Chapter 8
'Self' Pollen Rejection Through the Intersection of Two Cellular Pathways in the Brassicaceae: Self-Incompatibility and the Compatible Pollen Response

M.A. Samuel, D. Yee, K.E. Haasen, and D.R. Goring

Abstract The sporophytic self-incompatibility (SI) system, which operates in the Brassicaceae is primarily controlled by two multi-allelic loci, encoding the SP11/SCR pollen ligand, and the stigma-specific *S* Receptor Kinase (SRK). Haplotype-specific recognition of SP11/SCR by SRK triggers the activation of SRK's intracellular kinase domain. This is predicted to cause the phosphorylation-mediated recruitment of the ARC1 E3 ubiquitin ligase. ARC1 is predicted to inhibit its substrate by ubiquitination, and recent work suggests that Exo70A1 is a target of ARC1. Exo70A1 is predicted to regulate targeted secretion and is required in the stigma to promote compatible pollen hydration, germination and pollen tube growth. SRK is also known to interact with a number of other proteins, including the *M* locus protein kinase (MLPK), which may function with SRK to co-regulate ARC1. Here we review our present knowledge of the various cellular components that act in concert during the SI response. We also discuss the cellular mechanisms of how SI can cause pollen rejection through the inhibition of stigmatic factors that promote compatible pollen acceptance.

Abbreviations

ARC1	Arm repeat containing; an E3 ubiquitin ligase
ARM	Armadillo
BAK1	BRI1 Associated receptor Kinase 1
BnExo70A1	*Brassica napus* gene encoding a protein with sequence similarity to Exo70
BRI1	BRASSINOSTEROID-INSENSITIVE 1
BY2 cells	Bright Yellow 2 cells

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$[Ca^{2+}]_i$	Cytosolic free intracellular calcium concentration
ER	Endoplasmic reticulum
Exo70	A subunit of the exocyst complex
KAPP	Kinase-associated protein phosphatase
MLPK	*M* Locus Protein Kinase
mod	Modifier
mRNA	Messenger RNA
PCPs	Pollen coat proteins
RLCK	Receptor-like cytoplasmic kinase
RNAi	RNA interference
SCR/SP11	*S*-locus cysteine-rich protein (the pollen *S*-determinant in Brassica)
SI	Self-incompatibility
SLG	*S*-locus glycoprotein
S	locus Self-incompatibility locus
SLR1	*S*-locus related-1 (a homologue of SLG)
SRK	*S*-locus receptor kinase (the pistil *S*-determinant in Brassica)
THL1 and THL2	Thioredoxin *h* proteins
U-box	A motif present in a family of E3 ligases
UND	A novel N-terminal domain present in a number of predicted U-box/ARM proteins

8.1 Introduction

In the Brassicaceae, a conserved sporophytic self-incompatibility (SSI) system is present, and detailed genetic studies have resulted in the identification of highly polymorphic *S* genes that confer this trait. The SSI system has been best characterised in the genus *Brassica*, and is primarily controlled by a receptor–ligand system encoded in two tightly linked and multi-allelic genes: the *S* Receptor kinase (SRK), and the small cysteine-rich secreted protein, SP11/SCR (see Chap. 6 for a discussion of SRK and SP11/SCR polymorphism and Chap. 7 for an introduction to the discovery of the *S*-locus determinants and other components involved in *Brassica* SI). The co-evolved SRK and SP11/SCR alleles constitute different *S*-haplotypes, and 'self' pollen rejection occurs when the *S*-haplotype of the pollen parent matches the pistil *S*-haplotype (Boyes and Nasrallah 1993). The haplotype-specific interaction between the pollen-specific SP11/SCR and stigma-specific SRK elicits a rapid rejection response. Successful fertilisation can occur only if the *S*-haplotype of the pollen parent and the pistil do not match. Thus, by rejecting self pollen, the outcome of self-incompatibility is an increased propensity towards out-crossing.

Although the interactions between SRK and SP11/SCR has been well mapped out, the subsequent signalling pathway that leads to the rejection response is relatively less well known. In this chapter, we discuss the various intracellular signalling components identified downstream of SRK and elaborate on their possible cellular

mechanisms. Recent research reinforces the concept that SI functions by blocking the compatible pollen response (Roberts et al. 1980; Dickinson 1995), and so it is appropriate to first start with a review of the literature on cellular factors and changes governing compatible pollen–pistil interactions. The early stages of interactions between the compatible pollen and the stigmatic surface at the top of the pistil involve pollen capture, adhesion and hydration as well as pollen tube penetration of the stigmatic barrier. These events have been largely studied in the *Brassica* and *Arabidopsis* genera and are reviewed below as these are the stages targeted by the *Brassica* SI response. Following these early stages, the compatible pollen tube enters the expanded stigmatic papillar cell wall, grows down to enter the intercellular space below the papilla and then grows to the ovary where fertilisation occurs (Elleman et al. 1992). The complex interactions that occur between the pollen tube and the pistil are reviewed in detail elsewhere (Edlund et al. 2004; Swanson et al., 2004).

