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Molecular analysis of the S-RNase in self-incompatible *Solanum chacoense*

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Sommaire

L'AI est la capacité génétiquement déterminée d'une plante fertile de rejeter son propre pollen pour prévenir l'autofécondation et l'inbreeding conséquent. Ce phénomène de reconnaissance cellulaire, implique des interactions fines pollen-pistil, sous le contrôle des éléments d'un locus multigénique, nommé locus-S. Le résultat de ces interactions est que les plantes auto-incompatibles rejettent le pollen "self", mais acceptent le pollen "non-self". Chez les Solanacées, Rosacées et Scrophulariacées, l'AI est de type gamétophytique, c'est-à-dire le phénotype incompatible du pollen est déterminé par son propre génotype. La composante pistillaire chez ces familles est une glycoprotéine polymorphe exprimée au niveau du tissu de transmission du style et sécrétée dans la matrice extracellulaire. La composante pollinique a été identifiée comme étant une protéine de type F-box, désignée S-locus F-box (SLF) ou S-F-Box haplotype-specifique (SFB) par les différents auteurs.

Dans notre première étude, nous avons voulu comprendre pourquoi le pollen diploïde ayant deux composantes polliniques différentes est auto compatible. Nous avons généré des tétraploïdes $S_{11}S_{11}S_{13}S_{13}$ et pollinisé avec leur pollen diploïde des plantes transgéniques exprimant une S-RNase a double spécificité $S_{11/13}$. Si la compatibilité était due au manqué d'expression, ces croisements devraient être compatibles. Si, par contre, il y a compétition, ces croisements devraient être incompatibles. Puisque l'analyse génétique a montré que les croisements étaient incompatibles, nous avons conclu que les deux allèles du gène-S pollinique sont exprimes normalement dans le pollen diploïde.

Dans notre deuxième étude, nous avons analysé la fonction de la région C4 des S-RNases. L'accumulation des S-RNases à l'intérieur des tubes polliniques compatibles et incompatibles implique l'existence dans le pollen d'un inhibiteur ou d'un mécanisme de dégradation des S-RNases. Mais comment celles-ci pénètrent dans les tubes polliniques ? Interagissent-elles avec un inhibiteur?

Possèdent-elles des lysines conservées qui pourrait constituer la cible d'ubiquitination ? Notre attention s'est portée tout naturellement sur la région C4 des S-RNases, car elle possède quatre résidus chargés K D R D exposés à sa surface, dont une lysine. Nous voulions déterminer si la région C4 pourrait être impliqué:

(i) soit dans l'entrée des S-RNases dans les tubes polliniques,

(ii) soit dans la liaison avec l'inhibiteur à l'intérieur du tube pollinique,

(iii) soit (via sa lysine conservée) dans l'ubiquitination menant à la dégradation des S-RNases lors des croisements compatibles. Pour départager entre ces différentes possibilités, nous avons généré des plantes transgéniques exprimant des mutants clés de la région C4 de la S11-RNase. L'analyse de ces mutants nous a permis d'exclure un rôle de la région C4 dans l'entré de la S-RNase dans les tubes polliniques, ainsi que dans la liaison avec un inhibiteur ou encore dans l'ubiquitination. Nous proposons à la place que cette région joue un rôle essentiel dans la stabilisation de la structure tridimensionnelle de la protéine.

Dans notre troisième étude, nous avons déterminé la quantité minimale (valeur seuil) nécessaire pour le rejet du pollen incompatible. Nous avons utilisé quatre génotypes, G4 ($S_{12}S_{14}$), VF60 ($S_{12}S_{12}$), 314 ($S_{11}S_{12}$), L25 ($S_{11}S_{12}$) pour déterminer la quantité de S_{12}-RNase contenue dans leurs styles. Nous avons tout d'abord développé des techniques pour pouvoir faire des analyses style par style sur ces lignés. Les analyses Western ont révélé que G4 et VF60 ont en moyenne 301 ± 93 ng et 269 ± 55 ng de S_{12}-RNase par style, respectivement, alors que les génotypes 314 et L25 ont 136 ± 86 ng et 86 ± 55 ng par style, respectivement. Ces valeurs expliquent pourquoi les génotypes L25 et 314 possèdent une compatibilité sporadique, alors que celle-ci n'a jamais été observée chez G4 et VF60. Quant au seuil lui-même, en se basant sur la formation de fruits après pollinisations, il semble se situer autour de 80 ng par style.

Dans notre dernière étude, nous voulions vérifier si la compatibilité de *N. sylvestris* est vraiment attribuable à une mutation qui a introduit un site de glycosylation dans la région hypervariable de son S-RNase, ce qui ne permettrait plus la reconnaissance du pollen. La S_{11}-RNase de *S. chacoense* possède un seul site de glycosylation dans la région conservée C2. Nous avons ajouté par mutagenèse dirigée un site de glycosylation dans la région HVa. Nous avons été surpris de voir le pollen S_{11} rejeté par les plantes transgéniques exprimant fortement le transgène. Puisque les analyses ont révélé que la glycosylation du nouveau site n'était pas complète, et pour favoriser la glycosylation du site en HVa, nous avons fait une autre construction où le site conservé endogène en C2 était aboli. Parmi les transgéniques analysés, une plante s'est révélée capable de rejeter non seulement le pollen S_{11}, mais aussi S_{13} et même le pollen diploïde hétéroallelique.

Mots-clés : *Solanum chacoense*, auto-incompatibilité gamétophytique, S-RNase, région conservée C4, mutagenèse dirigée un site, pollen diploïde hétéroallelique, glycoprotein, et valeur seuil.

Abstract

Self-incompatibility (SI) is the inherited ability of a fertile hermaphrodite flowering plant to prevent self-fertilization by blocking the growth of pollen tubes on or inside the pistil. SI constitutes a prezygotic barrier present in about half of the Angiosperm families, and is one of the most widespread evolutionary devices used by flowering plants to prevent inbreeding depression by promoting gene exchange within a species. This cell-cell recognition mechanism, by far more complex than previously imagined, involves finely tuned pollen-pistil interactions under the control of elements of the S-locus. As a result, self-incompatible plants reject self- but accept non-self pollen. Solanaceae, Rosaceae and Scrophulariaceae families display gametophytic SI (GSI), where the breeding behavior of the pollen is determined by its own haploid genotype. Controlled by a single multigenic S-locus, the pistillar-expressed determinant to SI in these families is a polymorphic RNase (S-RNase), a glycoprotein secreted in the extracellular matrix of the style. The pollen determinant to S-RNase-mediated SI has only recently been identified as an F-box encoded by the so-called *S*-haplotype-specific F-box gene (SFB) or *S*-locus F-box gene (SLF), according to the various authors.

In our first study we tried to understand why diploid pollen containing two different pollen determinants is typically self-compatible. We generated $S_{11}S_{11}S_{13}S_{13}$ tetraploids and analyzed the behavior of their pollen on pistils of transgenic plants expressing a dual-specific $S_{11/13}$-RNase. If the compatibility were due to lack of pollen-S gene expression (silencing), the $S_{11}S_{13}$ pollen produced by the $S_{11}S_{11}S_{13}S_{13}$ tetraploids would be compatible with the transgenic plants. Alternatively, if competitive interaction between S_{11} and S_{13} pollen-S components present together in diploid pollen takes place, the $S_{11}S_{13}$ pollen should be rejected by the transgenic plants, because our dual-specific $S_{11/13}$-RNase rejects both S_{11} and S_{13} pollen. Since the genetic analysis revealed full rejection of $S_{11}S_{13}$ pollen, we concluded that the pollen components of the SI system are fully expressed in diploid heterozygous pollen.

In a second study we analyzed the function of the conserved C4 domain of the S-RNase, which had not been experimentally determined. We wanted to know if the C4 region is involved either:

i) in S-RNase entry into the pollen tubes or

ii) in binding a general S-RNase inhibitor or

iii) being the target for ubiquitination

To test these hypotheses, we mutated the C4 region of the S_{11}-RNase of *Solanum chacoense* in three different constructs. In the first construct, the 4 charged amino acids K D R D were substituted with the uncharged G G G G. All transgenic plants fully accepted S_{11} pollen. When western blots were performed with an anti-S_{11} antibody, no S_{11}-RNase could be detected in the pistils of these transgenic plants. In the second construct, the arginine was substituted with glycine. The transgenic plants rejected S_{11} pollen. However, since they accepted other pollen types, this demonstrates that C4 is neither involved in S-RNase entry into the pollen tubes (otherwise S_{11} pollen would be accepted), nor in binding an S-RNase inhibitor (otherwise all pollen types should be rejected). In the last construct, the lysine was substituted with arginine. Here again, the transgenic plants rejected S_{11} pollen, but they accepted S_{13} pollen. If the lysine in C4 region represents the ubiquitination site for S-RNase degradation via an F-box protein, the transgenic plants should reject all pollen types, because the substitution would block the ubiquitin degradation pathway. Since the transgenic plants rejected S_{11} pollen but accepted other pollen types, this demonstrates that the lysine in C4 is not the target for ubiquitination.

In our third study we determined the minimum levels (threshold) of S-RNase required for pollen rejection. Four genotypes, G4 ($S_{12}S_{14}$), VF60 ($S_{12}S_{12}$), 314 ($S_{11}S_{12}$), L25 ($S_{11}S_{12}$) were used for quantifying the S_{12}-RNase contents in individual styles. The Western blots from individual styles revealed that G4 and VF60 have an average of 301 ± 93 ng and 269 ± 55 ng of S_{12}-RNase per style, respectively, whereas genotypes 314 and L25 have 136 ± 86 ng and 86 ± 55 ng per style, respectively. These values can explain why L25 and 314 show sporadic

self-compatibility, whereas in G4 and VF60 this never happens. Surprisingly, the amount the S_{12}-RNase accumulated in different styles of the same plant can differ by over twenty-fold. We have measured a low level of 160 ng S-RNase in individual styles of fully incompatible plants, and a high value of 68 ng in L25 line during a bout of complete compatibility, suggesting that these values bracket the threshold level of S-RNase needed for pollen rejection. Remarkably, correlations of S_{12}-RNase values to average fruit sets in 314 and L25 lines to different extents as well as to fruit set in immature flowers, are all consistent with a threshold value of 80 ng S_{12}-RNase.

In our last study, we wanted to verify if the reported compatibility of *N.sylvestris* was really due to a mutation that allows glycosylation to occur inside one of the hypervariable regions of its S-RNase. Since pollen recognition occurs at the HV regions of the S-RNases, it is possible that the sugar moiety hampers pollen recognition, hence the observed compatibility. The authentic S_{11}-RNase in *Solanum chacoense* has only one N-glycosylation site in the conserved C2 region. We reasoned that an additional site, added at its hypervariable region (HVa), should influence the S-RNase recognition capacity, and therefore we expected the S_{11} pollen to be accepted by transgenic plants harboring such mutated S-RNase. We were surprised to see that the plants highly expressing the transgene fully rejected S_{11} pollen. The analyses revealed that styles from these plants did not uniformly glycosylate both sites of the mutated S_{11}-RNase. This induced us to make another construct where the conserved, endogenous glycosylation site was removed in order to augment glycosylation at the HVa site. Surprisingly, one transgenic individual was found that rejected not only S_{11} pollen, but also S_{13} pollen, and even the otherwise compatible diploid heteroallelic pollen.

Key words: *Solanum chacoense*, gametophytic self-incompatibility, S-RNase, C4 conserved region, site-directed mutagenesis, S-heteroallelic pollen, glycoprotein, and threshold.

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List of Abbreviations

bp: base pair(s)

C4: conserved region 4

cDNA: complementary DNA

DBA: days before anthesis

DNA: deoxyribonucleic acid

DTT: dithiothreitol

ER: endoplasmic reticulum

EDTA: ethylene diamine tetra acetic acid

GSI: gametophytic self-incompatibility

HAP: S-heteroallelelic pollen

HV: hypervariable region

HVa: hypervariable region a

HVapb-RNAse: dual-specific S-RNase

HVb: hypervariable region b

IPTG: isopropyl-beta-D-thiogalactopyranoside

kb: kilobase(s)

kDa: kilodalton(s)

M: molar or moles/liter

ml: milliliter(s)

mg: milligram(s)

mM: millimolar or millimoles/liter

mRNA : messenger RNA

ng: nanogram(s)

PAGE: polyacrylamide gel electrophoresis

PCR: polymerase chain reaction

PMSF: Phenylmethylsulphonylfluoride

PNGase: peptide-N-glycosidase

RNA: ribonucleic acid

RNase: ribonuclease

S_{11}: S-haplotype 11

S_{12}: S-haplotype 12

S_{13}: S-haplotype 13

S_{14}: S-haplotype 14

SC: self-compatibility

***S. chacoense* :** *Solanum chacoense*

SD: standard deviation

SDS: sodium dodecyl sulfate

SI: self-incompatibility

SLB: S-haplotype-specific F-box gene

SLF: S-locus F-box gene

SLG: S-locus glycoprotein

S-RNase: S-ribonuclease

SSC: sporadic-self-compatibility

UV: ultraviolet

µlg : microgram(s)

µl : microliter(s)

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Chapter I. Introduction

I.1 Definition

In flowering plants, seed set is dependent on the mutual recognition of compatible interacting partners: the pollen grain, carrying the male gametes, and the pistil, containing the female gametes. During the pollination process, the pollen is transported by wind or animal vectors to the stigma, which constitutes the receptive surface of the pistil. The pistil possesses the ability to accept or reject the different types of pollen it receives. Since about 95% of the angiosperms species carry male and female organs on the same individuals, and the vast majority of these species have hermaphroditic flowers, self-pollination is expected to be likely to occur (Robert et al., 1994). In order to avoid self-pollination and the resulting negative effects of inbreeding, i.e. the decline in fitness of selfed individuals compared to outcrossed ones, the flowering plants have developed several strategies, including dioecism, proterandry, protogyny, and heterostyly. The most common barrier to selfing, however, is self-incompatibility (SI), present in more than 50% of the angiosperm species (Gaude and Cabrillac, 2001). SI represents an inherited device that promotes outbreeding and therefore generates and maintains genetic diversity within species (de Nettancourt, 2001). According to the most widely accepted definition, self-incompatibility in higher plants is "the inability of a fertile hermaphrodite seed-plant to produce zygotes after self-pollination"(de Nettancourt, 1977).

In SI species, the recognition and rejection phases are governed by elements from pistil and from pollen. This recognition mechanism can be compared to the defense mechanisms in plants and to the immunological system in animals, with the important difference that in SI rejection is directed

towards the "self", whereas in the defense mechanisms and animal immune response the rejection is towards the "non-self ".

I.2 Self-Incompatibility systems

I.2.1 Heteromophic and homomorphic SI

A variety of self-incompatibility systems have been recognized in flowering plants. Based on floral morphological differences, these systems can be divided into two groups, heteromorphic and homomorphic self-incompatibility (Stone, 2002). In the heteromorphic SI system, flowers of the same species have different floral morphological types. Present in at least 24 families, there are two classes of stylar polymorphism: distyly and tristyly, that differ for the number of floral morphs that occur within populations. These differences provide a physical barrier to self-pollination. Crosses succeed only when they involve individuals with different morphs (de Nettancourt, 2001). In a homomorphic SI system, flowers of the same species have the same floral morphological type. Present in about 90 families, the interactions between pollen and pistil are generally determined by elements of one of the most polymorphic loci in plants, the *S*-locus (de Nettancourt, 2001). This locus is constituted by a multigene complex inherited as one segregating unit. Thus, the term *S*-haplotype is used to designate variants of the locus, whereas the term allele is used to designate variants of a given polymorphic gene of the S-locus (McCubbin and Kao, 2000).

I.2.2 Genetic basis of homomorphic SI: sporophytic and gametophytic types

Classic genetic studies have revealed that the homomorphic SI system could

be further divided into two types, based on the genetic control of pollen breeding behavior: the sporophytic self-incompatibility (SSI) and gametophytic self-incompatibility (GSI).

In sporophytic SI, the breeding behavior of the pollen is determined by the genotype of the pollen-producing plant. This type is characterized by stigmatic rejection and is usually found in species with dry stigma and trinucleate pollen. So far, SSI has been reported in only six families, and has been investigated at the molecular level in Brassicaceae and Convulvulaceae (de Nettancourt, 2001).

