Chapter 21
Self-Incompatibility in the Brassicaceae

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Abstract Self-incompatibility (SI) in angiosperms prevents inbreeding and promotes outcrossing to generate genetic diversity. SI in the Brassicaceae is controlled by the *S*-haplotype-specific interaction between pollen ligand (*S*-locus protein 11, SP11 or SCR) and its stigmatic receptor (*S*-receptor kinase, *SRK*). SP11/SCR binding to cognate SRK induces autophosphorylation of SRK, which triggers a signaling cascade leading to the rejection of self-pollen. However, the mechanism of self-pollen rejection downstream of this ligand–receptor interaction is unknown. Here, we generated self-incompatible *Arabidopsis thaliana* accession C24 for the forward-genetic approach and live-cell imaging of SI in the Brassicaceae. Furthermore, for reverse-genetic analysis, we extended the Arabidopsis Targeting Induced Local Lesions IN Genomes (TILLING) resources by developing a new population of ethyl methanesulfonate (EMS)-induced mutant lines in *A. thaliana* accession C24. We believe that the reverse-genetic approach is a useful tool for identifying genes that function in the SI signaling pathway of the Brassicaceae.

Keywords Arabidopsis • Brassicaceae • Self-incompatibility • TILLING

21.1 Introduction

Angiosperms have developed self-incompatibility (SI) as a genetic system to prevent inbreeding and thereby promote outcrossing to generate genetic diversity. SI is based on the self/non-self discrimination between male and female. In many angiosperms, SI is controlled by a single locus, designated *S*, with multiple haplotypes (de Nettancourt 2001). Each *S*-haplotype encodes both male-specificity and

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female-specificity determinants (*S*-determinants), and self/non-self discrimination is accomplished by the *S*-haplotype-specific interaction between these *S*-determinants. Both the male and female determinants are polymorphic and are inherited as one segregating unit. The variants of this gene complex are called *S*-haplotypes. Self/non-self recognition operates at the level of protein–protein interactions between the two determinants, and an incompatible response occurs when both determinants originate from the same *S*-haplotype.

21.2 Self/Non-Self Recognition System in the Brassicaceae

In the Brassicaceae, the self/non-self discrimination between male and female occurs on papilla cells covering the stigma surface of the pistil. When cross-pollen lands on the papilla cell, the pollen hydrates and germinates. The pollen tube penetrates the surface of the papilla cell and enters the style, ultimately resulting in cross-fertilization. By contrast, when self-pollen lands on the papilla cell, pollen hydration and germination are inhibited (Fig. 21.1).

The female determinant is *S*-receptor kinase (SRK) (Takasaki et al. 2000). SRK consists of an SLG-like extracellular domain, a transmembrane domain, and an intracellular serine/threonine kinase domain. SRK spans the plasma membrane of the stigma papilla cell. The male determinant is *S*-locus protein 11 (SP11; also called *S*-locus cysteine-rich protein, SCR) (Schopfer et al. 1999; Takayama et al. 2000). SP11 is a small basic cysteine-rich protein that is predominantly expressed in the anther tapetum and accumulates in the pollen coat during pollen maturation (Iwano et al. 2003) (Fig. 21.2). Upon pollination, SP11/SCR penetrates the papilla cell wall and binds SRK in an *S*-haplotype-specific manner. This binding induces the autophosphorylation of SRK, triggering a signaling cascade that results in the rejection of self-pollen (Takayama et al. 2001; Takayama and Isogai 2005; Iwano and Takayama 2012) (Fig. 21.3).

The self-recognition, that is, the *S*-haplotype-specific interaction between SP11 and its cognate SRK, has been shown by a series of biochemical studies in *Brassica rapa*. A binding experiment using ^{125}I-labeled-S_8-SP11 suggested that it strongly

