before and after pollination with both self-incompatible W1 and self-compatible Westar. These general trends suggest that there are electrical responses associated with pollination regardless of the pollen compatibility (Dearnaley *et al*., 1997).

STIGMATIC CYTOSKELETAL ORGANIZATION DURING SELF-INCOMPATIBLE AND SELF-COMPATIBLE POLLINATIONS

Since the plant cytoskeleton plays an important role in plant morphogenesis, including processes such as pollen tube growth (Fowler and Quatrano, 1997), we were interested in determining whether changes to the stigmatic papillae cytoskeleton occurred during pollen–pistil interactions. In compatible pollinations, one of the steps following pollination involves the loosening of the stigmatic papilla cell wall to allow for pollen tube growth which could involve the cytoskeleton for the movement of secretory vesicles. In addition, cytoskeletal changes in response to receptor kinase signalling have been well documented in animals (Hunter, 1998; Schenk and Snaar-Jagalska, 1999). Thus the cytoskeletal organization was examined in stigmatic papillae before and after pollination with both incompatible W1 and compatible Westar pollen (Dearnaley *et al*., 1999).

In incompatible W1 pollinations, there is generally little pollen adhesion and germination occurring at normal growth conditions (low relative humidity). Therefore, to study pollen–pistil interactions in W1, high relative humidity conditions were used, and the resulting W1 pollinations showed pollen hydration levels similar to self-compatible Westar. In addition, a much greater number of the W1 pollen grains germinated to form pollen tubes. However, only a very low percentage of these pollen tubes penetrated the papillar cell wall and these pollen tubes tend to be curled. In most of the self-incompatible W1 pollinations where the pollen tubes had contacted the papillae, some papillar cell wall loosening was observed. In the compatible Westar pollinations, papillar cell wall loosening to accommodate the growing pollen tube was also observed. However, no accumulation of organelles in the papilla next to the growing pollen tube was detected (the pollen tubes showed the typical accumulation of vesicles at the growing tips) (Dearnaley *et al*., 1999).

The organization of the cytoskeleton was studied by labelling actin microfilaments (MF) with rhodamine phalloidin and labelling microtubules (MT) with an anti-human α tubulin antibody. Unpollinated papillae were compared to pollinated self-compatible Westar and self-incompatible W1 papillae. In all cases, no changes in the organization or abundance of the papillar MTs or MFs could be observed before and after pollination. Thus, signalling events occurring during compatible and incompatible pollinations do not appear to involve obvious changes to the cytoskeleton (Dearnaley *et al*., 1999). The process of cell wall loosening is not due to local accumulation of secretory vesicles in the papilla. Papillar cell wall loosening may result from enzymes present in the pollen (Albani *et al*., 1991; Robert *et al*., 1993; Hiscock *et al*., 1994), and somehow these enzymes are inhibited in the incompatible pollinations. Part of a plant's defence to pathogen attack involves cytoskeletal changes to deposit defence related compounds, and the lack of any changes to the papillar cytoskeleton during pollen tube growth suggests that the defence responses are suppressed during pollen–pistil interactions (Dearnaley *et al*., 1999).

THE ROLE OF CALLOSE IN SELF-INCOMPATIBLE POLLINATIONS

A correlation has existed between the self-incompatibility response and the deposition of callose in the stigmatic papillae (Dumas and Knox, 1983; Kerhoas *et al*., 1983), though in more recent years this has been questioned (Singh and Paolillo, 1990; Elleman and Dickinson, 1996; Dearnaley *et al*., 1997). In the self-incompatible W1 cultivar an intense staining of callose with aniline blue is detected when self-pollinated, while self-compatible Westar does not show the same level of staining when self-pollinated. Therefore, we were interested in determining if callose was an essential component of the self-incompatibility response in W1. The approach taken was to produce transgenic plants which expressed a tobacco β-1,3-glucanase cDNA in the stigma. The β-1,3-glucanase enzyme could then degrade any callose that was produced in the stigmatic papillae. Two different transgenic W1 lines were produced which expressed β-1,3-glucanase in the stigma and showed little or no staining of aniline blue following W1 pollinations. However, the self-incompatibility system was not affected by the absence of callose. As expected, seed sets with essentially no seed were produced in crosses with self-incompatible W1 and ample seed produced in crosses with self-compatible Westar. Thus, callose was not found to be essential for the rejection of the incompatible W1 pollen (Sulaman *et al*., 1997). In *B. oleracea*, the use of chemical

inhibitors to block callose deposition also had no effect on the self-incompatibility response (Singh and Paolillo, 1990; Elleman and Dickinson, 1996).