In gametophytic SI, the compatible or incompatible phenotype of the pollen is determined by the S-haplotype of its haploid genome. Usually found in species with wet stigma and binucleate pollen, this type of SI is characterized by pollen rejection at the stylar level, based on the matching of its haploid *S*-haplotype with one of the two *S*-haplotypes of the diploid pistil. In this case pollen is recognized as self and rejected, and the cross will be incompatible. Conversely, if the *S*-haplotype of the pollen differs from those of the pistil, the pollen is accepted for fertilization and the cross will be compatible (Figure I.1). Normally, after compatible crosses, the pollen grains germinate very rapidly on the stigma surface producing pollen tubes that penetrate and grow inside the style through the transmitting tissue and finally reach the ovary, where they accomplish fertilization. However, if the *S*-haplotype of the pollen grain matches with either of the *S*-alleles of the diploid tissue of the style, the cross is incompatible. In that event, the pollen tube initially elongates, but it will be arrested in the upper region of the style. Observations of an incompatibility reaction with the UV microscope reveal that the pollen tube growth becomes irregular and slows down, its walls thicken, the tip of the tube swells and frequently bursts (Figure I. 2). In most cases, the arrest of the pollen tube's growth occurs at the upper third of the style.

GSI is far more common than SSI, is found in numerous families and has been particularly studied at the molecular level in Solanaceae, Rosaceae, Scrophulariaceae and Papaveraceae (Cappadocia, 2003). This last family, with dry stigma, stigmatic rejection and binucleate pollen, has a SI system that appears to differ at the molecular level from that of the other three families that are evolutionarily related (Igic and Kohn, 2001) and share a common SI mechanism often referred to as Solanaceae type GSI based on S-RNases (see below) (Igic and Kohn, 2001). These differences in SI mechanism strongly supports the hypothesis that GSI is not monophyletic, and together with the molecular data available for the sporophytic system, suggests that SI evolved independently several times in different lineages of the flowering plants (Takayama and Isogai, 2005).

I.3 Molecular basis of Solanaceae type GSI: identification of the female determinant

During the past two decades, much progress has been made in identifying and characterizing the *S*-locus genes that are responsible for the SI reaction in the families mentioned above. The studies aiming at identifying the S-gene encoding for the female determinant to SI, assumed that S-specific gene products should co-segregate with *S*-alleles (although the fact that proteins co-segregate with *S*-alleles does not necessarily mean that they are products of the *S*-alleles, because they could also be produced by genes very closely linked to the *S*-locus), should be expressed in the pistil and display allele-specific sequence differences. The first evidence of S-specific proteins present in both stigma and style of mature flowers, and that co-segregated with *S*-alleles in inbred and cross progenies of *Nicotiana alata*, was provided in the early 80s (Bredemeyer and Blaas, 1981). The molecular breakthrough,

however, occurred a few years later, with the first cloning, in *Nicotiana alata,* of a cDNA encoding for a 32-kDa S_2-glycoprotein co-segregating with the S_2-allele (Anderson et al., 1986). This work opened the way to the rapid identification and cloning of many related cDNAs encoding for S-proteins from other members of the Solanaceae (Singh and Kao, 1992). Further comparison of the sequences of the *S*-locus gene expressed in the pistil of other SI species belonging to the Rosaceae and Scrophulariaceae families, has revealed that these families as well share the same female S-determinant molecule as the Solanaceae, a glycoprotein with ribonuclease activity termed S-RNase.

I.3.1 Structure-function relationship of S-RNases

S-RNases are glycoproteins with molecular masses ranging between 20 and 40 kDa and pI values between 6.2 and 9.5 (Green, 1994). They are relatively abundant in the pistil, being secreted by the cells of the transmitting tissue of the style, and their concentration in the extracellular matrix of the transmitting tissue is estimated in mg per style (McCubbin and Kao, 2000; Qin et al., 2006). Their denomination as S-RNases came with the discovery that they have ribonuclease activity and that the specific activities of the purified *N. alata S*-glycoproteins were comparable with those of fungal RNase T2 from *Aspergillus oryzae* (McClure et al., 1989). In addition, sequence data confirmed that the S-glycoproteins shared sequence homology with two regions of the T2 ribonuclease and those of the ribonuclease Rh from *Rhizopus niveus*, each of which contains two conserved catalytic histidines (McClure et al., 1989). Evidence that S-RNases control the rejection of pollen came from further experiments in *N. alata* showing that pollen RNA degradation, in particular rRNA degradation, took place in self-pollinated styles, but did not occur after compatible pollinations (McClure et al., 1990). It has, however, been argued that degradation of pollen rRNA could be the

result of pollen tube death rather than its cause (Kao and Tsukamoto, 2004b).

The role of the S-RNases in SI and the demonstration that they are necessary for the rejection of self-pollen was established via *Agrobacterium*-mediated transformation experiments (Lee et al., 1994; Murfett et al., 1994). Thus, in a gain-of-function experiment, the expression of the S_3-RNase transgene in S_1S_2 plants of *Petunia inflata*, enabled the transgenic plants to reject S_3 pollen (Lee et al., 1994). Conversely, in a loss-of-function experiment, the expression of an antisense S_3-RNase transgene in S_2S_3 plants, made the transgenic plants unable to reject S_3 pollen (Lee et al., 1994). Similar results were obtained with the introduction of the S_{A2} gene from *Nicotiana alata* into a *N.langsdorffii* x self-compatible *N.alata* hybrid, which conferred to the transgenic plants the ability to reject the S_{A2} pollen (Murfett et al., 1994). The *Agrobacterium*-mediated transformation approach was also used to demonstrate that ribonuclease activity is essential for the role that S-RNases play in SI (Huang et al., 1994). Here, the codon of one of the two conserved histidines of the catalytic domain of the S_3-RNase gene of *P.inflata* was replaced with the codon of an asparagine and this mutated S-RNase gene was introduced into S_1S_2 plants. The transformed plants expressing this transgene had no S_3-RNase activity and failed to reject the S_3 pollen. This, together with the observation that a mutant self-compatible line of *L. peruvianum* lacks one of the conserved histidines of the catalytic site and produces an inactive S-RNase (Royo et al., 1994), led to the conclusion that rejection of incompatible pollen involves the cytotoxic action of the RNase activity of S-RNases (Huang et al., 1994).

Taken together, all the results obtained from plant transformation experiments proved that the stylar S-RNases are necessary for the recognition and rejection of incompatible pollen. In addition, since the pollen behavior of the transgenic plants remained always unaltered, these studies provided evidence for the hypothesis that the *S*-locus must contain at least

two distinct genes. One is the pistillar S-RNase gene, which codes for the female determinant to SI, while the other is the pollen *S*-gene that codes for the male determinant to SI.

I.3.2 The three-dimensional structure of S-RNases

The crystal structure of the ribonuclease Rh from *Rhizopus niveus* has been determined at 2 Å resolution (Kurihara et al., 1992). The overall three-dimensional structure consists of six α-helices and seven β-strands. The two histidine residues, which were predicted to be important for catalysis, are located on the central β-sheet consisting of four β-stands and a α-helix running parallel to those strands (Parry et al., 1997). The crystal structure of the S_{F11}-RNase from *Nicotiana alata*, as well as that of the S_3-RNase from *Pyrus pyrifolia* have also been determined (Ida et al., 2001a; Matsuura et al., 2001b). Both have a structure very similar to the Rh and T2 RNases with eight α-helices and seven β-strands. Further, both possess two pockets where the two conserved histidines of the active site are located, and both have the carbohydrate moiety bound at the conserved Asn at the border of the C2 region protruding from the surface of the molecule, far away from both the active site and the HVa and HVb regions (see later). These two HV domains are close to each other and are exposed at the molecular surface, so they both are expected to form a domain interacting with the product of the pollen *S*-gene. Since the sequence alignments of S-RNases show that most of the differences occur in loop regions rather than in regions of secondary structure, this has suggested that despite their widely different primary sequences, the S-RNases produced by different *S*-alleles have similar three-dimensional structures (Parry et al., 1998).

I.3.3 General features of S-RNases

Sequence identity among solanaceous S-RNases ranges from 38% (Tsai et al., 1992) to 95% (Saba-El-Leil et al., 1994), with S-RNases of different species being sometimes more closely related than those of the same species. This has led to the suggestion that S-RNase polymorphism in the Solanaceae predates species divergence, a suggestion confirmed by molecular phylogenetic studies of S-RNase sequences from *Nicotiana, Petunia* and *Solanum*, genera that diverged some 27 million years ago (Ioerger et al., 1990). Similar data and conclusions have also been obtained in the Rosaceae (Sassa et al., 1996) and in the Scrophulariaceae (Xue et al., 1996).

Despite the high sequence diversity, the S-RNases display a number of important common features (some of which will be discussed later in more detail). In particular they have:

(1) an N-terminal signal peptide, not present in the mature protein, indicating that they enter into the secretory pathway (Cornish et al., 1987; Singh and Kao, 1992). This signal peptide is usually 22 amino acids long in the Solanaceae (Singh and Kao, 1992), 26-27 amino acids long in Rosaceae (Sassa et al., 1996), and 32-33 amino acids long in Scrophulariaceae (Xue et al., 1996);

(2) several conserved cysteines (usually eight, although their number can vary from seven to ten), involved in disulfide bridges (Ishimizu et al., 1996a);

(3) an Asn-X-Ser/T motif (usually one, although up to six have been found) for a potential attachment of N-linked glycosyl chains (Ishimizu *et al.*, 1998);

(4) several hydrophilic and hydrophobic regions (Clarke and Newbigin, 1993);

(5) a distinct pattern of short conserved regions and longer variable regions. In particular, five conserved regions, designated C1 through C5 are very conserved among the solanaceous S-RNases (Ioerger et al., 1991). The C1 and C5 contain mostly hydrophobic residues and are thought to be involved

in stabilizing the core of the protein (Kao and McCubbin, 1996). The C2 and C3 regions are homologous to the catalytic domain of the T_2 and Rh ribonucleases from the fungi *Asperigillus orizae* and *Rhizopus niveus*, respectively (McClure et al., 1989). The two conserved histidines in C2 and C3 form the active site and are essential for S-RNase activity (Huang et al., 1994; Royo et al., 1994). The function of the C4 region has been recently reevaluated (Qin et al., 2005). In the Rosaceae, an RC4 region takes the place of the C4 region of Solanaceae but shares no homology with it (Sassa et al., 1996; Ishimizu et al., 1998), whereas such a region seems absent in the Scrophulariaceae (Xue et al., 1996);

(6) two highly hydrophilic hypervariable (HV) regions, termed HVa and HVb, with the highest degree of diversity among the sequences analyzed. They were predicted to be the prime candidates for the determinant of S-haplotype specificity and to form the domains that interact with the pollen *S*-gene product (McCubbin and Kao, 2000). Only one hypervariable region (RHV) has been identified in the Rosaceae (Takayama and Isogai, 2005);

(7) one conserved short intron common to all S-RNases from Solanaceae, Rosaceae and Scrophulariaceae, except those in the genus *Prunus* of the Rosaceae family, where two introns are present (Igic and Kohn, 2001).

I.3.3.1 Role of hypervariable regions in *S*-allele specificity

The amino acid residues of the two hypervariable regions (HVa and HVb) are typically hydrophilic and thus predicted to be exposed on the surface of the molecule, where they presumably interact with the product of the pollen *S*-gene (Anderson et al., 1989). Domain swapping experiments have induced some authors to suggest that the hypervariable regions are necessary but not sufficient for *S*-allele specificity (Kao and McCubbin, 1996; Zurek et al., 1997). Thus, in *P. inflata*, either both HVa and HVb regions of the S_3-gene and the region between them were replaced with the corresponding region of

the S_1-gene, or only the HVb region of the S_3-gene was replaced with the corresponding HVb region of the S_1-gene. Each chimeric *S*-gene was introduced into S_2S_2 *P.inflata* plants. Both hybrid S proteins produced by the transgenic plants were found to have an S-RNase activity comparable to that of the wild type S_3 protein. However, transgenic plants expressing the hybrid S proteins failed to reject either S_1 or S_3 pollen. This indicates that both hybrid S proteins had lost their S_3-allele specificity but had not gained the S_1-allele specificity, although they maintained the ability to reject S_2 pollen. Similar experiments have allowed other authors to suggest that the *S*-allele specificity is also located outside of the HV regions (Zurek et al., 1997). In this last study, nine chimeric S-RNases were constructed, combining various sequences of the S_{A2} allele with those of the S_{C10} allele of *N. alata*, and all were introduced into a *N. langsdorffii* x self-compatible *N. alata* hybrid. The genetic analysis revealed that none of the transformants harboring any of the chimeric genes could recognize and reject either S_{A2} and S_{C10} pollen. Since the chimeric S-RNases had normal RNase activity, the authors concluded that the *S*-allele specificity is not restricted to the HV regions of the S-RNase but scattered throughout the protein.

The demonstration that the hypervariable regions of the S-RNase are both necessary and sufficient for *S*-allele specificity came from studies using site directed mutagenesis rather than the swapping domain approach (Matton et al., 1997). The molecular characterization of S_{11} and S_{13}-RNases of *S. chacoense*, which reject pollen haplotypes S_{11} and S_{13}, respectively, revealed that the mature proteins differ by only 10 amino acids, three of which found in the HVa region and one in the HVb region (Saba-El-Leil et al., 1994). These S-RNases thus constitute an ideal system to evaluate the role of the HV regions in pollen recognition.

In a first study, when an S_{11}-allele was introduced into a $S_{12}S_{14}$ genotype, the transformed plants expressing the transgene acquired the S_{11} phenotype. When the HV regions of the S_{11}-allele were substituted by site

directed mutagenesis with those of the S_{13}-allele, the transformants highly expressing the transgene acquired the ability to reject pollen S_{13}, but no longer recognized pollen S_{11} (Matton et al., 1997). This represents the first instance of an experimentally induced change of *S*-allele specificity, and confirmed that the HV regions of the S-RNase do control pollen recognition. In a subsequent study, an *S*-allele where three amino acids of the HV regions of the S_{11}-RNase were substituted with those of the S_{13}-RNase was constructed. The introduction of this allele into an $S_{12}S_{14}$ genotype resulted in transgenic plants with a new *S* phenotype, that was termed dual-specific since these plants could reject both S_{11} and S_{13} pollen simultaneously (Matton et al., 1999).

All the experiments reported above by the various authors indicate that the hypervariable regions are necessary for the allelic specificity. However, the conclusions differ on whether the hypervariable regions alone are sufficient for allelic specificity. In this regard, it has been noted that the level of amino acid sequence identity between the *S. chacoense* S_{11} and S_{13} alleles (95%) is much higher compared to the other S-RNases, and it cannot be excluded that other regions that are identical between the S_{11} and S_{13}-RNases may also be involved in the determination of *S*-allele specificity (Verica et al., 1998).

I.3.3.2 S-RNases are glycoproteins with N-linked glycans

More than 200 S-RNases analyzed so far are glycoproteins with N-linked glycans (de Nettancourt, 2001). Potential N-glycosylation sites with the consensus sequence Asn-X-Ser/Thr (X any amino acid except Pro or Asp) are present in variable numbers in different *S*-alleles (de Nettancourt, 2001). Thus, for example, the S_{11} and S_{12}-RNases cloned in our laboratory have one and four potential glycosylation sites, respectively (Saba-El-Leil et al., 1994; Qin et al., 2001). Early studies showed that enzymatic removal of the glycan

chains had no effect on RNase activity of native S-RNases (Broothaerts et al., 1991). Considering the important role played by the glycan chains of many animal cell proteins in cell-cell recognition, the possibility that the glycan chain of the S-RNases could be involved in self/non-self recognition has been examined (Karunanandaa et al., 1994). In that study, the codon for Asn 29, which constitutes the only potential N-glycosylation site of the S_3-RNase in *P. inflata*, was replaced with the codon for Asp, and this mutated S_3-gene was introduced into S_1S_2 plants by *Agrobacterium*-mediated transformation. The transgenic plant producing high levels of the non-glycosylated S_3 protein was able to fully reject S_3 pollen. The authors concluded that the carbohydrate moiety of the S-RNases does not constitute the recognition site for rejection of self-pollen and is not involved in SI. This latter view, however, is not universally accepted. Since the transformed plants had only been tested for rejection of S_1, S_2 and S_3 pollen, but not for rejection of other pollen haplotypes, it could not be excluded that the removal of the conserved N-glycosylation site might have abolished the specificity of the SI reaction, thereby enabling the transgenic plants to reject pollen of any haplotype (Oxley et al., 1996). Furthermore, observing that all solanaceous S-RNases possess a conserved potential glycosylation site at the border of the C2 region (rosaceous S-RNases also have one conserved glycosylation site at Asn 121 located in the RC4 conserved region), and that this site is absent in S-like ribonucleases (see later) known not to be involved in SI (Green, 1994), Oxley argued that the glycans must play some still unknown biological role in SI (Oxley et al., 1998). It has been suggested that the N-glycans may influence the three-dimensional structure of the S-RNases which is important for the proper folding and stabilization of their structure (Ishimizu et al., 1999).