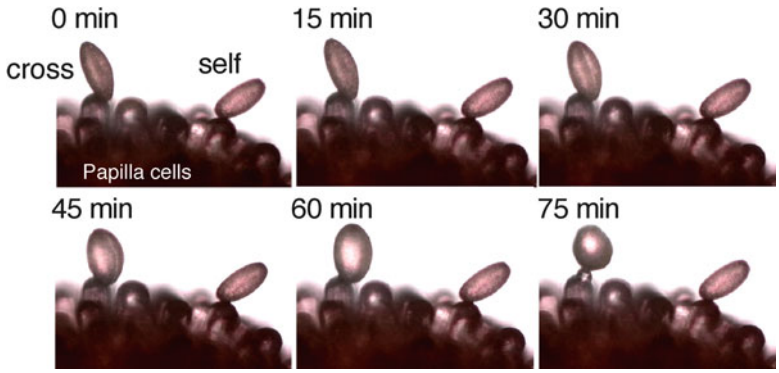

Fig. 21.1 Self- and cross-pollination in *Brassica rapa*

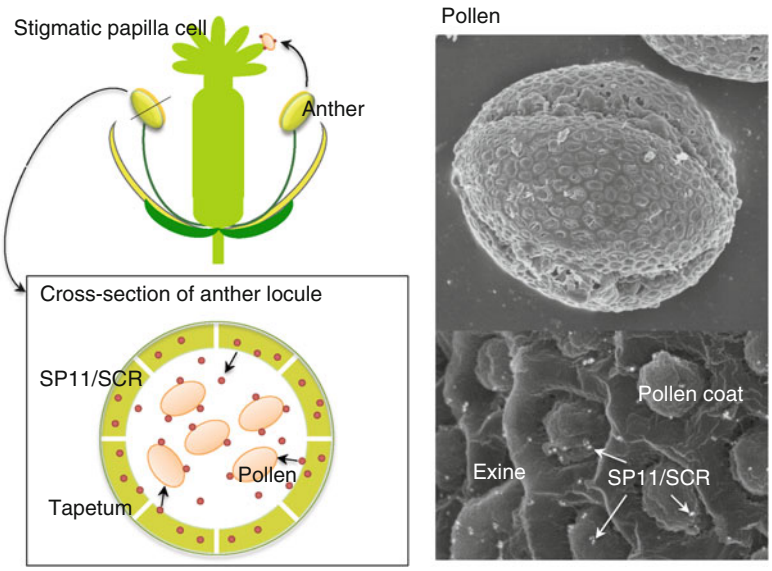

Fig. 21.2 Expression of SP11/SCR in the anther locule and localization of SP11/SCR on the pollen surface

binds to the stigmatic membrane of S_8-haplotype ($K_d = 0.7$ nM) but not of the *S*-haplotype (Takayama et al. 2001). Cross-linking and immunological analyses suggested that ^{125}I-labeled-S_8-SP11 directly binds to S_8-SRK and a 60-kDa protein in the stigmatic membrane of S_8-haplotype (Takayama et al. 2001). Affinity purification and LC-MS/MS analysis of SP11-binding stigmatic proteins have revealed that the 60-kDa protein is a truncated form of SRK (tSRK) containing the extracellular, transmembrane, and part of the intracellular juxtamembrane domains (Shimosato et al. 2007). Interestingly, an artificially expressed dimerized form of eSRK exhibited high-affinity binding to SP11. Another recent study suggested that two regions in the extracellular domain of SRK mediated the homo-dimerization of eSRK (Naithani et al. 2007). Taken together, these studies suggested that SRK on the stigmatic membrane is in an equilibrium between the inactive monomeric or dimeric low-affinity forms and the dimeric active high-affinity form, and that the SP11/SCR binding to its cognate SRK stabilizes its dimeric active form, which is expected to trigger the SI responses in the papilla cell (Shimosato et al. 2009) (Fig. 21.3).

21.3 SI Signaling Cascade Leading to Rejection of Self-Pollen

To date, the only candidates for signaling molecules acting downstream of SP11/SRK have been MLPK, the membrane-anchored *M*-locus protein kinase (Murase et al. 2004), and ARC1, an arm repeat-containing protein with E3 ubiquitin-ligase

