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Mutational Origin of New Mating Type Specificities in Flowering Plants

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Many hermaphroditic plants avoid self-fertilization by rejecting pollen that express genetically-determined specificities in common with the pistil. Self-incompatibility systems typically show extremely high genetic diversity, some maintaining hundreds of specificities. This article addresses the genetic and evolutionary mechanisms through which new mating specificities arise. Recent investigations of the genetic and physiological basis of self-incompatibility are reviewed. Two evolutionary pathways are considered: one which requires full expression of self-incompatibility in all intermediates and one in which new mating specificities arise through episodes of partial breakdown and restoration of self-incompatibility.

INTRODUCTION

Perhaps half of all species of flowering plants express some form of self-incompatibility (SI), which inhibits fertilization by pollen produced by the same plant (de Nettancourt 1977). In heteromorphic systems of SI, different mating types have different floral morphologies which discourage pollen transfer within type. Examples of this form of SI include cowslip (*Primula veris*), in which flowers produced by an individual plant have either the thrum form, in which the pollen-producing anthers extend above the stigmatic surface on which pollen grains germinate, or the pin form, in which the relative positions are reversed. In homomorphic systems, different mating types have similar floral morphologies, with a highly specific rejection response inhibiting fertilization by pollen that express the same mating specificity as the pistil. In gametophytic SI (GSI) systems, the pollen expresses the specificities encoded in its own haploid genome and in sporophytic SI (SSI) systems, the specificities encoded in the genome of the plant that produced it.

Homomorphic SI systems typically show extraordinarily high polymorphism, with the number of distinct specificities maintained within species ranging from a dozen to nearly two hundred (Lawrence 2000). Classical questions have addressed the structure of natural populations and mutation rates required for the maintenance of such diversity (Wright 1939). In this article, I review current

findings on the genetic and physiological basis of self-incompatibility in flowering plants and address the evolutionary processes through which new mating specificities may arise.

GENETIC BASIS OF SI

Despite their disparate phylogenetic origins, most systems of SI exhibit single-factor regulation at the level of classical genetics. This *S*-locus controls both pollen specificity and the rejection response of the pistil. X-irradiation experiments have succeeded in generating mutations that separately disrupted these two functions, demonstrating the bipartite nature of the *S*-locus (reviewed by Golz et al. 2000). Crossing experiments (Lewis 1949) detected no recombination between the two components, which presumably would have generated self-compatible haplotypes in which the specificity expressed by pollen differed from the specificity rejected by the pistil.

Considerable progress towards the elucidation of the physiological and genetic mechanisms of homomorphic SI has been made in the 15 years since the isolation and characterization of the gene that regulates the rejection response of the pistil in two major systems (Nasrallah et al. 1985; Anderson et al. 1986). Even so, the mechanism for the determination of mating type specificity awaits full resolution.

In the form of SSI expressed by a number of species in the Brassicaceae (cabbage), recognition by the stigma of incompatible pollen grains results in the withholding of

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hydrating factors necessary for germination of pollen grains. Protein determinants of pollen specificity are borne in the pollen coat and recognized by a membrane-bound protein kinase in the stigmatic epidermal cells (Stephenson et al. 1997; Schopfer et al. 1999; Takasaki et al. 2000; Takayama et al. 2000). SSI appears to entail expression of several genes within the extensive region of the genome that segregates with the *S*-locus (Franklin-Tong and Franklin 2000). The extensive structural differences observed among haplotypes segregating within populations suggest that recombination would likely generate only unbalanced, nonfunctional recombinants, if it can occur at all (Boyes et al. 1997).

In the form of GSI expressed in the Solanaceae (potato, tomato), Rosaceae (apple), and Scrophulariaceae (snapdragon), rejection by the pistil entails inhibition of the growth of incompatible pollen tubes by an extracellular ribonuclease (*S*-RNase) secreted into the stylar transmitting tract leading to the ovary (McClure et al. 1990; Lush and Clarke 1997). The gene that controls pollen specificity, provisionally called *pollen-S*, has not yet been identified. As in Brassica, the *S*-locus appears to comprise an extensive, nonrecombining region of the genome (McCubbin and Kao 1999).