I.3.3.3 Plant S-like RNases and "relic" S-RNases

The discovery of the S-RNases has led to the identification of a large number of plant RNases not involved in SI, called S-like RNases (for a review see (Bariola and Green, 1997)). They were initially found in pistils of both SI and SC species of the Solanaceae, Rosaceae and Scrophulariaceae and have later been described in SC species of other families (Kao and Tsukamoto, 2004b). In general, they have molecular masses between 21 and 29 kDa, and while some have been either shown or predicted to be secreted proteins, the role of most of them remains unknown. Comparison of the amino acid sequences of these proteins revealed that they do not exhibit allelic diversity, and in particular, numerous residues are conserved among S-like RNases but not among S-RNases, mainly between the C2 and C3 regions, as well as at the N-terminal end (Green, 1994). Some S-like RNases have been proposed to be derived from S-RNases, possibly selected for during the breeding process leading to the generation of SC cultivars from their SI ancestors. This seems to be the case of S_x and S_o RNases found in one SC cultivar of *Petunia hybrida* (Ai et al., 1992). Another interesting category of RNases concerns the so-called "relic" S-RNases also found in SI and SC species of the Solanaceae, Rosaceae and Scrophulariaceae (Kao and Tsukamoto, 2004b). Similar to S-like RNases, they are exclusively found in the pistils and do not exhibit allelic sequence polymorphism. A specially interesting case is represented by the "relic" S-RNase found in *N. sylvestris*, a SC species that possesses a stylar level of RNase activity comparable to that of its related SI species (Golz et al., 1998). Phylogenetic analyses reveal that this "relic" S-RNase is derived from functional S-RNases. More important, sequence analyses show that such a glycoprotein is unique in having the carbohydrate side chain within the first hypervariable region, and not at the border of the C2 region as found in all functional S-RNases sequenced to date (Golz et al., 1998). It is not clear, however, whether the presence of the carbohydrate chain in such position is responsible for the SC

character of *N. sylvestris*, which it might well do by sterically disrupting the self-recognition process.

I.3.4 The hypothesis of S-RNase threshold for expression of the SI phenotype

S-haplotype-specific pollen rejection requires high levels of S-RNase expression; conversely, plants with low levels of S-RNase are not able to reject self-pollen (Takayama and Isogai, 2005). This has been further observed in transformation experiments, where only the transformants producing S-RNase amounts similar to wild type acquire the new phenotype. Thus, for example, when the *S. chacoense* S_{11}-S-RNase was expressed in a $S_{12}S_{14}$ genetic background, S_{11}-pollen was not rejected by those transgenic plants producing low amounts of the protein (Matton et al., 1997; Matton et al., 1999; Qin et al., 2005). In addition, accumulation of the S-RNases in the style during flower development has been shown to be temporally regulated and the increase in S-RNase levels correlates with the acquisition of the incompatibility phenotype (Clark et al., 1990). Thus, bud pollination has been widely used for the production of progenies containing S-homozygotes necessary for research purposes. The success of the method derives from the fact that immature buds have not yet accumulated enough S-RNase for rejection of self-pollen. The incomplete SI response, associated with a reduced level of S-RNases in the pistil, has led to the hypothesis that a threshold level of the RNase is required for full expression of the SI phenotype (Clark et al., 1990). Support for this hypothesis has been provided by studies comparing the expression of the S4-protein in two Japanese pear cultivars, the self-compatible (SC) Osa-Nijisseiki and the self-incompatible (SI) Nijisseiki (Zhang and Hiratsuka, 1999). According to those studies, the SC of Osa-Nijisseiki was due to a reduced expression level, in mature styles, of the S4-protein, representing one quarter that of the S4-protein normally

found in mature styles of Nijisseiki. In addition, as predicted by the threshold hypothesis, the immature styles of Nijisseiki were characterized by low levels of the S4-protein and weak incompatibility (Zhang and Hiratsuka, 1999). Further support for the hypothesis has been provided by work with *S. chacoense* displaying a special type of partial incompatibility called sporadic self-compatibility, i.e. occasional fruit formation after crosses expected to be incompatible (de Nettancourt, 1977; Qin et al., 2001). Interestingly, sporadic self-compatibility has been observed in some but not all S_{12}-containing genotypes of *Solanum chacoense*, and is characterized by occasional bouts of compatibility with S_{12} pollen that can affect from 10 to 60 % of the styles on a given plant. An analysis of the S_{12}-allele expression in several plant lines showed genotype-specific differences in the amount of the corresponding mRNA and protein. As sporadic self-compatibility occurred only in those genotypes with the lowest average S_{12}-RNase levels, it was proposed that that style-to-style variations in S-RNase levels could explain sporadic compatibility (Qin et al., 2001) if levels of S-RNase below the threshold were expressed in some individual styles. This hypothesis was confirmed by the experimental determination of style-to-style variation in individual styles (Qin et al., 2006).

It must be noted that other studies in which values of S-RNase content per style were reported (Jahnen et al., 1989a; Jahnen et al., 1989b), did not permit the determination of the RNase threshold. The reason for this is that the analyses were not conducted on individual styles, thus providing only averaged RNase levels, and furthermore, the genotypes used were fully incompatible (i.e., all S-RNase levels exceeded that threshold). In *N. alata*, for example, calculations of the RNase levels of S_2- and S_6- glycoproteins based on the amount of S-RNases purified from hundreds of styles yielded estimates of about 8 to 10 µg of protein per style, while the amount of the S_3-glycoprotein was significantly lower (0.5 to 1.0 µg). Since even the styles containing the S_3-glycoprotein were fully self-incompatible, this determination

indicates only that the threshold lies below the lowest values (Jahnen et al., 1989a; Jahnen et al., 1989b).

I.4 The molecular basis of Solanaceae type GSI: identification of the male determinant

The identity of the male determinant and the molecular mechanisms by which S-RNase degrades pollen RNA in an *S*-haplotype-specific manner has remained for a long time a mystery in S-RNase-mediated GSI. The predicted characteristics of the *S*-gene representing the male determinant are:

(1) it must belong to the *S*-locus;

(2) its allelic sequence should display a level of polymorphism comparable to that of the S-RNase gene;

(3) it must be expressed in pollen and not in styles;

(4) the encoded pollen-*S* protein and the stylar S-RNase protein should interact in an allelic- specific manner leading to rejection of incompatible pollen tubes.

Initially, the finding that S-RNases are also expressed in developing pollen seemed to indicate that the same *S*-gene would encode both male and female determinants to RNase-based SI (de Nettancourt, 1977; Dodds et al., 1993). Loss-of function and gain-of-function experiments, however, have argued against this idea, since expression in transgenic plants of S-RNase allele of *P.inflata* in either an antisense or a sense orientation was found to alter the self-incompatibility phenotype of the pistil, but not that of the pollen (Lee et al., 1994). Further evidence that pollen-expressed S-RNases are not involved in SI came from transformation experiments in *L. peruvianum*, where sense and antisense S_3-RNase constructs under control of a pollen-specific promoter did not alter the pollen incompatibility phenotype in the transgenic plants (Dodds et al., 1999). Finally, the pollen of a cultivar of *Pyrus serotina* with a style-specific SC mutation caused by deletion of the S4-RNase gene

was found to have a normal incompatibility phenotype (Sassa et al., 1997). These studies demonstrated that the pollen *S*-gene product must be encoded by a separate gene. The pollen S-gene has very recently been identified as an F-box (see later).

I.4.1 *S*-haplotype specific inhibition of pollen tubes: the receptor and inhibitor models

The two main and fundamentally different models that have been proposed to explain how S-RNase specifically inhibit the growth of self-pollen tubes are often referred to as the receptor and the inhibitor models (Figure II.3). The receptor model predicts that the pollen *S*-gene product is a receptor or gatekeeper, whereas the inhibitor model predicts that the pollen *S*-allele products are inhibitors of S-RNase (Haring et al., 1990; Thompson and Kirch, 1992). Since the RNase activity of S-RNases is essential for their function in pollen rejection, both 'receptor' and 'inhibitor' models assume that growth inhibition of self-pollen tubes results from specific degradation of pollen tube RNA by 'self' S-RNases. However, the two models differ in how *S*-haplotype-specific rejection is determined. In the receptor model, the recognition events occurring between S-RNase and pollen take place at the surface of the growing pollen tube. This model proposes that the product of pollen-*S* gene is a membrane-or wall-bound receptor, which allows extracellular S-RNases to enter pollen tubes in an *S*-haplotype-specific manner. The haplotype-specific uptake of the S-RNase would result in degradation of pollen RNA leading to inhibition of pollen tube growth (McCubbin and Kao, 2000). In contrast, the inhibitor model proposes that the product of pollen *S*-gene is a general S-RNase inhibitor located in the cytoplasm of the pollen tube. According to this model, all S-RNases enter freely inside the pollen tube. Here, the pollen-S product binds and inhibits all S-RNases except those of identical *S*-haplotype that will be allowed to degrade pollen RNA leading to inhibition of pollen tube

growth. Clearly, a major distinction between models is that in one case only specific S-RNases enter the pollen, while in the other, all S-RNases enter. This distinction has been made experimentally, and the results provide support for the inhibitor model (Luu et al., 2000). In these experiments, an anti-S_{11}-RNase antibody that is highly specific (Matton et al., 1999) was used in immunolocalization studies, and the results showed that when styles of an $S_{11}S_{13}$ plant were either self-pollinated (a fully incompatible cross), pollinated with S_{12} pollen (a fully compatible cross), or pollinated with S_{13} and S_{14} pollen (a semi-compatible cross), all pollen tubes appeared similarly labeled with the S_{11} antibody. This indicated that S-RNase accumulation in pollen tubes is genotype-independent and argue strongly against the receptor model (Luu et al., 2000).

I.4.2 A modified inhibitor model: the two-component model

The comparative analyses of S-RNases and the domain-swapping experiments have indicated that S-RNases contain two separate functional domains, the *S*-allele-specificity and the RNase activity domains (Kao and McCubbin, 1996). It has therefore been assumed that the pollen-*S* gene product might also possess two domains, one for binding in allelic-specific way to the recognition domain of its cognate S-RNase, the other for inhibiting the RNase activity of non-cognate S-RNases. Thus, the original inhibitor model assumes that binding to the two domains is mutually exclusive, and that binding to a cognate recognition domain is thermodynamically favored over binding to the RNase activity domain. More importantly, this model predicts that deletion or inactivation of pollen S-gene should be lethal. It is possible, however, that RNase inhibition and allele-specific recognition are carried by two separate proteins (Luu et al., 2001). In this modified model, a general RNase inhibitor is present in all pollen tubes, and it would bind to the RNase activity domain and block the activity of any S-RNase except those of

identical *S*-haplotype, whereas the product of the pollen *S*-gene would only bind to the recognition domain of its cognate S-RNase, leaving the RNase activity domain free to destroy the pollen tube RNA (Figure II.4). This modified model was suggested to explain the rejection of otherwise compatible heteroallelic diploid pollen (see below) by transgenic plants expressing a chimeric S-RNase with dual-specificity (Luu et al., 2001). According to this modified model, deletion or inactivation of pollen S-gene would produce viable and fully compatible pollen.

I.4.2.1 Breakdown of the self-incompatibility in heteroallelic diploid pollen

It is well known that in several families with S-RNase-based GSI, including Solanaceae and Rosaceae, natural or induced tetraploids from SI diploids are self-compatible (de Nettancourt, 1977). Genetic studies show differences in reciprocal crosses between SI diploids and their SC tetraploid counterparts, which indicates that the breakdown of self-incompatibility is due to the pollen and not to the pistillar component (Figure II.5). In particular, when diploid pollen produced by a heterozygous tetraploid (for example $S_1S_1S_2S_2$) is placed on a diploid pistil of identical *S*-haplotype (S_1S_2), SC is observed; conversely, when haploid pollen or diploid pollen from a homozygous tetraploid of a given S-haplotype (for example $S_1S_1S_1S_1$) is placed on a tetraploid pistil containing that *S*-haplotype (for example $S_1S_1S_2S_2$), SI is observed. Tetraploid individuals of $S_1S_1S_2S_2$ type are expected to produce diploid pollen at a 50:50 ratio of heterozygote: homozygote (Stone, 2002). With regard to the SI phenotype, diploid pollen homozygous for the *S*-locus responds exactly as does normal haploid pollen. By contrast, diploid pollen carrying two different *S*-loci, escape recognition and is compatible on any style because of the so-called competitive interaction (de Nettancourt, 1977; Chawla et al., 1997). Classic genetic

studies, confirmed by more recent molecular studies, have also shown that duplication of only the *S*-locus itself, and not of the entire genome, has the same effect. Indeed most of the self-compatible pollen mutants analyzed so far have been shown to possess a second S-locus on a centric chromosomal fragment, which would result in the production of pollen carrying two *S*-alleles at the *S*-locus (de Nettancourt, 1977; Golz, 2000; Golz et al., 2001). Although the biochemical basis of the competitive interaction remains unknown, it has been proposed that the presence of two different *S*-alleles in the same pollen grain would establish a state of competitive interaction perhaps analogous to co-suppression leading to their silencing, which would result in SC (de Nettancourt, 2001). This explanation, however, has been ruled out, since heteroallelic $S_{11}S_{13}$ diploid pollen was rejected by transgenic plants expressing a dual-specific chimeric $S_{11/13}$-RNase, i.e. able to reject simultaneously both S_{11} and S_{13} pollen (Matton et al., 1999), suggesting that both *S*-alleles are expressed in heterozygous diploid pollen (Luu et al., 2001). The rejection of the otherwise compatible *S*-heteroallelic pollen by the dual-specific $S_{11/13}$-RNase was explained by assuming that: (1) the active form of the pollen *S*-allele product is a homotetramer; (2) the pollen S-allele product contains only the *S*-specificity domain and would therefore bind exclusively to its cognate S-RNase; and (3) a general inhibitor present in the pollen is responsible for the inactivation of all non-cognate S-RNases. According to the modified inhibitor model, the general inhibitor would bind and inactivate all S-RNases, except those bound to their cognate pollen S-allele product via the *S*-specificity domain. In *S*-heteroallelic pollen, the pollen S-products would form mainly heterotetramers, which could not bind efficiently their cognate S-RNases, leaving the general inhibitor free to inactivate such S-RNases. In addition, since the heterotetramers could also not bind efficiently to any other S-RNase, this would explain why heteroallelic pollen would be compatible on any pistil. Since the dual-specific S-RNase was able to reject both S_{11} and S_{13} pollen separately, it could still bind to both RNases when

present in heterotetramers. Pollen-S would thus still prevent binding of the general inhibitor, leading to the rejection of heteroallelic $S_{11}S_{13}$ pollen (Luu et al., 2001).

