Chapter 25
Self-Incompatibility System of *Ipomoea trifida*, a Wild-Type Sweet Potato

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Abstract Diploid *Ipomoea trifida* (Convolvulaceae) is a close relative of the cultivated hexaploid *Ipomoea batatas*, the cultivated sweet potato. These plants have sporophytic self-incompatibility that is regulated by a single multiallelic locus, designated as the *S*-locus. Genetic analyses of *I. trifida* plants collected from Central America identified about 50 different *S*-haplotypes with a linear dominance hierarchy having some codominance relationships. A linkage map of DNA markers around the *S*-locus indicated that the *S*-locus is delimited to 0.23 cM. Within the *S*-locus genomic region, a hypervariable genomic region of 35–95 kbp was identified, and we designated this region SDR (*S*-locus-specific divergent region). Of the several genes located within the SDR, one anther-specific gene, *AB2*, and three stigma-specific genes, *SE1*, *SE2*, and *SEA*, are candidate *S*-genes that may encode male and female *S*-determinants of self-incompatibility.

Keywords *Ipomoea trifida* • Self-incompatibility • Sweet potato

25.1 Introduction

Self-incompatibility (SI) is a genetic mechanism to prevent self-fertilization (and thus encourage outcrossing) in angiosperms. In self-incompatible plants, when a pollen grain is recognized as the same type as self, some stage of pollen germination, pollen tube elongation, ovule fertilization, or embryo development is halted, and no seeds are produced. SI is classified into several groups: homomorphic SI, heteromorphic SI, cryptic SI (CSI), and late-acting SI. Heteromorphic SI is classified into two groups: distyly is determined by a single locus, which has two alleles,

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and tristyly is determined by two loci, each with two alleles. Heteromorphic SI is sporophytic, in that both alleles in the male plant determine the SI response in the pollen. CSI exists in a limited number of taxa (for example, there is evidence for CSI in *Silene vulgaris* in the Caryophyllaceae; Glaettli 2004). In this mechanism, the simultaneous presence of cross- and self-pollen on the same stigma results in higher seed set from cross-pollen (Bateman 1956). Late-acting SI is also termed ovarian SI. In this mechanism, self-pollen germinates and reaches the ovules, but no fruit is set (Seavey and Bawa 1986; Sage et al. 1994).

Homomorphic SI is classified into sporophytic SI (SSI) and gametophytic SI (GSI) . The SSI system is found in species of several plant families, such as the Brassicaceae, Asteraceae, Malvaceae, Betulaceae, Sterculiaceae, Polemoniaceae, and Convolvulaceae (de Nettancourt 2001; Allen and Hiscock 2008). In the plants in these families, SI is genetically regulated by a single multi-allelic locus, the *S*-locus. Within the *S*-locus, a pair of genes (named *S*-genes), one encoding the male-determinant molecules and the other the female-determinant molecules, is localized. These genes are essential for the SI reaction, because these gene products contribute to self/non-self recognition. The sets of *S*-genes are tightly linked at the *S*-locus, and the *S*-locus (also called *S*-haplotype: Nasrallah and Nasrallah 1993), is inherited as a single unit to maintain the SI system. In the SSI system, self-pollen rejection is observed as the arrest of pollen germination, or pollen tube penetration into the stigma cell, and therefore self/non-self recognition occurs on the surface of the stigma. The male *S*-gene is sporophytically expressed in the tapetum of the anther, and the product of the male *S*-gene (*S*-protein) is deposited onto the pollen surface. The male phenotype of the SSI plant is determined by the diploid *S*-haplotypes of the pollen-producing plant; therefore, determination of the male *S*-phenotype is under the control of dominant–recessive relationships. On the other hand, the female *S*-gene is expressed in the papilla cells of the stigma. SSI of the Brassicaceae is well characterized at the molecular level; *SP11*/*SCR* and *SRK* are the male and female *S*-genes, respectively. SP11/SCR acts as a ligand of the membrane-anchored protein kinase SRK; these two determinants interact when self-pollination occurs, which induces a phosphorylation pathway and inhibits self-pollen germination.

The GSI system is found in the Solanaceae, Rosaceae, Fabaceae, Papavaraceae, and Poaceae, and in most cases it is regulated by a single *S*-locus, except in the Poaceae, which has both *S*- and *Z*-loci. In *S*-RNase-mediated GSI, the female *S*-determinant, *S*-RNase, is taken up into the elongating pollen tube and degrades RNA molecules that are recognized as self. In the Papavaraceae, the female *S*-determinant, PrsS, acts as a ligand of the pollen tube membrane-anchored receptor/channel male *S*-determinant, PrpS. After recognition of the pollen grain as self, the Ca^{2+} concentration in the pollen tube is increased, and actin is depolymerized, resulting in programmed cell death.

Ipomoea trifida is a close relative of the cultivated sweet potato *Ipomoea batatas*. Self-incompatible plants of the genus *Ipomoea* show SSI; however, this SI is strong, and is active even in hexaploid species, such as *I. batatas* (Fig. 25.1). Moreover, the SSI of *Ipomoea* may be regulated by a different mechanism than SSI in *Brassica*. In this chapter, this unique SSI system of *I. trifida* is described.

Fig. 25.1 Flowers of diploid *Ipomoea trifida*. Floral structures of plants in the Convolvulaceae are similar to each other, with funnel-shaped, radially symmetrical corolla, five sepals, five fused petals, and five epipetalous stamens. The flowering of this plant is enhanced under short-day conditions with relatively high temperature

25.2 Origin, Domestication, and Compatibility of *Ipomoea* Plants

Cultivated sweet potato, *I. batatas*, and its wild relatives belong to the section Batatas. The center of the origin and domestication of the sweet potato is thought to be either in Central America or South America. In Central America, domestication of the sweet potato might have started at least 5,000 years ago, and in South America, Peruvian sweet potato remnants dating as far back as 8000 BC have been found. Austin (1988) postulated that the center of origin of *I. batatas* was between the Yucatán Peninsula of Mexico and the mouth of the Orinoco River in Venezuela. Zhang et al. (1998) provided strong supporting evidence that the geographic zone postulated by Austin is the primary center of diversity. The much lower molecular diversity found in Peru and Ecuador suggests this region should be considered a secondary center of sweet potato diversity. The sweet potato was also grown in Polynesia. Sweet potato was cultivated in the Cook Islands in 1000 AD, and it may have been brought to central Polynesia around 700 AD, and spread across Polynesia to Hawaii and New Zealand from there (van Tilburg 1994; Bassett et al. 2004). A theory that the plant could have spread by seeds floating across the ocean is not supported by evidence. Another point is that the sweet potato in Polynesia is the cultivated *I. batatas*, which is generally spread by vine cuttings and not by seeds (Fig. 25.2).