IDENTIFICATION OF STIGMATIC PROTEINS WHICH INTERACT WITH THE SRK KINASE DOMAIN

In animal receptor kinase signalling pathways, ligand binding to the extracellular domain leads to activation of the kinase domain and phosphorylation of specific residues. The phosphorylated amino acids then become specific binding sites for the substrates of the receptor kinase. Receptor kinases can bind to more than one substrate leading to the activation of multiple pathways (Hunter, 1998; Schenk and Snaar-Jagalska, 1999). While little is known about receptor kinase signalling in plants, it is thought that plant receptor kinases could function in a similar fashion. Thus, one approach to finding components of the SRK signalling pathway(s) is to identify proteins which bind to the kinase domain. If the interaction is dependent on receptor activation, one would expect that the interaction would require a phosphorylated kinase domain. Also, the haplotype specificity in the *Brassica* self-incompatibility system would not be seen at this level. It is generally accepted that the haplotype specificity is found in the extracellular domain which is predicted to interact with a haplotype specific pollen *S* factor leading to activation of the kinase domain. Once the receptor kinase is activated, it would then lead to the activation of common downstream pathways. Thus, any interactions with the SRK kinase domain should be conserved between different SRKs, although it is possible that some SRKs can only activate a subset of the pathways leading to some of the differences in phenotypes that have been observed, such as early acting *vs*. later acting self-incompatibility responses (Dickinson, 1995).

To identify proteins which interact with the SRK kinase domain, the yeast two-hybrid system, a method for detecting protein–protein interactions (Vidal and Legrain, 1999) was used. A W1 pistil cDNA library was constructed in a VP16 activation domain plasmid, and the SRK-910 kinase domain was cloned into a lexA DNA binding domain plasmid. These plasmids were co-transformed into a yeast strain with two reporter genes, HIS3 and lacZ, which are only expressed when an interaction occurs between the SRK-910 kinase domain and the protein encoded by the library plasmid. Screening of the library led to the identification of three proteins which interact with the SRK-910 kinase domain as detected by expression of the HIS3 (growth in the absence of histidine) and lacZ (β-galactosidase activity) reporter genes. Two of these proteins belong to the thioredoxin h family of proteins and were called THL1 and THL2 (thioredoxin-H like; Fig. 4) (Bower *et al*., 1996). The third protein is a novel plant protein called ARC1 (arm repeat containing; Fig. 4) due to the presence of arm repeats in the C-terminal region (Gu *et al*., 1998).

THL1 and THL2

In the yeast two-hybrid system, both THL1 and THL2 showed the same pattern of interactions. In addition to interacting with the SRK-910 kinase domain, they were also found to interact with the SRK-A14 kinase domain. However, when tested with a catalytically inactive form of the SRK-910 kinase domain, there was no interaction suggesting that phosphorylation was required. When tested with the kinase domains from two *Arabidopsis* receptor kinases, RLK4 and RLK5, these clones did not interact. Thus from the two-hybrid results, it was found that THL1 and THL2 interact specifically with SRK kinase domains in a phosphorylation-dependent manner. *In vitro* binding studies with THL1 confirmed the specific interactions with the SRK-910; however, THL1 was also able to bind to the catalytically inactive form of SRK highlighting some discrepancies on the requirements for phosphorylation. RNA blot analysis showed that THL1 and THL2 are expressed in a variety of tissues with THL2 showing higher levels of expression in floral tissues (Bower *et al*., 1996).