8.2 The Early Stages of Compatible Pollen–Stigma Interactions in the Brassicaceae

When a random pollen grain lands on a stigma of another plant species, it cannot successfully complete fertilisation. This is due to an active recognition system in place to allow successful fertilisation following compatible pollinations. In plant species with dry stigmas, as found in the Brassicaceae, this recognition starts at the earliest stages of pollen capture and adhesion (Roberts et al. 1980; Dickinson 1995; Swanson et al. 2004).

8.2.1 Pollen Capture and Adhesion

Once a compatible pollen grain comes in contact with the stigma, the pollen is captured, and a strong connection forms between the pollen grain and the stigmatic papilla (Fig. 8.1a). This initial stage is mediated by the 'glue-like' surface properties of both the pollen grain and stigma (Clarke et al. 1979; Roberts et al. 1980; Zinkl et al. 1999). In the Brassicaceae, the surfaces of the stigmatic papillae are coated with a waxy cuticle and a thin proteinaceous pellicle. It is the pellicle that has the adhesive properties and has been proposed to be important for pollen–stigma recognition events, since removal of the pellicle disrupts these events (Mattison et al. 1974; Stead et al. 1980). The pollen grain has a highly sculptured exine outer layer with a pollen coat composed of lipids and proteins filling in the cavities of the exine (Mayfield et al. 2001; Murphy 2006). Pollen capture is mediated by the exine and does not appear to be dependent on the pollen coat, since this initial stage is not affected when the pollen coat is absent (Zinkl et al. 1999).

After pollen capture, the pollen coat mobilises and spreads towards the pollen–stigma interface to form a meniscus-shaped 'foot', and the lipidic and proteinaceous

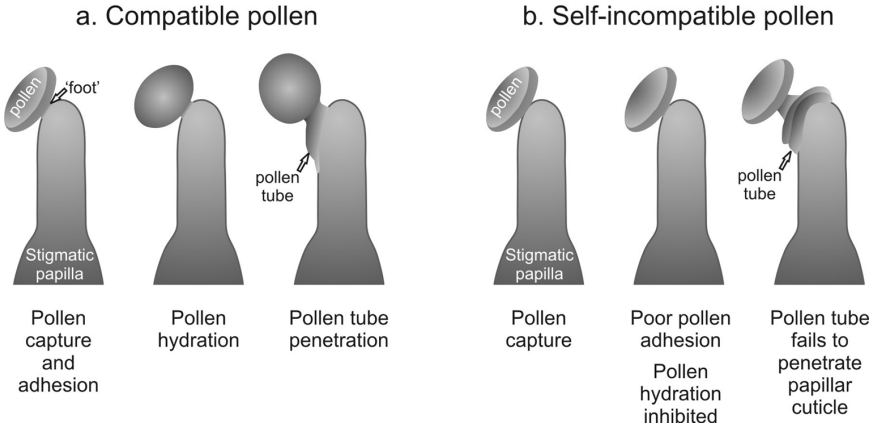

Fig. 8.1 Illustrations of early events following a compatible (**a**) or self-incompatible (**b**) pollination. Images are based on micrographs published in Ockendon (1972) and Dickinson (1995). See text for more details

contents of both surfaces mix (Elleman and Dickinson 1990; Preuss et al. 1993). It is at this stage where the pollen coat mediates pollen adhesion, and there is a cross-linking of the pollen grain to the stigmatic papilla (Elleman et al. 1992; Zinkl et al. 1999). Two stigma-specific proteins, the *S*-locus glycoprotein (SLG) and the *S*-locus Related-1 (SLR1) protein, have been proposed to be required for pollen adhesion in *Brassica* (Fig. 8.2a). Decreased pollen adhesion was observed in *Brassica* transgenic lines with reduced SLR1 expression through transgenic suppression, and when *Brassica* stigmas were pre-treated with antibodies to SLR1 and SLG (Luu et al. 1997b, 1999). Both SLG and SLR1 have been found to bind to Class **A** pollen coat proteins (PCPs) and are thought to mediate pollen adhesion through these interactions (Fig. 8.2a) (Doughty et al. 1993; Hiscock et al. 1995; Takayama et al. 2000b). Consistent with this model, the pre-treatment of *Brassica* stigmas with pollen coat proteins also decreased adhesion (Luu et al. 1999).

8.2.2 Pollen Hydration

Once the pollen grain has adhered to the stigmatic papilla, hydration follows (Fig. 8.1a). Since the Brassicaceae stigma is of the 'dry' type, pollen hydration occurs through the passage of water from the stigma to the pollen (Heslop-Harrison 1979; Roberts et al. 1984). Pollen grains are quiescent and desiccated upon maturation, and can only reactivate and germinate following rehydration on the stigma (Heslop-Harrison 1979; Zuberi and Dickinson 1985). In both *Brassica* and *Arabidopsis*, the cuticular layer of the dry stigma serves as a barrier to regulate water transfer during selective pollen hydration. As a result, this minimises the occurrence of indiscriminate pollination, allowing for species-specificity in this process (Sarker et al. 1988; Hulskamp et al. 1995; Zinkl et al. 1999).