I.4.3 Identification of the pollen *S*-gene product as F-box protein

Recently, several pollen *S*-genes have been identified through genomic analysis of the region containing the *S*-locus in three plant families, namely Scrophulariaceae, Rosaceae and Solanaceae. The first clue for the identification of the pollen *S*-gene was obtained in *Antirrhinum hispanicum*, a member of the Scrophulariaceae. The analysis of a 63.7-kb BAC clone of the S_2-locus has revealed the presence of a gene encoding for an F-box protein, *AhSLF-*S_2 (for *A. hispanicum S*-locus F-box of S_2-haplotype), located 9kb downstream of the S2-RNase gene, that was specifically expressed in anthers and pollen grains, as predicted for the pollen *S*-gene (Lai et al., 2002). However, since no other sequences allelic to *AhSLF-*S_2 were identified in other *S*-haplotypes, except one cDNA encoding for a *AhSLF-*S_2 homolog with an extremely high sequence similarity (97.9% deduced amino acid sequence identity), it was initially unclear whether *AhSLF-*S_2 represented the true pollen *S*-gene. Later on, genomic analysis of the *S*-locus of *Prunus mume* (Japanese apricot), a member of the Rosaceae, revealed that the 62.5-kb genomic region around the S-RNase gene contained as many as four F-box genes (Entani et al., 2003). Among them, only one, termed *Pm*SLF, fulfilled the characteristics of a pollen *S*-gene: (*a*) it was located within the *S*-locus, (*b*) its product exhibited *S*-haplotype-specific diversity (78% to 81% deduced amino acid identity), and (*c*) it was specifically expressed in pollen (Entani et al., 2003). Around the same time, other polymorphic F-box genes were found in the *S*-locus of *P.dulcis* (Japanese almond), *P.avium* (sweet cherry), and *P. cerasus* (sour cherry), and were independently named SFB (S-haplotype-specific F-box) (Ushijima et al.,

2003; Yamane et al., 2003a). SLF/SFB from *Prunus* species fulfill most conditions required for the pollen *S*-gene. Aligning deduced amino acid sequences of SLF/SFBs of these *Prunus* species revealed the presence of two hypervariable regions, HVa and HVb, at the C-terminus (Ushijima et al., 2003). The conclusive evidence that SLF/SFB encodes the pollen *S*-gene was finally obtained using the transgenic approach in *Petunia inflata* (Sijacic et al., 2004). A thorough analysis of the sequence in a large region around the *S*-locus (328-kb BAC derived from the S_2-haplotype) revealed the presence of a polymorphic F-box gene, named *PiSLF,* 161-kb downstream of the S_2-RNase gene. To confirm that *PiSLF* encodes the true pollen *S*-gene, S_1S_1, S_1S_2 and S_2S_3 plants were transformed with the S_2-allele of *PiSLF* ($PiSLF_2$) under the control of its own promoter. The transformation of *PiSLF* caused breakdown of their pollen function in SI, equivalent to the same competitive interaction observed in tetraploid plants (de Nettancourt, 1977). In addition, genotypic analysis of the progeny from self-pollinations of $S_1S_2/PiSLF_2$ and $S_2S_3/PiSLF_2$ revealed that only S_1 and S_3 pollen carrying the $PiSLF_2$ transgene (corresponding to S-heteroallelic pollen) had become self-compatible, but not S_2 pollen carrying the $PiSLF_2$ transgene (corresponding to S-homoallelic pollen), that remained incompatible (Sijacic et al., 2004). These results provided conclusive proof that SLF/SFB is the long-sought pollen *S*-gene representing the male determinant to S-RNase-based GSI.

Two additional studies have provided additional evidence that the SLF/SFB is the pollen *S*-gene. Four self-compatible mutants of *P.avium* and *P.mume* have recently been described with mutations in their SLF/SFB gene causing loss of pollen-*S* function. In one particular mutant, both HVa and HVb regions of the SLF gene were missing (Ushijima et al., 2004), whereas in another, the entire *S*-haplotype-specific F-box gene was deleted (Sonneveld et al., 2005). This last mutant is particularly interesting because according to the original inhibitor model this mutant should be lethal, whereas it is

predicted to be viable and self-compatible by the modified two-component inhibitor model (Luu et al., 2001).

1.4.3.1 Involvement of F-box proteins and a new model for SI

The F-box domain was first described as part of an enzyme complex required for ubiquitin-mediated protein degradation in mammalian cells (Bai et al., 1996). In particular, the role of this sequence motif was to interact specifically with proteins destined for degradation. Degradation of the target protein in this process requires several steps and involves, among others, three components of the ubiquitination enzymatic system, namely E1, (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin-ligase), that together catalyze the formation of polyubiquitin chains on specific protein substrates which marks them for degradation by the 26S proteasome. In the first step, the E1 activates a small 76 residue polypeptide called ubiquitin by coupling ATP hydrolysis to the formation of an E1-Ub intermediate in which the C-terminal Gly residue of ubiquitin links itself covalently to E1 via high-energy thiol ester bond. The activated ubiquitin is then transferred to an E2 which in turn delivers the Ub moiety to the specific target protein using an E3 complex as catalyst. The E3 selects the substrate and positions it for optimal transfer of the ubiquitin moiety, and then initiates the conjugation. In the final step, the carboxyl group of the C-terminal Gly residue of ubiquitin is transferred via an isopeptide bond to an ε NH_2 group of a target Lys of the substrate protein to be degraded (Smalle and Vierstra, 2004).

Through several rounds of conjugation, multiple ubiquitination of a specific Lys is achieved, and the targeted protein is now able to be degraded by the 26S proteasome. The 26S proteasome is a large proteolytic complex that degrades Ub-conjugated cytosolic proteins. It is formed by two sub-complexes, the 20S complex responsible for the core protease activity, and

the 19S regulatory complex that confers specificity. The ubiquitination pathway represents a protein quality control system that is essential for cellular housekeeping purposes since it is involved in removing both defective or short-lived regulatory proteins (Smalle and Vierstra, 2004).

There are five classes of E3 described to date, and four have been found in plants. One class, SCF E3s, may be particularly relevant to RNase-based GSI, as all candidates for the pollen-*S* identified to date in *Antirrhinum* (Lai et al., 2002; Qiao et al., 2004a), *Prunus* (Entani et al., 2003; Ushijima et al., 2003; Yamane et al., 2003b; Sonneveld et al., 2005), and *Petunia* (Sijacic et al., 2004) contain a motif 40 to 50 amino acids long typical of F-box proteins. F-box proteins are part of the SCF complex (from Skp1, Cullin1 and F-box, three of the four proteins it contains). Together with a RING-HC finger protein called Rbx1, the four polypeptide SCF complex has ubiquitin-ligase activity (Smalle and Vierstra, 2004). Based on this role of F-box proteins in ubiquitination, a new model for SI has been proposed, where it is not the inhibition, but rather the stability of S-RNases that is controlled by the interaction between the S-RNase and the pollen *S*. According to this model (Figure I.6), SLF/SFB is proposed to function as an inhibitor of non-self S-RNases by mediating their degradation in compatible pollinations, whereas *S*-haplotype-specific interaction between SLF/SFB and its cognate S-RNase protects the latter from ubiquitin-mediated degradation in incompatible pollinations (Qiao et al., 2004a). As a consequence, levels of S-RNase in pollen should decrease after compatible but not incompatible pollination. Although this has been reported in *Anthirrhinum* (Qiao et al., 2004a), it has not been observed in other studies (Luu et al., 2000; Goldraij et al., 2006).

Thus, although there is little doubt that *SLF/SFB* gene is involved in self-incompatibility, the details of the mechanism underlying *S*-haplotype-specific pollen rejection remain a mystery. Recent biochemical studies, for example, suggest the involvement of AhSLF-S_2 in the protein degradation pathway, although a number of features remain quite obscure. AhSLF-S_2 has

been reported to interact with the S-RNase in pull-down and immunoprecipitation experiments, but no biochemical evidence has been provided for an S-specific interaction (Qiao et al., 2004a). In fact, AhSLF-S_2 appears to interact with both self and non-self S-RNases, including an S_3-RNase from Petunia, but it mediates degradation of only non-self S-RNases (Qiao et al., 2004a; Qiao et al., 2004b). So far, however, such interactions and degradation have not been reported in either *Prunus* or in *Petunia*. Thus, in spite of the fact that both S-RNase and pollen *S*-gene have been identified, the molecular mechanisms concerning how these molecules interact and specifically inhibit self-pollen growth remains unclear, as is how S-RNases penetrate inside the pollen tubes.

1.4.4 Additional genes that modulate the SI response and the most recent model for SI

In addition to the S-RNase gene and the pollen *S*-gene that determine the specificity of the SI interaction, genetic and molecular analyses have revealed that other genes at other loci are required for full manifestation of the SI response. These genes, located outside the *S*-locus, are collectively called modifier genes and are responsible for the observed breakdown of SI in particular genetic backgrounds. The analysis of a natural population of self-incompatible *Petunia axillarix*, for example, has shown the presence of self-compatible individuals (Tsukamoto et al., 1999). The molecular analyses revealed that these individuals carried a pistil-part defective S_{13}-haplotype that did not produce any S_{13}-RNase (Tsukamoto et al., 2003a). The progeny of the selfed individuals, however, contained SI plants expressing the S_{13}-RNase, which indicated that the S_{13}-RNase was not defective. This finding suggested that its expression could be suppressed by an unidentified modifier gene in some genetic backgrounds (Tsukamoto et al., 2003a). A similar case was reported in *S. chacoense*, where the S_{12}-RNase is

expressed at different levels in various genotypes, leading to sporadic SC in some of them (Qin et al., 2001).

Modifier genes can be identified by isolating pistil and pollen proteins that interact with S-RNases. In this way, a pollen-expressed protein of *Petunia hybrida* has been identified through a yeast two-hybrid assay and appears to bind to the N-terminal part of the S-RNase, although not in an allelic-specific fashion (Sims and Ordanic, 2001). This protein, named PhSBP1, contains a RING-finger domain and, as with other RING-finger proteins, is thought to function as part of a E3 ubiquitin ligase complex. This has led to the suggestion (Sims and Ordanic, 2001) that PhSBP1 could be the general inhibitor of S-RNase predicted by the two-component inhibitor model (Luu et al., 2000; Luu et al., 2001). As such, PhSBP1 would bind to the activity domain of any S-RNase, whereas the product of pollen-S gene, if present, would bind to the recognition domain of its cognate S-RNase. The latter S-haplotype-specific binding, being thermodynamically favored over the general binding between S-RNase and PhSBP1, would prevent self S-RNases from being ubiquitinated, so that only non-self S-RNases would be degraded by the 26S proteasome (Sims and Ordanic, 2001).

Modifier genes can also be identified by comparing pollen- or pistil-expressed genes present in SI species but not in closely related SC species, and recently a number of pistil-expressed factors have been shown to be involved in pollen rejection, although they do not contribute to S-haplotype-specificity *per se.* The most interesting of such modifier genes has been initially identified in *Nicotiana*, where a gene encoding for a small 8.6-kDa asparagine-rich protein, called HT-B, has been shown to be required for pollen rejection (McClure et al., 1999). The *HT-B* gene was found to be expressed in the style of self-incompatible *N.alata* but not in self-compatible *N. plumbaginifolia* (McClure et al., 1999), and homologs of *HT-B* have since been identified in *Lycopersicon esculentum* (Kondo et al., 2002) and *Solanum chacoense* (O'Brien et al., 2002). Reduction or suppression of HT-

B transcripts in individuals transformed with an antisense RNA or by RNA interference have resulted in the loss of S-haplotype-specific rejection of pollen in both *N. alata* (McClure et al., 1999) and *S. chacoense* (O'Brien et al., 2002). In these self-compatible plants, however, the S-RNase levels were not affected, and the HT-B protein was not found to bind directly to the S-RNase in yeast two-hybrid system analyses.

Most recently, a new model for S-RNase-based GSI has been proposed (Figure II.7), where HT-B plays a central role in S-specific pollen rejection. In this model, compatible pollen tubes avoid rejection by degrading HT-B via a hypothetical pollen-expressed protein and sequestering the S-RNases in the vacuole. In contrast, after incompatible pollinations a complex S-RNase-SLF of identical haplotype would inhibit the hypothetical pollen protein, prevent HT-B degradation, and lead to a breakdown of the membrane surrounding the vacuole. This in turn would release the cytotoxic S-RNases in the cytoplasm, thus leading to pollen rejection (Goldraij et al., 2006).

1.4.5 The present project objectives

The present study includes four projects:

(i) The behavior of heteroallelic pollen on transgenic plants expressing a dual-specific S-RNase;

(ii) The characterization of the role of the C4 domain of the S-RNase;

(iii) The determination of the level of S-RNase required for pollen rejection;

(iv) The effect of adding a glycosylation site in the HV region of the S_{11}- RNase.

The first objective was to characterize the behavior of S-Heteroallelic pollen (HAP) on transgenic plants expressing a dual-specific S-RNase. HAP are usually diploid and contain two different S- alleles. HAP produced by tetraploids derived from self-incompatible diploids are typically self-compatible. The HAP effect has so far remained poorly understood. Two different hypotheses have

been proposed to explain the compatibility of HAP: (i) the lack of pollen-S gene expression and (ii) the " competition effect" between two pollen-S gene products when expressed in a single pollen grain. The objective of the present project was to distinguish between these two hypotheses by generating $S_{11}S_{11}S_{13}S_{13}$ tetraploids and using a unique S-RNase with dual specificity.

The second objective was to characterize the role of the C4 domain in S-RNases, as the function of this conserved region had not been previously determined experimentally. The C4 region is particularly interesting, as four out of the eight conserved amino acids are charged and exposed at the surface of the molecule, and could potentially be a high affinity protein binding site. Furthermore, lysine is among these conserved amino acids, suggestive of a possible target for ubiquitination. This could thus be part of a mechanism whereby the S-RNases may be destroyed through an F-box protein degradation pathway, as suggested by the recent literature. We wanted to know if the C4 region is involved in either S-RNase entry into the pollen tubes, binding a general S-RNase inhibitor or in being the target for ubiquitination. These hypotheses were addressed using three different constructs, all mutated at the C4 region of the S_{11}-RNase of *Solanum chacoense*.

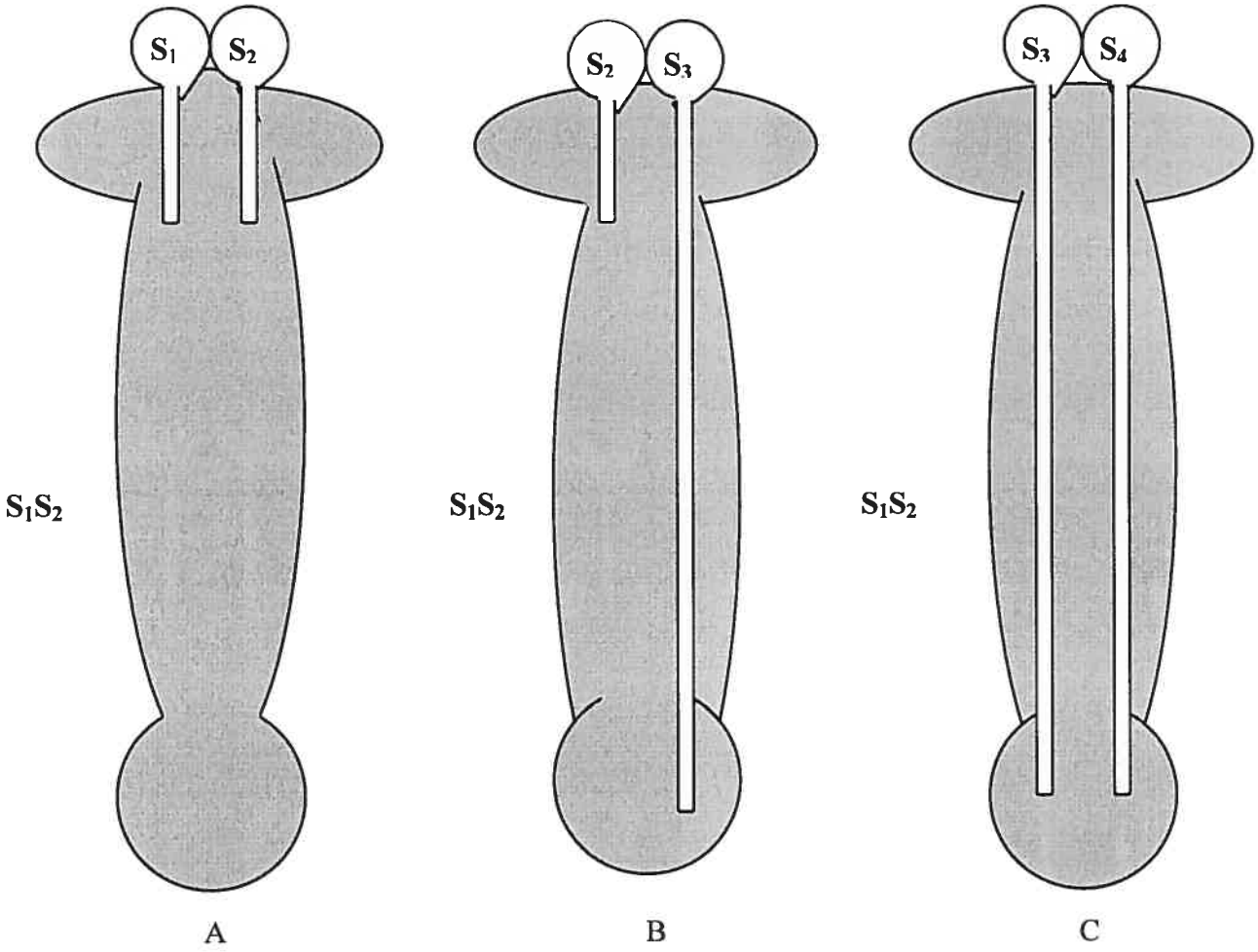
The third objective was to determine the level of S-RNases actually required for pollen rejection. It is known that pollen rejection does not occur at low levels of S-RNase, which has led to the idea that a minimum level (a threshold) of S-RNase must be bypassed for expression of the SI phenotype. Our goal was to experimentally determine this threshold. This was made possible thanks to the availability of plant genotypes expressing variable levels of S-RNases in their styles and by devising a method for quantifying S-RNase amounts in single styles.

