

Roles of Proteolysis in Plant Self-Incompatibility

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Key Words

S-locus, ubiquitin-proteasome, U-box protein, SCF, vacuole, PCD

Abstract

Self-incompatibility (SI) is a genetically controlled system adopted by many flowering plants to avoid inbreeding and thus to maintain species diversity. Generally, self-pollen rejection occurs through active pollen and pistil recognition and subsequent signaling responses. So far, three different molecular controls of pollen and pistil recognition have been characterized and are exemplified by three families: the Solanaceae, the Papaveraceae, and the Brassicaceae. With more components involved in these SI systems coming to light, recent studies have provided intriguing insights into the downstream reactions that follow the initial SI signal perception. The process of pollen rejection is closely associated with rapid and effective proteolytic events, including the ubiquitin-proteasome pathway and the vacuolar sorting pathway. Here, we review our current understanding of the roles of proteolysis in SI responses of flowering plants.

Contents

INTRODUCTION	22
Self-Incompatibility:	
The Fundamentals	22
Proteolysis, an Extremely Versatile Mediator for Signaling	22
SELF-INCOMPATIBILITY IN THE BRASSICACEAE AND UBIQUITIN-MEDIATED PROTEOLYSIS	25
SELF-INCOMPATIBILITY IN THE PAPAVERACEAE AND PROGRAMMED CELL DEATH-RELATED PROTEOLYSIS	29
S-RNASE-BASED SELF-INCOMPATIBILITY AND PROTEOLYSIS	30
The Ubiquitin-Proteasome Pathway and S-RNase Degradation	30
The Vacuole Compartmentalization Pathway, HT-B Degradation, and S-RNase Restriction	32
The Function of S-RNase: An Initiator for Programmed Cell Death in Self-Pollen Inhibition?	35

Self-incompatibility (SI): the genetic system adopted by flowering plants to prevent inbreeding and promote outcrossing

S-locus: a single polymorphic locus that contains at least two genes to control SI specificity on the pistil and pollen side

S-haplotype: representation of S-locus variants; pollen inhibition occurs when the same S-haplotype is expressed by both pollen and pistil

INTRODUCTION

Self-Incompatibility: The Fundamentals

Self-incompatibility (SI) is a widespread mechanism in flowering plants to prevent self-fertilization and thereby to generate and maintain genetic diversity within a species (13). Classical genetic studies established that SI in most species is controlled by a single polymorphic locus known as the self-incompatibility (S)-locus. This locus contains at least two separate genes: one that controls the self and nonself pollen recognition specificity on the pistil side (pistil *S*) and another on the pollen side (pollen *S*); thus, the term haplotype has been adopted to describe variants of the *S*-locus and the alleles for pollen *S* or pistil *S*. Pollen rejection

occurs when the same *S*-haplotype is expressed by both pollen and pistil (13). Since the early 1980s, the molecular nature of the *S*-locus has been extensively studied in several species with different genetic features. Broadly speaking, three types of SI systems exist in terms of their distinctive *S*-locus-encoded components: the sporophytic SI (SSI) in Brassicaceae, the gametophytic SI (GSI), in Papaveraceae, and the *S*-ribonuclease (*S*-RNase)-based GSI shared by Solanaceae, Rosaceae, and Plantaginaceae (for recent reviews see References 103 and 116).

With the eventual elucidation of the molecular nature of the *S*-locus products, great efforts have been made to define how self-pollen rejection is achieved. Several major proteolytic pathways are actively involved in all three types of SI systems.

Proteolysis, an Extremely Versatile Mediator for Signaling

A continuous turnover of intracellular proteins is essential for the maintenance of cellular homeostasis and for the regulation of multiple cellular functions (34, 97). Two well-characterized routes are mainly responsible for intracellular proteolysis: the ubiquitin-proteasome pathway and the autophagy-lysosome/vacuole pathway (9, 10, 88).

Proteasomes are barrel-shaped multiprotein complexes that predominantly degrade aberrant or short-lived nuclear and cytosolic proteins (9). The substrates destined for degradation by the 26S proteasome are covalently attached to ubiquitin, a small molecule of 76 amino acids. Ubiquitin is activated by a ubiquitin-activating enzyme (E1) in an ATP-dependent manner and is transferred to a ubiquitin-conjugating enzyme (E2). E2, with the participation of a ubiquitin-protein ligase (E3), specifically catalyzes the formation of a covalent bond between the C terminus (Gly-76) of ubiquitin and the ϵ -amino group of a lysine residue of the target protein (**Figure 1a**). The ubiquitin chain is lengthened to at least four sequentially attached ubiquitins through Lys-48 of the previously bound ubiquitin molecule,

a signal that typically allows the ubiquitylated target protein to be recognized and degraded by the 26S proteasome (106). Additional types of mono- or polyubiquitination through Lys-29 or -63 of the previously bound ubiquitin are involved in protein transport, stress response, and DNA repair (33, 75, 101). Usually, the ubiquitin moiety is removed by deubiquitylating enzymes, which are of great significance for physiology and pathology (62).

Substrate specificity is largely conferred by the substrate-recruiting E3 ligases, which compose a large and diverse gene superfamily; at least 1300 annotated genes in the *Arabidopsis* genome encode putative E3 ligases (106, 110). In general, those E3 ligases can be grouped into two classes on the basis of the E2-interacting region: The homologous to E6-AP C terminus (HECT) type and the really interesting new gene (RING) type (**Figure 1a**). HECT E3s are single polypeptides characterized by the presence of a conserved 350-amino-acid region at the C terminus designated the HECT domain, which acquires the ubiquitin from the E2 and serves as the ubiquitin donor for substrates during the ligation reaction (42). The RING E3s contain a subunit or domain with a RING motif, which coordinates a pair of zinc ions. RING E3s are thought to function as adaptors to facilitate the transfer of the ubiquitin from E2s to substrates (55, 111). The RING E3 ligases come in a large variety of forms, including monomeric E3 ligases, which contain the substrate-binding and E2-binding domains in a single protein, and the multiple-subunit complexes, such as the anaphase promoting complex (APC) (80) and Cullin (CUL)-RING ligases (CRLs) (35, 81, 96), which have these domains on separate proteins (**Figure 1a**). Three types of CRLs have been described in plants, each with a different Cullin subunit (CUL1, CUL3a/3b, or CUL4). The Cullin protein forms the scaffold that binds an invariant RING protein at its C terminus and a substrate-recognition module at the N terminus (**Figure 1b**). The paradigm for this class of E3 ligases is the SKP1 (suppressor of kinetochore protein1)-CUL1-F-box protein

(SCF) complex, in which CUL1 interacts at its C terminus with the RING-domain protein ROC1 (regulator of Cullins)/RBX1 (RING-BOX1) (forming the core catalytic domain) and at its N terminus with the adaptor protein SKP1, which in turn binds an F-box protein. In addition to a loosely conserved F-box motif that binds to SKP1, F-box proteins usually bear one of a variety of typical protein-protein interaction domains at the C terminus that confers substrate specificity to the SCF complexes. Strikingly, nearly 700 putative F-box proteins have been annotated in the *Arabidopsis* genome, suggesting that plants have the potential to assemble a multitude of SCF complexes, which could possibly control the stability of a large number of substrates implicated in various biological processes (59). Additional types of modified RING domains, differing in either the spacing between metal ligands or bearing substitutions at one or more of the metal ligand positions, have also been identified (55, 111). U-box ubiquitin ligases compose an important family of modified RING E3s, which accounts for more than 60 members in the annotated *Arabidopsis* genome (3, 134). The U-box domain has only a few of the conserved Cys/His residues in the RING domain and does not coordinate zinc ions. However, hydrogen bonds and salt bridges formed by residues at metal ligand positions render the U-box domain a similar structure to that of the RING domain. The plant U-box (PUB) family can be generally divided into five groups on the basis of the presence of other distinctive domains, such as the ubiquitin fusion degradation protein-2 (UFD2), Armadillo (ARM) repeats, Ser/Thr kinase, and leucine-rich domains. Most U-box proteins exhibit ubiquitination activity on their own and have been implicated in a variety of biological processes (3, 134). Except for those ubiquitination components mentioned above, various ubiquitin-proteasome-like and related systems have also been discovered during the past two decades and have been demonstrated to possess indispensable and diverse functions (14, 53, 99).