Charles Darwin described outcrossing in higher plants and the diversity of reproduction modes (Darwin 1876). He described inbreeding depression based on his experiments with morning glory, *Ipomoea purpurea*. The family Convolvulaceae contains 55 genera and more than 1,500 species (Austin 1997), and the genus *Ipomoea* is the largest member of the Convolvulaceae, with more than 500 species. The genus occurs throughout the tropical and subtropical regions of the world, including both annual and perennial herbaceous plants, and most species are twining climbing plants. The floral structure of the genus *Ipomoea* is almost the same, a

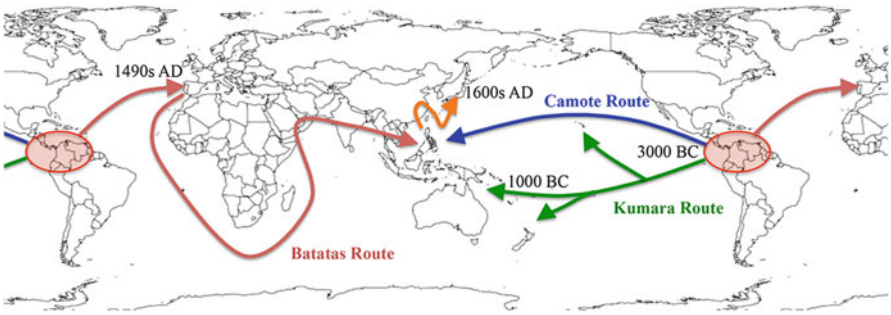

Fig. 25.2 Domestication and distribution of sweet potato. The center of origin and domestication of the sweet potato is thought to be in either Central America or South America. In Central America, sweet potatoes were domesticated at least 5,000 years ago. During the ancient era, sweet potato was thought to be distributed from Central America to the Pacific Islands by two routes, the Camote and the Kumara routes. After Columbus introduced sweet potato to Europe, the sweet potato was distributed to South and Southeast Asian countries, including Japan

gamopetalous flower, resembling that of the morning glory. This genus *Ipomoea* is classified into several sections based on reproductive and morphological traits (Austin and Huáman 1996). For instance, the section Pharbitis includes both the self-incompatible *Ipomoea serifera* and the self-compatible species *I. purpurea*; both horticultural varieties are known as morning glory. This classification is also supported by genetic analysis based on chloroplast DNA restriction site variations (McDonald and Mabry 1992) in section Batatas, which includes sweet potato and its wild relatives, series Pharbitis, which includes Japanese morning glory, and section Tricolor, which includes western morning glory, and others.

About 50 species belong to section Batatas. The site of origin of these plants is considered to be the same region as sweet potato. Plants in the section Batatas can be classified into two groups, one cross-compatible with sweet potato, *I. batatas*, and the other not. For example, South American species *Ipomoea triloba* ($2n=2x=30$), *Ipomoea tiliacea* ($4x$), and *I. trifida* ($2x$, $4x$, $6x$) are cross-compatible with *I. batatas* ($6x$); however, *Ipomoea umbraticola* ($2x$) is not (Table 25.1). Plants belonging to section Batatas can be alternatively classified as belonging to a self-incompatible (group A) and a self-incompatible (group B) (Table 25.2) (Nishiyama et al. 1975). In group B, the diploid species *I. trifida* and the tetraploid species *Ipomoea tabascana* are most closely related to the hexaploid sweet potato *I. batatas* (Rajapakse et al. 2004). However, according to its chromosome number and SI, *I. trifida* seems to be the wild species most closely related to *I. batatas*, and Huang and Sun (2000) revealed that *I. trifida* is the ancestral species of *I. batatas*, according to inter-simple sequence repeat and restriction analyses of chloroplast DNA. Artificial hexaploid *I. trifida* has also been produced (Shiotani and Kawase 1987); currently, it is understood that *I. batatas* is an autohexaploid of diploid *I. trifida*, produced by several chromosomal duplication and crossing events (Fig. 25.3) (Shiotani and Kawase 1989).

Table 25.1 Cross-incompatibility of sweet potato (*Ipomoea batatas*) with its wild relatives

	Origin		
	North America	South America	Caribbean region
Cross-compatible with *I. batatas* (6*x*)	*I. lacunosa* (2*x*) *I. cordatotriloba* (2*x*)		*I. triloba* (2*x*) *I. tiliacea* (4*x*) *I. trifida* (2*x*, 4*x*, 6*x*)
Cross-incompatible with *I. batatas* (6*x*)	*I. tenuissima* (2*x*)	*I. ramosissima* (2*x*) *I. grandifolia* (2*x*)	*I. umbraticola* (2*x*)

The wild population of plants that belong to section Batatas are classified into two major groups, one cross-compatible with cultivated sweet potato, and the other cross-incompatible. *I. trifida*, the close relative to cultivated sweet potato, is cross-compatible with sweet potato, despite the varied ploidy level

Table 25.2 Classification of several species of *Ipomoea* in section Batatas according to their self-incompatibility

	Scientific name	Chromosome numbers
Group A: self-compatible	*I. lacunosa*	$2n=30$
	I. cordatotriloba	$2n=30$
	I. tenuissima	$2n=30$
	I. triloba	$2n=30$
	I. ramosissima	$2n=30$
	I. cynanchifolia	$2n=30$
Group B: self-incompatible	*I. perviana*	$2n=30$
	I. gracilis	$2n=30$
	I. tiliacea	$2n=60$
	I. trifida	$2n=30, 60, 90$
	I. batatas	$2n=90$

Plants belonging to section Batatas are also classified under their mode of reproduction: self-compatibility (group A) and self-incompatibility (group B)