In poppies, which lack a style, expression of GSI entails recognition of incompatible pollen on the stigma. The stigmatic *S* component is a glycoprotein that induces an increase in calcium ion concentration in incompatible pollen tubes, followed by the arrest of pollen tube growth (Franklin-Tong et al. 1997).

ANALYSIS OF SEQUENCE VARIATION

As in any structure-function analysis, sequence conservation across *S*-RNases may reflect shared requirements, while differences between specificities may contribute to the determination of specificity. Within the *S*-locus, the pattern of variation is expected to bear the signature of the form of natural selection imposed by the expression of self-incompatibility.

Evolutionary expectations: Selectively overdominant mutations confer higher fitness in heterozygous form than in homozygous form. Because rare mutations occur primarily in heterozygous form, overdominance reduces the rate of extinction by genetic drift relative to selective neutrality. As a consequence, the rate of substitution at overdominant sites in a sequence is expected to exceed the rate of mutation (Maruyama and Nei 1981; Takahata 1990; Sasaki 1992). Rate acceleration does not extend to neutral sites, irrespective of linkage to overdominant sites (Birky and Walsh 1988).

Loci within the mammalian major histocompatibility complex (MHC) constitute one of the most significant examples of balancing selection. Within Class I and

Class II MHC molecules, nonsynonymous substitution rates in excess of synonymous rates are observed only in the peptide binding regions essential to their immune system function (Hughes and Nei 1988). Other aspects supporting the hypothesis of the operation of some form of balancing selection include high heterozygosity and coalescence times on the order of tens of millions of years (Takahata and Nei 1990).

Pollen that express rare SI specificities encounter incompatible plants with lower frequency. This transmission advantage engenders balancing selection of a greater intensity than that of any form of overdominant selection (Vekemans and Slatkin 1994).

Patterns of variation: Stigmatic *S* proteins segregating in poppy populations show amino acid sequence similarities of 50% to 70%, with variable sites distributed throughout the coding region (Kurup et al. 1998). While *S*-RNases also exhibit low similarity (40% to 80%), with variation throughout the protein, Ioerger et al. (1991) recognized two hypervariable regions, designated HVa and HVb. Figure 1 presents *Solanum chacoense* *S*-RNases *S*₁₁ and *S*₁₃ (Saba-El-Leil et al. 1994), which show the highest sequence similarity among *S*-RNases demonstrated to encode distinct specificities. Regions corresponding to HVa and HVb are indicated in boldface.

The observed association between antigen recognition sites in MHC molecules and significant excess of nonsynonymous over synonymous differences suggested a means of identifying sites involved in the determination of SI specificity. Ishimizu et al. (1998) examined the pattern of variation among *S*-RNases from the three plant families known to exhibit RNase-mediated GSI. They observed a significant excess of nonsynonymous over synonymous substitution in four regions (PS1, PS2, PS3, and PS4 in Figure 1), two of which correspond to HVa and HVb.

S-RNases show homology to the fungal RNase T₂ (Kawata et al. 1988). This family of RNases shows a very broad phylogenetic distribution, with amino acid sequences available for fungal, animal, and plant representatives. Crystal structures have been determined for fungal RNase Rh (Kurihara et al. 1996) and two plant RNases: RNase MC1 (Nakagawa et al. 1999), isolated from seeds of bitter melon (*Momordica charantia*), and RNase LE (Tanaka et al. 2000), which is induced upon phosphate starvation in tomato (*Lycopersicon esculentum*).

Based on an alignment of *S*-RNases against RNase Rh, Ishimizu et al. (1998) inferred that the region homologous to PS1 corresponds to a surface loop that extends over the loop and helix region homologous to PS2. Regions corresponding to PS3 and PS4 are juxtaposed on the opposite surface of the molecule, with the substrate binding cleft located between PS1/PS2 and PS3/PS4.

While most plant RNases show four disulfide bridges

Fig. 1. Structural features of *S*-RNases from *Solanum chacoense*. Hypervariable regions HVa and HVb are indicated in bold, regions PS1, PS2, PS3, and PS4 by asterisks, and disulfide bonds by solid lines. L (loop), E (sheet), and H (helix) indicate secondary structures predicted for *S*₁₁ by PredictProtein.