The plant vacuole, where various proteases reside, is another major place for proteolysis,

SSI: sporophytic self-incompatibility

GSI: gametophytic self-incompatibility

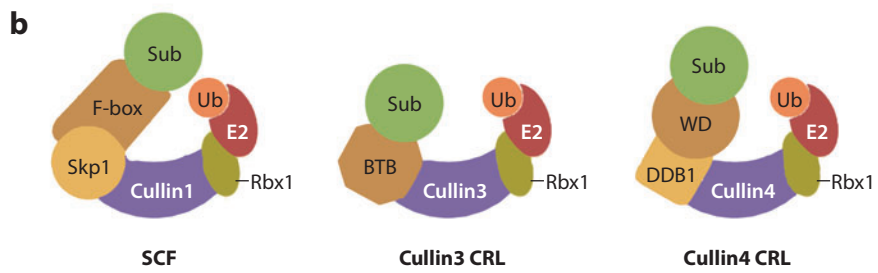
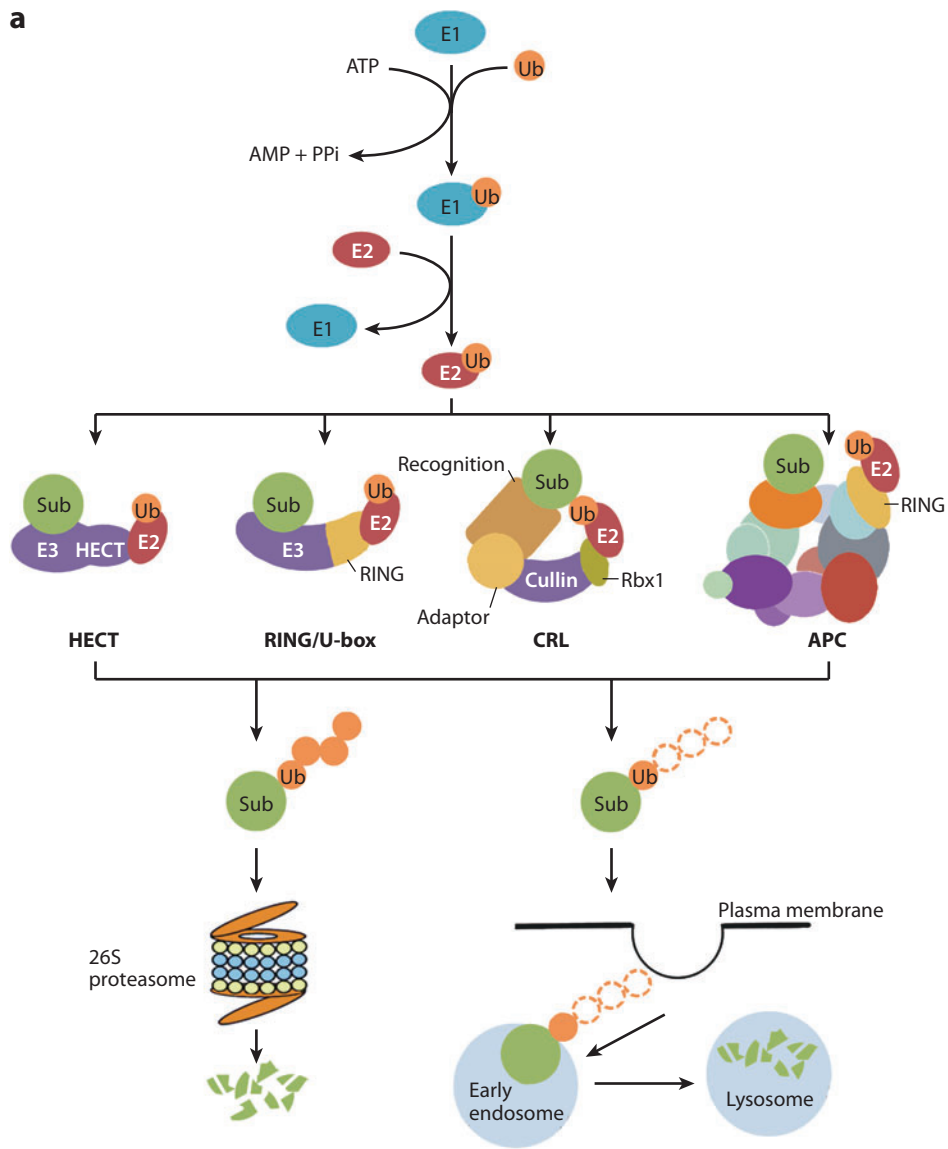
Ubiquitin: a 76-amino-acid peptide covalently attached to proteins destined for the proteasome or for another purpose such as subcellular targeting

Proteasome: a large multisubunit protease complex that selectively degrades multi-ubiquitylated proteins; composed of a 20S catalytic particle and two regulatory 19S particles

Vacuole: an organelle in plants that contains various proteases and is mainly classified into two types, the lytic vacuole and the storage vacuole

SCF: a multi-subunit ubiquitin ligase complex composed of F-box protein, CUL1, SKP1, and RBX1

PUB: plant U-box



and the substrates include protein complexes and organelles (4, 76, 122). In addition to known functions in nutrient recycling, plant vacuoles play active diverse roles in response to changes in developmental or environmental conditions and notably in protein degradation during reversible senescence and programmed cell death (PCD).

Analogous to transport systems in animals and yeast, the traffic of intracellular proteins for plant vacuole degradation can be achieved through the autophagic engulfment of cytoplasm and organelles (4, 122), whereas plasma membrane proteins are usually translocated into the vacuole via endocytosis (58, 78) (Figure 2). Strikingly, modification by ubiquitin or a ubiquitin-like moiety is the prerequisite for some types of protein transport into the vacuole (4, 33, 78, 122).

In addition to the two major proteolytic pathways described above, plants also utilize families of other proteases and alternative pathways for protein degradation (89, 125). For example, under starvation conditions and during PCD, a variety of proteases in the cytosol or organelles are activated for scavenging, one of which is the caspase family protease. Caspases are key proteases that cleave numerous sub-

strates during apoptosis in animals, but have no obvious homologs in plants (6). However, good evidence exists for caspase-like activity in plants (6, 57, 84), specifically in the poppy SI response (121).

Recent studies have clearly shown that proteolysis is a fine-tuned process that is involved in a variety of important biological responses. Thus, it is perhaps not unexpected that the SI system is closely associated with proteolytic events, given that the SI response leads to the eventual death of the cognate male gametophyte. In this review, we attempt to summarize the current knowledge pertinent to the important roles of proteolysis in the three types of SI systems, and discuss their possible functional implications. Because we consider the S-RNase-based SI to be particularly instructive example, these processes are reviewed in a greater detail for this type of SI.