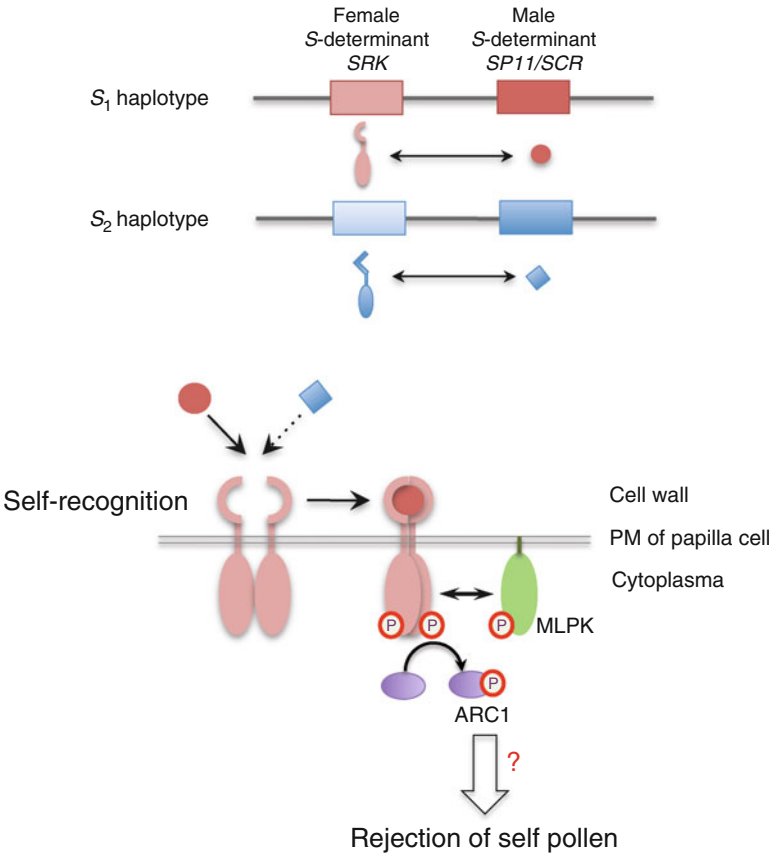

Fig. 21.3 Self-recognition self-incompatibility (SI) in Brassicaceae. The *S*-locus encodes female and male *S*-determinants, designated SRK and SP11 (or SCR), respectively. In self-pollination, the self (same *S*-haplotype)-specific SP11 binding to its cognate SRK stabilizes SRK in an active dimeric form on plasma membrane (PM), which triggers SI responses in the stigmatic papilla cell. MLPK and ARC1 were the only SP11/SRK downstream signaling molecular candidates

activity (Stone et al. 1999) (Fig. 21.3). MLPK was identified as a positive mediator of SI signaling in a genetic analysis of a self-compatible mutant of *B. rapa* var. Yellow Sarson (Murase et al. 2004). Upon self-pollination, MLPK is thought to interact directly with SRK to form an SRK-MLPK receptor complex on the plasma membrane and enhance SI signaling (Kakita et al. 2007). ARC1 is a potent positive mediator of this signal transduction pathway (Stone et al. 2003), and can be phosphorylated in vitro by both SRK and MLPK, suggesting that ARC1 is recruited to an SRK-MLPK complex at the plasma membrane (Samuel et al. 2008). ARC1 is predicted to promote self-pollen rejection in the self-incompatibility response by negatively regulating Exo70A1 and blocking the delivery of secretory vesicles to the pollen contact site (Samuel et al. 2009). However, the observation that

suppression of *ARC1* expression results in incomplete breakdown of SI in both *Brassica napus* and *Arabidopsis lyrata* (Stone et al. 1999; Indriolo et al. 2012) might suggest the existence of another unknown signaling pathway acting downstream of SP11/ SRK.

Pollen hydration is the earliest step of cross-pollination. The regulation of water transport from a papilla cell to a pollen grain is one of the most important steps in the rejection of self-pollen. The lipid and proteinaceous components of the pollen coat are essential to pollen hydration during pollen-foot formation in *Brassica oleracea* (Elleman and Dickinson 1986). In *B. rapa*, monitoring transiently expressed GFP-mTalin, and rhodamine-phalloidin staining showed the concentration of actin bundles at the cross-pollen attachment site and actin reorganization at the self-pollen attachment site (Iwano et al. 2007). Additionally, the application of cross-pollen coat induces actin polymerization in the apical region of the papilla, whereas the application of self-pollen coat is associated with a decrease in actin filaments in the apical region. The actin-depolymerizing drug cytochalasin D (CD) significantly inhibited pollen hydration and germination during cross-pollination, further emphasizing a role for actin in these processes. Furthermore, electron tomography using ultrahigh-voltage electron microscopy revealed the close association of the actin cytoskeleton with an apical vacuole network. Self-pollination disrupted the vacuole network, whereas cross-pollination led to vacuolar rearrangements toward the site of pollen attachment. Taken together, these data suggested that self- and cross-pollination differentially affect the dynamics of the actin cytoskeleton, leading to changes in vacuolar structure that might be associated with hydration and germination (Iwano et al. 2007). In *B. napus* and *Arabidopsis thaliana*, immunostaining using anti-tubulin antibodies found that moderate changes in the microtubule network were observed after self-incompatible pollinations, but a more distinct localized breakdown of the microtubule network was observed during compatible pollinations (Samuel et al. 2011). Visualization over time of the morphological and physiological changes in the stigmatic papilla cell during self- and cross-pollination is a useful method for understanding SI. However, the application of live-cell imaging to *B. rapa* and *B. oleracea* has been difficult because of the low efficiency of transformation and the variability of pollination timing. To investigate the SI downstream signaling cascade leading to rejection of self-pollen in the Brassicaceae, generation of SI *Arabidopsis* was thought to be useful.