Thioredoxin h proteins are cytoplasmic proteins involved in the reduction of disulphide bonds (Buchanan, 1991). Thus, the importance of this reducing activity in the interaction between THL1 and SRK was examined. The THL1 protein was purified from *E. coli*, tested in an insulin assay, and found to have reducing activity (Bower *et al*., 1996). Subsequently, a conserved cysteine near the end of the transmembrane domain was identified in SRK which is required for this interaction with THL1 and THL2 (Mazzurco and Goring, unpubl. res.). Lastly, site-directed mutagenesis of the two cysteines in the active site of THL1 produced results which are in keeping with the reducing activity. The active site in THL1 consists of a C_{45}–P–P–C_{48} (Fig. 4). According to the model of thioredoxin function (Holmgren, 1995), mutation of C_{45} (to serine) is predicted to knock out the reducing activity, and in our system was found to abolish the interaction with the SRK kinase domains. Mutation of C_{48} (to serine) is predicted to lock the thioredoxin in an intermediate step with its target, and in our system it was found to interact as effectively or even better than the wild type THL1 (Mazzurco and Goring, unpubl. res.). Thus, these results indicate that the nature of the thioredoxin interaction involves its function to reduce disulphide bonds. While we have identified one of the

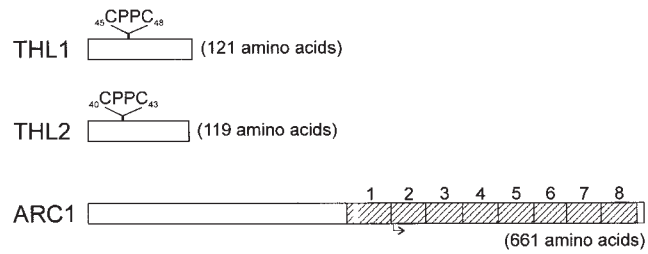

FIG. 4. Predicted features of the THL1, THL2 and ARC1 proteins. THL1 and THL2 are predicted to belong to the thioredoxin h family of proteins and the active site (CPPC) is shown for each (Bower *et al*., 1996). ARC1 is predicted to have eight arm repeats in the C-terminal region. The arrow represents the start of the original ARC1 clone isolated from the yeast two-hybrid system (Gu *et al*., 1998).

cysteines in SRK, there is presumably a second cysteine participating in a disulphide bond, the identity of which is not known. Recently, Trotochaud *et al.* (1999) have shown that the CLV1 receptor kinase is found *in vivo* as a disulphide linked dimer which raises the possibility that the identified cysteine is involved in SRK dimer formation. Thus, the *in vitro* experiments with THL1 and THL2 have aided to further define the nature of their interaction with SRK, and now *in vivo* experiments are required to test the biological importance of this interaction.

Thioredoxins have also been implicated in the *Phalaris coerulescens* self-incompatibility system. Isolation of the putative pollen *S* led to the identification of a thioredoxin-h domain in the C-terminal region of the predicted amino acid sequence (Li *et al.*, 1994). This domain was found to have thioredoxin activity when produced in *Escherichia coli* (Li *et al.*, 1995), and the pollen *S* protein from a self-fertile mutant was found to have significantly reduced thioredoxin activity (Li *et al.*, 1996).

ARC1

Similarly to THL1 and THL2 in the yeast two-hybrid system, ARC1 specifically interacted with only the SRKs and was unable to interact with kinase inactive SRK-910, again suggesting that the interaction was phosphorylation dependent. *In vitro* binding studies confirmed these results, including the requirement for a phosphorylated kinase domain. Treatment of the SRK-910 kinase domain with a phosphatase abolished the interaction with ARC1, and the addition of a phosphatase inhibitor restored the interaction. Lastly, only the C-terminal half of ARC1 is required for binding to the SRK kinase domains and this region is predicted to consist of arm repeats (Gu *et al.*, 1998; Fig. 4).