SELF-INCOMPATIBILITY IN THE BRASSICACEAE AND UBIQUITIN-MEDIATED PROTEOLYSIS

Genetic studies in the early 1950s revealed two distinct forms of SI: GSI, for which pollen recognition specificity is determined by the

Programmed cell death (PCD): a mechanism used by organisms to destroy unwanted cells in a regulated manner; essential for development and defense

Caspase: cysteine protease that cleaves substrates after aspartic acid residues

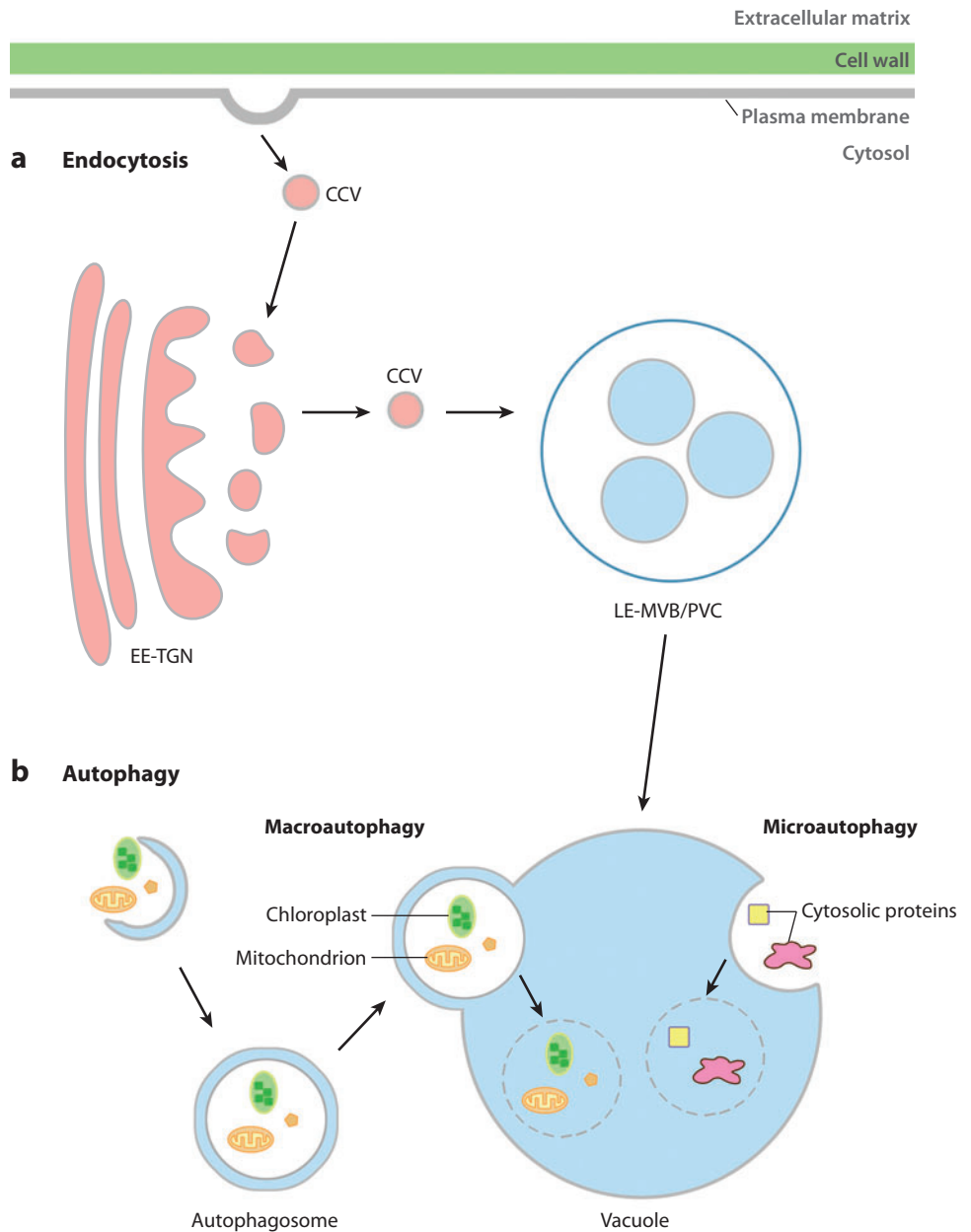
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Figure 1

Overview of the ubiquitination pathway. (a) Cascade of the ubiquitination pathway. Ubiquitin (Ub) is activated by a ubiquitin-activating enzyme (E1), then transferred to a ubiquitin-conjugating enzyme (E2), and finally attached to a substrate protein (Sub) with or without the help of ubiquitin ligase (E3). Four major classes of E3s exist; one class is homologous to E6-AP C terminus (HECT)-domain E3s, and the other three classes of E3s have a domain or a subunit with a really interesting new gene (RING) motif. These are named RING/U-box E3s, CRL (Cullin RING ligase) E3s, and APC (anaphase promoting complex) E3s. HECT-domain E3s and RING/U-box E3s are both monomeric E3s, whereas CRLs and APCs are complexes with several subunits. A common CRL contains a small RING domain-containing protein, a Cullin protein, a recognition subunit (Recognition) to recognize special substrate, and an adaptor subunit (Adaptor). APCs are composed of multiple subunits and include at least 13 subunits and three related adaptors, most of which are identified in mammals. Ubiquitinated proteins have different fates that depend on the type of ubiquitination. In general, the attachment of a single ubiquitin to a substrate can alter its localization and the attachment of a polyubiquitin chain marks the substrate for degradation by 26S proteasome. However, the attachment of a polyubiquitin chain can also alter function and localization of the substrate depending on the linkage of the polyubiquitin chain. (b) Schematic representation of the three types of CRLs in plants based on the different subunits of Cullin. Cullin1-based CRLs are called SCF complexes, and contain four subunits: Cullin1, an F-box protein, Skp1, and Rbx1. Cullin3 CRLs contain Rbx1 and BTB (broad-complex, tramtrack, and bric-a-brac)/POZ (poxvirus and zinc finger) and lack an adaptor protein, which is different from other CRLs. Cullin4-based CRLs contain Rbx1, an adaptor protein DDB1 (DNA damage binding protein 1), and a recognition protein that contains a WD-repeat domain (WD).

haploid male gametophyte (pollen) *S*-haplotype of the polymorphic *S*-locus, and SSI, for which the pollen SI phenotype is controlled by its parental (sporophyte) *S*-haplotypes (13). SI in the Brassicaceae belongs to SSI, in which the

recognition and rejection of self pollen occurs on the stigmatic surface before pollen germination or pollen tube penetration of the papillar cells of the stigma (for recent reviews see References 91 and 132).



During the past two decades, extensive molecular studies have shown that the *S*-locus of this family encodes two highly polymorphic proteins: the *S*-locus receptor kinase (SRK) (107, 115), a single-pass transmembrane serine/threonine kinase, and the *S*-locus cysteine-rich (SCR) protein, also designated SP11 (95, 100, 112, 117), which is a small protein (ca. 50 amino acids) that is a component of the pollen coat. The male determinant (SP11/SCR) physically interacts with the female determinant (SRK) in an *S*-haplotype-specific manner (48, 118), but details of how this initial specific interaction is transduced into the rejection of self pollen are relatively less known.

Another key factor that regulates the *Brassica* SI response is the *M* locus protein kinase (MLPK), discovered following positional cloning of a modifier (*mod*) mutation of SI in the *Brassica rapa* yellow sarson variety (77). MLPK is also a membrane-anchored cytoplasmic serine/threonine protein kinase that positively regulates SI signaling (77). Two isoforms of MLPK have been identified in *Brassica* and are produced by alternative transcriptional initiation sites, and both are localized to the plasma membrane and could independently complement the homozygote mutation. The membrane localization is indispensable for the SI response because their mutant forms that lack the membrane targeting motif fail to complement the mutation (49). Furthermore, the efficient phosphorylation of MLPK by the kinase domain of SRK and colocalization of

MLPK and SRK in a bimolecular fluorescence complementation assay in tobacco protoplasts suggested that following the recognition between SRK and SP11/SCR, MLPK is activated by SRK to promote subsequent SI signaling (49, 50).