Lastly, we wished to determine if a role could be attributed to the glycosylation of the S_{11}- RNase. It has been reported that *N.sylvestris* strongly expresses an S-RNase, and yet it is self-compatible possibly due to a mutation in its sequence that allows glycosylation to occur inside one of its hypervariable

regions. Since pollen recognition occurs at the HV regions of the S-RNases, it is possible that the sugar moiety hampers pollen recognition, hence the observed compatibility. The authentic S_{11}-RNase in *Solanum chacoense* has only the N-glycosylation site conserved in the C2 region. We reasoned that an additional site, added at its hypervariable region (HVa), should influence the S-RNase recognition capacity, and we expected the S_{11} pollen to be accepted by our transgenics. We were surprised to see that the plants highly expressing the transgene fully rejected S_{11} pollen. The analyses revealed that styles from these plants did not uniformly glycosylate both sites of the S_{11}-RNase. This induced us to make another construct where the conserved, endogenous glycosylation site was removed in order to augment glycosylation at the HVa site.

Figure Legends

Figure I.1 In **GSI** system, the genotype of the individual pollen determines whether the interaction is compatible or not. If the genotype of a pollen grain (shown inside the pollen grains) matches either alleles of the diploid stylar tissue (indicated on the side of the pistil), the elongation of the pollen tube is arrested in the style. **A**. Pollen grains S_1 and S_2 match either alleles S_1S_2 of the diploid stylar tissue and are rejected. The cross is fully incompatible. **B** Pollen S_3 does not match alleles S_1S_2 of the diploid stylar tissue and is accepted, whereas pollen S_2 is rejected. The cross is semi-compatible. **C**. Pollen S_3 and S_4 match neither alleles S_1S_2 of the diploid stylar tissue. They are accepted and the cross is fully compatible.



A. S_1S_2 Selfed Incompatible cross
B. S_1S_2x S_2S_3 Semi-compatible cross
C. S_1S_2x S_3S_4 Fully compatible cross

Figure I.2. Rejection of incompatible pollen tube at the upper third of the style observed by means of fluorescence. Note how the tip of the tube swells and bursts.

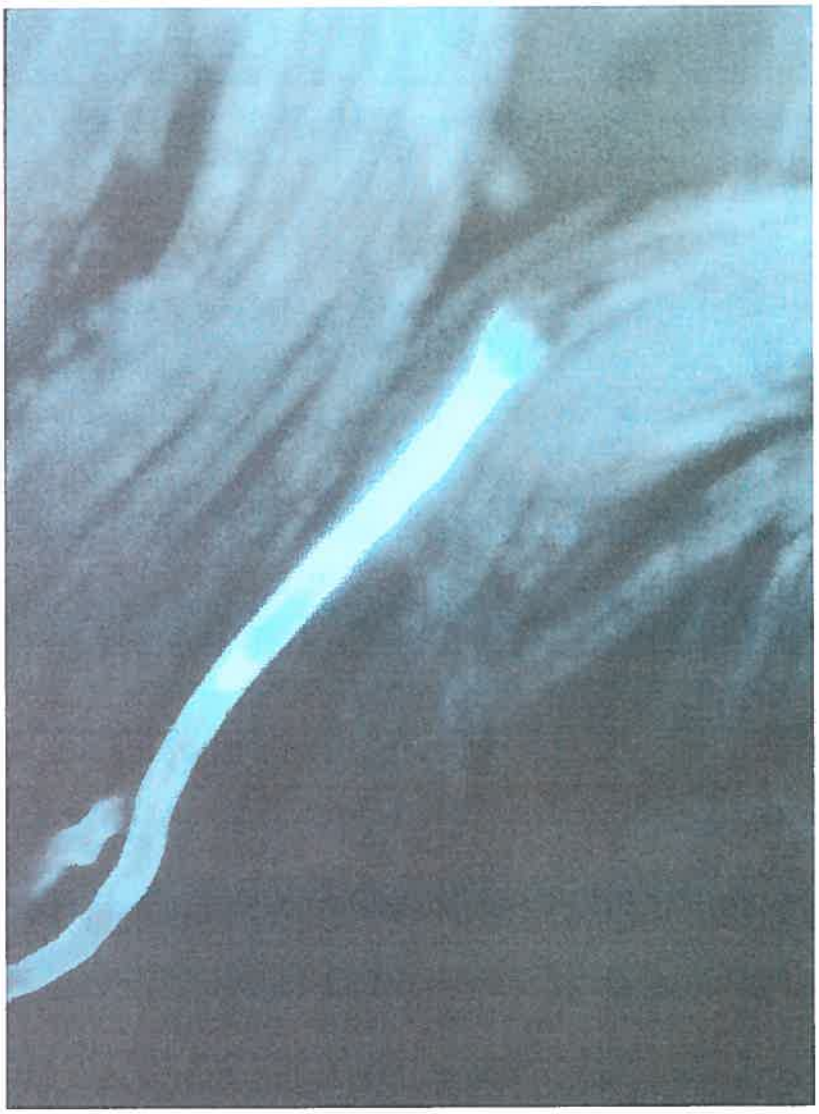


Figure I.3 Models of Receptor and Inhibitor for S-RNase-mediated pollen rejection (from Cappadocia, 2003). The crosses, represented in A and B, involve pistils of an S_1S_2 plant pollinated with pollen from an S_1S_3 plant. The behavior of individual pollen tubes (Cheptou et al.) is either compatible (left; no active RNase inside pollen tubes) or incompatible (right; active RNase inside pollen tubes). (A) Receptor model. A pollen-S protein recognizes and allows entry only of its cognate S-RNase into pollen tubes. (B) One component inhibitor model. Pollen-S proteins have two binding domains, one for the allelic specificity determinants on the S-RNases and one for the RNase activity domain. Binding to the two domains is assumed to be mutually exclusive, and although a pollen-S could bind to either domain of its cognate S-RNase, binding to the allelic specificity determinants is assumed to be thermodynamically favored.

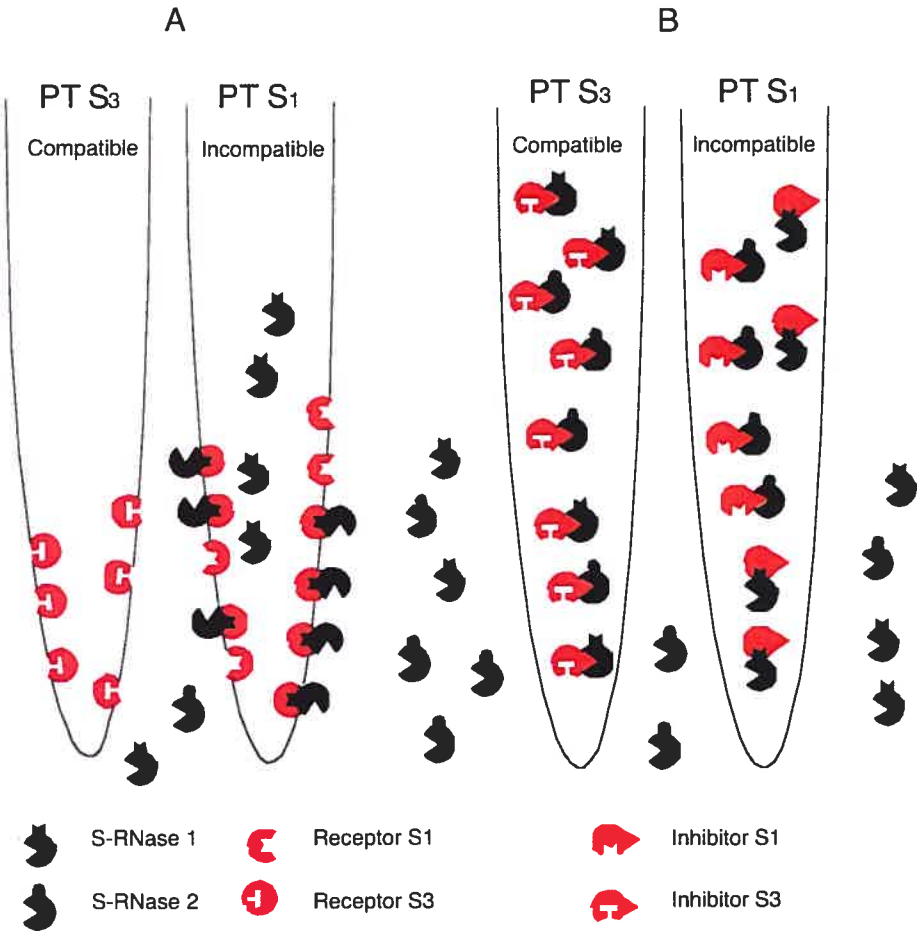

A
B
PT S3
Compatible
PT S1
Incompatible
PT S3
Compatible
PT S1
Incompatible
S-RNase 1
S-RNase 2
Receptor S1
Receptor S3
Inhibitor S1
Inhibitor S3

Figure I.4 Two-component inhibitor model (from Cappadocia, 2003). Pollen-S, binding to the allelic specificity determinants of the S-RNase, is a different protein from a general inhibitor binding to the RNase activity domain. Once again, binding to the two domains is assumed to be mutually exclusive and binding to the allelic specificity determinants is assumed to be thermodynamically favoured.

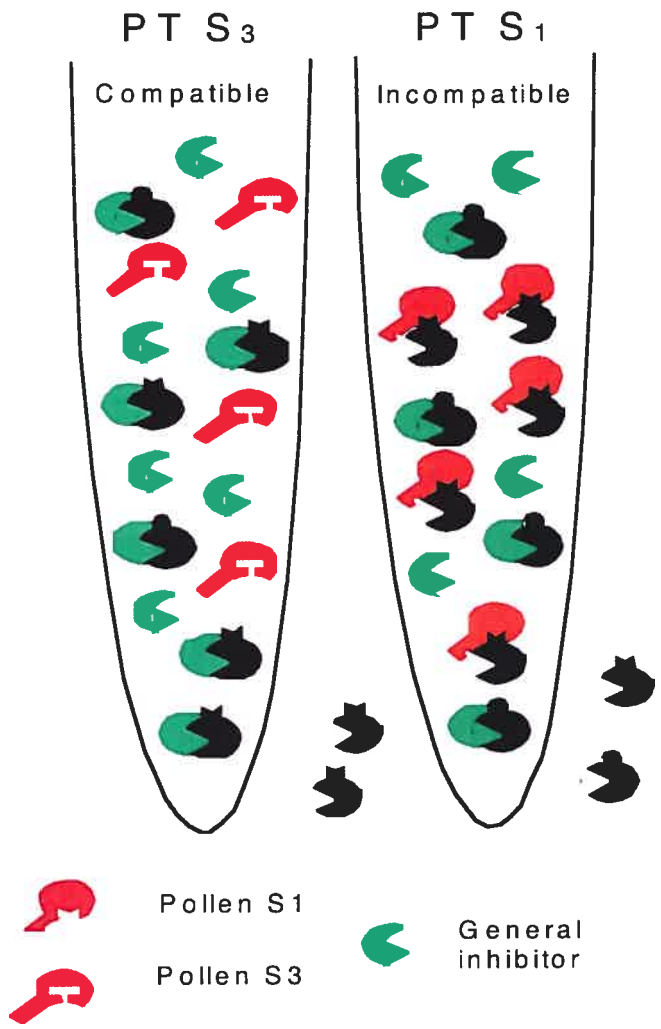
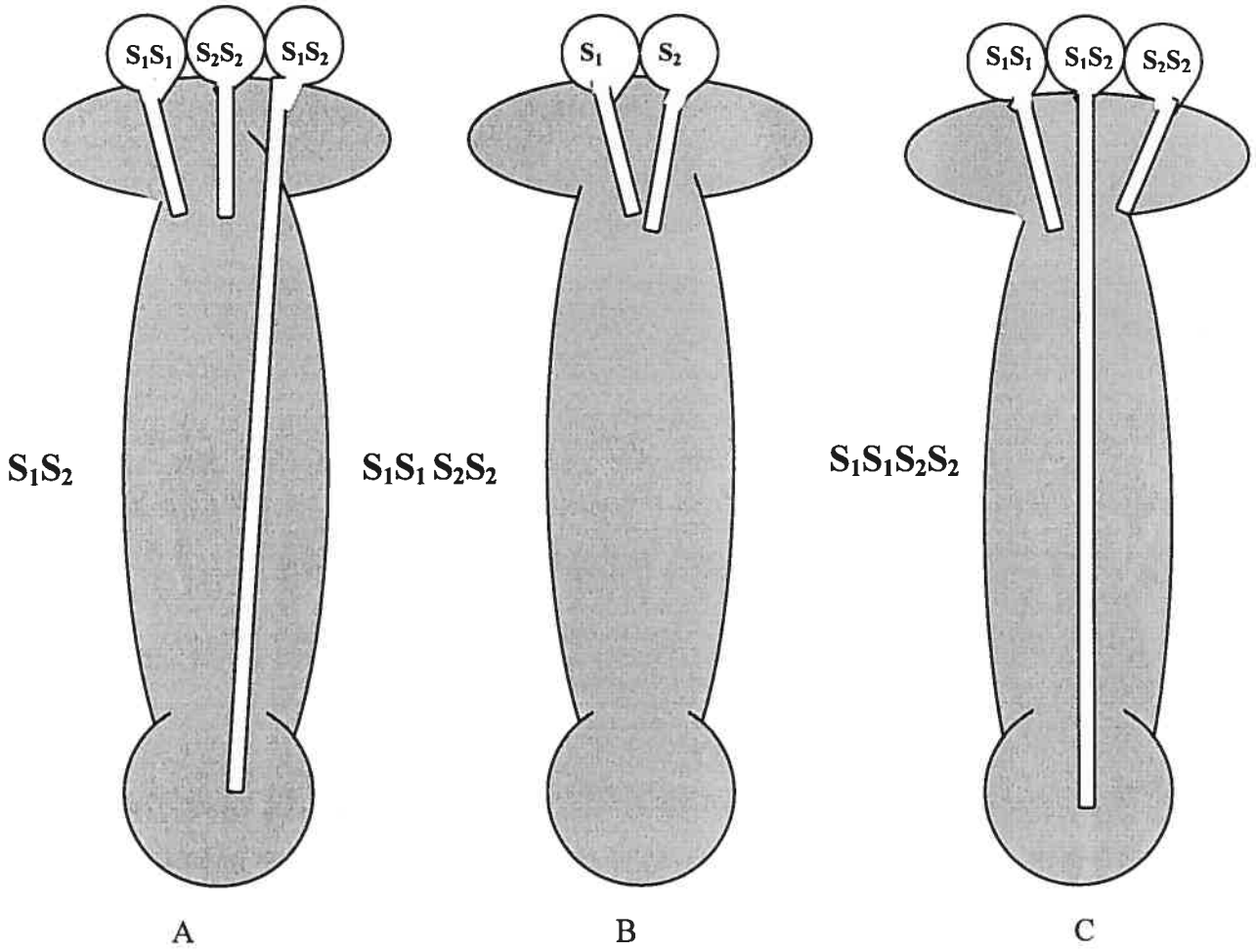
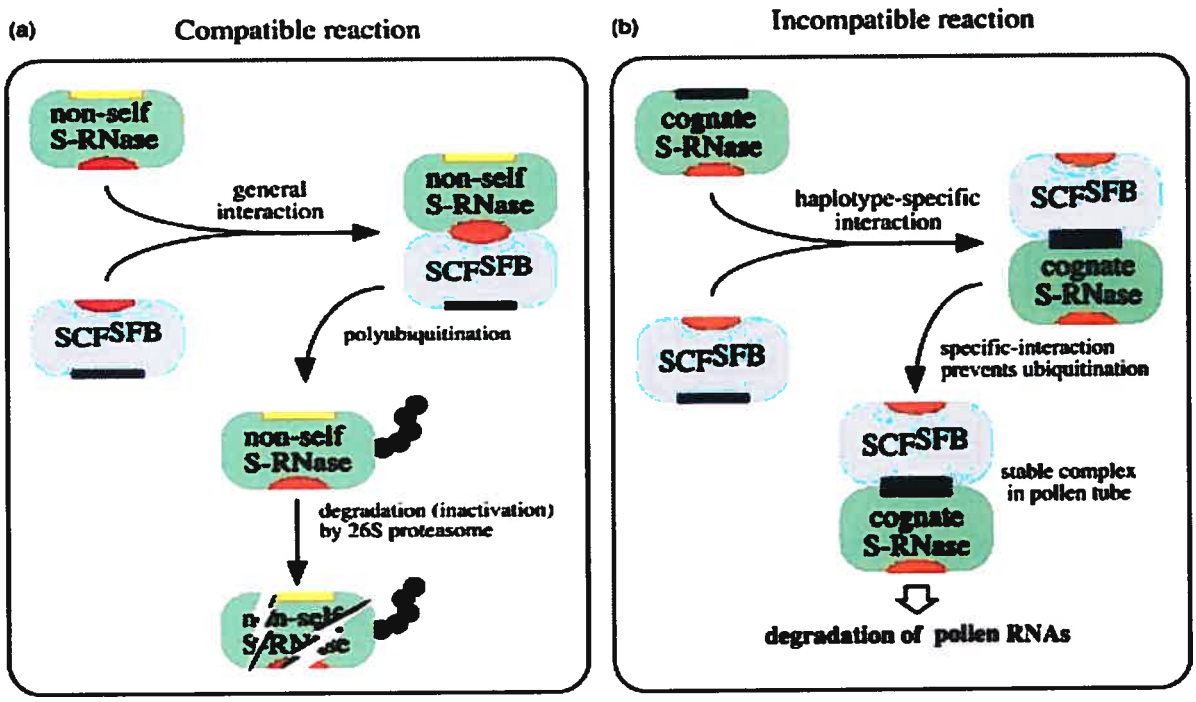