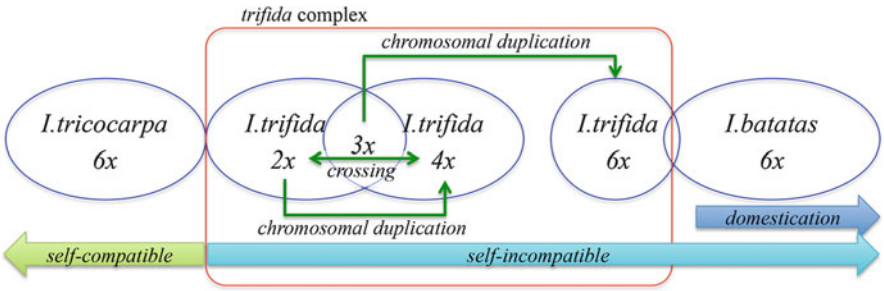

Fig. 25.3 Establishment of *Ipomoea batatas* from *Ipomoea trifida*. *I. batatas* ($2n=6x=90$) is thought to be an autohexaploid of diploid *I. trifida*. Hexaploid *I. trifida* was produced through two chromosomal duplications and crossing in the wild population, and the resulting plant became the ancestral species of *I. batatas*, the cultivated sweet potato. During these stages, self-incompatibility did not break down. However, once self-incompatibility was broken down, the reproduction mode was not resumed, and a self-compatible species was established

In the practical breeding of sweet potato, cross-incompatibility between parental plants is a genetic barrier to producing hybrids because the choice of parents for cross-pollination is sometimes limited to a small number of lines. *I. trifida*, a diploid species of the section Batatas, is a useful genetic resource in sweet potato breeding (Shiotani and Kawase 1987). *I. trifida* is an herbaceous insect-pollinated weed native to Central America and has a relatively small genome (532 Mbp, 831 Mbp per haploid) (Arumuganathan and Earle 1991; Ozias-Akins and Jarret 1994).

25.3 SI of *Ipomoea* Plants

Compatible cross-pollinating pollen grains germinate about 10–20 min after pollination (Kowyama et al. 2008), with pollen grains attaching to stigma surface cells called papilla cells (Fig. 25.4). However, in the case of incompatible self-pollination, pollen germination is arrested, not resulting in seed formation. In the SI system of *I. trifida*, the self-recognition reaction between male and female *S*-determinants (*S*-gene products) occurs rapidly after pollination. Kowyama et al. (1980, 1994) reported that the SI phenotype of *I. trifida* segregates as a single multi-allelic *S*-locus, and the pollen phenotype of SI is regulated sporophytically with clear dominant–recessive relationships between *S*-haplotypes. These features are consistent with the general features of SSI. In *Brassica*, CO_2 treatment and bud pollination can permit self-pollination (Hinata et al. 1994); however, these artificial techniques are not applicable in *Ipomoea*. From this point of view, it is conceivable that the SSI mechanism of *Ipomoea* is stronger and clearer than in *Brassica* plants.

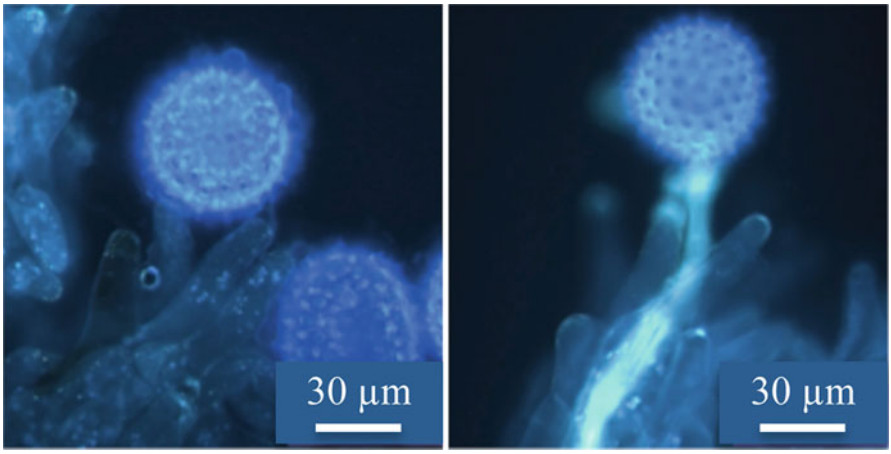

Fig. 25.4 Pollen behavior during self- and cross-pollination of *I. trifida*. Pollen tube germination is completely arrested during self-pollination (*left*), including crossing between plants with the same *S*-phenotype; however, pollen tube germination is not inhibited during cross-pollination (*right*)

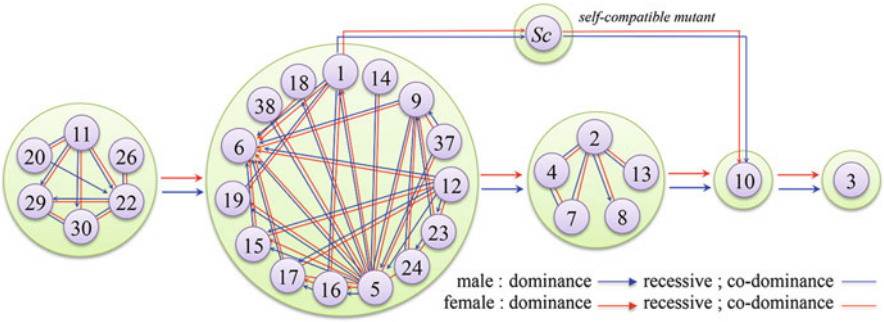

Fig. 25.5 Dominant–recessive hierarchy among 28 self-incompatible and 1 self-compatible mutant of *I. trifida*. Twenty-eight *S*-haplotypes are classified into five linear dominant–recessive groups. In each group, dominant–recessive relationships are slightly different between *S*-haplotypes in male and female reproductive organs. *Arrows* are drawn from dominant to recessive; *lines* indicate codominant relationships. The original *S*-haplotype of the self-compatible (Sc) allele is unknown; however, *Sc* is recessive to the S_1 haplotype and dominant over the S_{10} haplotype. (Figure is redrawn from Kowyama et al. 2008)

Kowyama et al. (1994) identified 49 different *S*-haplotypes from 224 individuals collected from six natural populations in Central America. *S*-haplotypes of *I. trifida* showed a linear dominant–recessive hierarchy between *S*-alleles and could be placed into five classes (Fig. 25.5). The *S*-alleles belonging to each class are the same for the male and female sides of the interaction (Fig. 25.5); however, several pairs of *S*-alleles show different interactions on the male and female sides. For example, S_{22} and S_{29} are codominant on the female side (stigma), and S_{22} is dominant over S_{29} on the male side (pollen). The linear hierarchy of dominant–recessive relationships suggests that new dominant *S*-haplotypes were created over recessive *S*-haplotypes, and the variation in allelic interaction suggests that the genetic mechanism for determination of the dominant–recessive hierarchy is regulated in different ways on the male and female sides.