Recent identification of the ubiquitination pathway implicated in this process has provided an important insight into downstream events after initial pollen recognition. ARM-repeat-containing1 (ARC1), a potential ubiquitin ligase, was originally identified in a yeast two-hybrid screen as a stigma protein interacting with the kinase domain of SRK via a C-terminal ARM repeat domain and phosphorylated in turn by SRK (27). In addition, a recent study suggested that MLPK can also phosphorylate ARC1 very efficiently *in vitro*, at a much higher level than SRK (90). Downregulation of ARC1 by antisense resulted in a partial breakdown of SI in the transgenic plants, suggesting a positive effect for ARC1 in SI signaling (109). Sequence analysis revealed that ARC1 contains a U-box motif, which is a modified RING finger, and further analyses confirmed its U-box-dependent E3 ubiquitin ligase activity. In the pistil, ubiquitinated protein levels increased specifically after incompatible pollination, but this effect was not apparent in the ARC1 antisense-suppressed pistil (108). Therefore, it has been proposed that following the perception of the cognate SP11/SCR interaction, SRK functions in a complex with MLPK to activate ARC1 (90), which promotes

ARC1: Armadillo-repeat-containing 1

Figure 2

The major protein digestive processes mediated by the vacuole in plants. (a) Endocytosis. Specific receptor-mediated endocytosis and nonspecific engulfment of cytosolic droplets containing extracellular fluid are shown. The trans Golgi network (TGN) serves as an early endosome (EE) in the endocytic pathway, whereas the prevacuolar compartment (PVC)/multivesicular body (MVB) in the secretory pathway serves as a late endosome (LE) that eventually delivers cargos to the vacuole. The protein traffic between the TGN and PVC/MVB or the plasma membrane (PM) is believed to be mediated by clathrin-coated vesicles (CCVs). (b) Micro- and macroautophagy of intracellular proteins and organelles. Microautophagy proceeds by invagination of the tonoplast to engulf portions of the cytosol to create autophagic bodies within the vacuole. Conversely, during macroautophagy, a cage-like structure consisting of preautophagic structures (PASs) encircles cytosolic components, and then coalesces to seal the region in a double-membrane-bound compartment called an autophagosome, which fuses with the tonoplast to release its contents into the vacuolar lumen.

the ubiquitination and degradation of a compatibility factor(s) in the pistil, eventually leading to self-pollen rejection (108). Thus, identifying and characterizing ARC1 substrates would be an essential next step in further dissecting the process of self-pollen rejection.

Recently, Samuel and coworkers (reviewed in Reference 91) identified *B. napus* Exo70A1 (*BnExo70A1*) as a potential substrate for ARC1. *BnExo70A1* was isolated as an interactor of the N terminus of ARC1 in a yeast two-hybrid assay using a *B. napus* pistil cDNA library and was shown to be ubiquitinated by ARC1 in an in vitro assay. Amino acid sequence comparison revealed that *BnExo70A1* is similar to Exo70, a subunit of the exocyst complex, which is well known in mammals and yeast as a regulator of specialized secretory events, including polarized exocytosis (36). In plants, Exo70 mutants showed defects in root hair elongation, pollen germination, polar growth, and cell morphogenesis (11, 29). Consistent with these findings, RNAi suppression of *BnExo70A1* in the stigma resulted in a severe reduction in seed production (91), suggesting an indispensable role of *BnExo70A1* in compatible pollen tube growth. Furthermore, previous microscopy studies have identified distinct changes in the endomembrane system in the stigmatic papillae after self or compatible pollinations (46), and a potential cellular role has been suggested for Exo70A1 (91). However, because it is difficult to discern whether these subcellular events are the consequence or the cause of the SI response, the exact role of Exo70A1 is still not clear, and more extensive functional dissections along with the identification of more players would be helpful.

An inevitable question now facing us is how pollen rejection specificity is achieved at the molecular level following the initial specific SRK/SCR recognition. Recent cytological observations may provide a promising clue to this issue (91, 108). Sequence analysis of ARC1 has revealed several motifs that might influence its subcellular localization, and the roles of these motifs were validated by subsequent cellular examinations. ARC1 can shuttle among the nucleus, cytosol, and the proteasome/COP9 sig-

nalosome (CSN) when expressed in tobacco suspension-cultured cells (108). However, localization of ARC1 and *BnExo70A1* to the ER-associated proteasome occurs only in the presence of an active SRK (91, 108). On the basis of these findings, a mechanistic hypothesis has been proposed, in which Exo70A1 functions as a positive regulator through facilitating specialized secretion of stigmatic factors following compatible pollination. In contrast, during the SI response, SRK and MLPK together phosphorylate ARC1, which would lead to an ARC1-mediated suppression of Exo70A1 function, resulting in pollen rejection (91, 108). Nevertheless, more work is needed to test this hypothesis and to clarify the mechanism underlying rejection specificity. For example, does SRK specifically phosphorylate ARC1 following self-pollination? Is Exo70A1 not ubiquitinated in the compatible pollen tube? Is the phosphorylation of ARC1 the prerequisite for Exo70A1 ubiquitination? What is the fate of ubiquitinated Exo70A1: degradation, membrane sorting, or both?

Clearly, these recent findings have provided some new clues about the mechanism of the *Brassica* SI response and suggest an intriguing connection with proteolysis that involves protein modification, endomembrane trafficking, and developmental signaling. Given that genes involved in the ubiquitination pathway account for a large percentage of the whole genome, these studies undoubtedly provide valuable information not only to the dissection of the rejection specificity involved in the SI response, but also to our knowledge about the mechanisms of ubiquitination. For example, interaction patterns similar to those of SRK and ARC1 are conserved in tobacco (54) and in *Arabidopsis* (90), in which the S-domain receptor kinase family members interact with and phosphorylate representative U-box/ARM-repeat (PUB-ARM) E3 ligases to modulate different signaling pathways, and the phosphorylation-dependent relocalization has also been observed in several additional cases (90). Further analysis of these pathways in *Arabidopsis* will certainly be helpful for our understanding of the

biochemical and cellular mechanisms of Brassicaceae-type SI.

SELF-INCOMPATIBILITY IN THE PAPAVERACEAE AND PROGRAMMED CELL DEATH- RELATED PROTEOLYSIS

SI in the Papaveraceae (poppy) family is of the GSI variety (20, 70). The pistil *S* encodes a polymorphic glycoprotein secreted by stigma cells, which, even when expressed in *Escherichia coli*, still has the ability to cause the arrest of self pollen tubes but not of nonself tubes as measured by in vitro assays (17). The exact molecular nature of the pollen *S* remains elusive. Some biochemical studies have suggested the presence of a candidate receptor, termed SBP (S-protein binding protein) in pollen, that binds stigmatic *S* proteins and was proposed to be associated with channels that conduct Ca^{2+} (21, 32). Mutations that reduce the interaction between SBP and the pistil *S* displayed a significant reduction in their ability to inhibit self-pollination (47), suggesting a key role for SBP in SI signaling. However, no *S*-allele specific interaction between the pistil *S* and SBP was detected (32). Although the existence of multiple binding domains in the SBP with different affinities for the pistil *S* has been suggested (32), there is no direct support for such a hypothesis as yet. Most recently, *PrpS1*, a pollen gene tightly linked to the pistil *S*, was isolated (19). *PrpS1* displays the polymorphism typical of an *S*-locus component. *PrpS1* encodes a small putative transmembrane protein with no homology to any known protein. Functional dissection suggested that *PrpS1* has an *S*-specific pollen inhibitory effect and probably functions as a channel, which would fit well with reports about the signaling events that relate to the pollen SI response (19).

