

# The recognition and rejection of self-incompatible pollen in the *Brassicaceae*

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**ABSTRACT.** Sporophytic self-incompatibility in the *Brassicaceae* is a reproductive strategy that prevents self-fertilization, and as a result, allows genetic diversity to be maintained. The conserved ‘self’ pollen recognition system is necessary due to the close proximity of male and female reproductive organs in the flower. This elaborate system is regulated by two tightly-linked polymorphic *S* genes encoding the pollen-coat protein *S* Cysteine-Rich/*S* Protein-11 (SCR/SP11) and the stigma-specific *S* Receptor Kinase (SRK). When the pollen is recognized as ‘self’, SCR/SP11 binds and activates SRK, and a signaling cascade is triggered in the stigma. While it is known that this signaling pathway leads to rejection of ‘self’ pollen, the cellular events downstream of SRK are less well understood. More recent research has identified new signaling proteins functioning downstream of SRK, such as the *M* Locus Protein Kinase (MLPK) and the ARM-Repeat Containing-1 (ARC1) an E3 ubiquitin ligase. As well, changes in the actin cytoskeleton have been documented. Finally, with the premise that pollen rejection occurs by blocking cellular responses in the stigma required for the acceptance of compatible pollen, Exo70A1 has been identified as a ‘compatibility’ factor that is negatively regulated by the self-incompatibility pathway.

**Keywords:** Actin; ARC1; Exo70A1; MLPK; SCR/SP11; Self-incompatibility; SRK.

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## INTRODUCTION

Members of *Brassicaceae*, *Brassica* and *Arabidopsis*, have become primary model systems for investigating pollen-pistil interactions in species with ‘dry’ stigmas (reviewed in Hiscock and Allen, 2008). The investigations have included extensive research on the perception and rejection of ‘self’ pollen during the self-incompatibility response as well as during compatible pollen-pistil interactions although less is known about the cellular system regulating compatible pollen recognition. With the *Brassica* family having ‘dry’ stigmas, pollen grains are

reliant on the stigma for water transfer to facilitate pollen hydration, and as a result, pollen recognition begins at the point of contact between the stigmatic papilla and pollen surface (Roberts et al., 1980; Dickinson, 1995; Swanson et al., 2004; Wei et al., 2007). Having a selective mechanism in the stigma to control the passage of water to rehydrate the pollen for germination allows for species-specificity, as a dry stigma can selectively regulate water transfer to the pollen grain (Sarker et al., 1988; Zinkl et al., 1999). With these recognition systems in place, if a random pollen grain lands on the stigma from an unrelated flowering species, it is not recognized, and no fertilization occurs (reviewed more extensively in Edlund et al., 2004; Swanson et al., 2004; Hiscock and Allen, 2008; Samuel et al., 2008b).

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## POLLEN RECOGNITION AT THE STIGMATIC SURFACE

When a related pollen grain lands on the stigmatic surface, the pollen grain interacts with a stigmatic papilla, setting off a series of events leading to the acceptance of compatible pollen or the rejection of self-incompatible pollen. The exine of the pollen grain has been implicated in this initial stage of pollen capture while the pollen coat, present in the cavities of the exine, is required for the subsequent step of adhesion to the stigmatic papilla (Zinkl et al., 1999; Mayfield et al., 2001; Murphy, 2006). On the surface of the stigmatic papillae, the waxy cuticular layer and the overlying proteinaceous pellicle are also required for mediating pollen capture and adhesion (Mattson et al., 1974; Stead et al., 1980). The *Brassica* stigmatic proteins, the S-locus Related 1 (SLR1) and S-locus glycoprotein (SLG), have been implicated in the early stages of pollen adhesion; with pollen adhesion reduced when the stigmatic SLR-1 expression was suppressed in transgenic lines and when antibodies were used to mask SLR1 and SLG binding sites on the stigma (Luu et al., 1999). The roles of SLR1 and SLG in pollen adhesion are thought to be mediated through their interactions with pollen coat proteins (SLG binds PCP-A1 and SLR1 binding SLR1-BP) (Doughty et al., 1998; Takayama et al., 2000b). Interestingly, the SLR1 and SLG proteins share similarities to the extracellular domains of the S-Domain Receptor Kinases (e.g., SRK; Shiu and Bleecker, 2003); thus, raising the question of whether some type of receptor signaling event is activated at this stage in the stigmatic papilla to accept the compatible pollen.