(indicated by solid lines in Figure 1), fungal RNase Rh lacks the bridge closest to the N-terminus. *S*-RNases show marginally higher amino acid sequence similarities to the plant RNase MC1 (on the order of 25%) than to the fungal RNase Rh (20%). The regions of MC1 homologous to PS1, PS2, PS3, and PS4 are similar in secondary and tertiary structure to those proposed by Ishimizu et al. based on Rh (results not shown). Figure 1 indicates structural features assigned by PredictProtein (Rost 1996) to the highly similar *S. chacoense* *S*-RNases *S*₁₁ and *S*₁₃.

Caveats: While sequence comparisons provide an essential tool for the exploration of function, strong balancing selection may affect interpretation of the observations. Because genes under balancing selection are also subject to deleterious and neutral mutation, distinguishing between reduced functional constraints and overdominant selection as the cause of high rates of nonsynonymous substitution may pose difficulties. Unlike mammalian MHC genes, the sites within SI proteins that determine speci-

ficity are unknown; in fact, a prime objective of sequence comparisons has been to identify candidate sites to be tested by direct experimental manipulation.

Further, many neutral substitutions are expected to accumulate over the course of the long coalescence times that typically separate lineages subject to strong balancing selection. In particular, the extreme form of balancing selection imposed by SI appears to have maintained *S*-haplotype lineages for periods in excess of 27 million years (MY) in the Solanaceae (Ioerger et al. 1990) and perhaps 40 to 50 MY in Brassica (Uyenoyama 1995). Underestimation of substitution rates at sites that have experienced multiple substitutions becomes more problematic as divergence times grow, reducing the power of sequence comparisons to recognize regions of accelerated substitution. Comparison of highly similar but functionally distinct *S*-haplotypes may reduce this problem (Matton et al. 1997). To address whether sites identical between a recently diverged pair also contribute to the determination of specificity (Verica et al. 1998) would appear

to require examination of several sets of highly similar haplotypes which show variation in different parts of the protein.

Subject to such caveats, sequence analyses of *S*-RNases have provided evidence of positive selection within the gene encoding a component of SI rejection expressed by the pistil. Regions subject to positive selection appear to extend over much of the protein, in contrast with the more sharply delimited peptide binding regions of Class I and Class II MHC molecules.

EXPERIMENTAL MANIPULATIONS

Full-function SI haplotypes must coordinate at least three functions: expression of pollen specificity, recognition of pollen specificity by the pistil, and rejection of incompatible pollen. Because the pollen gene has only recently been characterized in Brassica and is unknown in GSI systems, experimental analyses have to date approached SI function primarily from the pistil side.

Loss of specificity: Kawata et al. (1990) identified two histidine residues essential for RNase activity in the RNase T₂ family, which includes the *S*-RNases. Royo et al. (1994) showed that a self-compatible *Lycopersicon peruvianum* *S*-allele, derived by Rick (1986) from a natural population in Peru and lacking RNase activity, carries a substitution at one of the key histidine sites. Huang et al. (1994) used site-directed mutagenesis to substitute the other essential histidine in a functional *S*-RNase sequence. Plants transformed with this construct failed to acquire the ability to reject the specificity of the unmutated *S*-haplotype, in spite of expression of the mutant protein, which lacked RNase activity. These studies indicate that RNase activity is fundamental to the rejection response in the *S*-RNase-mediated system of GSI.

Transgenic plants bearing chimeric constructs, comprising regions of two highly divergent *Nicotiana alata* *S*-RNase sequences, expressed mutant proteins which failed to reject either donor specificity in spite of retaining RNase activity (Zurek et al. 1997). A similar experiment, conducted on *Petunia inflata*, also demonstrated the failure of mutant proteins with RNase activity to reject pollen (Huang, S., McCubbin, A. G., and Kao, T.-h., unpublished results described by Kao and McCubbin 1996). The retention of RNase activity by the mutant proteins suggests that the substitutions altered stylar recognition, although the observations cannot distinguish between loss of function and change in specificity.