In surveys for self-compatible (Sc) mutants to understand the genetic mechanism underlying SI in *I. trifida*, only one self-compatible plant (MX1) has been found as a spontaneous mutant from a natural population in Central America; its *S*-haplotype is designated *Sc* (Kakeda et al. 2000). Genetic analysis of the F_1 progeny derived from crosses between MX1 and several *S*-homozygous plants indicated that the *Sc-allele* is also within the dominant–recessive hierarchy; *Sc* is recessive to S_1 and dominant over S_{10} (Kakeda et al. 2000). The original *S*-allele is unknown; however, the fact that the *Sc* haplotype is within the dominant–recessive hierarchy suggests that *Sc* lacks one or more genes that contribute to SI but maintains genes that contribute to determination of dominance among *S*-haplotypes. Analyses of *Sc* mutants have provided important information for understanding the genetic features of SI in *Brassica* (Watanabe et al. 1997), *Pyrus* (Sassa et al. 1997), *Solanum* (Royo et al. 1994), and other plant species. In the case of *Ipomoea*, analyzing the *Sc* mutant may provide information to allow elucidation of the genetic features of SI.

25.4 Several Approaches to Identifying *S*-Genes in *I. trifida*

Because *I. trifida* has SSI, the *S*-genes have to meet several conditions. Male and female *S*-genes have to be expressed in the tapetum of the anther and papilla cells of the stigma, respectively, and these *S*-genes have to be linked tightly to maintain the *S*-haplotype at the *S*-locus. Moreover, these *S*-genes have to be present in a single copy per genome, and *S*-genes have to be polymorphic between *S*-haplotypes. In initial studies of gene products correlating with SI of *I. trifida*, reproductive organ-specific genes and genes similar to *S*-genes of other plant species were surveyed. *Ipomoea* stigma protein 11 (ISP11) has been isolated from a mature stigma cDNA library; however, this gene cannot be an *S*-gene, because it is expressed not only in stigma but also in the anther, and it is not linked to the *S*-locus, although it is a single-copy gene (Kowyama et al. 1995b). Because *I. trifida* has the same genetic SI system as *Brassica* species, it might be expected that products of genes homologous to *SLG* or *SRK* would be present in the reproductive tissues of *I. trifida*. *SRK* is known as a female *S*-gene (Stein et al. 1991; Takasaki et al. 2000), and *SLG* is an *S*-gene-related gene in *Brassica* (Kandasamy et al. 1989). *Ipomoea secreted glycoprotein* genes (*ISG1*, *-2*, and *-3*) were isolated from a mature stigma cDNA library, and the sequences of these genes showed structural similarities to *Brassica* SLGs. However, these genes were not linked to the *S*-locus of *I. trifida*, and these cDNAs have been considered truncated derivatives or modified genes of membrane-anchored protein kinase genes that are expressed predominantly in various vegetative tissues of *I. trifida* (Kowyama et al. 1995a; Kakeda and Kowyama 1996), similar to the *SLR3* gene in *Brassica oleracea* (Cock et al. 1995). *Ipomoea receptor kinase 1* (*IRK1*) was isolated as a gene homologous to *SRK* in *Brassica*, and its predicted amino-acid sequence was more similar to that of *SRK6* (Stein et al. 1996) in *Brassica*, *ARK1* (Tobias et al. 1992) in *Arabidopsis* than to *ZmPK1* (Walker and Zhang 1990) in *Zea mays*. However, both the pattern of gene expression and the results of RFLP analysis indicate that the *IRK1* gene does not have any major involvement in the SI system of *Ipomoea* (Kowyama et al. 1996).

In initial studies of the gene products associated with SI, an *S*-locus glycoprotein (SLG) in *Brassica* (Nishio and Hinata 1977; Nasrallah et al. 1985) and an *S*-locus ribonuclease (*S*-RNase) in *Nicotiana* (Anderson et al. 1986) were identified as pistil proteins that co-segregate with *S*-haplotypes. SLG and *S*-RNase are major proteins that are expressed abundantly in the pistil and are detectable by protein electrophoresis of tissue extracts. Currently, the functional role of SLG is somewhat controversial, because some functional *S*-haplotypes of *Brassica rapa* lack the *SLG* gene (Suzuki et al. 2003). Because these pioneering studies stimulated molecular studies on SI in many plant species, a similar approach was attempted with *Ipomoea*. After two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of proteins extracted from mature stigmas of *I. trifida*, one highly polymorphic protein spot was detected (Kowyama et al. 2000). This protein is about 70 kDa, with a pI of 4–6. The protein spots were associated with *S*-haplotypes, so we designated them *S-locus-linked* stigma proteins (SSPs). The *SSP* gene encodes a short-chain alcohol dehydrogenase family protein, and this gene is expressed abundantly in mature papilla

cells of the stigma. However, the amino-acid sequence of the SSPs from several *S*-haplotypes showed more than 95 % identity, and the *SSP* gene is located about 1.1 cM from the *S*-locus of *I. trifida* (Tomita et al. 2004a). Our surveys of 2D-PAGE profiles from stigma and pollen extracts have so far identified no *S-haplotype-specific* proteins other than SSP, which suggests that the *S*-locus gene products of *I. trifida* are minor proteins that might be present in amounts too small to be detectable by standard 2D-PAGE analysis.