At the interface of the compatible pollen-papilla interaction, the contents of both the pollen and stigmatic surfaces mix, leading to the formation of a meniscus-shaped foot (Elleman and Dickinson, 1990; Preuss et al., 1993). Now pollen hydration can occur, through the passage of water from the stigma papilla to the pollen grain at the point of contact (Heslop-Harrison, 1979; Roberts et al., 1984; Elleman et al., 1992; Dickinson, 1995). The pollen coat also plays an essential role in this hydration step, and removal of the pollen coat leads to loss of pollen hydration (Elleman and Dickinson, 1986, 1990; Preuss et al., 1993). For example, GRP-17 pollen coat protein was also found to be required for normal pollen hydration (Mayfield and Preuss, 2000). Interestingly, Iwano et al. (2007) have observed vacuolar movement in the stigmatic papilla in response to compatible pollen, with the vacuolar network re-orienting toward the compatible pollen attachment site. Re-orientation of the vacuolar network required the establishment of filamentous actin bundles in close proximity to the pollen attachment site in the stigmatic papilla (Iwano et al., 2007). Once the hydration of the pollen grain is complete, the pollen germinates, and a pollen tube emerges. The foot at the pollen-papilla contact is also the site of pollen tube penetration into the stigmatic surface (Elleman et al., 1992; Dickinson, 1995). Pollen tube growth through the stigma requires degrading

enzymes to breakdown both the stigmatic cuticle and cell wall, and consistent with this, a number of enzyme activities have been detected in the pollen and stigma (reviewed in Hiscock and Allen, 2008).

## SELF-INCOMPATIBILITY: A PROCESS OF SELF-PERCEPTION AND REJECTION

In the *Brassicaceae*, the self-incompatible response causes pollen arrest at the stigmatic surface, and pollen rejection is controlled by the stigmatic papillae, which effectively disrupt pollen adhesion, hydration, and pollen tube penetration into the stigma (Dickinson, 1995). Upon self-pollination, pollen capture and some pollen adhesion to the stigmatic papilla first occurs prior to the activation of the self-incompatibility pathway. Following the activation of this pathway, 'self' pollen can be inhibited at stages from pre-pollen hydration to pollen tube penetration of the stigmatic surface. Inhibition of hydration is detectable by a decreased axis expansion of the pollen grain when compared to a compatible pollination event (Roberts et al., 1980; Zuberi and Dickinson, 1985). When partial hydration of 'self' pollen is observed, this can result if the pollen grain has achieved sufficient adhesion to the stigmatic papilla to allow for some water transfer; however, it can also result from increased environmental humidity. Pollen germination may not subsequently occur if the degree of hydration is not sufficient (Dickinson and Lewis, 1973). With sufficient pollen hydration, germination and pollen tube formation occurs, but an additional level of 'self' pollen inhibition take place, and the pollen tube is unable to penetrate the stigmatic surface. This indicates that many checks are in place to ensure undesired pollen does not complete the fertilization process (Ockendon, 1972; Carter et al., 1975; Zuberi and Dickinson, 1985).

## The receptor kinase signaling pathway activated in the self-incompatibility response

Extensive research on the genetics of self-incompatibility in *Brassica* species, and more recently for *Arabidopsis lyrata*, have resulted in the identification of highly-polymorphic and tightly-linked pollen and stigma specific *S* genes regulating this system (reviewed in Takayama and Isogai, 2005; Watanabe et al., 2008; Sherman-Broyles and Nasrallah, 2008). In *Brassica*, the first locus to be identified was the stigma-specific *S-locus glycoprotein* (*SLG*; Nasrallah et al., 1985), and while *SLG* appears to have a role in pollen adhesion as described above, it is not required for the self-incompatibility response (Suzuki et al., 2000; Takasaki et al., 2000; Silva et al., 2001; Nasrallah et al., 2004; Boggs et al., 2009c). The second locus identified was the *S Receptor Kinase* (*SRK*) (Stein et al., 1991; Goring and Rothstein, 1992), and this gene confers the self-incompatibility trait in the