21.4 Generations of SI *Arabidopsis*

Arabidopsis thaliana is a popular model plant in the Brassicaceae. *A. thaliana* is the first plant to have its genome sequenced and is a popular tool for understanding the molecular biology of many plant traits. Its small size, rapid life cycle, and easy genetic transformation are also advantageous for research. In addition, this plant is well suited for live-cell imaging during pollination. However, *A. thaliana* is a self-compatible species; by contrast, *Arabidopsis lyrata* is a self-incompatible species in

genus *Arabidopsis*. Previously, a comparative analysis of the *S*-locus region of *A. lyrata* and its homologous region in *A. thaliana* (Col-0) identified orthologues of the *SRK* and *SCR* genes (Kusaba et al. 2001). However, none of the three candidate SCR orthologues was predicted to encode full-length SCR proteins; therefore, they were designated *ΨSCR1, ΨSCR2*, and *ΨSCR3*. The predicted *SRK* orthologue was also thought to be inactive because it contains a premature stop codon. Thus, self-compatibility in *A. thaliana* is associated with the inactivation of SI specificity genes. Introduction of functional *SP11*/*SCR* and *SRK* gene pairs isolated from *A. lyrata* into *A. thaliana* accession C24 conferred stable SI responses (Nasrallah et al. 2004). In the resulting SI *Arabidopsis*, pollen hydration and germination were arrested after self-pollination with SP11/SCR pollen, but normal pollen germination and pollen tube penetration were observed after pollination with wild-type (WT) pollen (SC). The establishment of the monitoring systems using this SI *Arabidopsis* is a useful tool to visualize the physiological events during SI- and SC-pollination.

21.5 A TILLING Resource for Functional Genomics in *Arabidopsis thaliana* Accession C24

Many reverse-genetic resources have been developed for functional genetic studies. Because site-directed mutagenesis is not effective in plants, random mutagenesis approaches, including insertional (Wisman et al. 1998; Alonso et al. 2003), chemical (McCallum et al. 2000), and fast neutron mutagenesis (Li et al. 2001), have been used to establish reverse-genetic platforms. In *Arabidopsis*, insertional mutagenic techniques using T-DNA or transposons have become popular tools for functional genomics. However, insertional mutagenesis often leads to complete gene knockouts, making it difficult to associate nuanced phenotypes with essential genes (Jander et al. 2002). Similarly, radiation mutagenesis, for example, fast neutrons, often induces large genomic deletions that affect multiple genes (Li et al. 2001). By contrast, classical chemical mutagenesis using a mutagen such as ethyl methanesulfonate (EMS) induces an array of interesting point mutations with different impacts on gene function. Such allelic series are desirable because they generate a wide repertoire of mutant phenotypes covering a range of severity, which provide more insight into a gene's function. Moreover, individual plants carrying point mutations can be identified easily through a powerful method called TILLING (Targeting Induced Local Lesions IN Genomes).