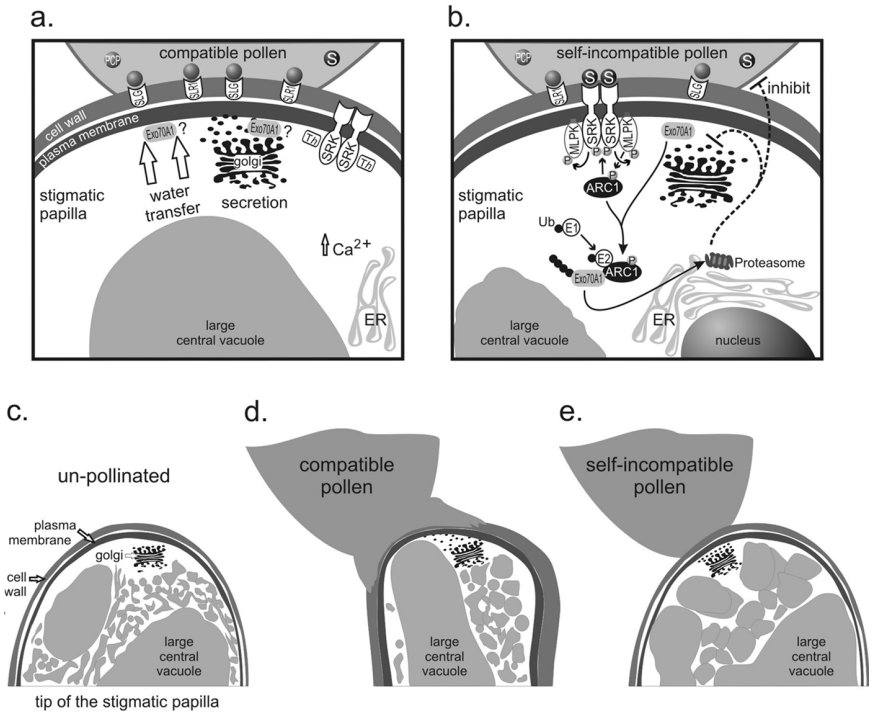

Fig. 8.2 Models of cellular signalling events following a compatible or self-incompatible pollination. (**a**) Compatible pollen-stigmatic papillar interactions: Pollen coat proteins (PCP) bind to SLG and SLR1 to promote pollen adhesion. This may lead to other signalling events, such as calcium signalling, in the stigmatic papilla. Exo70A1 may have a role in targeting the vacuolar network and/or secretory vesicles to the plasma membrane to promote water transfer for pollen hydration and enzyme secretion to loosen the papillar surface for pollen tube growth. SRK remains inactive due to the inhibitory effects of thioredoxin h (T*h*), and the absence of the haplotype-specific SP11/SCR (S) pollen ligand. (**b**) Self-incompatible pollen-stigmatic papillar interactions: The haplotype-specific SP11/SCR (S) pollen ligand binds to SRK and activates the SRK/MLPK complex. A phosphorylation cascade activates the ARC1 E3 ubiquitin ligase, leading to the ubiquitination (Ub) and inactivation of Exo70A1. Both water transfer and loosening of the papillar surface are blocked causing pollen rejection. (**c**) The vacular network in the un-pollinated stigmatic papilla. (**d**) Following a compatible pollination, the vacuolar network in the stigmatic papilla appears to become directed towards the pollen grain, possibly for water transfer. Secretory events lead to the expansion of the papillar cell wall prior to pollen tube penetration. (**e**) Following a self-incompatible pollination, the vacuolar network in the stigmatic papilla appears to be disrupted, possibly blocking pollen hydration. Vesicles are observed to be accumulating next to the unexpanded papillar cell wall, perhaps due to an inhibition of vesicle docking by the SRK signalling pathway shown in (**b**). Images in (**c**–**e**) are based on data and micrographs published in Iwano et al. (2007) and Elleman and Dickinson (1996)

The lipid-rich pollen coat and the cuticular layer of the stigma at the point of pollen contact are believed to form a unique hydraulic conduit for water flow from the stigma to the pollen grain (Elleman et al. 1992; Dickinson, 1995). The mechanism that allows hydrophobic lipids to form such a liquid conduit is not known, but

numerous studies show that lipids from the pollen coat and stigma surface are both necessary and sufficient for proper pollen hydration. For example, the *Arabidopsis fiddlehead* mutant has altered cuticle properties from a β-ketoacyl CoA synthase mutation, which is thought to alter long-chain lipid biosynthesis, and results in aberrant organ fusion (Lolle et al. 1997; Yephremov et al. 1999; Pruitt et al. 2000). The *fiddlehead* mutants also show inappropriate pollen hydration on non-stigmatic surfaces indicating that proper lipid content is critical for the control of pollen hydration (Lolle and Cheung 1993). Other *Arabidopsis* mutants with defects in the long-chain lipid synthesis, such as the *cer* mutants, have altered pollen coat lipid contents and cannot hydrate properly (Preuss et al. 1993; Hulskamp et al. 1995).