PT S3
Compatible
PT S1
Incompatible
Pollen S1
Pollen S3
General inhibitor

Figure I.5. Breakdown of self-incompatibility in heteroallelic diploid pollen. **A.** Only heteroallelic diploid pollen S_1S_2 is accepted, which results in breakdown of self-incompatibility, whereas homoallelic diploid pollen S_1S_1 and S_2S_2 are rejected. **B.** Haploid S_1 and S_2 pollen are rejected by tetraploid S_1S_1 S_2S_2 styles. **C**. Same as in **A** only heteroallelic diploid pollen S_1S_2 is accepted by the S_1S_1 S_2S_2 style.



A. S_1S_2 x $S_1S_1S_2S_2$ Compatible cross
B. S_1S_1 S_2S_2 x S_1S_2 Incompatible cross
C. $S_1S_1S_2S_2$ (selfed) Compatible cross

Figure I.6. A model for S-RNase degradation by F-box ubiquitination (modified from Ushijima et al., 2004). Haplotype-dependent degradation of S-RNase is assumed to be regulated by SCF^{SFB} and the 26S proteasome pathway. (a) In the compatible reaction, S-RNase are recognized by general interaction domain (red color) of SCF^{SFB} resulting in polyubiquitination and degradation of S-RNase by the 26S proteasome pathway. (b) In the incompatible reaction, SCF^{SFB} and its cognate S-RNase interact with the haplotype-specific interaction domain, which consists of HV region(s) (blue color). This interaction prevents S-RNase from being polyubiquitinated. As a result, the cognate S-RNase remains active and degrades pollen RNA, which results in the arrest of the pollen tube growth.

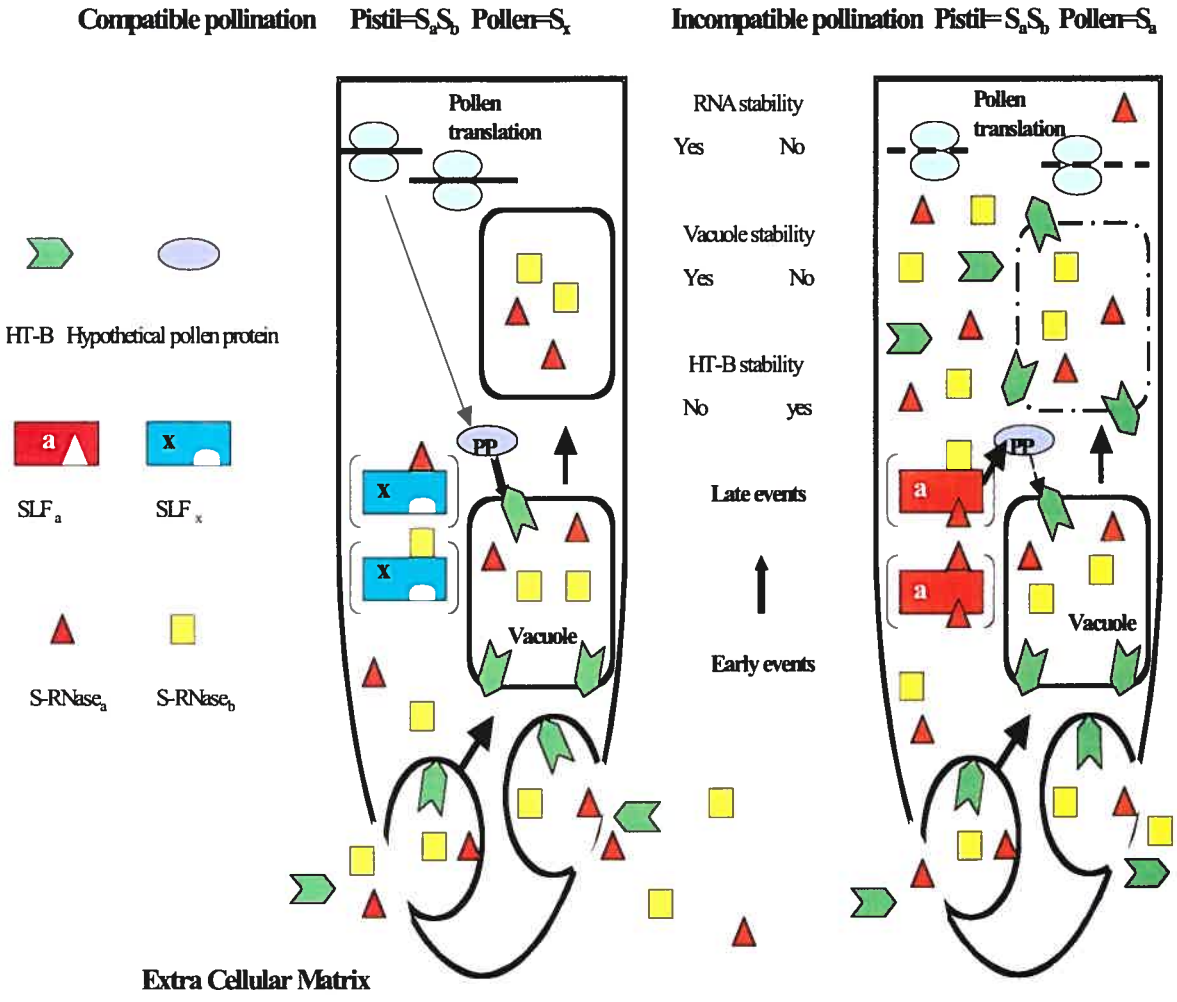


The yellow- and blue-colored boxes are the regions for the *S* haplotype-specific interaction, which could be mainly comprised of the hypervariable region(s). Different colors represent different *S* genotypes.

The red region is for the general interaction.

The black circle is mono ubiquitin.

Figure I.7. The model for S-RNase-based SI (Goldraij et al., 2006) In compatible pollinations (top), pollen overcomes rejection by degrading HT-B and compartmentalizing S-RNase (vacuole). HT-B degradation is shown by a hypothetical pollen protein (PP), but other mechanisms are possible. Some S-RNase may escape and interacts with SLF. In incompatible pollinations (bottom), the self-interaction ($S\text{-}RNase_a$–SLF_a) stabilizes HT-B, perhaps by inhibiting a factor such as PP. In the late stages of inhibition, the compartment breaks down, releasing the S-RNase. RNA degradation prevents replacement of factors needed for degradation of HT-B and maintenance of the compartment. Thus, pollen rejection is self-reinforcing.


Compatible pollination Pistil=S_aS_b Pollen=S_x
Incompatible pollination Pistil=S_aS_b Pollen=S_a
HT-B Hypothetical pollen protein
a
x
SLF_a
SLF_x
$S\text{-RNase}_a$
$S\text{-RNase}_b$
Pollen translation
PP
Vacuole
RNA stability
Yes No
Vacuole stability
Yes No
HT-B stability
No yes
Late events
Early events
Extra Cellular Matrix

Chapter II. Rejection of S-heteroallelic pollen by a dual specific S-RNase in *Solanum chacoence* predicts a multimeric SI pollen component

Objectives

1) **To explain the compatibility of S-heteroallelic pollen (HAP) produced by tetraploids**
2) **To distinguish between two hypothesis: lack of pollen-S expression or competition effect**

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Dr. D.Luu supervised my work and was at the origin of this study. I did most of the experimental procedures, except the production of tetraploids (G.Laublin) and the analysis of some tetraploids (Q.Yang). Dr Morse helped with the interpretation of the results and discussions

Rejection of S-heteroallelic pollen by a dual specific S-RNase in *Solanum chacoense* predicts a multimeric SI pollen component

Running title: Rejection of S-heteroallelic pollen

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ABSTRACT

S-heteroallelic pollen (HAP) grains are usually diploid and contain two different S-alleles. Curiously, HAP produced by tetraploids derived from self-incompatible diploids are typically self-compatible. The two different hypotheses previously advanced to explain the compatibility of HAP are the lack of pollen-S expression and the "competition effect" between two pollen-S gene products expressed in a single pollen grain. To distinguish between these two possibilities, we have used a previously described dual specific $S_{11/13}$-RNase, termed HVapb-RNase, which can reject two phenotypically distinct pollen (P_{11} and P_{13}). Since the HVapb-RNase does not distinguish between the two pollen types (it recognizes both), $P_{11}P_{13}$ HAP should be incompatible with the HVapb-RNase in spite of the competition effect. We show here that $P_{11}P_{13}$ HAP is accepted by $S_{11}S_{13}$ styles, but is rejected by the $S_{11/13}$-RNase, which demonstrates that the pollen-*S* genes must be expressed in HAP. A model involving tetrameric pollen-*S* is proposed to explain both the compatibility of $P_{11}P_{13}$ HAP on $S_{11}S_{13}$ containing styles and the incompatibility of $P_{11}P_{13}$ HAP on styles containing the HVapb-RNase.

Key words: S-RNase, Solanum chacoense, self-incompatibility, heteroallelic pollen

INTRODUCTION

Self-incompatibility (SI) is a cell-cell recognition phenomenon used by higher plants to prevent inbreeding. In the most widespread type of SI (gametophytic SI, or GSI), the self-incompatibility phenotype is specified by a highly multiallelic *S*-locus, and the genotype of the haploid pollen determines its own incompatibility phenotype (de Nettancourt, 1977; de Nettancourt, 2001). In the Solanaceae, the identity of the pollen component of the GSI is unknown, whereas the stylar product has been identified as an extracellular ribonuclease, *S*-RNase (McClure et al., 1989) expressed in the transmitting tissue of the style (Anderson et al., 1986). Gain-of-function experiments have shown that expression of an S-RNase transgene is necessary and sufficient to alter the SI phenotype of the pistil but does not change the pollen phenotype (Lee et al., 1994; Murfett et al., 1994; Matton et al., 1997), and thus the identity of the pollen *S*-gene must be different from the S-RNase (Kao and McCubbin, 1997). S-RNases appear to contain two domains, an RNase activity domain essential for expression of the SI phenotype(Huang et al., 1994) and a recognition domain involved in the specificity of the cell-cell recognition phenomenon. In closely related S-RNases, such as the S_{11}- and S_{13}-RNases (Saba-El-Leil et al., 1994), the recognition domain includes the amino acids found in the so-called hypervariable (HV) regions (Ioerger et al., 1991). The HV regions of these two S-RNases differ by only four amino acids, and transgenic plants where these four residues in the S_{11}-RNase were replaced with those of the S_{13}-RNase displayed an S_{13} rather than an S_{11} phenotype (Matton et al., 1997). Curiously, replacement of only three of these four amino acids has produced RNases which are either non-functional (Matton, 2000) or have the unusual property of dual specificity (i.e. able to reject both the phenotypically distinct P_{11} and P_{13} pollen; (Matton et al., 1999).

The availability of this unique dual specific S-RNase (termed HVapb-RNase) allowed us to re-evaluate the S-heteroallelic pollen (HAP) effect (also known as competitive interaction in diploid HAP. In many diploid species with monofactorial GSI, naturally or artificially produced tetraploids often display self-compatibility (Lewis, 1947; Brewbaker, 1954; de Nettancourt, 1977; de Nettancourt, 2001). Differences in reciprocal crosses between SI diploids and their tetraploid counterparts indicate that the breakdown of SI is due to the pollen and not the stylar component (de Nettancourt, 1977). As first noted by LEWIS (1947), only pollen which contains two different S-loci can bypass the SI barrier, an observation fully confirmed by recent molecular analyses in both *Lycopersicon peruvianum* (Chawla et al., 1997) and *Nicotiana alata* (Golz et al., 1999). The HAP effect requires only a second different S-locus whether carried by a centric fragment or not (van Gastel, 1976; de Nettancourt, 1997; Golz et al., 1999; Golz, 2000).

In spite of more than 50 years of research since its first description, the HAP effect remains poorly understood. In particular, it is not known if it is caused by some peculiar features of a distinct pollen-S gene (still unknown), by some other component of the S-locus (McCubbin and Kao, 1999), or by gene inactivation (Lewis, 1961; van Gastel, 1976) resulting in non-expression of the pollen-S components. Current models for the biochemical role of pollen-S, however, cannot readily explain HAP compatibility. Immunocytochemical analyses showing that S-RNases can enter pollen tubes of any genotype (Luu et al., 2000) have provided experimental evidence for models involving RNase inhibitors (Thompson and Kirch, 1992; Kao and McCubbin, 1996). These models postulate that all S-RNases can enter a pollen tube and that their RNase activity is inhibited, except for that corresponding to the S-haplotype of the pollen. Models where the pollen-S and the RNase inhibitor are on the same molecule (Kao and McCubbin, 1996) or on separate molecules (Luu et al., 2000) have both been proposed. All versions of the inhibitor model assume that pollen-S binding to the recognition

domain of its cognate S-RNase is thermodynamically favored over binding to the RNase activity domain, so that it permanently precludes activity domain binding and permits RNase activity (Kao and McCubbin, 1997). In HAP, the two pollen-S should each preferentially bind to the recognition domains of their respective S-RNases, leaving the RNases active. The inhibitor models thus predict incompatibility for HAP, in contrast with experimental observations.

We report here that $P_{11}P_{13}$ HAP is accepted by styles containing the S_{11} and S_{13} RNases, but rejected by styles expressing the dual $S_{11/13}$ HVapb-RNase. This demonstrates that pollen SI components are functional in HAP, thus ruling out gene inactivation. We propose that pollen-S acts as a tetramer and that heterotetramers, such as would be produced in HAP, are unable to block inhibitor binding and thus produce compatible pollen.

MATERIALS AND METHODS

Solanum chacoense Bitt (2n=2x=24) plants of various S-constitutions were produced by crosses (Veronneau et al., 1992; Birhman et al., 1994; Van Sint Jan et al., 1996) (Birhman *et al.* 1994; Van Sint Jan *et al.* 1996; Veronneau *et al.* 1992). Diploid genotypes V22 ($S_{11}S_{13}$), V28 ($S_{12}S_{13}$) and G4 ($S_{12}S_{14}$) were selected from crosses of two parental lines PI458314 ($S_{11}S_{12}$) and PI230582 ($S_{13}S_{14}$) (Potato Introduction Station, Sturgeon Bay, WI), whereas L25 ($S_{11}S_{12}$) resulted from crosses between V22 and V28 (pollen parent). The dual-specific S-RNase that rejects both P_{11} and P_{13} pollen was produced by site-directed mutagenesis and is expressed as a transgene introduced into host plant G4 ($S_{12}S_{14}$; (Matton et al., 1999). HVapb plants thus reject four different pollen haplotypes, the P_{12} and P_{14} via the endogenous S-RNases from the untransformed host, and the P_{11} and P_{13} using the dual-specific HVapb-RNase. All plants with a dual-specific phenotype contain wild-type levels of the HVapb-RNase in the styles (Matton et al., 1999).