25.5 Isolating and Analyzing the *S*-Locus in *I. trifida*

Fine-scale mapping of a gene locus is necessary to start positional cloning to identify a gene of interest. To obtain DNA markers, AFLP (amplified fragment length polymorphism: Vos et al. 1995) and AMF (AFLP-based mRNA fingerprinting: Money et al. 1996) methods are useful to identify molecular markers that are tightly linked to or co-segregate with a genetic trait (Agrama et al. 2002; Simoes-Araujo et al. 2002). These methods were attempted in *I. trifida* for identification of DNA markers around the *S*-locus with DNA and cDNA from 10 to 15 plants for each of the four *S* genotypes in the F_1 progeny from a single cross of $S_1S_{22} \times S_{10}S_{29}$. Based on the AFLP and AMF analyses, eight DNA markers were linked to the *S*-locus, and three were mapped closely to the *S*-locus (SAM-23, AAM-68, and AF-41; Fig. 25.6: Tomita et al. 2004a). The SAM-23 marker, derived from the stigma AMF analysis, contains a partial sequence of the *SSP* gene. The AAM-68 marker was obtained from the anther AMF analysis, and is tightly linked to the *S*-locus, with no recombinants among 873 F_1 plants. The AAM-68 marker is a partial sequence of a glycosyltransferase family member and is expressed in the anther and pollen; however, the predicted amino-acid sequences of this gene from different *S*-haplotypes exhibit high similarity. This finding suggests that the *AAM-68* gene is unlikely to be the *S*-gene. One AFLP marker, AF-41, was located on the opposite side of the *S*-locus to SAM-23 at an interval of 0.11 cM, and the sequence of AF-41 was similar to that of the histone deacetylase gene of *Arabidopsis*. Using these DNA markers as probes, BAC, cosmid and lambda phage clones covering the *S*-locus were screened from genomic libraries of the S_1 haplotype. The terminal sequences of the BAC clones 682-T7 and 681-SP6 map to 0.74 and 0.46 cM from the *S*-locus, respectively.

Up to now, no recombination between male and female determinant genes of SI has been observed in any plant species. If recombination between these two genes occurs, it leads to breakdown of SI. In the case of *I. trifida*, no recombination between the two components was observed among 873 F_1 plants, a result similar to that observed in *Brassica* (Casselman et al. 2000) and sweet cherry (Ikeda et al. 2005), so the *S*-locus may be located within a region where recombination is suppressed. Analysis of the DNA sequences amplified near the DNA markers among 873 F_1 siblings allowed identification and mapping of four recombination breakpoints (Fig. 25.6; Rahman et al. 2007a). According to DNA marker analysis, the *S*-locus of *I. trifida* is delimited to between 0.23 and 0.57 cM. The physical size of the *S*-locus may be estimated at about 212 kb in the S_1 haplotype (Kowyama et al. 2008).

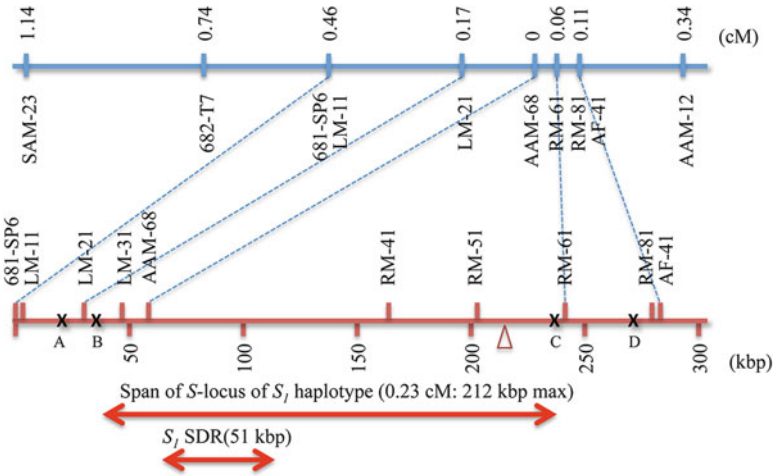

Fig. 25.6 Linkage map around the *S*-locus and location of recombination breakpoints in the S_1 haplotype. *Upper*: Linkage map of DNA markers derived from AFLP and AMF analyses and from PCR-amplified fragments. *Numbers* above the line indicate genetic distances (in cM) from the *S*-locus. *Lower*: Physical map of the DNA markers and positions of the recombination breakpoints. The *numbers* below the line indicate physical distance (in kbp). Breakpoints A–D are marked with *X*s on the map. The physical span of the *S*-locus (about 212 kbp) was estimated from the distance between breakpoints B and C. The *S*-haplotype-specific divergent region (SDR) was estimated to be about 51 kbp on the basis of sequence comparison between the S_1 and S_{10} haplotypes. (Figure redrawn from Rahman et al. 2007a)

In the case of *Brassica campestris*, *S*-locus of the S_8 haplotype is 70 kb long (Casselman et al. 2000). Within this region, two recombination breakpoints were identified that are 0.3 cM apart. The relative amount of recombination on the chromosome is calculated by the ratio of DNA length per recombination unit (kb/cM). A ratio of about 920 kb/cM was calculated for the *S*-locus of the S_1 haplotype (Rahman et al. 2007a). This value is higher than in *B. campestris*, in which the ratio appears to be 233 kb/cM (Casselman et al. 2000), so recombination within the *S*-locus is highly suppressed in *I. trifida*. In the *S*-locus region of *Petunia inflata*, the ratio was calculated as 17.6 Mb/cM because of its centromeric localization (Wang et al. 2003). Fluorescence in situ hybridization analysis of metaphase chromosomes in *I. trifida* indicated that the *S*-locus region is localized at the distal end of a chromosome (Suzuki et al. 2004). This result supports the idea that suppression of recombination at the *S*-locus of *I. trifida* is not caused by the location of the locus. Prevention of recombination within the *S*-locus may be regulated because of the necessity of preventing outbreeding through SI. Therefore, the two genes that encode recognition molecules for SI have to be located within a delimited *S*-locus, SP11/SCR [*S*-locus protein 11/*S*-locus cysteine (Cys)-rich protein] and SRK (*S*-receptor kinase) for *Brassica* plants, and two unidentified genes for *I. trifida*. The suppression of recombination around the *S*-locus might contribute to the maintenance of the gene complex as a single genetic unit.