stigma (Takasaki et al., 2000; Silva et al., 2001). Finally, *S Cysteine-Rich/S Protein 11 (SCR/SP11)* was the last *S* locus to be identified, and it confers the self-incompatibility trait in the pollen (Schopfer et al., 1999; Takayama et al., 2000a; Shiba et al., 2001). These co-evolved and multi-allelic loci are inherited by progeny as units termed *S* haplotypes. Therefore, in addition to ‘self’ pollination, a self-incompatible pollination occurs when the *S* haplotype of a pollen parent matches that of the female recipient. Conversely, ‘non-self’ or compatible pollination occurs when the *S* haplotypes for the pollen parent and the female recipient are different (Boyes and Nasrallah, 1993; Takayama and Isogai, 2005). Similar *SCR/SP11* and *SRK* loci have been identified in self-incompatible *Arabidopsis lyrata*, and interestingly, these loci carry inactivating mutations in the self-fertile *Arabidopsis thaliana* (Kusaba et al., 2001; Schierup et al., 2001; Boggs et al., 2009c). Furthermore, the transformation of *Arabidopsis lyrata SCR/SP11* and *SRK* genes into *Arabidopsis thaliana* can confer the self-incompatibility trait, but only in some ecotypes (Nasrallah et al., 2004; Boggs et al., 2009b, c).

In the *Brassica* stigmatic papillae, SRK has been found to be localized to endosomes and the plasma membrane and where it is positioned to perceive and bind to the SCR/SP11 protein present in the pollen coat of the self-incompatible pollen (Kachroo et al., 2001; Takayama et al., 2001; Ivanov and Gaude, 2009). Interestingly, in the absence of the SCR/SP11 ligand, SRK forms ligand-independent dimers (Giranton et al., 2000; Shimosato et al., 2007). The extracellular region contains hypervariable subdomains for ligand-specificity as well and presents a high affinity binding site at the plasma membrane for haplotype-specific SCR/SP11 ligand binding (Kemp and Doughty, 2007; Shimosato et al., 2007; Boggs et al., 2009a). Binding of the SCR/SP11 ligand results in the phosphorylation and activation of the SRK kinase domain, which in turn leads to further signaling within the stigmatic papilla to produce the pollen rejection response (Kachroo et al., 2001; Takayama et al., 2001; Shimosato et al., 2007).

Receptor kinase activation typically results in downstream signaling proteins interacting with the activated kinase domain, and consistent with this, several proteins have been found to interact with the SRK kinase domain (Bower et al., 1996; Gu et al., 1998; Vanoosthuyse et al., 2003; Kakita et al., 2007a, b). Two *Brassica* thioredoxin *h* proteins, THL1 and THL2, were isolated first (Bower et al., 1996). The thioredoxin *h* proteins were found to negatively regulate SRK as well as the self-incompatibility response and were proposed to maintain SRK in an inactive state in unpollinated stigmas (Cabrillac et al., 2001; Haffani et al., 2004). Interestingly, THL1 was found to partially co-localize with SRK in the endosomes, but could not be detected at the plasma membrane (Ivanov and Gaude, 2009); thus, whether thioredoxin *h* inhibition of SRK occurs at the plasma membrane is not clear. Nevertheless, this inhibition is proposed to be released

with SCR/SP11 ligand binding. A recessive *mod* mutation which causes a breakdown in *Brassica* self-incompatibility led to the discovery of the *M Locus Protein Kinase (MLPK)* encoded by the *mod* locus (Murase et al., 2004). MLPK was found to have serine/threonine activity, and membrane localization of MLPK is required for the self-incompatibility response (Murase et al., 2004; Kakita et al., 2007a). MLPK is predicted to interact with SRK at the plasma membrane, and this interaction was demonstrated in BY-2 cells as well as phosphorylation studies (Kakita et al., 2007a, b). Thus, MLPK and SRK may form a complex to activate downstream signaling proteins to set the self-incompatibility cellular cascade in motion.