Dual-specificity: Matton et al. (1997, 1999) generated the first unequivocal examples of *S*-RNases with altered specificity recognition. Only 10 amino acid residues, including three in HVa and one in HVb, differ between

the naturally occurring *S. chacoense* *S*-RNases *S*₁₁ and *S*₁₃ (Figure 1; Saba-El-Leil et al. 1994). Plants transformed with an *S*₁₁ sequence in which the four residues in HVa and HVb had been substituted to match the *S*₁₃ sequence acquired the ability to reject *S*₁₃ pollen (Matton et al. 1997), and those in which the three HVa residues but not the HVb residue had been substituted rejected neither *S*₁₁ nor *S*₁₃ pollen (Matton et al. 2000). Substitution at the HVb site and two of the three HVa sites generated dual-specificity transformants, which rejected both *S*₁₁ and *S*₁₃ pollen (Matton et al. 1999). The authors hypothesized that new *S*-specificities might originate from such dual-specificity mutations.

MODELS

Although virtually all models of SI incorporate a single parameter to represent the mutation rate to new, full-function mating specificities, experimental evidence indicates that the process entails coevolved changes between the components of the bipartite *S*-locus. Recent theoretical studies have addressed the fate of evolutionary intermediates prior to the acquisition of full function. Evolutionary constraints may require the preservation of SI in all intermediates; alternatively, new specificities may arise through episodes of partial breakdown and restoration of SI.

Classical formulation: To address the maintenance of the large numbers of *S*-alleles observed in natural populations, Wright (1939) developed the fundamental model of GSI, which postulates the generation of new *S*-specificities by single mutations. Under the small population size estimates then available, the magnitude of *S*-allele diversity implied very high mutation rates (10^{-3}). In contrast, the complete absence of observations of new *S*-specificities in experimental studies designed for their detection indicated mutation rates of at least five or six orders of magnitude lower (Lewis 1949). Wright's (1939) suggestion that effective population size may significantly exceed present-day census size subsequently received support from studies of natural populations, which indicate significant population substructuring due to restricted dispersal of seeds and pollen (Levin and Kerster 1974).

Even under higher estimates of effective population size, the undetectable rate of origin of new *S*-specificities remains problematic. The recent findings on the genetic structure of the *S*-locus summarized here suggest that the origin of new specificities may in fact entail a series of mutations. Possible evolutionary pathways may include intermediates that retain SI (Uyenoyama and Newbigin 2000) or that show impaired SI function (Uyenoyama et al. submitted).

Intermediates with full SI: Not all nonsynonymous

substitutions disrupt functional interactions between the pistil and pollen components of a given *S*-haplotype. In the poppy system, for example, substitution of a number of residues in certain hydrophilic surface loops of the stigmatic *S* protein had no detectable effect on the activity or specificity of the rejection response (Kakeda et al. 1998). In their analysis of the origin of new specificities under the strict maintenance of GSI, Uyenoyama and Newbigin (2000) distinguished between selectively neutral variation of this kind and functional variation, which affects communication between pollen and pistil.

Figure 2 depicts two pathways through which successive mutations in pistil (*A*) and pollen (*B*) genes may give rise to a new *S*-specificity. Asterisks indicate mutations that were neutral at the time of their appearance. Pathway I begins with neutral variation in *B* (B_1 B_2^*), followed by a functional mutation in *A* (A_1 A_2), which enables the pistil to distinguish between the *B* locus variants. Because A_1 rejects both B_1 and B_2^* but A_2 rejects only B_2^* , styles bearing the ancestral haplotype (A_1B_1) reject pollen expressing the new haplotype ($A_2B_2^*$), while the reciprocal pollination is compatible. In pathway II, neutral variation first arises in *A*, followed by a functional mutation in *B*, which causes pollen to accept inhibition only from the *A* variant encoded by the same haplotype (A_1B_1 $A_2^*B_1$ $A_2^*B_2$). Styles bearing the new haplotype ($A_2^*B_2$) exhibit nonreciprocal rejection of pollen that express the ancestral haplotype.

Uyenoyama and Newbigin (2000) presented a simple argument indicating that nonreciprocal rejection constitutes a strong selective force which favors the haplotypes that express more rejection. SI permits random assortment of *S*-haplotypes into egg cells and inhibits only pollen transmission. Natural selection favors haplotypes borne by pollen that encounter fewer incompatible

pistils. This force permits the ancestral haplotype to drive both derived haplotypes to extinction in pathway I, and the new haplotype $A_2^*B_2$ to eliminate both the ancestral haplotype and the intermediate in pathway II. Consequently, pathway II can yield new *S*-specificities while pathway I is inherently conservative.