8.2.3 Pollen Germination and Pollen Tube Penetration

Following hydration, the pollen grain germinates and a pollen tube emerges to grow into the hydrophobic interface corresponding to the foot (Fig. 8.1a). It is believed that the ability of the pollen grain to sense a water gradient through the lipid conduit determines the initial germination orientation and polarity. Thus, the site of the pollen–stigma contact becomes the site of pollen tube penetration into the stigmatic surface (Elleman et al. 1992; Dickinson 1995; Edlund et al. 2004). Secreted enzymes from either the pollen tube or the stigma are thought to selective breakdown the papillar surface, allowing pollen tube entry. Accordingly, both pollen and stigma serine esterases have been identified, and treatment of *Brassica* stigmas with a serine esterase inhibitor blocks pollen tube invasion (Hiscock et al. 2002). Various other enzymes, including cutinases, polygalacturonase and pectin esterases, have also been identified in *Brassica* pollen and/or stigma (Hiscock et al. 1994; Kim et al. 1996; Dearnaley and Daggard 2001). Similarly, cell wall modifying enzymes (e.g. pectin methylesterases) may also play a role in loosening the papillar cell wall in preparation for pollen tube penetration (Micheli 2001). The involvement of so many types of enzymes is not surprising considering that breaching the papillar surface would logically implicate the breakdown, or at the very least the modification, of the waxy cuticle, its surrounding proteinaceous pellicle and the underlying complex epidermal cell wall.

Calcium appears to serve as a second messenger during this stage during pollination in *Arabidopsis*, and may be related to secretory events associated with papillar cell wall loosening (Elleman and Dickinson 1996; Hiscock et al. 2002). In *Arabidopsis*, imaging of cytosolic-free intracellular calcium levels ($[Ca^{2+}]_i$) in the stigmatic papilla revealed three intervals of cytosolic calcium increases. The first occurred at the pollen attachment site following pollen hydration, followed by a second increase prior to pollen germination and a final third increase just prior to pollen tube penetration of the stigmatic surface (Iwano et al. 2004). Increases in $[Ca^{2+}]_i$ have also been observed following compatible pollinations in *Brassica* (Dearnaley et al. 1997; Elleman and Dickinson 1999).

8.3 The SI Response Causes Pollen Arrest at the Stigmatic Surface

In the Brassicaceae, self pollen rejection is controlled by the female side through an organised cascade of cellular events in the stigmatic papillae, leading to pollen arrest. With the dry type stigma, this response starts early by disrupting pollen adhesion, pollen hydration and stigma penetration by the pollen tube if necessary (Dickinson 1995). Following self pollination, pollen capture rapidly occurs and pollen adhesion is also initiated, but is diminished relative to compatible pollinations (Stead et al. 1980; Luu et al. 1997a). One could speculate that both the 'species'-compatible and self-incompatible pollen recognition events have been initiated, but a small 'delay' in relaying the SI signal to the stigmatic papilla allows pollen capture and some pollen adhesion to occur (Fig. 8.1b).

In terms of pollen hydration, axis expansion through hydration was significantly decreased in *Brassica* self pollen compared to cross-pollen (Roberts et al. 1980; Zuberi and Dickinson 1985). Considering that adhesion is reduced in self pollinations, a suitable lipid interface may not form between the pollen and the stigmatic papillae and as a result water transfer from the stigma is impaired. Hydration can occur in some *Brassica* self pollen, which may be attributable to environmental humidity, though some pollen may achieve sufficient adhesion to allow for water transfer from stigma (Zuberi and Dickinson 1985; Luu et al. 1999). Self pollen that successfully hydrate either do not germinate a pollen tube or germinate a pollen tube that curls at the stigmatic surface without penetrating the cuticle (Fig. 8.1b) (Ockendon 1972; Carter et al. 1975; Zuberi and Dickinson 1985). Stigma-derived compatibility factors, such as secreted enzymes (to breakdown the stigmatic papillar surface), could be targeted by SI to prevent pollen tube entry (Hiscock et al. 2002). As well, any stigma-to-pollen permissive signals needed for pollen tube growth could be potentially denied; for example, chemo-attractants such as GABA identified in *Arabidopsis* (Palanivelu et al. 2003). A key area in the field has focused on identifying the cellular events in the stigmatic papilla, which disrupt these pollen–stigma interactions during a SI response, and very exciting progress has been made in recent years in uncovering this pathway (Fig. 8.2).

8.3.1 The S Receptor Kinase Activates a Cellular Signalling Pathway in the Stigmatic Papilla to Trigger Self Pollen Rejection