25.6 *S*-Locus of *I. trifida*

The structure of the *S*-locus has been analyzed in several plant species by genomic sequencing. These analyses provide evidence to determine male- and female-determinant genes at the *S*-locus that contribute to the self/non-self recognition in SI, and to polymorphism between *S*-haplotypes among these genes. *S*-determinant genes (*S*-genes) of the SSI system in *Brassica* species, *SP11*/*SCR* and *SRK*, show polymorphism between *S*-haplotypes (Sherman-Broyles and Nasrallah 2008). To determine the genomic sequence of the S-locus region of *I. trifida*, sequence contigs of about 300 kbp were constructed by map-based cloning using BAC, cosmid, and lambda libraries from S_1 homozygotes (Tomita et al. 2004b), and 68 kbp from S_{10} homozygotes (Rahman et al. 2007a). Comparison of the S_1 and S_{10} *S*-locus regions revealed high variability in the *S*-locus region, which was designated the *S-haplotype-specific* divergent region (SDR; Fig. 25.7). The length of the SDR is about 50 kbp in the S_{10} haplotype, 35 kbp in the S_1 haplotype, and about 95 kbp in the S_{29} haplotype. The flanking region of the SDR is highly conserved between *S*-haplotypes. In other self-incompatible plant species, a highly divergent region has been identified. In *Brassica* species, highly divergent regions from 30 to 56 kbp are present in the *S*-locus region, and this region is flanked by sequences with high similarity (Fukai et al. 2003; Shiba et al. 2003). This polymorphic region is also observed in *Prunus*

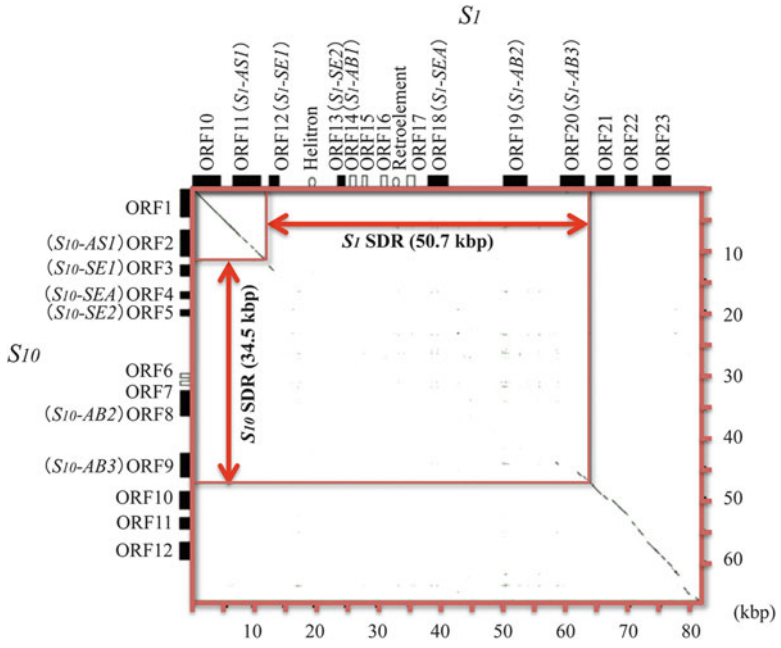

Fig. 25.7 Comparison of the nucleotide sequences spanning the *S* loci of the S_1 and S_{10} haplotypes by ARR plot analysis. Highly polymorphic regions are shown as SDRs. Estimated sizes of the SDRs are 50.7 and 34.5 kbp for the S_1 and S_{10} haplotypes, respectively. (Figure from Rahman et al. 2007a)

and *Malus* (Ushijima et al. 2001; Entani et al. 2003; Sassa et al. 2007). In these plants, male- and female-determinant genes involved in SI are located within these polymorphic regions in the *S*-locus region. Therefore, the *S*-determinant genes of *I. trifida* may be located in the SDR of the *S*-locus region.

The size differences in the SDR of *I. trifida* among *S*-haplotypes may be caused by the insertion of transposon-like sequences, retroelement-like sequences, and simple sequence repeats that accumulate in the larger SDRs (Rahman et al. 2007a). An interesting correlation between allelic dominance and SDR size has emerged from sequence comparisons of *S*-locus regions: the more dominant the *S*-haplotype, the larger the SDR. This finding suggests that acquisition of sequence complexity by recessive *S*-haplotypes is responsible for the differentiation of more dominant *S*-haplotypes.

25.7 Genes Located at or Near the *S*-Locus of *I. trifida*

Reproductive organ-specific genes at the *S*-locus may be directly correlated with self/non-self recognition in SI, one acting as male and the other as female determinant genes. In *S*-RNase-based GSI, *S*-RNases are abundantly expressed in the transmitting tract of the style, and they directly inhibit the growth of self-pollen tubes (Lee et al. 1994). The pollen determinant genes, *SFB*/*SLF* and related genes, are expressed in developing pollen grains and pollen tubes (Ushijima et al. 2003; Entani et al. 2003; Sijacic et al. 2004; Sassa et al. 2007, 2010; Meng et al. 2010; Kakui et al. 2011), and they recognize self or non-self *S*-RNases (Meng et al. 2010; Sassa et al. 2010). In *Papaver* plants, the female *S*-gene product, PrsS, is an extracellular signaling molecule that acts as a ligand to the self male *S*-gene product, PrpS, a transmembrane ion-channel/receptor. When self PrsS is recognized by self PrpS, Ca^{2+} flux is triggered and results in the depolymerization of actin fibers (Geitmann et al. 2000), activating several signaling pathways (Li et al. 2007) and inducing programmed cell death through activation of caspase-like activity in the growing pollen tube (Bosch and Franklin-Tong 2007). In the SSI system of *Brassica*, *SLG* and *SRK* genes are expressed in the mature papilla cells of the stigma (Nasrallah and Nasrallah 1993). The expression of *SRK* is significantly lower than *SLG*; however, *SRK* plays a key role in the self-pollen recognition of *Brassica* (Takasaki et al. 2000). The male *S*-gene in *Brassica*, *SP11*/*SCR*, is tightly linked to the *SRK* gene at the *S*-locus and is expressed in the anther tapetum (the sporophytic tissue) and microspores at a late developmental stage of pollen grains (Schopfer et al. 1999; Takayama et al. 2000; Shiba et al. 2002). According to previous experiments, the *S*-genes are specifically expressed in reproductive organs, and the expression pattern of these two components coincides with the characters and types of SI.