TILLING is a reverse-genetic method that takes advantages of classical mutagenesis, sequence databases, and high-throughput PCR-based screening for point mutations in a targeted sequence (Henikoff et al. 2004). The key advantage of TILLING over competing methods is that it can be applied to any plant species, regardless of ploidy level, genome size, or genetic background (Kurowska et al. 2011). TILLING extends genomic resources, particularly in organisms lacking reverse-genetic tools, where mutants with a range of phenotypic severity are highly desirable. Since the inception of TILLING, this method has been applied to various organisms including

Table 21.1 Ethyl methanesulfonate (EMS)-mutagenized TILLING resources in the Brassicaceae

Species	Ploidy level	Population size (line)	Mutation density (kb^{-1})	References
Arabidopsis thaliana (Col-1)	2*x*	3,072	1/300	Greene et al. (2003)
Arabidopsis thaliana (Ler)	2*x*	3,712	1/89	Martín et al. (2009)
Arabidopsis thaliana (C24)	2*x*	3,509	1/345	Lai et al. (2013)
Brassica oleracea	2*x*	2,263	1/447	Himelblau et al. (2009)
Brassica rapa	2*x*	9,216	1/60	Stephenson et al. (2010)

Cucumis melo L. (González et al. 2011), *Solanum lycopersium* (Minioa et al. 2010), *B. napus* (Wang et al. 2008; Harloff et al. 2012), *B. oleracea* (Himelblau et al. 2009), *B. rapa* (Stephenson et al. 2010), *Lotus japonicus* (Perry et al. 2009), *Zea mays* (Till et al. 2004), *Oryza sativa* (Till et al. 2007), *Drosophila* (Winkler et al. 2005), and zebrafish (Wienholds et al. 2003).

To date, *Arabidopsis* TILLING resources are only available in accessions Columbia (Col-0) (Greene et al. 2003) and Landsberg *erecta* (L*er*) (Martín et al. 2009). Reverse genetic tools for many commonly used *Arabidopsis* accessions are still limited, in particular accession C24, which is genetically distinct from accession Col-0 (Barth et al. 2002; Törjek et al. 2003). C24 is distinguished physiologically from other familial accessions in terms of tolerance to drought (Bechtold et al. 2010), ozone (Brosche et al. 2010), and frost (Rohde et al. 2004), and enhanced basal resistance to pathogens (Bechtold et al. 2010). The transgenic *A. thaliana* accession C24 also exhibited a robust and stable self-incompatible (SI) phenotype (Rea et al. 2010), which served as a good model for understanding SI signaling. In addition, a large portion of its genomic sequence was available (Schneeberger et al. 2011), making accession C24 an excellent alternative tool for plant research.

To take advantage of this tool, the Arabidopsis TILLING resources from the GAH molecules by developing a new population of EMS-induced mutant lines in *A. thaliana* accession C24 X (Lai et al. 2013). From approximately 8,000 *A. thaliana* C24 seeds treated with 25 mM EMS, 3,620 M1 seedlings were obtained, all of which were used to generate the M2 population. An M2 population with a total of 3,509 individual plants was successfully recovered for use in TILLING. This M2 population also contained 77 partial-seed set lines (semi-sterile) and 125 very low seed set lines (sterile). This population, including semi-sterile and sterile phenotypes, represents a valuable genetic resource for use in forward-genetic screens aimed at isolating novel genes affecting reproduction. Each M2 plant sampled for DNA used in TILLING was originally isolated from a distinct individual M1 plant to ensure independence of the mutations within the population. Ultimately, DNA from M2 plants and M3 seeds from 3,509 lines were stored for TILLING analysis (Lai et al. 2013). The TILLING collection represents the third TILLING resource reported for *A. thaliana* to date (Table 21.1). TILLING for selected genes from this new collection successfully identified allelic series of induced point mutations, including sense, missense, and nonsense mutations.

21.6 Conclusion

Self-recognition in the Brassicaceae is clearly mediated by a haplotype-specific interaction between pollen ligand (SP11/SCR) and its stigmatic receptor kinase (SRK). To clarify the downstream signaling pathway leading to self-pollen rejection, SI *Arabidopsis* was generated by the introduction of functional SP11/SCR and SRK gene pairs isolated from *A. lyrata* into *A. thaliana* accession C24 and conferred stable SI responses. The SI *Arabidopsis* is useful for the forward-genetic approach and live-cell imaging. In addition, TILLING resource was established in *A. thaliana* accession C24 for reverse genetic approach. The combination of forward- and reverse-genetic approaches with live-cell imaging will be a useful tool for identifying genes that function in the SI signaling pathway in the Brassicaceae.

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