During the past decade, great efforts were made to elucidate the downstream signaling events transduced by the initial recognition, and it is now clear that cascades of physiological and biological processes are involved in this type of SI. The female determinant interacts

with incompatible pollen, triggering a Ca^{2+} -dependent signaling network associated with rapid depolymerization of the actin cytoskeleton and microtubules and phosphorylation of a soluble inorganic pyrophosphatase, Prp26.1, both of which are involved in the rapid inhibition of pollen tube growth, and ultimately the death of incompatible pollen via PCD (for recent reviews see References 8 and 19).

PCD is perhaps one of the most important findings relating to events triggered by SI in *Papaver* pollen. PCD is a mechanism used by many organisms to destroy unwanted cells in a precisely regulated manner (120). PCD in plants is essential for development and defense, which occur at all stages of the life cycle (84, 86, 126, 127). Several lines of evidence strongly suggest that PCD plays a role in *Papaver rhoeas* SI signaling (121). The hallmarks of PCD, i.e., nuclear DNA fragmentation and cytochrome C leakage, could be sequentially induced by artificially increasing Ca^{2+} in pollen tubes, suggesting that these steps are all a part of the series of reactions that make up the Ca^{2+} -mediated SI signaling cascade (121). A caspase-like activity, which is a major characteristic of PCD in plants, was consistently confirmed in the self-pollen tube of poppy. Pretreatment of incompatible pollen tubes with the peptide DEVD, a caspase-3-specific inhibitor, significantly reduced DNA fragmentation associated with SI in *Papaver* and the SI-induced inhibition of pollen tube growth could also be overcome (121). Use of a fluorescent caspase-3 substrate, Ac-DEVD-AMC, that acts as a fluorogenic indicator for DEVDase activity, has provided direct evidence for the SI-induced DEVDase activity (7, 60). Furthermore, the cleavage product of poly (ADP-ribose) polymerase (PARP), which is a typical substrate for caspase-3, was detected only in extracts from incompatible pollen tubes in which SI had been induced (121). These data implicate a caspase-like activity in mediating PCD stimulated by the SI pollen response in *Papaver* pollen.

PCD in plants is different from that in animals and yeast in several respects, and the progress of the study of PCD in plants is

SLF: *S-locus F-box*

SFB: *S-haplotype-specific F-box*

SSK1: *SLF-Binding SKP1*

relatively slow, with few analogous pathways to those extensively studied systems (74, 84). The study in poppy SI has broadened our knowledge concerning PCD in plants, and it would be interesting to further examine the roles of other plant PCD elements in this process. Additionally, is the PCD pathway unique to the poppy SI system? Are there some parallel pathways between different types of SI signaling? Answers to these questions will not only help us understand this SI system better, but also provide useful information for the association of the SI mechanism with PCD.

S-RNASE-BASED SELF-INCOMPATIBILITY AND PROTEOLYSIS

The Ubiquitin-Proteasome Pathway and S-RNase Degradation

S-RNase-based SI appears to be the most phylogenetically widespread form of SI found in angiosperms; it is under GSI control and has been studied thoroughly in three families: Solanaceae, Plantaginaceae, and Rosaceae (20, 67, 70, 104). These three families share the similar female S-determinants, S-RNases (1, 44, 45, 93, 135), which are secreted by the pistil (2, 12, 135) and taken up by pollen tubes in a non S-haplotype-specific manner (66). S-RNases act as cytotoxins to degrade the RNA of self-pollen tubes for growth inhibition (71), but the exact mechanism of how this specific inhibition is achieved still remains largely unclear. The study of the role of the pollen S has provided some revealing information. Genetic evidence suggested that the pollen S in the Solanaceae functions as an S-RNase inhibitor because no pollen S deletion mutants had ever been found based on exhaustive induced mutagenesis screens, suggesting that the pollen S is essential for inhibiting S-RNase activity (13, 25, 26). Recent molecular studies have revealed that the pollen S in these three families belongs to a class of F-box genes: *SLFs* (*S-locus F-box*) in the Solanaceae (102, 130) and Plantaginaceae (56, 82) or *SFBs* (*S-haplotype-specific F-box*) in the Rosaceae (15, 123).

Given that an F-box protein often serves as an adaptor that binds a specific substrate protein to the SCF E3 ubiquitin ligase complex (59), it was obvious to assume that the putative pollen S product SLF/SFB could participate in an SCF complex and target S-RNase for degradation (51, 56, 83, 124). Because no biochemical or physiological evidence for the role of SFB in Rosaceae has yet been reported, we mainly discuss our current knowledge about the molecular and biochemical mechanisms of SLFs involved in S-RNase restriction in Solanaceae and Plantaginaceae, in which the presence of such an SCF complex and its interaction with S-RNases are clearly evident from several recent studies (38, 41, 83). *SLF-interacting SKP1-like1* (*SSK1*), a homolog of *SKP1*, was isolated in *Artbrinium hispanicum* through a yeast two-hybrid screening against a pollen cDNA library using *AbSLF-S₂* as bait (41). The results of pull-down assays suggested that *SSK1* could be an adaptor that connects SLF to CUL1-like protein (41). Therefore, SLF and *SSK1* are likely to be recruited into a canonical SCF complex that would be responsible for S-RNase ubiquitination.

Typically, ubiquitinated proteins are delivered to the 26S proteasome (106), so the role of the proteasome in S-RNase degradation was investigated. Proteasomal inhibitors could specifically block compatible pollen tube growth *in vitro*, but had little effect on incompatible pollen tubes (83). Theoretically, if SLF could selectively interact with and ubiquitinate nonself S-RNases, the issue of pollen rejection specificity would readily be explained. However, no specificity was apparently detected in either the interaction between SLF and S-RNase or S-RNase ubiquitination (38, 83); thus, the mechanism underlying SI signaling specificity is still far from clear. Nonetheless, recent biochemical data shed some light on this issue. On the basis of yeast two-hybrid and *in vitro* binding assays, Hua & Kao (38) reported that the physical interaction between S-RNase and nonself SLF was much stronger than that between S-RNase and self SLF. To define the biochemical basis for this differential

interaction, Hua and coworkers (40) identified three PiSLF-specific regions, compared them with ten PiSLF-like proteins, designated functional domains 1–3 (FD1–3), and analyzed the contribution to S-RNase binding activity of the truncated SLFs containing one or two of the three domains. The results suggested that FD2 was sufficient for general S-RNase interaction with high affinity, the interaction was even stronger than that between the full length SLF and S-RNase, and this interaction could be weakened with the addition of FD1, FD3, or both. Because FD1 and FD3 each contain one of the two variable regions of PiSLF, they also measured the S-RNase binding affinity for chimeric SLFs generated by FD domain swaps between different allelic SLFs. The results suggested that FD1 and FD3 together would be sufficient to confer S-haplotype specificity. These observations provide a plausible explanation for rejection specificity: SLF could interact with nonself S-RNase with high affinity and mediate S-RNase ubiquitination, whereas following self-pollination, the interaction between SLF and self S-RNase would be so weak that no ubiquitination event could occur (40). However, with no *in vivo* evidence, the results entirely based on *in vitro* pull-down assays are insufficient to draw a solid conclusion. Thus, it would be extremely interesting to find out whether similar interaction patterns exist in other species that possess this type of SI, and further transgenic experiments with SLF domain swaps would be indispensable to validate these observations.