The SSI system in the *Brassicaceae* has been best characterised in the genus *Brassica*, and is regulated by the *S* Receptor kinase (SRK) small secreted SP11/SCR protein (see Chap. 7 for a review of the discovery of the *S*-locus determinants and Chap. 6 for a discussion of SRK and SP11/SCR polymorphism). SRK is expressed solely in the stigma and is the female determinant of this system (Takasaki

et al. 2000; Silva et al. 2001) while SP11/SCR is a pollen-specific coat protein and the male determinant of this system (Schopfer et al. 1999; Suzuki 1999; Takayama et al. 2000a, b). During early anther development, SP11/SCR is secreted from the anther tapetal cells into the locule, where it is integrated into the pollen coat deposited on the pollen surface (Iwano et al. 2003). As predicted for a plant receptor kinase, SRK is an integral plasma membrane protein with a functional serine/threonine kinase (Goring and Rothstein 1992; Stein and Nasrallah 1993; Giranton et al. 2000). SRK exists as a pre-formed dimer in unpollinated pistils and provides a high-affinity ligand binding site for SCR/SP11 (Giranton et al. 2000; Shimosato et al. 2007). Following a self-incompatible pollination, there is a *S*-haplotype-specific interaction between SP11/SCR and SRK (Kachroo et al. 2001; Takayama et al. 2001). An intracellular signalling pathway is then activated in the stigmatic papilla, which rapidly leads to self pollen rejection (Fig. 8.2b).

SP11/SCR and *SRK* genes have also been identified in other genera in the Brassicaceae, including *Arabidopsis lyrata* (Kusaba et al. 2001; Schierup et al. 2001), *Capsella grandiflora* (Paetsch et al. 2006) and *Raphanus sativus* (Sato et al. 2004). Interestingly, *Arabidopsis thaliana,* which is naturally self-fertilising, carries non-functional *SP11/SCR* and *SRK* genes (Kusaba et al. 2001). However, some ecotypes can become fully self-incompatible with the addition of the *Arabidopsis lyrata SP11/SCR* and *SRK* genes (Nasrallah et al. 2004). This indicates that while *Arabidopsis thaliana* has lost its SI due to inactivation of the *S* genes, the downstream signalling components required for conferring SI has remained intact in some instances. The nature of the downstream signalling events has become much clearer in recent years and involves a novel receptor kinase signalling pathway (Fig. 8.2).

8.3.2 The M Locus Protein Kinase acts Together with the S Receptor Kinase to Promote SI

The *M* locus protein kinase (MLPK) was discovered following positional cloning of a modifier (*mod*) mutation in the *Brassica rapa* yellow sarson variety (Murase et al. 2004). This recessive mutation led to a complete breakdown of the stigmatic SI response (Murase et al. 2004). MLPK encodes a predicted kinase belonging to the receptor-like cytoplasmic kinase (RLCK) family, and was found to have serine/threonine kinase activity. As well, transient expression of a functional MLPK in the stigmatic papillae of *Brassica rapa* var. yellow sarson was sufficient to restore the SI response (Murase et al. 2004).

Brassica MLPK, and its *Arabidopsis* orthologue, can exist as two isoforms produced through alternate transcriptional start sites, and both forms are targeted to the plasma membrane by either an N-terminal hydrophobic domain in one isoform or by myristoylation of the other isoform (Kakita et al. 2007b). Membrane localisation of MLPK is required for conferring SI, and this likely facilitates interactions with SRK, as demonstrated through bi-molecular fluorescence complementation in tobacco protoplasts (Kakita et al. 2007b). Finally, the active SRK kinase domain was

shown to efficiently phosphorylate MLPK in vitro, further supporting that MLPK functions with SRK to promote SI (Kakita et al. 2007a).

Given MLPK's plasma membrane location and interactions with SRK, one can speculate that MLPK functions in a complex with SRK to promote downstream signalling events following SRK activation by the SP11/SCR ligand (Fig. 8.2b). The theme of receptor kinases functioning with 'cytoplasmic' kinases is quite common in animal receptor kinase systems (Pawson 2002). The SRK/MLPK complex may also have some parallels to the BRI1/BAK1 complex involved in brassinosteroid signalling (Gendron and Wang 2007).

8.3.3 The SRK Kinase Domain can Interact with a Range of Intracellular Proteins

Kinase domains of receptor kinases can often interact with a number of intracellular proteins to activate intracellular signalling pathways and regulate receptor signalling (Pawson 2002). Thus, it is not surprising that protein–protein interaction screens have identified a number of interacting partners for the SRK kinase domain. At least six interacting partners have been identified: two thioredoxin *h* proteins, THL1 and THL2 (Bower et al. 1996); the E3 ubiquitin ligase, ARC1 (Gu et al. 1998; Stone et al. 2003); the Kinase-Associated Protein Phosphatase (KAPP); a sorting nexin; and calmodulin (Vanoosthuyse et al. 2003). Of these six interacting partners, only ARC1 has been shown to act downstream of SRK as a positive regulator of SI (Stone et al. 1999). Thioredoxin *h* has been shown to be a negative regulator of SRK and SI (Cabrillac et al. 2001; Haffani et al. 2004). Both KAPP and sorting nexin are known to be negative regulators in other systems. KAPP has been implicated in the down-regulation of various receptor kinases following activation (Johnson and Ingram 2005), and sorting nexins are known to participate in the sorting of endosomal-localised receptors for either recycling or degradation (Carlton et al. 2005).