Tetraploids of genotypes L25 ($S_{11}S_{11}S_{12}S_{12}$), V28 ($S_{12}S_{12}S_{13}S_{13}$) and G4 ($S_{12}S_{12}S_{14}S_{14}$) were produced by leaf disc culture from the corresponding diploids as described (Veronneau et al., 1992), whereas tetraploids F 20 ($S_{11}S_{11}S_{12}S_{13}$), F38 and F55 ($S_{11}S_{11}S_{13}S_{13}$), and F44 ($S_{12}S_{12}S_{12}S_{12}$) were selected from progeny of crosses between tetraploids L25 and V28 (pollen parent) (Qin et al., 2001Tetraploid plant 1022 ($S_{11}S_{11}S_{13}S_{13}$) was produced by leaf disc culture of a plant issued from a cross between V22 ($S_{11}S_{13}$) as pollen donor and SP10 ($S_{13}S_{13}$), an individual obtained by obligate selfing of parental line PI230582 ($S_{13}S_{14}$) (Rivard et al., 1994). The genotype of all plants used was verified by Western analyses of stylar extracts using antibodies against S_{11}, S_{12} and S_{13}-RNases (Matton et al., 1999; Qin et al., 2001), by Southern blot analyses and by PCR analyses using S-allele specific primers. All plants used were true tetraploids and not chimaeric, as assessed by chloroplast number in stomatal guard cells (L1 layer), pollen size and chromosome number in pollen mother cells (L2 layer) and chromosome number in root meristems (L3 layer). All cytological analyses were performed as described (Cappadocia, 1984). Compared with their diploid relatives, most autotetraploids showed some reduction in pollen fertility, as generally reported (Singh, 1993).

Crosses were performed under greenhouse conditions, and were classified as compatible when almost all pollinations resulted in fruit formation and incompatible when no fruits developed. Because the nature of the study required a precise assessment of pollen tube behavior after pollinations, tube growth inside the styles was routinely monitored by UV fluorescence microscopy as described (Matton et al., 1997). Incompatibility defined by the pollinations corresponded in all cases to pollen tube growth arrest in the style.

RESULTS

Tetraploids derived from self-incompatible diploids are known to produce compatible S-heteroallelic pollen (HAP; (de Nettancourt, 1997), and this was also observed with our *S. chacoense* tetraploids. As an illustration, the breeding behavior of tetraploids containing two different S-loci (plants G4, L25, V28, F38, F55 and 1022) or three (F20) is shown in Table II.1. These plants all produce diploid pollen, about two-thirds of which contains two different S-alleles and is thus fully self-compatible (see pollinations along the diagonal). This behavior is in sharp contrast to the breeding behavior of diploid pollen containing only one type of S-allele, such as that produced by the S-homozygous tetraploid F44 (Table II.1) which is incompatible with any plant containing the S_{12} allele. In agreement with all previous studies, the compatibility of these tetraploids is due to their pollen, as their styles continue to block haploid pollen containing corresponding S-alleles (Table II.2). It is important to note that pollen produced by the plant V22 (S_{11} S_{13}) is rejected by all the tetraploids expressing both S_{11}- and S_{13}-RNases, by transgenic plants expressing the HVapb-RNase (an $S_{11/13}$ specificity), but is accepted by the untransformed host plant G4 (S_{12} S_{14}). Note also that the pollen produced by the HVapb-plants behaves identically to the pollen produced by the untransformed host since transgene expression is restricted to the style.

The dual-specific HVapb-RNase provides a unique tool to distinguish between gene inactivation and competition models for the HAP effect. If the HAP effect were caused by gene inactivation (Lewis, 1961; Van Gastel., 1976) the $P_{11}P_{13}$HAP would be as compatible with HVapb plants as with V22 ($S_{11}S_{13}$). In contrast, if competition between P_{11} and P_{13} pollen-S components present together in diploid pollen takes place, the $P_{11}P_{13}$ HAP pollen should be rejected by HVapb plants (just like normal haploid pollen) since our dual-specific HVapb-RNase rejects both P_{11} and P_{13} pollen. As shown in Table II.3,

no fruits are formed when pollen from plants with an $S_{11}S_{11}S_{13}S_{13}$ genotype (F38, F55 or 1022) is tested on styles of HVapb plants, and microscopic examination of these pollinated styles confirms full rejection of $P_{11}P_{13}$ HAP at mid-style (not shown). Since plants F38, F55 and 1022 all have different genetic backgrounds, indicating that HAP rejection is not restricted to a particular genotype, we conclude that the pollen components of the SI system must be fully expressed in HAP.

The genetic analysis also demonstrates that the dual-specific HVapb-RNase alone is responsible for HAP rejection. First, there is nothing unusual about plants F38, F55 and 1022, as their HAP is self-compatible (Table II.1), compatible on V22 styles (Table II.3), and their styles reject pollen from V22 (TableII.2). Second, there are no breeding differences between the five independent transgenic plants expressing the HVapb-RNase, as $P_{11}P_{13}$ HAP was fully rejected by their pistils (Table II.3). All the HVapb plants used here express wild type levels of their transgene S-RNase (Matton et al., 1999), and accept all other HAP combinations such as $P_{11}P_{12}$, $P_{12}P_{14}$ or $P_{12}P_{13}$ (Table II.1). In addition, HVapb transgenic plants which do not express the transgene behave like the untransformed host G4 and do not reject HAP (not shown). Lastly, the rejection of the $P_{11}P_{13}$ HAP is unrelated to expression of more than two different S-RNases in the style, as neither tetraploid F20 (Table II.1) nor a transgenic plant of $S_{12}S_{14}$ genotype expressing an additional S_{11}-RNase (Matton et al., 1997) reject $P_{11}P_{13}$ HAP (not shown).

DISCUSSION

Model for pollen-S action: Any model for GSI must now explain the normal compatibility of HAP, as well as its incompatibility with the cognate dual-specific S-RNase, as shown here. To develop a working model, however, two additional observations must be taken into account. First, screens for

compatible pollen produced after mutagenesis have uncovered a variety of pollen part mutants, some of which contained what was referred to as an additional S-allele while others apparently lack any S-allele (Pandey, 1967; Van Gastel., 1976; de Nettancourt, 1977; Golz et al., 1999). Clearly, while an additional S-allele (pollen-S) could be analogous to HAP, deletion of pollen-S must be different. Thus, any model for SI must predict a compatible pollen phenotype either when two different pollen-S are expressed, or when none is expressed. Second, as discussed above, at least part of the function of the SI system inside pollen tubes is likely to involve RNase inhibitors (RI). Although not yet reported for plants, RI are well known in animal systems (Hofsteenge, 1997).

We recently proposed a model for GSI with two pollen components, one a general RNase inhibitor (RI) which can inactivate any S-RNase, and the other an S-allele-specific product which maintains the activity of a specific S-RNase inside the pollen tube by blocking RI binding (Luu et al., 2000). Separation of the pollen-S blocker from the general RI was proposed in order to explain the compatibility of pollen mutants possibly lacking pollen-S (Pandey, 1967; Van Gastel., 1976; Golz et al., 1999). Interestingly, Dr. T. Sims has recently identified in *P.hybrida*, by the two-hybrid system, a non polymorphic S-RNase-binding protein with a RING-HC domain that could represent a possible candidate for the general inhibitor (T. Sims and M. Ordanic, unpublished results).

The multimeric nature of pollen-S: From the results shown here, we deduce that only a multimeric pollen-S blocker can explain all aspects of the HAP phenotype. First consider the incompatibility reaction of haploid P_{11} pollen growing in an $S_{11}S_{13}$ style (Figure II.1A). The RI components are drawn as shaded arcs, to mimic the structure of the mammalian RNase inhibitor (Hofsteenge, 1997), the P_{11} blockers as small shaded circles and the S-RNases as large white ovals. S-RNases enter the pollen tubes from the styles

(Luu et al., 2000) and, in this illustration, we assume that eight S-RNases of any type present in the style will enter. We also assume that there are sufficient blockers in a pollen tube to bind to their cognate S-RNases, and therefore the P_{11} pollen tube contains eight P_{11} tetramers in addition to the S_{11}- and S_{13}-RNases. All the S_{11}-RNases bind the P_{11} blocker (favored thermodynamically over RI binding; (Kao and McCubbin, 1997). Since blocker binding precludes RI binding, S_{11}-RNase remains active and incompatibility results. The RI binds the S_{13}-RNase because no P_{13} blocker is present in the P_{11} pollen, but inhibition of the S_{13}-RNase activity has no effect on the incompatibility phenotype since the active S_{11}-RNase causes pollen rejection.

The multimeric nature of the blocker is irrelevant for the incompatibility phenotype of normal (haploid) pollen, but is essential to explain the compatibility of HAP (see next section for the choice of tetramers over dimers). When $P_{11}P_{13}$ HAP grows in $S_{11}S_{13}$ styles (Figure II.1B), S_{11} and S_{13}-RNases enter the pollen tube as before. Once again, blockers will compete with the RI for binding to their cognate S-RNases. However, even if HAP produces the same number of P_{11} (small circles) or P_{13} blockers (small squares) as would haploid, the random assembly of monomers into tetramers would produce homotetramers and heterotetramers in binomial proportions, similar to the 1:4:6:4:1 ratio observed for lactate dehydrogenase tetramers (Markert, 1963). In this case, only one out of the sixteen blocker tetramers in $P_{11}P_{13}$ HAP would be a P_{11} homotetramer and thus only one of the S_{11}-RNases entering the pollen tube would remain active. If heterotetramers were inactive, the other S_{11}-RNases would be inhibited because the hybrid blockers would no longer outcompete RI binding. The same argument holds for assembly of a P_{13} homotetramer blocker and its binding to the S_{13}-RNase. Therefore, only a fraction (one quarter) of the amount of RNase active in haploid pollen (Figure II.1A) would be active in HAP (Figure II.1B). Is this reduction in active RNase sufficient to cause compatibility? It is generally

accepted that a minimum threshold of S-RNase is required for pollen rejection. The threshold idea is derived from experiments in transgenic *Petunia inflata* (Lee et al., 1994) data from natural SC Japanese pear mutants (Hiratsuka et al., 1999), where S-RNase expression at one-third the normal level results in self-compatibility. Thus, a reduction in the amount of active S-RNases to one quarter normal levels could indeed result in HAP compatibility.

How, then, might the dual specific HVapb-RNase reject HAP? Since this S-RNase can bind either P_{11} or P_{13} (Matton et al., 1999), it is unlikely to discriminate between any of the heterotetramers in $P_{11}P_{13}$ pollen (Figure II.1C). Blocker binding then would be unaffected by the formation of heterotetramers and this RNase would remain fully active and reject the pollen. For clarity, we have drawn only the HVapb RNase in Figure II.1C, although it must be kept in mind that these pollen tubes will also contain S_{12}- and S_{14}-RNases which are present in the styles of the transformed plants at the same (wild type) levels as the HVapb-RNase.

Theoretical support for a trameric pollen-S: To buttress the intuitive argument provided above, we have also analyzed the predictions of a mathematical formulation for the amount of pollen-S, which takes into account the possibility of fractional activity of heteromers relative to homomers (b) and relative expression of pollen-S in diploid compared with pollen (a). In the expression defined below for k-mers, the amount of active pollen-S in diploid pollen (x_d) is a function of the amount of pollen-S normally expressed in haploid pollen (x_h):

$$x_d = 2ax_h\,((1/2)^{k-1} + b(1-(1/2)^{k-1})) \qquad (1)$$

This equation takes into account situations where pollen-S expression levels are less than in haploid plants ($a<1$) as well as cases where heteromers are partially active ($0<b<1$). In the section above, we assumed that expression of

pollen-S was the same in diploid and in haploid pollen (a=1) and that heteromers are totally inactive (b=0).

The quantity of active pollen-S in HAP must be less than x_h for compatible crosses (with $S_{11}S_{13}$ plants) and greater than or equal to x_h in incompatible crosses (with HVapb plants). To visualize the main conclusions of this model, we calculated the range of values of pollen-S expression (a) that satisfy these two requirements for various values of (k) and (b) (Table II.4). Note that b=1 for HVapb plants, since the dual specific RNase cannot distinguish between P_{11} and P_{13}. Two important conclusions can be unequivocally drawn from this analysis. First, the pollen-S cannot be a monomer (Table II.4). Second, heteromers cannot be as active as homomers, as no value of a can produce compatibility with $S_{11}S_{13}$ and incompatibility with HVapb plants if b=1.

The analysis also allows us to describe the conditions required for dimeric or tetrameric blocker activity. Were pollen-S dimeric, its expression in HAP would be restricted to $1/2 \leq a < 1$. Only two values of b are shown, but it is clear that as the activity of the heteromers (b) increases, the a value must decrease. If pollen-S were tetrameric, a wider range of pollen-S expression levels is permitted ($1/2 \leq a < 4$). Thus, if pollen-S is a dimer, this model would require a reduced expression in diploid pollen. While pollen-S is as yet unknown and cannot be assayed, reduction of allele expression in polyploids has been reported for some genes (Birchler, 1981). However, we do not as yet have any evidence for a reduction in the levels of S-RNases (presumably tightly linked to the pollen-S gene at the S-locus) in tetraploids. Epigenetic silencing, resulting from the increase in ploidy level, could also account for a reduced level of pollen-S, although this phenomenon is usually restricted to the silencing of one of the original alleles (Scheid, 1996). Furthermore, it is unclear why gene silencing would preferentially occur in HAP as opposed to homoallelic pollen. We therefore conclude that the blocker is probably a tetramer.

The model proposed here supports the proposal that the S-RNase-based GSI evolved from an RNase-based defense mechanism (Kao and McCubbin, 1996). Indeed, extracellular S-like RNases not involved in GSI have been described and their role in host defense suggested (Lee et al., 1992). If extracellular S-RNases can enter pollen tubes indiscriminately (Luu et al., 2000) then inhibitors for S-like RNases must have been necessary in pollen. GSI could indeed have derived stepwise from an ancestral self-compatible system as proposed (Uyenoyama, 1988). Initially, pollen tube RI would block the cytotoxicity of extracellular S-like RNases. These extracellular S-like RNases may already have evolved polymorphisms which, if selected for a role in pathogen defense, would be neutral for pollen RI binding. Thus, we see self-incompatibility as having arisen by the development of an allele-specific recognition domain on a pollen protein which binds a particular stylar RNase and blocks RI binding.

Finally, our model suggests that the term "competition effect" may not accurately reflect the mechanism of HAP compatibility. Earlier interpretations of the phenomenon were that two different pollen components competed with each other for some limiting factor (de Nettancourt, 1997). In our view, the reduced activity of heteromers compared to homomers points to pollen-S itself as the limiting factor.

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Table II.1 Breeding behavior of diploid pollen from tetraploids with various S-genotypes

		Pollen parent							
Plant Style	Genotype	G4	L25	V28	F20	F38	F55	1022	F44
G4	$S_{12}S_{12}S_{14}S_{14}$	12/15	9/11	9/13	10/11	19/22	7/9	10/10	0/7
L25	$S_{11}S_{11}S_{12}S_{12}$	14/16	11/12	12/17	15/17	11/12	11/14	14/16	0/12
V28	$S_{12}S_{12}S_{13}S_{13}$	12/13	14/15	16/19	12/13	13/13	13/14	10/10	0/12
F20	$S_{11}S_{11}S_{12}S_{13}$	6/11	6/6	8/10	11/12	14/15	12/16	13/13	0/19
F38	$S_{11}S_{11}S_{13}S_{13}$	9/10	9/11	7/9	11/13	16/19	6/6	10/12	8/8
F55	$S_{11}S_{11}S_{13}S_{13}$	11/11	7/8	6/7	11/12	6/8	12/13	9/9	14/18
1022	$S_{11}S_{11}S_{13}S_{13}$	6/6	8/8	6/7	10/11	10/11	8/8	8/9	7/7
F44	$S_{12}S_{12}S_{12}S_{12}$	7/8	11/12	9/10	10/10	13/13	7/9	12/13	0/11

The pollen rejection phenotype, given as number of fruits set /number of flowers pollinated, is deemed incompatible when no fruits are set and compatible when the majority of pollinated flowers set fruit. All plants except F44 ($S_{12}S_{12}S_{12}S_{12}$) produce heteroallelic diploid pollen, which accounts for the observed compatibility.