Despite the finding that no haplotype specificity was detected for S-RNase ubiquitination in both *A. bispanicum* and *Petunia inflata* (38, 83), these data were based on an *in vitro* system and further direct evidence is urgently required. Alternatively, both self and nonself S-RNases could be ubiquitinated, but different types of ubiquitination could be involved. Mono- and polyubiquitination lead to different fates for targets, and different forms of ubiquitination may also be the reason why some ubiquitinated proteins are degraded and others are not (75). Identifying the residues responsible for ubiqui-

nitination and the types of ubiquitin attached to S-RNase would be a key solution to the question of pollen rejection specificity. Recently, major ubiquitination sites of S₃-RNase in *P. inflata* were reported (39) based on the cell-free system used previously (38). Mutagenesis of these lysine residues appeared to reduce both S-RNase ubiquitination and the rate of degradation *in vitro*. Because ubiquitination was not abolished in the mutated form of S-RNase, lysines other than those major sites identified could also be responsible for S-RNase ubiquitination. In particular, this S-RNase degradation assay was conducted with the application of nonself pollen tube extracts; whether some distinction in the ubiquitination of S-RNase exists when using self pollen tube extracts would be an interesting issue to examine. Further *in vivo* data involving mutagenesis of these putative ubiquitination sites would provide *in vivo* information as to the relationship between ubiquitination and SI signaling specificity.

Furthermore, another potential E3 ligase, S-RNase binding protein1 (SBP1) also appeared to display a possible role in S-RNase ubiquitination and degradation (79, 105). SBP1 was originally identified on the basis of a yeast two-hybrid approach in *Petunia hybrida* designed to isolate pollen proteins that could interact with S-RNase. Sequence comparison revealed that SBP1 contains a RING-finger domain (105). Interestingly, *P. inflata* SBP1 (PiSBP1) can interact with PiSLFs, PiCUL1, and a ubiquitin-conjugating enzyme; thus a putative novel E3 ligase complex was hypothesized, with the possibility that PiSBP1 plays the combined role of SKP1 and RBX1 (38). However, because the *SBP1* gene displayed no haplotype polymorphism and was unlinked to the *S*-locus, it is unlikely to be responsible for the specificity (38, 105). Therefore, a new model was proposed in which SBP1 functions as the general S-RNase inhibitor, which accounts for the basal degradation of S-RNase, whereas SLF together with SBP1 preferentially recognizes and targets nonself S-RNases (38). In addition, a recent *in vitro* ubiquitination assay suggested that PiSBP1 alone could act as an E3 ligase

that, in conjugation with E1 and E2, had the capacity to ubiquitinate S-RNases (39). However, according to this model, the deletion of SLF would be tolerable, which is inconsistent with the lack of a pollen *S* deletion mutation observed in the Solanaceae (13, 25, 26). Because more than one E3 ligase seems to be involved in S-RNase ubiquitination, it would be difficult, solely on the basis of an in vitro system, to distinguish whether ubiquitinated S-RNases result from the action of SCF^{SLF} or some other E3 complex. If both SLF and SBP1 could target S-RNase for ubiquitination, then do any differences exist between the ubiquitination types involved and, if so, what would be their possible functional implication? Do they work together or sequentially to ubiquitinate S-RNase? More careful experiments with additional controls such as pollen with a knockout or knock-down expression of SLF and/or SSK1 or SBP1 would be necessary to dissect the biochemical function of these potential E3 components.

Nonetheless, *S*-haplotype-specific S-RNase restriction appears to be a complicated process that could involve a complex network of proteolytic events. Many unknowns exist; for example, a notable feature for this type of the *S*-locus is that several *SLF*-like (*SFL*) genes reside in the vicinity of *SLF/SFB* in the Solanaceae (40, 131, 133), in the Plantaginaceae (136), and in the Rosaceae (15, 92, 123). These *SFL*s display little or no interaction with S-RNases compared with the obvious interaction between *SLF*s and S-RNases (40, 83). Moreover, no interaction has been detected between these *SFL*s and SSK1 in *A. hispanicum* (41). Genetic studies suggested that transferring a different copy of *SLF* into self-incompatible plants could result in SI breakdown (82, 102), whereas the introduction of the *SFL* genes produced no apparent effect on SI behavior (40). Therefore, the function of those *SFL*s is still obscure. Recently, Sassa and coworkers (92) identified multiple pollen-specific F-box genes termed *S*-locus *F*-box brothers (*SFBB*s) that display *S*-haplotype-specific polymorphisms in apple and Japanese pear. The authors suggested that these gene products could work together or have some

functional redundancy to determine pollen *S* specificity. One possibility is that the pollen *S* protein functions as a multimer (65), as some F-box proteins do in humans and yeast (31, 113, 119). However, a specific N-terminal D domain has been identified as responsible for the homo- or heterodimerization of those F-box proteins in humans and yeast (31, 113, 119), and this domain apparently is not present in *SFBB*s, so further study is needed to find out whether some analogous domain is present. However, the assumption that multiple *SFBB* genes function redundantly as the pollen *S* would provide a reasonable interpretation for the lack of a pollen *S* mutant based on classical genetic screens in those species (13, 92), because the deletion of a single redundant gene is tolerable for pollen *S* function. If this was the case, the specificity involved would be more complicated than what is suggested by simple genetics of the *S*-locus. Further functional experiments and comparative analyses would be required to shed more light on the mechanism and evolution of the pollen *S*. Interestingly, clustering of F-box proteins is a common phenomenon in plants; for example, nearly 40% of the ~700 predicted *Arabidopsis* F-box genes are arranged in tandem repeats of two to seven genes with the implication that the tandem duplications of chromosomal regions played a major role in creating the large array of F-box loci in *Arabidopsis* (22, 129). The elucidation of the role of those *SFL*s could also provide further insights into the function and evolution of F-box gene clusters in plants.

The Vacuole Compartmentalization Pathway, HT-B Degradation, and S-RNase Restriction

Despite the proposal of the proteasome-mediated degradation pathway as the major reason for S-RNase restriction, another scenario emerged from a recent study at the cytological level. Goldraij and coworkers (24) performed elegant immunolocalization experiments to demonstrate that S-RNase is transported to the pollen vacuole after its uptake from the stylar extracellular matrix. Most

compartments remained intact to sequester the S-RNases in compatible pollen tubes, whereas the vacuole-like endomembrane system for S-RNases appeared to break down in latter stages in incompatible pollination, likely releasing the S-RNase into the cytosol. To trace the cause of this breakdown, Goldraij and coworkers (24) used a similar approach to investigate several plant materials with defects in the stelar factors, which are known to be indispensable for the SI response (30, 69, 73). In these self-compatible transgenic plants in which the pistil does not express H-top band (HT-B), S-RNase appeared to remain sequestered during all stages of self-pollination. This result prompted them to examine the HT-B level in the pollen tubes growing in wild-type styles. A large-scale HT-B degradation preferentially occurred in compatible pollen tubes, but not in incompatible pollen tubes, suggesting that HT-B is required for pollen rejection, although the underlying mechanism is still speculative (24, 67, 68).