Another interesting feature is the nature of the interactions between these proteins and the SRK kinase domain in vitro. Both ARC1 and KAPP show phosphorylation-dependent interactions, suggesting that they may only bind to the SRK kinase domain following receptor activation (Gu et al. 1998; Vanoosthuyse et al. 2003). The thioredoxin *h* proteins (THL1/2), the sorting nexin and the calmodulin were able to bind both the active and inactive forms of the SRK kinase domain, indicating that their regulatory functions are likely unrelated to receptor activation (Bower et al. 1996; Vanoosthuyse et al. 2003). This feature implicates a complexity to the dynamics of SRK interactions during the SI response. In unpollinated stigmas, SRK is likely maintained in an inactive state with reduced basal activity, potentially through interactions with thioredoxin *h* and/or calmodulin. In response to self pollen, SRK is activated through conformational changes and released from these potential negative regulators. The phosphorylated SRK kinase domain recruits downstream signalling partners, such as ARC1, to activate the cellular response

for pollen rejection. SRK returns to an inactive state when dephosphorylated by KAPP. Sorting nexin may also be involved at this stage if receptor endocytosis has occurred to regulate SRK-signalling through endosome-mediated degradation or recycling.

8.3.4 Thioredoxin h Inhibits SRK Activity in the Absence of Self Pollen

The thioredoxin *h* proteins, THL1 and THL2, were identified as SRK interactors through screening a *Brassica napus* pistil cDNA yeast two-hybrid library with SRK kinase domain (Bower et al. 1996). These proteins were able to bind both the active and inactive forms of the SRK kinase domains and required a cysteine at the end of the transmembrane domain for this interaction (Bower et al. 1996; Mazzurco et al. 2001). The role of thioredoxin *h* as an inhibitor of SRK was elegantly demonstrated by Cabrillac et al. (2001). They found that SRK was maintained in an inactive state in unpollinated pistils, yet became constitutively active when immuno-purified due to the loss of a soluble stigmatic inhibitor during the extraction procedure. Through a series of experiments, the inhibitor was identified as a thioredoxin-type protein, and the addition of recombinant thioredoxin *h* (THL1) was able to reconstitute this activity (Cabrillac et al. 2001). Subsequently, Haffani et al. (2004) demonstrated that transgenic *Brassica napus* Westar lines carrying an antisense-THL1 construct exhibited constitutive low levels of pollen rejection, possibly through the increased activity of an endogenous SRK. Interestingly, similar parallels have been observed in pathogen signalling with the tomato Cf-9 receptor system. A thioredoxin protein, CITRX, was found to interact with the C-terminal tail of the Cf-9 receptor, and function as a negative regulator of Cf-9 signalling (Rivas et al. 2004). Recently, it was shown that CITRX functions as an adaptor between Cf-9 and the ACIK cytoplasmic serine/threonine kinase, a positive regulator of disease resistance (Nekrasov et al. 2006). Whether a similar conserved arrangement exists between SRK-thioredoxin *h*-MLPK does remain to be seen. Given the function of thioredoxins in alleviating oxidative stress and their potential to be modified by redox imbalances (Vieira Dos Santos and Rey 2006), it is reasonable to postulate that a possible localised oxidative burst near the site of pollen attachment could modify thioredoxin *h* and relieve the inhibition of SRK (Fig. 8.2a, b).

8.3.5 ARC1 Functions Downstream of SRK to Promote SI

One of the most interesting SRK interactor to come from the yeast two-hybrid screen was the Armadillo repeat containing protein, ARC1 (Gu et al. 1998). ARC1 is a modular protein with a novel N-terminal domain (UND), followed by a U-box, and

an ARM repeat domain (Samuel et al., 2006). UND is a conserved domain present in a number of predicted U-box/ARM proteins, though its function is unknown (Samuel et al. 2006). The U-box is a conserved motif present in the U-box family of E3 ubiquitin ligases, and is responsible for binding to the E2 ubiquitin conjugating enzyme as part of the ubiquitination reaction (Hatakeyama and Nakayama 2003). The ARM repeat domain mediates binding of ARC1 to the phosphorylated SRK kinase domain (Gu et al. 1998). ARM repeat domains are composed of tandemly repeated 42 amino acid ARM repeats, which form a super helix of alpha-helices, and provide binding sites for interacting partners (Samuel et al. 2006).

ARC1 is specifically expressed in the stigma, a pattern which mimics the expression of SRK, and indicates a function specific to this tissue (Gu et al. 1998). Antisense suppression of ARC1 in self-incompatible W1 plants resulted in a partial breakdown of SI, demonstrating a positive role for ARC1 during SI response (Stone et al. 1999). These ARC1 antisense W1 plants had functional *SP11/SCR* and *SRK* genes present, and thus, indicated that ARC1 was functioning downstream of SRK as predicted. The lack of complete breakdown in these ARC1 antisense W1 plants is likely attributed to the incomplete suppression of the ARC1 mRNA, though one can not rule out that other potential intracellular signalling proteins were partially compensating for the loss of ARC1 (Stone et al. 1999).