An example of a downstream player is another *Brassica* interacting protein, ARC1, an E3 ubiquitin ligase also required for the self-incompatibility response (Gu et al., 1998; Stone et al., 1999). The ARC1 protein contains a novel N-terminal domain (UND), followed by a U-box domain and an ARM repeat domain (Samuel et al., 2006). The binding of ARC1 to the phosphorylated SRK kinase domain *in vitro* is mediated by the ARM repeat domain (Gu et al., 1998), but interestingly, ARC1 serves as a much better substrate for *in vitro* phosphorylation by MLPK, supporting the idea of an SRK-MLPK complex activating ARC1 (Samuel et al., 2008a). Because ARC1 is proposed to target a substrate for degradation in the self-incompatibility response, it is reasonable to speculate that ARC1 functions as an inhibitor of compatibility factors in pollination events as a pollen rejection mechanism (Stone et al., 2003). Not as much is known about the cellular events occurring in a stigmatic papilla during a compatible pollen interaction. Whether activation of this ARC1 E3 ubiquitin degradation pathway is directly responsible for the rejection of self-incompatible pollen has yet to be confirmed, but recently, a new compatibility factor, Exo70A1, which may be the substrate for ARC1’s ubiquitin-mediated degradation pathway in this self-incompatibility response, was identified in both *Brassica* and *Arabidopsis* (Samuel et al., 2009).

### The intersection of the compatible and self-incompatible pollen response pathways in the stigma

*Exo70A1* was pulled out as a potential interactor of ARC1, and RNAi inhibition of *Exo70A1* in *Brassica* stigmatic papillae resulted in a constitutive rejection of compatible pollen (Samuel et al., 2009). In addition to the self-incompatibility response, defects in pollen hydration, germination, and penetration of the pollen tube through the papillar surface were observed, and this phenotype was conserved in *Arabidopsis exo70A1* mutants (Samuel et al., 2009). Furthermore, the expression of an *RFP:Exo70A1* fusion construct in the stigma of the *exo70A1* mutants restored the acceptance of compatible pollen. Interestingly, RFP:Exo70A1 was found to be localized to the plasma membrane in mature stigmatic papillae before

it disappeared from this location following contact with compatible pollen. In other systems, Exo70 has been found to be a subunit of the exocyst complex involved in tethering vesicles to the plasma membrane during polarized or regulated secretion (He and Guo, 2009). Thus, perhaps Exo70A1's role in the stigmatic papillae in response to a compatible pollination is to promote pollen acceptance through the targeted secretion of stigmatic factors at the pollen attachment site. In contrast, during a self-incompatible response, Exo70A1's function is blocked resulting in pollen rejection (Samuel et al., 2009).

## FUTURE OUTLOOK

In recent years, significant progress has been made in strengthening our understanding of the molecular and cellular steps behind the self-incompatibility response in the *Brassicaceae*. However, there are areas that still require further dissection, and current research is aimed at more fully understanding the specific signaling events that take place in the stigmatic papillae following pollination. For example, efforts have focused on identifying residues within the SRK extracellular domain that are essential for its activation by the SCR/SP11 ligand. It appears that a surprisingly small number of residues are required, and that the three-dimensional conformation of these regions is a determining factor (Boggs et al., 2009a). Outstanding questions also encompass the regulatory role of MLPK, in relation to SRK, on downstream signaling steps including ARC1 and perhaps other unknown signaling proteins or events. For example, what proteins are responsible for the observed decrease in actin bundles and disruption of the vacuolar network in the stigmatic papilla following self-incompatible pollinations (Iwano et al., 2007)? As well, the relationship between ARC1 and Exo70A1 and their cellular roles in the stigmatic papillae need to be better defined. For example, how does Exo70A1, in the context of exocyst and polarized secretion, promote pollen hydration and pollen tube penetration? In summary, the dissection of cellular events activated in the self-incompatibility response in the *Brassicaceae* has started to uncover steps in the compatible pollen response pathway that are inhibited (Exo70A1, actin bundles), and future investigation of their mechanisms of action will result in more important discoveries in the general regulation of pollen-pistil interactions.

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