These findings associate for the first time the SI response with the vacuole compartment, opening an illuminating avenue in light of the subcellular fate of S-RNases after uptake by the pollen tubes. Despite a well-defined proteolysis role for the vacuole (4, 76, 122), no obvious distinction in S-RNase quantity could be detected between self-pollinated or cross-pollinated pollen tubes (24), although one might argue that the degraded S-RNase could be offset by the S-RNase that is entering the pollen tube constantly. Therefore, it is not yet possible to draw a definitive conclusion as to whether the S-RNase could be degraded inside the vacuole. Furthermore, these cytological observations have provided an alternative explanation for the specificity underlying the SI response compared with the S-RNase ubiquitination model. However, some unanswered questions demand urgent answers. First of all, the role of SLF in this compartmentalization process is uncharacterized and it is difficult to discriminate whether the compartment breakdown is a direct effect of the specific recognition between cognate SLF and S-RNase or a con-

sequence of the SI response. Moreover, no interaction between HT-B and S-RNase or SLF was detected (Y. Zhang, G. Chen, B. Zhang, Q. Li & Y. Xue, unpublished data); thus, the reason for HT-B-specific degradation in compatible pollen tubes and its relationship with compartment breakdown remain to be determined.

On the basis of recent findings, several mechanistic models for S-RNase-based SI have been proposed and discussed, but no consensus has emerged (37, 68). The center point for the discrepancy is the method of S-RNase restriction, is it compartmentalized or ubiquitinated? If both ways could occur, what kind of a relationship does that imply? Do these two events take place in parallel or sequentially? To address these issues, it would be really interesting to find out the answers to the following questions: First, what is the intrinsic cellular route for self and nonself S-RNase? For example, do they gain access to the pollen tube in different manners or show some distinction at subsequent cellular sorting steps? Second, where do the S-RNase recognition and ubiquitination events take place? Third, does the ubiquitination of S-RNase have some effect on its subcellular localization?

Some clues for the first two questions have already emerged. Our recent data indicate that S-RNases enter the pollen tube via endocytosis and are present in small endosomes in both self and nonself pollen tubes (Y. Zhang, G. Chen, B. Zhang, Q. Li & Y. Xue, unpublished result). Because SLF has been reported to be associated with the membrane system (128), the specific interaction and ubiquitination likely occur in the vicinity of an endomembrane system. On the basis of the available evidence, we propose a new hypothetical S-RNase sorting model, in which S-RNases enter both self and nonself pollen tubes via endocytosis. S-RNases in compatible pollen tubes are ubiquitinated by SCF^{SLF} in early endosomes and sorted into the vacuole-like structures, whereas in self-pollen tubes, S-RNases are released into the cytoplasm by an unknown mechanism, where they exert their cytotoxic effect (**Figure 3**). This new model is essentially consistent with previous

H-top band (HT-B): a small asparagine-rich protein expressed in the style that is required for self-pollen rejection

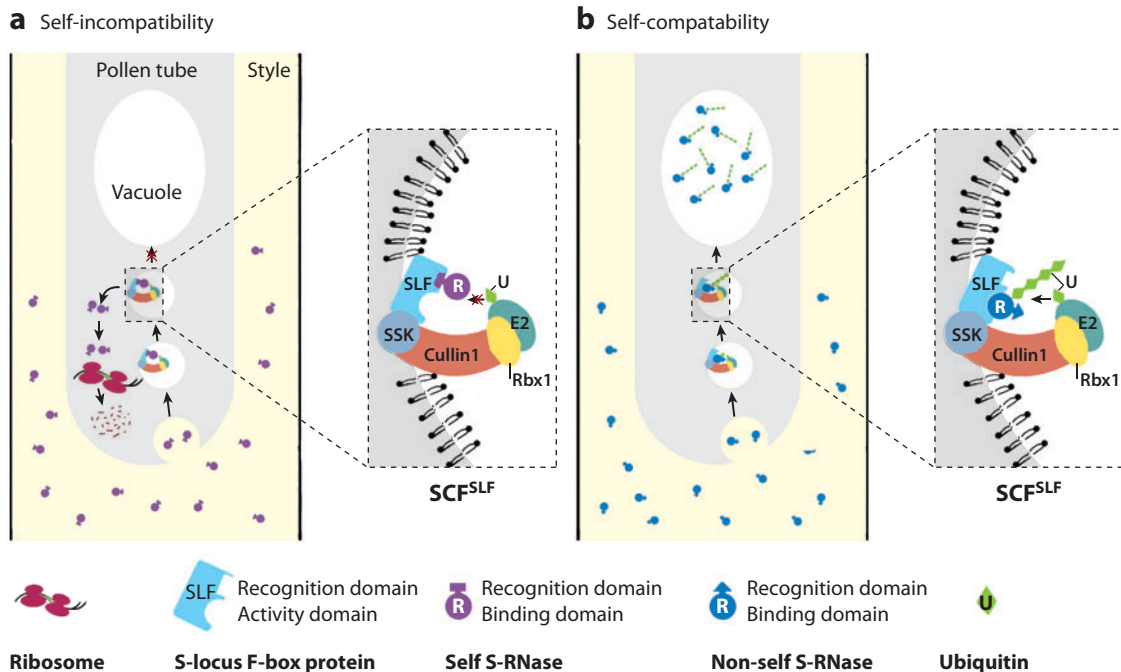


Figure 3

A hypothetical model for S-ribonuclease (S-RNase)-mediated gametophytic self-incompatibility in Solanaceae and Plantaginaceae.

(a) Self-incompatibility reaction. When a pollen tube germinates and grows in a self style, self S-RNases (R) are taken up into the pollen tube through endocytosis and recognized by a recognition domain of the pollen S, S-locus F-box (SLF), which recruits SLF-interacting SKP1-like1 (SSK1), Cullin 1 (CUL1), and RING-BOX1 (RBX1) (SCF) to form an SCF^{SLF} complex. However, this self recognition would prevent the formation of such a functional SCF complex for self S-RNase modification by ubiquitin (Ub). The nonubiquitinated S-RNases are somehow translocated into the cytoplasm of the pollen tube to inhibit the elongation of the pollen tube by destroying ribosome RNA. However, this self recognition could also lead to other types of ubiquitination not involved in protein degradation. This possibility is not shown in the model.

(b) Self-compatibility reaction. When a pollen tube germinates and grows in a nonself style in a manner similar to that of the SI reaction, nonself S-RNases (R) are also taken up into the pollen tube through endocytosis. But, nonself S-RNases are not recognized by a recognition domain, instead they are recognized by an activation domain of SLF, which leads to the formation of a functional SCF complex to ubiquitinate S-RNases. Polyubiquitinated S-RNases are subsequently sorted into the vacuole instead of the cytoplasm of the pollen tube. Thus, the pollen tube can elongate normally and complete fertilization.

findings and provides a cellular and molecular interpretation for the specificity of the pollen determinant. Nevertheless, the third key question regarding the relationship between ubiquitination and subcellular sorting presented in the model remains unanswered. It would be helpful if we could figure out the mechanism for the release of S-RNase into the cytoplasm from the vesicle, such as the breakdown of the vesicle or some protein transport pathway involving mono-ubiquitination. Furthermore, we could not exclude the possibility that the SLF together with SSK1 or the SLF alone would

be sufficient for S-RNase targeting, because in several cases F-box proteins have the ability to traffic independent of the formation of the SCF complex (16, 23, 98, 114). With a combined approach of molecular genetics, biochemistry, and cell biology, we can expect a better understanding of how the proteolytic pathways work together to determine the distinct fates of S-RNases after self-pollination and compatible pollination. So far, only a few substrates for E3 ligases have been identified in plants, and nearly no fates other than proteasome degradation have ever been reported for ubiquitinated

proteins. The elucidation of the signaling cascades active during the SI response would be of great significance for our understanding of the diverse functions of the large number of E3s in plants.

The Function of S-RNase: An Initiator for Programmed Cell Death in Self-Pollen Inhibition?