ARC1 can function as an E3 ubiquitin ligase, and increases in the levels of ubiquitinated proteins were observed 30 min following a self pollination (Stone et al. 2003). In keeping with the role of ARC1 as an E3 ligase during the SI response, pre-treatments of pistils with a proteasomal inhibitor also reduced the SI response, leading to a large increase in pollen adhesion and pollen tube growth down the pistil. Finally, transient expression of ARC1 and the active SRK kinase domain in tobacco BY2 cells resulted in targeting of ARC1 to ER-associated proteasomes in the perinuclear region (Stone et al. 2003). Altogether, these results point to a model where activated SRK directs ARC1 to re-localise to the proteasomes, where ARC1 targets various substrates for degradation (Fig. 8.2b).

Recently, we have found that MLPK can have a similar effect on ARC1 localisation in tobacco BY2 cells, and MLPK can also phosphorylate ARC1 very efficiently in vitro, at a much higher level than that seen with SRK (Samuel et al. 2008a). This is consistent with MLPK functioning in a complex with SRK to activate downstream events and raises the possibility that MLPK functions with SRK to activate ARC1 (Fig. 8.2b). ARC1 'activation' by phosphorylation could include directing ARC1 to a specific sub-cellular location (such as the proteasome) and/or causing ARC1 to have increased binding affinity for its substrate.

In keeping with the general model that SI functions by blocking compatible pollen–stigma interactions (Dickinson 1995), ARC1 substrates are hypothesised to be stigmatic factors, which would normally promote events required for compatible pollen. *Activated* ARC1 would inhibit these factors, possibly by proteasomal degradation, and cause pollen arrest on the stigma surface. The possibility of ARC1 functioning as an inhibitor of compatibility factors brings us to an important crossroad in understanding where SI meets the compatible pollen pathway.

8.3.6 *BnExo70A1 is a Potential Substrate for ARC1 and is Required for Compatible Pollen–Stigma Interactions*

To identify potential substrates for ARC1, the N-terminus of ARC1 was used to screen a *Brassica napus* pistil cDNA yeast two-hybrid library, and *Bn*Exo70A1 was identified as a potential interactor (Samuel et al. 2008b). The interaction between ARC1 and *Bn*Exo70A1 was confirmed through in vitro pull down assays and an in vitro ubiquitination assay of *Bn*Exo70A1 by ARC1. As well, transient expression of an active SRK kinase domain, ARC1 and *Bn*Exo70A1 in tobacco BY2 cells resulted in targeting of ARC1 and *Bn*Exo70A1 to ER-associated proteasomes in the perinuclear region. This indicated the possibility that *Bn*Exo70A1 could be targeted for inactivation by ARC1 during the SI response (Fig. 8.2b).

*Bn*Exo70A1 displays sequence similarity to the conserved eukaryotic protein, Exo70, a subunit of the exocyst complex. In yeast and mammalian cells, the exocyst complex is comprised of eight subunits (Sec3, 5, 6, 8, 10 and 15; Exo70 and 84), and functions in tethering and docking selected secretory vesicles to specific sites on the plasma membrane. Thus, the exocyst regulates specialised secretory events, and functions include polarised exocytosis during yeast budding and neurite outgrowth, insulin-stimulated trafficking of the Glut4 transporter, and selective tethering of vesicles to the apical or basolateral membranes in polarised epithelial cells (Munson and Novick 2006). Plant genomes contain all the predicted exocyst genes, and have specifically expanded the Exo70 gene family, but whether they function in a similar manner to other systems is not known. In *Arabidopsis*, exocyst subunit mutants have been identified with defects in root hair elongation, pollen germination and polar growth and development (Cole and Fowler 2006).

A potential role for *Bn*Exo70A1 in regulating polarised secretion is very consistent with previous studies documenting cellular changes in the stigmatic papilla following compatible pollinations, and raises a very exciting new development in our understanding of these processes. *Bn*Exo70A1 would be predicted to play a role in the stigmatic papilla promoting events such as pollen adhesion, pollen hydration or penetration of the pollen tube through the stigmatic surface, following a compatible pollination. Consistent with this, the RNAi suppression of *Bn*Exo70A1 in the stigma of compatible *Brassica napus* Westar plants resulted in a severe reduction in seed production (Samuel et al. 2008b). This was a consequence of decreased pollen adhesion and hydration of what normally should be compatible pollen. As well, any pollen tubes that formed were incapable of penetrating the papillar surface, essentially phenocopying the SI response. A role for Exo70A1 in promoting compatible pollen–stigma interactions was also found to be conserved in *Arabidopsis*, where null *At*Exo70A1 mutants were also found to display defects in pollen hydration and germination (Samuel et al. 2008b).

From our results, we propose that during compatible pollen–stigma interactions, Exo70A1 functions as a positive regulator by facilitating targeted secretion of stigmatic factors in response to compatible pollen (Fig. 8.2a). In contrast, during the SI response, SRK-MLPK activation leads to ARC1-mediated suppression of Exo70A1

function, resulting in pollen rejection (Fig. 8.2b). In concurrence with this hypothesis, previous microscopy studies have identified changes in the endomembrane system following self or cross pollinations, which may point to potential cellular roles for Exo70A1 (Fig. 8.2c–e).