Arm repeats were first identified in the Drosophila armadillo (β-catenin) protein and have been found in animal proteins with a variety of functions from signal transduction to nuclear import where the arm repeats represent protein interaction domains (Hatzfeld, 1999). The three-dimensional structure of the arm repeat domain in β-catenin has been determined (Huber *et al.*, 1997). Each arm repeat has a hydrophobic core and consists of three short α-helices. The arm repeats pack together to form a right-handed superhelix with a positively charged region lying in a shallow groove. A minimum of six arm repeats are thought to be required to make up an arm repeat domain, and the positively charged region is predicted to interact with an acidic region on the interacting protein. Based on β-catenin binding regions, Huber *et al.* (1997) proposed that Ser–Ser–Leu may represent a β-catenin binding motif, and that phosphorylation would increase the acidity and enhance interactions with the arm repeat domain. In plants, the only class of arm repeat proteins that have been identified belong to the importin family of nuclear targeting factors (Hatzfeld, 1999). When compared to the various classes of arm repeat proteins in plants and animals, ARC1 does not show any sequence similarity outside of this arm repeat region; thus, ARC1 represents a novel class. In addition, ARC1 is the first arm repeat protein that has been implicated as an effector of receptor kinase signalling. Interestingly, RNA blot analysis showed that ARC1 has a very tissue specific pattern of expression and is only expressed in the stigma (Gu *et al.*, 1998).

While the *in vitro* data suggest that ARC1 binds in a phosphorylation-dependent manner to the SRK kinase domain, it does not give any clues to the biological function of ARC1. Two possible roles are (1) ARC1 is involved in promoting *Brassica* pollen adhesion and germination, and the interaction with activated SRK negatively regulates ARC1; or (2) ARC1 is a positive effector of SRK signalling in response to the incompatible pollen. To differentiate between these two models, a transgenic approach was used to study the effects of ARC1 overexpression and ARC1 RNA suppression on self-compatible Westar and self-incompatible W1 pollinations. The most striking phenotype was a partial breakdown of the self-incompatibility phenotype in the ARC1 antisense W1 transgenic plants resulting in some seed set. The self-incompatibility system appeared to be most affected at the early stages with a large amount of pollen germination occurring. Thus, ARC1 is a positive effector of the self-incompatibility response and appears to have a role in the early stages of pollen rejection (Stone *et al.*, 1999).

CONCLUSIONS

Our understanding of the molecular events occurring during pollen–pistil interactions in *Brassica* is still very rudimentary. Our results suggest that calcium may play some role during pollination, perhaps related to hydration. As well, there are some electrical responses detected with pollination (Dearnaley *et al.*, 1997). Progress is starting to be made in dissecting the molecular events leading to the rejection of incompatible pollen. Loss-of-function studies have supported a role for both SLG and SRK as components of the self-incompatibility response in the pistil (Toriyama *et al.*, 1991; Nasrallah *et al.*, 1992, 1994; Goring *et al.*, 1993; Shiba *et al.*, 1995; Conner *et al.*, 1997; Watanabe *et al.*, 1997; Stahl *et al.*, 1998). More recently, we have produced transgenic Westar plants expressing SLG-910 and SRK-910. These plants, as expected, are not self-incompatible; however, when pollinated with incompatible W1 pollen, the transgenic plants also produced ample seed set. Failure to reject W1 may have been due to low expression levels of the transgenes. Interestingly, a reduction in W1 pollen adhesion and germination as well as pollen tubes was observed in the transgenic plants suggesting that a very weak self-incompatibility response may have been occurring (Nunes, Sulaman, Arnoldo, Rothstein and Goring, unpubl. res.).

A model integrating the various factors that may play a role in the self-incompatibility response is presented in Fig. 5. The SLG is present throughout the cell wall of the stigmatic papillae (Kandasamy *et al.*, 1989) and presumably has a role related to the plant cell wall. SLG-like molecules appear to be a plant specific feature and are not generally associated with receptor kinase systems in animals (which lack cell walls). Thus, SLG may have evolved as a mechanism to aid the pollen *S* factor in crossing the cell wall to reach SRK. The SLG may also form a complex with