It is noteworthy that the precise role of S-RNase remains unclear; this is a fundamental question for understanding the SI mechanism. Because the sequences of S-RNases share extensive similarity with that of the RNase-T₂ (two catalytic histidines are conserved in all functional S-RNases), that S-RNase has been widely accepted to function as a ribonuclease in the SI response (43, 52, 72). However, although rRNA degradation in incompatible pollen tubes is strongly correlative with the expression of SI, it is difficult to discriminate whether it is the direct cause or secondary effect of incompatibility (71). In addition, because no substrate specificity of S-RNase had been detected *in vitro*, how S-RNase activity could be

restricted in compatible pollen tubes remained unclear (71). Further, incompatible pollen tubes could revert to normal growth if grafted onto compatible styles; thus, the cytotoxic effect of S-RNase is not permanent (64). Recently, several new functions unrelated to the ribonuclease activity were uncovered for S-like RNases (5, 28, 63, 85, 94), indicating that the exact cytotoxic effect of S-RNase potentially could be on proteins rather than on RNA. For example, a fungal T₂-RNase, ACTIBIND, possesses actin-binding activity, which is associated with antiangiogenic and anticarcinogenic characteristics and the induction of cell apoptosis in colonic tumors (87). Additionally, the *Pyrus pyrifolia* stylar S-RNase induces alterations in the actin cytoskeleton in self-pollen tubes *in vitro* and the alterations occur prior to the arrest of pollen tube growth (61). It would be interesting to examine whether S-RNases could act as initiators for some form of PCD during self-pollen tube growth inhibition, similar to what happens in the Papaveraceae-type SI, because the uncontrolled cell death is rather harmful to both the pollen tubes and surrounding style tissues.

SUMMARY POINTS

1. Self-incompatibility (SI) signaling represents a unique mechanism for self/nonself recognition between pollen and pistil. As a result, the self or genetically related pollen is unable to germinate or grow in the style to complete fertilization. Recent studies have shown that proteolytic events play important roles in both self-pollen rejection and compatible pollen growth during SI signaling.
2. In Brassicaceae-type SI, a U-box protein ARM-repeat-containing1 (ARC1) participates in the SI response as a functional E3 ligase after the specific recognition between pollen and pistil. In addition, its likely substrate, Exo70A, which acts as a potential pollen compatibility factor, has been identified.
3. In Papaveraceae-type SI, the pistil *S* selectively interacts with self pollen, which in turn triggers a signaling cascade that culminates in the programmed cell death (PCD) of the self-pollen tube.
4. In Solanaceae-type SI, which represents the most phylogenetically widespread form of SI, both the ubiquitin-proteasome pathway and the vacuole pathway appear to take active parts in SI responses.

FUTURE ISSUES

1. The factors involved in proteolysis account for a large number of annotated proteins in eukaryotes and specifically, in plants. These factors do not act alone, but instead form cascades, circuits, and networks that all dynamically interconnect to form a proteolytic web. Their substrates and activation mechanisms are elusive, however, and represent a challenging topic for future research.
2. Researchers have made great progress during the past two decades toward elucidating the molecular mechanism for self-incompatibility; one exciting finding is the identification of the linkage between different types of SI response and specific proteolysis. Whether some analogy in proteolysis exists between these forms of SI would be an interesting issue for further research.
3. The elucidation of the target for a specific ubiquitin ligase remains a challenging task given the apparent redundancy of these E3s and some form of posttranslational modification as the prerequisite for recognition in many cases; thus, only a few E3 targets have been identified compared with the thousands of E3s already annotated in plants. The findings in the SI response have clearly demonstrated several E3-substrate partners. Moreover, SI could serve as a model system to study the relationship between specific protein modifications and E3-substrate recognition through comparing the different signaling events following self and cross pollination.
4. The study of the SI response has made an important contribution to the understanding of proteolysis in plants, which in turn will provide further insights into the evolution and mechanism of SI signaling.
5. Despite recent exciting advances, we are perhaps only on the brink of appreciating the regulatory complexity of self-incompatibility through proteolysis. How the regulated changes in cellular protein composition and trafficking translate into the physiological SI responses will doubtless continue to draw substantial research interest for many years to come.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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Contents

My Journey From Horticulture to Plant Biology <i>Jan A.D. Zeevaart</i>	1
Roles of Proteolysis in Plant Self-Incompatibility <i>Yijing Zhang, Zhonghua Zhao, and Yongbiao Xue</i>	21
Epigenetic Regulation of Transposable Elements in Plants <i>Damon Lisch</i>	43
14-3-3 and FHA Domains Mediate Phosphoprotein Interactions <i>David Chevalier, Erin R. Morris, and John C. Walker</i>	67
Quantitative Genomics: Analyzing Intraspecific Variation Using Global Gene Expression Polymorphisms or eQTLs <i>Dan Kliebenstein</i>	93
DNA Transfer from Organelles to the Nucleus: The Idiosyncratic Genetics of Endosymbiosis <i>Tatjana Kleine, Uwe G. Maier, and Dario Leister</i>	115
The HSP90-SGT1 Chaperone Complex for NLR Immune Sensors <i>Ken Shirasu</i>	139
Cellulosic Biofuels <i>Andrew Carroll and Chris Somerville</i>	165
Jasmonate Passes Muster: A Receptor and Targets for the Defense Hormone <i>John Browse</i>	183
Phloem Transport: Cellular Pathways and Molecular Trafficking <i>Robert Turgeon and Shmuel Wolf</i>	207
Selaginella and 400 Million Years of Separation <i>Jo Ann Banks</i>	223
Sensing and Responding to Excess Light <i>Zhirong Li, Setsuko Wakao, Beat B. Fischer, and Krishna K. Niyogi</i>	239
<i>Aquilegia</i> : A New Model for Plant Development, Ecology, and Evolution <i>Elena M. Kramer</i>	261

Environmental Effects on Spatial and Temporal Patterns of Leaf and Root Growth <i>Achim Walter, Wendy K. Silk, and Ulrich Schurr</i>	279
Short-Read Sequencing Technologies for Transcriptional Analyses <i>Stacey A. Simon, Jixian Zhai, Raja Sekbar Nandety, Kevin P. McCormick, Jia Zeng, Diego Mejia, and Blake C. Meyers</i>	305
Biosynthesis of Plant Isoprenoids: Perspectives for Microbial Engineering <i>James Kirby and Jay D. Keasling</i>	335
The Circadian System in Higher Plants <i>Stacey L. Harmer</i>	357
A Renaissance of Elicitors: Perception of Microbe-Associated Molecular Patterns and Danger Signals by Pattern-Recognition Receptors <i>Thomas Boller and Georg Felix</i>	379
Signal Transduction in Responses to UV-B Radiation <i>Gareth I. Jenkins</i>	407
Bias in Plant Gene Content Following Different Sorts of Duplication: Tandem, Whole-Genome, Segmental, or by Transposition <i>Michael Freeling</i>	433
Photorespiratory Metabolism: Genes, Mutants, Energetics, and Redox Signaling <i>Christine H. Foyer, Arnold Bloom, Guillaume Queval, and Graham Noctor</i>	455
Roles of Plant Small RNAs in Biotic Stress Responses <i>Virginia Ruiz-Ferrer and Olivier Voinnet</i>	485
Genetically Engineered Plants and Foods: A Scientist's Analysis of the Issues (Part II) <i>Peggy G. Lemaux</i>	511
The Role of Hybridization in Plant Speciation <i>Pamela S. Soltis and Douglas E. Soltis</i>	561
Indexes	
Cumulative Index of Contributing Authors, Volumes 50–60	589
Cumulative Index of Chapter Titles, Volumes 50–60	594

Errata

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