8.3.7 Endomembrane Changes in the Stigmatic Papillae Following Compatible and Self-Incompatible Pollinations in the Brassicaceae

The *Brassica* papillar cytoplasm is a thin layer surrounding a large central vacuole as well as a network of smaller tubular or round vacuoles (Dearnaley et al. 1997; Iwano et al. 2007). The papillar cytoplasm also utilises small vesicles to secrete proteins into the cell wall (Roberts et al. 1984). Following contact with a compatible pollen grain, structural changes in the cell wall occur with the outer wall expanding beneath the pollen grain. This expansion has not been observed following self-incompatible pollinations (Elleman and Dickinson 1990; Dickinson et al. 2000). While changes in secretory vesicles could not be observed with the addition of compatible or self-incompatible *Brassica* pollen grains, the addition of isolated pollen coat to the stigma resulted in multiple secretory vesicles being targeted to the stigmatic cell wall from the cytoplasm (Elleman and Dickinson 1996). Following pollen coat treatment, there was a rapid expansion of the stigmatic outer cell wall, and the cytoplasm under the extended stigmatic cell wall region frequently had extensive ER and Golgi structures as well as plasma membrane vesiculation. Elleman and Dickinson (1996) proposed a localised secretion event in the zone of pollen–stigma interaction, where pre-loaded vesicles carrying hydration factors and cell wall loosening enzymes discharge at the plasma membrane, resulting in a localised loosening of the cell wall matrix to allow pollen tube penetration (Fig. 8.2a, d). This phase could be regulated by the exocyst by marking the pollen attachment site for polarised exocytosis. During the SI response, vesicles were observed to be accumulating by the unexpanded cell wall (Elleman and Dickinson 1996). If SI leads to a rapid inactivation of Exo70A1 by ARC1, this may result in the accumulation of secretory vesicles, which are unable to dock to the stigmatic plasma membrane at the pollen–stigma interface, and rapid pollen arrest occurs (Fig. 8.2b, e).

More recently, Iwano et al. (2007) found changes in the vacuolar network in *Brassica* stigmatic papillae following pollination. With compatible pollinations, the vacuolar network appears to be directed towards the pollen while self-incompatible pollinations appeared to disrupt the vacuolar network in the stigmatic papillae (Fig. 8.2d, e). Iwano et al. (2007) proposed that these changes were related to promoting water and ion transport to compatible pollen grains while restricting transport to incompatible pollen grains. Interestingly, the vacuolar changes were also linked to altered actin dynamics in the compatible and incompatible pollinations. Compatible pollinations led to an accumulation of actin bundles in the apical region of the stigmatic papilla adjacent to the pollen grain at approximately the start

of pollen hydration. In contrast, self-incompatible pollinations resulted in decreased actin filaments in the apical region, suggesting actin depolymerisation was occurring (Iwano et al. 2007). Interestingly, Boyd et al. (2004) found that while yeast Sec3p and Exo70 arrive at the plasma membrane prior to exocyst assembly, the other six subunits are transported to this site with the secretory vesicles in an actin-dependent manner. The Iwano et al. (2007) study raises the intriguing question of whether the exocyst could play a role in regulating the vacuolar network during compatible pollinations (Fig. 8.2d, e). This would be a novel role for Exo70, but it might in part account for the observed plant-specific expansion of the Exo70 gene family (Cole and Fowler 2006).

8.4 Conclusions and Prospects

The emerging picture, in the pursuit of identifying the molecular mechanisms behind the SI response in the Brassicaceae, has brought us to a critical junction where this response intersects with and inhibits components of the compatibility pathway (with Exo70A1 identified as a key player). Although recent evidence has added to our general understanding of the signalling mechanism, a number of questions still remain unanswered. While the receptor–ligand interaction between SRK and SP11/SCR has been well studied, the biochemical role of MLPK in relation to SRK and ARC1 or how it participates in mediating downstream signalling is not clear. Since the loss of MLPK results in a complete failure of SI, SRK may impart its effects through MLPK and co-ordinately regulate ARC1. For example, activated SRK may bind ARC1 bringing it in close proximity to MLPK for phosphorylation and activation.

Similarly, a number of questions arise for ARC1 and Exo70A1 on their cellular roles and relationships: What is Exo70A1 regulating during a compatible pollination: secretory vesicles and/or the vacuolar network? The analysis of loss-of-function Exo70A1 plants suggests a role for Exo70A1 in the stigmatic papillae during multiple stages following compatible pollinations. This includes pollen hydration, pollen germination and pollen tube penetration of the stigmatic surface. If the vacuolar network is the target of Exo70A1, this may allow for the control of water release to compatible pollen grains. If Exo70A1 is targeting secretory vesicles for membrane fusion, what is the cargo in these vesicles: secretory enzymes for the modification of the papillar surface? Given Exo70A1's essential role in compatible pollen–stigma interactions, it does become a logical target for the SI response to elicit a rapid pollen rejection. How ARC1 regulates Exo70A1 in vivo during SI is still an outstanding question. Finally, a number of other SRK kinase-interactors have been identified such as calmodulin, KAPP and sorting nexin, but their in vivo biological functions in regulating SRK remain to be uncovered. Nevertheless, a number of exciting new players have been uncovered in recent years, and identifying how they participate in pollen–stigma interactions will continue to uncover some very exciting discoveries in the coming years.

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