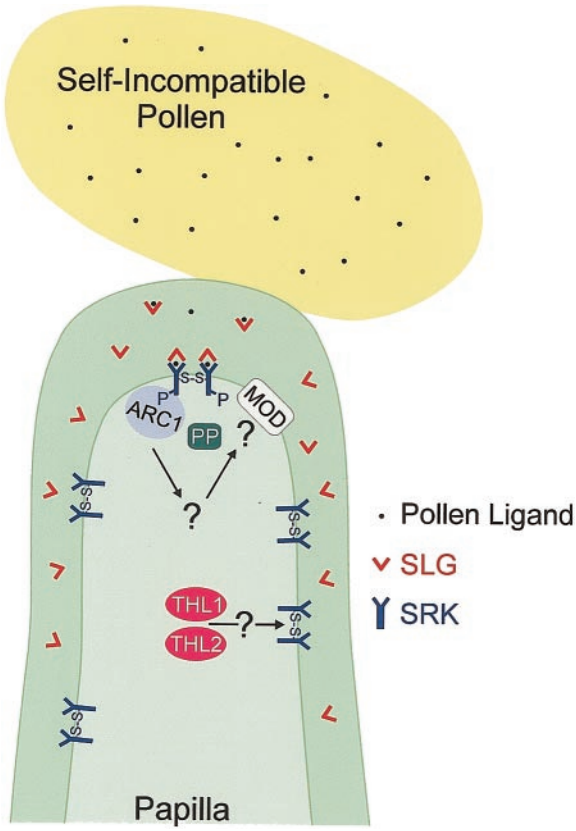

FIG. 5. A model for the SRK signalling pathway. See text for explanation. PP, Protein phosphatase.

the pollen *S* factor and SRK leading to receptor activation. The SRK has been drawn as a disulphide linked dimer based on work by Trotochaud *et al.* (1999) on the CLV1 receptor kinase and our work with THL1 and THL2. The *Arabidopsis* CLV1 receptor kinase has been found to exist as a disulphide linked dimer, though its partner may not be CLV1 based on the size of the complex (Trotochaud *et al.*, 1999). The interaction between the SRK kinase domain and THL1/2 involves the redox activity of the thioredoxins, and perhaps THL1/2 have a regulatory or housekeeping role in reducing the disulphide bonds in the SRK dimers. However, the significance of this interaction *in vivo* is not known. There is some evidence that disulphide linked complexes are detected with some animal receptor kinases (Crepaldi *et al.*, 1994; Wells *et al.*, 1999).

Protein phosphatases have been implicated in the self-incompatibility response through the use of protein phosphatase inhibitors, and the *in vitro* binding of KAPP to the SRK-A14 kinase domain (Rundle *et al.*, 1993; Scutt *et al.*, 1993; Braun *et al.*, 1997). It is not known if protein phosphatases are involved in the dephosphorylation of SRK following receptor activation or have a more direct role in the SRK signalling pathway(s).

For ARC1, we have convincing evidence that it is a positive effector of SRK signalling during the self-incompatibility response. ARC1 appears to have a role in the early stages of pollen rejection leading to the inhibition of pollen adhesion and germination. How ARC1 works downstream of SRK is not known. It may be some type of adaptor molecule where the C-terminal region binds to the phosphorylated kinase domain and the N-terminal region interacts with the next molecule in the pathway. Downstream of the pathway involving ARC1 may be MOD, an aquaporin-like protein which has been proposed to draw water away from the incompatible pollen (Ikeda *et al.*, 1997). The role of MOD in the W1 self-incompatibility system is uncertain since pollen hydration under high relative humidity conditions does not lead to a breakdown in self-incompatibility. Under these conditions, the rates of pollen germination are much lower than a compatible germination and the rare pollen tubes are unable to penetrate the papillar cell walls (Dearnaley *et al.*, 1999). Since the self-incompatibility system appears to operate at multiple stages, it is difficult to see how the loss of a protein which prevents hydration can lead to a complete breakdown in the self-incompatibility system. Receptor kinase signalling in animals can often involve more than one substrate binding to the activated receptor kinase leading to the activation of multiple pathways (Hunter, 1998; Schenk and Snaar-Jagalska, 1999). Thus, while ARC1 has been implicated in the pathway leading to a block in the early stages of pollination, it is possible that SRK can also activate other pathways that act at later stages such as blocking papillar cell wall loosening. If so, there are other SRK substrates which have not yet been identified.

NOTE ADDED IN PROOF

The identification of a gene linked to the *S* locus which fulfils the criteria for being the putative pollen *S* factor has been published recently (Schopfer *et al.*, 1999).

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