To identify the genes located in the *S*-locus region of *I. trifida*, Northern blot analyses were carried out with shotgun clones as probes that were used to determine the whole sequence of the S_1 *S*-locus (Fig. 25.8). More than ten genes were identified in the S_1 *S*-locus region; however, three stigma-specific (*SE1*, *SE2*, and *SEA*)

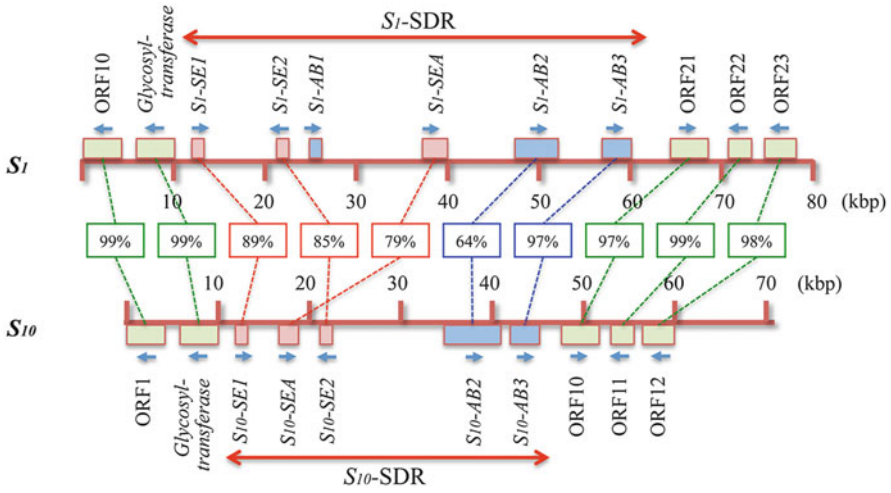

Fig. 25.8 Alignment of *S*-locus genes in the S_1 and S_{10} haplotypes showing locations of the SDRs in the S_1 and the S_{10} haplotype. *Red boxes* indicate stigma-specific genes (*SE1*, *SE2*, *SEA*) and *blue boxes* anther-specific genes (*AB1*, *AB2*, *AB3*) identified in the SDR. *Green boxes* indicate open reading frames (*ORF*s) outside the SDR. *Arrows* beside the boxes show orientation of transcription and *numbers* below each line show physical distances (in kbp). Genes or ORFs in common between the two *S*-haplotypes are connected by *dotted lines* with percentage nucleotide identity shown in *boxes*. (Figure redrawn from Rahman et al. 2007a)

and four anther-specific genes (*AB1*, *AB2*, *AB3*, and *AB4*) were located in the region (Rahman et al. 2007b). All three *SE* genes and three *AB* genes (*AB1–AB3*) were located in the SDR of the S_1 haplotype. Northern blot analyses using total RNA prepared from reproductive organs at several developmental stages and from vegetative organs indicated that all *AB* genes were expressed in the anthers 1–2 weeks before flowering. During this developmental period, pollen grains are at the microspore stage, and thus the tapetum of the anther is viable, not degraded. All *SE* genes were expressed in the stigma beginning 1 week before anthesis through the day before flowering. The transcripts of these six genes were not detected in other reproductive organs (*AB* genes were not expressed in the stigma, and *SE* genes were not expressed in the anther) or vegetative organs. According to Southern blot analysis, the *AB1* gene is present in at least two copies per genome; however, the other five genes are present as single copies. Therefore, these five genes (*AB2*, *AB3*, and three *SE* genes) are currently candidate *S*-genes based on their localization in the SDR, expression patterns, and copy number.

Among the anther-specific genes, S_1-*AB1* showed 95 % similarity to S_1-*AB3*; however, *AB1* is not located in the SDR of the S_{10}-haplotype. Furthermore, the sequence similarity of *AB1* and *AB3* genes was more than 95 % between *S*-haplotypes. From these results, *AB1* and *AB3* are not likely to be involved in the determination of *S*-haplotype specificity on the male side of *I. trifida*. On the other hand, *AB2* is located in the *S*-locus genomic region of all tested *S*-haplotypes, S_1, S_{10}, and S_{29}.

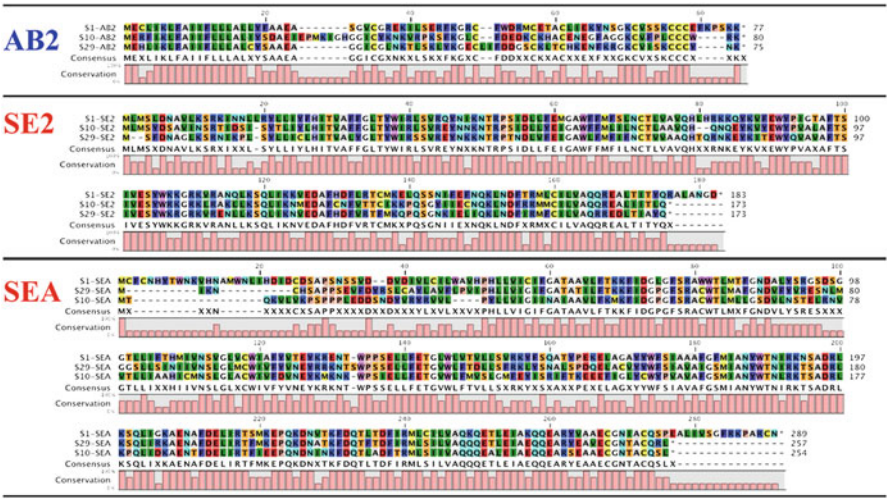

Fig. 25.9 Alignment of amino-acid sequences of *S*-candidates from S_1, S_{10}, and S_{29} alleles. Similar residues are indicated by the *same colors*

Sequence comparison of the predicted amino acid sequences of these genes showed 46–58 % identity between tested *S*-haplotypes (Fig. 25.9). According to temporal and spatial expression analyses, the *AB2* gene is only expressed in the tapetum of the anther at 14 to 7 days before anthesis, not in other reproductive organs or vegetative organs. A prediction program for protein structural features suggested that AB2 proteins have extracellular signaling domains at their N-termini that are highly conserved among *S*-haplotypes.

In addition, the predicted AB2 protein sequence shows homology to plant defensins, a class of Cys-rich proteins that are members of the gamma-thionin protein family. The defensins are small peptides (about 100 amino acids or less) with antimicrobial activities and are widely distributed in plants and animals (Boman 2003). Genes for defensin-like proteins such as PCP-A1 (Doughty et al. 1998) and SP11/SCR (Suzuki et al. 1999; Schopfer et al. 1999) are also expressed in the tapetum of developing *Brassica* anthers. Amino-acid sequence comparison of *Ipomoea* AB2 proteins with *Brassica* SP11/SCR proteins showed that only the eight Cys residues are conserved; however, sequences interlaid between the conserved Cys residues are not conserved in length or amino-acid residues (Fig. 25.10). Because the number of Cys residues is only conserved between AB2 and SP11/SCR, these two proteins may be structurally different. However, the possibility remains that this small AB2 protein acts as male *S*-determinant in the SI of *I. trifida*. If true, the AB2 protein may act as a ligand for the female *S*-determinant, which may be a receptor-like protein, as in *Brassica*.