Intermediates with impaired SI: Over the course of evolutionary time, both loss-of-function and change-of-specificity mutations are expected to appear in natural populations. Loss-of-function mutations (for example, those that abolish RNase activity in the *S*-RNase-mediated system of GSI) likely represent evolutionary dead-ends which, if not excluded, cause the permanent transition of the population to partial or complete self-compatibility. The *L. peruvianum* *S*-allele in which substitution of one of the essential histidine residues appears to have eliminated RNase activity (see preceding section) may represent such a loss-of-function mutant that increased to high frequency in a natural population (Rick 1986). In contrast, change-of-specificity mutations, which alter SI function in pistil or pollen, may induce a transient state of self-compatibility subject to reversion to full self-incompatibility upon the appearance of compensatory mutations.

Uyenoyama et al. (submitted) explored the effects of inbreeding depression and other selective pressures on prospects for the evolution of new, full-function *S*-specificities through intermediates with impaired SI. New *S*-specificities are unlikely to descend from mutations that cause the pistil to direct rejection against pollen specificities that are not present in the population while permitting self-compatibility. Such mutations preserve rejection of their own pollen while ceasing to reject other kinds of pollen. Under inbreeding depression levels sufficiently low to permit the invasion of pistil component mutants of this kind, the population evolves to a permanent state of partial or complete self-compatibility which excludes compensatory pollen component mutations that would restore SI.

Pollen expressing a mutation that permits disablement only by pistils expressing a specificity not yet present in the population escape rejection on any pistil. Whether such mutations successfully invade the population depends on the number of functional *S*-haplotypes presently segregating (*n*), the rate at which pistils receive pollen produced by the same plant (*s*), and the viability of offspring produced by self-fertilization relative to those produced by outcrossing (σ). Parameter combinations corresponding to regions P and S in Figure 3 permit the invasion of pollen-part mutations of this kind. Under parameter values in region S, the loss of self-incompatibility is irreversible, reflecting the extinction of all functional *S*-haplotypes and the active exclusion of pistil component mutations that would restore recognition and rejection of

Fig. 2. Two pathways for the derivation of a new specificity from ancestral haplotype A_1B_1 . Pollen bearing the haplotype at the base of an arrow may fertilize styles bearing the haplotype at the tip, while the reciprocal pollination is incompatible.

Fig. 3. Fate of a haplotype bearing a pollen component mutation that permits rejection only by a pistil specificity not present in the population in which $n = 10$ functional haplotypes segregate. The mutant haplotype increases both near extinction and near fixation in region S, increases when rare but not near fixation in region P, decreases near extinction but increases near fixation in region D, and decreases in both ranges in region E.

self-pollen. Parameter values corresponding to region P permit the stable segregation of full-function haplotypes together with haplotypes bearing the pollen component mutation. These conditions strongly favor subsequent mutations in the pistil component that restore SI. Region P specifies levels of inbreeding depression sufficiently intense to exclude pistil component mutations that would cause a permanent transition to partial or complete self-compatibility. It is only in region P that a succession of new specificities is expected to arise through transient intermediate states of self-compatibility.

Divergence of haplotype lineages: In the evolutionary scenarios discussed here, the rise of a new full-function S-haplotype entails the extinction within the population of the ancestral and intermediate haplotypes. Coevolution between the pollen and pistil components causes a given S-haplotype lineage to shift its specificity (anagenesis), rather than to branch into two mutually compatible lineages (cladogenesis). Bifurcation of haplotype lineages may require additional factors, including forms of population subdivision that would permit independent shifts of specificity may occur in different demes (Uyenoyama and Newbigin 2000; Uyenoyama et al. submitted). This scenario would appear to require rates of migration among demes sufficiently low to permit specificity shifts within demes in relative isolation but sufficiently high to permit the spread of the new haplotypes. Explorations of population structures conducive to the origin of new S-haplotypes are currently in progress.

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