cDNA clones of the stigma-specific genes (the *SE* genes), also showed a high level of allelic polymorphism among *S*-haplotypes and were located on the SDR of the *S*-locus. The predicted amino-acid sequences of the SE1, SE2, and SEA proteins

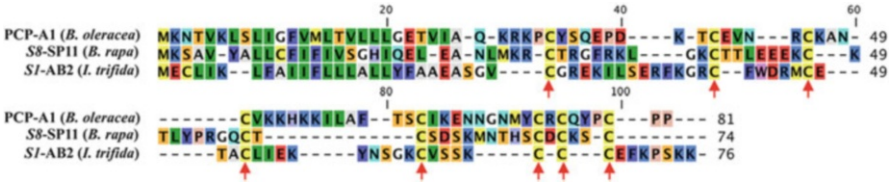

Fig. 25.10 Similarity between AB2 protein sequence and that of other PCP-A1 family proteins. Protein sequences of PCP-A1 from *Brassica oleracea* and S_8-SP11/SCR from *B. rapa*, and the predicted protein sequence of S_1-AB2 from *I. trifida*, were aligned. Conserved Cys residues in mature peptides of all three proteins are indicated with *arrows*

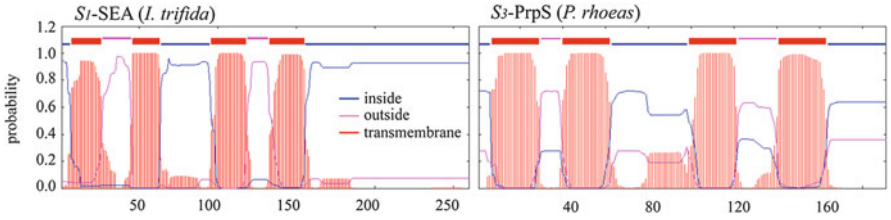

Fig. 25.11 Predicted structures of S_1-SEA protein of *I. trifida* and S_3-PrpS protein of *Papaver rhoeas*. Female *S*-candidate proteins, SE2 and SEA, of *I. trifida*, and male *S*-protein, PrpS, of *P. rhoeas* are predicted as membrane-anchored proteins with three or four membrane-spanning domains by the TMHMM program (http://www.cbs.dtu.dk/services/TMHMM/). However, these protein sequences showed no significant similarity to other reported proteins

are, respectively, 53–76 %, 67–69 %, and 52–62 % identical among the tested *S*-haplotypes (Fig. 25.9).

Expression analyses indicated that *SE1*, *SE2*, and *SEA* are expressed in the papilla cells of the stigma at 7 to 1 days before anthesis. The predicted amino-acid sequences of all *SE* genes showed no similarity to known proteins in the database. However, hydropathy plot analysis indicated that these proteins may share three to four membrane-spanning domains, and may localize to the plasma membrane of papilla cells, where the interaction between pollen and stigma cells occurs. The structural features of SE proteins are similar to the predicted structure of PrpS proteins, the female determinant of SI of *Papaver* (Wheeler et al. 2009), and the flower protein of *Drosophila* (Yao et al. 2009; Brose and Neher 2009), which may act as a Ca^{2+} channel in synaptic endocytosis (Fig. 25.11). In the GSI of *Papaver* plants, Ca^{2+} influx into self-pollen tubes is observed, and may regulated by PrpS. If the SE proteins play the same role in the SI of *I. trifida*, some ion, such as Ca^{2+}, may induce signal transduction to inhibit germination of self-pollen on the stigma surface, as in *Papaver* plants.

Taken together, the available data suggest that the *SE1*, *SE2*, and *SEA* genes that are expressed in the papilla cells of the stigma are candidates for the female *S*-determinant genes, and the *AB2* gene that is expressed in the tapetum of the

anther is a strong candidate for the male *S*-determinant gene in the SI of *I. trifida*. To determine the true *S*-genes of *I. trifida*, both functional analyses of these genes by creating transgenic plants expressing these genes and molecular–molecular interaction analyses of the gene products may be necessary to obtain definitive evidence. Further study is also necessary to determine an outline of the self-incompatible reaction in *I. trifida*.

25.8 Conclusion

SI of angiosperms is not regulated by a single and simple genetic mechanism. In the GSI system of the Plantaginaceae, Solanaceae, and Rosaceae, *S-RNase* has been identified as a female determinant and *SFB*/*SLF* and related genes as male determinants of recognition specificity. In this system, RNA and protein degradation are involved during pollen tube growth in the style (McClure and Franklin-Tong 2006). In another gametophytic system in the Papavaraceae, PrsS acts as a ligand from the stigma and PrpS as a receptor or channel molecule on the plasma membrane of the pollen tube, and their interaction induces a Ca^{2+}-mediated signaling cascade to induce programmed cell death and inhibit pollen tube growth (Franklin-Tong and Franklin 2003; Thomas and Franklin-Tong 2004; Wheeler et al. 2010). On the other hand, in SSI of the Brassicaceae, SRK acts as receptor kinase on the papilla cells of stigma and SP11/SCR as its cognate ligand on the pollen grain, resulting in self-pollen tube germination through activation of a phosphorylation pathway of downstream signaling molecules (Kachroo et al. 2002; Takayama and Isogai 2005). In the other plant species with SSI, such as the Convolvulaceae and Asteraceae, SRK-mediated self-recognition has been postulated (Hiscock and McInnis 2003). However, at least in *I. trifida* of the Convolvulaceae, the SRK-mediated signaling pathway is not recruited.

Interestingly, in the SI system in *I. trifida*, a candidate molecule for the male determinant, AB2, is similar to SP11/SCR protein, the male *S*-determinant molecule of *Brassica* plants with SSI; however, candidate molecules for the female determinant, SE1, SE2, and SEA, resemble PrpS protein, the male *S*-determinant of *Papaver* plants with GSI. This finding supports the hypothesis that SI systems may have arisen independently in the evolution of angiosperms (Allen and Hiscock 2008). Our research with *I. trifida* may provide important information about the evolution of the system of SI and may contribute to the breeding of crop plants in the Convolvulaceae, including cultivated sweet potato. The classification of SI into two types, namely GSI and SSI, is based simply on differences in gene expression of pollen determinants, not on the molecular mechanisms that underlie the SI systems in these plant families. Investigation of a wide range of plant taxa is required to understand the evolutionary lineage of the SI systems in flowering plants.

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