Table II. 2 Breeding behavior of haploid pollen from plants with various S-genotypes

		Pollen parent		
Plant Style	Genotype	V22	G4 (2X)	HVapb
G4(4x)	$S_{12}S_{12}S_{14}S_{14}$	15/19	0/17	0/14
L25	$S_{11}S_{11}S_{12}S_{12}$	13/13	9/10	11/12
V28	$S_{12}S_{12}S_{13}S_{13}$	14/16	9/10	11/12
F20	$S_{11}S_{11}S_{12}S_{13}$	0/32	8/9	11/11
F38	$S_{11}S_{11}S_{13}S_{13}$	0/11	8/8	8/10
F55	$S_{11}S_{11}S_{13}S_{13}$	0/14	12/13	7/8
1022	$S_{11}S_{11}S_{13}S_{13}$	0/12	12/12	10/11
F44	$S_{12}S_{12}S_{12}S_{12}$	13/13	11/11	8/9
V22	$S_{11}S_{13}$	0/23	15/16	23/24
G4(2x)	$S_{12}S_{14}$	14/14	0/9	0/9
HVapb	$S_{12}S_{14}S_{11/13}$	0/38	0/14	0/32

Data for the HVapb dual specific RNase is pooled from five independent transgenic lines.

Table II. 3 **Rejection of $S_{11}S_{13}$ heteroallelic pollen by a dual recognition specificity S-RNase**

Plant		Pollen parent							
Style	Genotype	G4(4x)	L25	V28	F20	F38	F55	1022	F44
V22	$S_{11}S_{13}$	11/12	13/13	15/19	10/13	23/25	17/19	21/23	11/12
G4(2x)	$S_{12}S_{14}$	11/13	8/9	9/9	16/20	11/12	13/15	14/14	0/13
HVapb	$S_{12}S_{14}S_{11/13}$	12/12	15/15	10/10	25/30	0/46	0/20	0/19	0/15

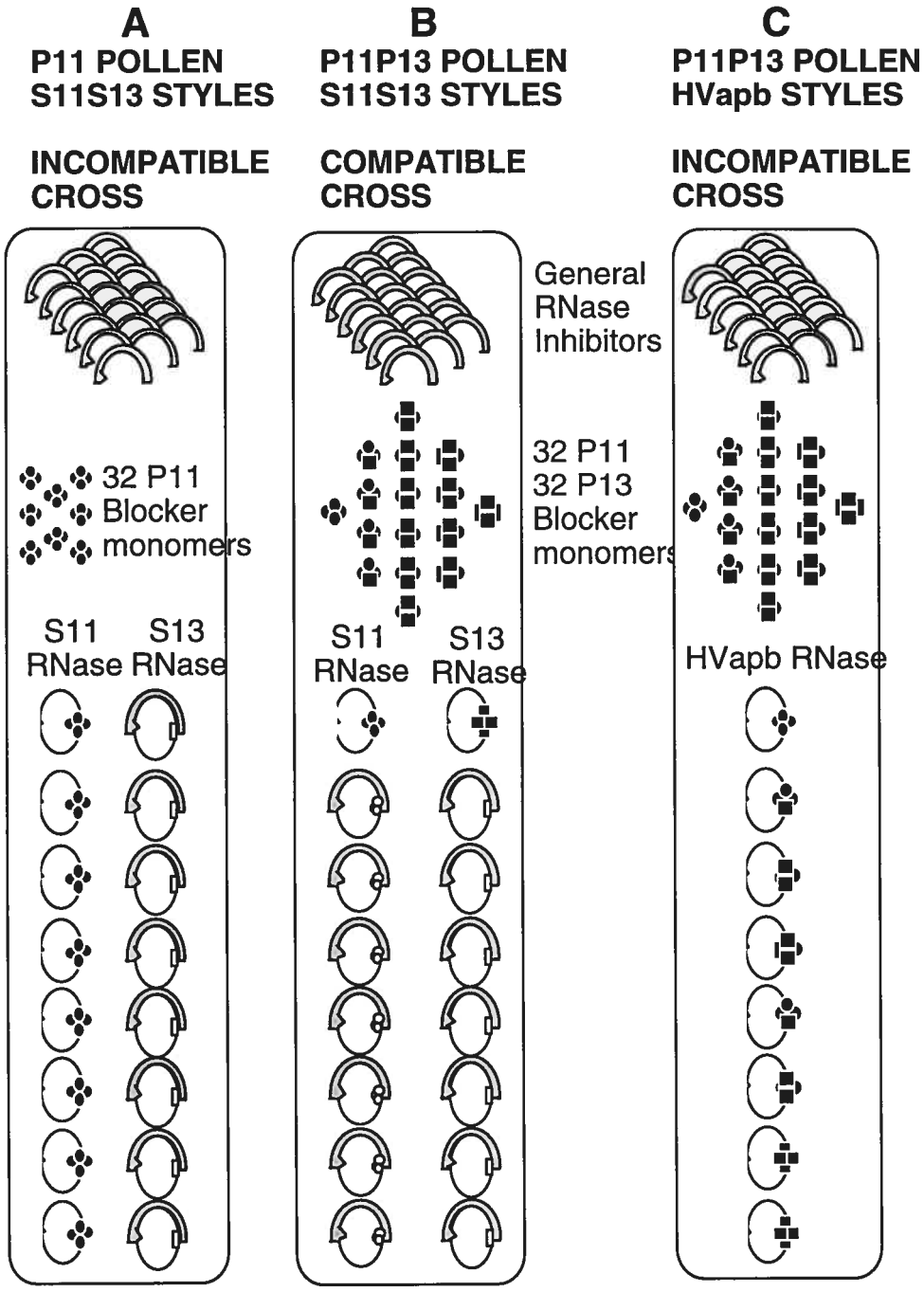
The pollen donors are tetraploids and their genotypes are shown in Table I. Data is given as number of fruits set /number of flowers pollinated. Data for the HVapb dual specific RNase is pooled from five independent transgenic lines.

Table II. 4 **Relative levels of pollen-S expression (a) in diploid *vs* haploid pollen as predicte from equation (1)**

	Expression levels of Pollen-S required		
k-mer	For compatibility in $S_{11}S_{13}$ plants	For incompatibility in Hvapb plants	Conclusion
Monomer (k=1)	a<1/2	a≥1/2	Impossible
Dimer (k=2)			
b=0	a<1	a≥1/2	Possible
b=1	a<1/2	a≥1/2	Impossible
Tetramer (k=4)			
b=0	a<4	a≥1/2	Possible
b=1	a<1/2	a≥1/2	Impossible

FIGURE LEGENDS.

Figure II.1 A model for GSI derived from the HAP effect. **(A)** Haploid P_{11} pollen growing in an $S_{11}S_{13}$ style is incompatible because the ribonuclease inhibitors (RI; shaded arcs) are prevented from binding to the S_{11}-RNases (white ovals at left), that have entered the pollen tubes from the styles, by the P_{11} blockers (small shaded circles) present in the pollen tubes. Since P_{13} blockers are absent, RI binds S_{13}-RNases (white ovals at right). **(B)** S_{11} and S_{13} RNases from an $S_{11}S_{13}$ style enter diploid $P_{11}P_{13}$ HAP, but RI binding can not be fully prevented because a binomial distribution of tetramer types results in a lower number of P_{11} and P_{13} homotetramers than would be found in either haploid pollen type. **(C)** The dual specific HVapb-RNase does not discriminate between P_{11} and P_{13} blockers and thus binds all of the heterotetrameric blockers. This results in incompatibility because RI is prevented from binding.


A
P11 POLLEN
S11S13 STYLES
INCOMPATIBLE
CROSS
32 P11
Blocker
monomers
S11
RNase
S13
RNase
B
P11P13 POLLEN
S11S13 STYLES
COMPATIBLE
CROSS
General
RNase
Inhibitors
32 P11
32 P13
Blocker
monomers
S11
RNase
S13
RNase
C
P11P13 POLLEN
HVapb STYLES
INCOMPATIBLE
CROSS
HVapb RNase

Chapter III. Molecular analysis of the conserved C4 region of the S_{11}-RNase of *Solanum chacoense*

Objectives

1) **To investigate if the C4 region is involved in S-RNase entry into pollen tube**

2) **To test if the lysine in C4 region is the target for protein degradation pathway**

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I did most of the experimental procedures, except several crosses, western blots and the 3d model (J. Soulard) and the production of transgenic plants (G.Laublin). Dr Morse helped with the interpretation of the results and discussions.

Molecular analysis of the conserved C4 region of the S_{11}-RNase of *Solanum chacoense*

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Abstract

The stylar component to gametophytic self-incompatibility in Solanaceae is an S-RNase. Its primary structure has a characteristic pattern of two hypervariable regions, involved in pollen recognition, and five constant regions. Two of the latter (C2 and C3) constitute the active site, while the highly hydrophobic C1 and C5 are believed to be involved in protein stability. We analyzed the role of the C4 region by site directed mutagenesis. A GGGG mutant, in which the four charged residues in the C4 region were replaced with glycine, did not accumulate to detectable levels in styles, suggestive of a role in protein stability. A R115G mutant, in which a charged amino acid was eliminated to reduce the potential binding affinity, had no effect on the pollen rejection phenotype. This suggests the C4 does not interact with partners such as potential pollen tube receptors facilitating S-RNase uptake. Finally, a K113R mutant replaced a potential ubiquitination target with arginine. However, this RNase acted as the wild type in both incompatible and compatible crosses. The latter crosses rule out a role of the conserved C4 lysine in ubiquitination.

Key words: self-incompatibility, site-directed mutagenesis, *Solanum chacoense*, S-RNase

Introduction

Self-incompatibility (SI) is a prezygotic reproductive barrier used by Angiosperms to promote outcrossing, assure intraspecific genetic variability and prevent inbreeding. This cell-cell recognition mechanism involves interactions between gene products expressed in the pollen, and those expressed in specialized cells of the pistil. As a result, self-incompatible plants reject self- but accept non-self pollen (de Nettancourt, 1977; de Nettancourt, 2001). Among the various SI systems, the most widespread is the gametophytic, or GSI, where the genotype of the haploid pollen determines its own incompatibility phenotype.

Generally characterized by stylar rejection and typically found in species with wet stigmas and binucleate pollen, GSI is present in more than 60 families, and has been studied at the molecular level in the Solanaceae, Rosaceae, Scrophulariaceae, Campanulaceae, Poaceae and Papaveraceae. With the exception of the Poaceae and Papaveraceae, all these families share an RNase-based GSI controlled by a highly complex *S*-locus with multiple S-haplotypes containing, among other elements, both the pistillar and the pollen determinants to SI (for recent reviews see (Franklin-Tong and Franklin, 2003; Kao and Tsukamoto, 2004a)). Pollen rejection occurs when the S-haplotype of the haploid pollen matches either of the two S-haplotypes of the diploid pistil. The components to the SI reaction must be tightly linked or the system would break down, suggesting that the style and pistil components are physically close.

The pistillar determinant to SI was identified almost 15 years ago as a highly polymorphic stylar extracellular ribonuclease (S-RNase) (McClure et al., 1989). The alignment of the deduced amino acid sequences of solanaceous S-RNases reveals a distinct pattern of five short conserved (designated C1 through C5) and two longer hypervariable (HV) regions. These latter contain

the S-haplotype-specific sequences that constitute the determinants for pollen recognition (Matton et al., 1997). The conserved C2 and C3 regions are hydrophilic and constitute the active site of the protein, as they contain the two conserved histidines essential for RNase activity (Green PJ, 1994). The conserved C1, C4 and C5 regions are thought to be involved in stabilizing the core of the protein (Ioerger et al., 1991). While this seems likely from the highly hydrophobic nature of the C1 and C5 regions, it is less evident for the C4 region where half of the residues are charged. Intriguingly, although the C1, C2, C3, C5 regions of the Solanaceae and Rosaceae S-RNases share similar positions and sequences, a region conserved among the Rosaceae, and named RC4, has no homology to its counterpart in solanaceous species (Ushijima et al., 1998). Thus, the role of the C4 region remains to be further investigated.

RNase activity is needed for self- pollen rejection (Huang et al., 1994; Royo et al., 1994). This finding suggests that SI is caused by the cytotoxic action of the S-RNases degrading pollen tube RNA in S-haplotype-specific manner. Two models have been proposed to account for this. The receptor model proposes entry of the S-RNases inside the pollen tube is S-haplotype-specific, i.e. does not allow entry of S-RNases of different haplotypes, and implies that the pollen determinant to SI (pollen S-gene product) is membrane- or cell wall-bound (Dodds et al., 1996). The inhibitor model permits penetration of the S-RNases inside the pollen tube in S-haplotype-independent manner, and implies that the pollen S-gene product acts as a cytoplasmic inhibitor of non-cognate S-RNases (Thompson and Kirch, 1992; Kao and McCubbin, 1996). The finding that an S-RNase of one haplotype accumulates in the cytoplasm of pollen tubes of different S-haplotypes has provided experimental support for the inhibitor model (Luu et al., 2000). This finding draws attention to the mechanism, still unknown, by which S-RNases penetrate inside the pollen tubes. It is tempting to speculate that a conserved domain of the protein might play such a role.

The pollen determinant to S-RNase-mediated SI has remained elusive for many years, but has finally been identified as an F-box family member (Sijacic et al., 2004). The general role of F-box proteins in ubiquitin-mediated protein degradation (for review see (Pickart, 2004)) suggests a novel biochemical basis for the SI reaction, in which inhibition of activity is replaced by protein degradation. This new model requires the F-box protein not to trigger degradation when bound specifically to the recognition domain of its cognate S-RNase (incompatible crosses) and to cause degradation when not bound in an allele-specific manner (compatible crosses). The breakthrough has occurred with the discovery, in Rosaceae and Scrophulariaceae, of several F-box genes physically linked to the S-RNase gene (Lai et al., 2002; Ushijima et al., 2003; Yamane et al., 2003b; Ikeda et al., 2004). Some were expressed in the pollen, and displayed levels of allelic sequence variability high enough to be considered good candidates for being the pollen *S* gene. These were designated as *S*-haplotype-specific F-box genes (SFB) (Ushijima et al., 2003; Ikeda et al., 2004) or *S*-locus F-box genes (SLF) (Lai et al., 2002; Entani et al., 2003). The formal implication of F-box genes in SI has very recently been provided by the transgenic approach in Petunia. In a first study, the transformation of plants S_1S_1, S_1S_2 and S_2S_3 haplotype with a transgene containing the pollen-expressed S_2 allele of the *P. inflata* SFL, has resulted in self-compatibility through the production of S-heteroallelic pollen (Sijacic et al., 2004). Similarly, the transfer of S_2 SLF from *Anthirrhinum hispanicum* into an SI line of *Petunia hybrida*, converted the latter into SC (Qiao et al., 2004b).

The aim of the present study was to investigate the role of the C4 region in solanaceous S-RNases. We were particularly interested in testing the hypothesis that the C4 region could be involved in S-RNase entry into the pollen tube or in mediating the compatibility response by either inhibiting S-RNase activity or by targeting the protein itself for degradation. In particular, assuming that ubiquitination is responsible for degradation of S-RNase in

Solanum, we wanted to see if substituting the lysine in the S_{11}-C4, one of the few lysine residues conserved among solanaceous S-RNases, would disrupt the pathway leading to S-RNase inhibition. In Anthirrhinum, degradation of S-RNases is proposed to occur through F-box protein-mediated ubiquitination (Qiao et al., 2004a). Our analyses showed no involvement of the C4 region in any of these functions, and support the idea that it plays a structural role.

Materials and methods

Plant materials

The diploid (2n=2x=24) *Solanum chacoense* self-incompatible genotypes used in the present study include two parental lines (obtained from the Potato Introduction Station at Sturgeon Bay, Wi) PI 458314 (called 314) which carries the S_{11} and S_{12} alleles and PI 230582 (called 582) which carries the S_{13} and S_{14} alleles. Other genotypes used here include plants L25 ($S_{11}S_{12}$), VF60 ($S_{12}S_{12}$) and G4 ($S_{12}S_{14}$,noted for its high regenerability *in vitro*) all of which have been described previously (Matton et al., 1997; Qin et al., 2001).

Transgenic plants and mutagenesis

The S_{11} genomic DNA was used as template for PCR-based mutagenesis. The mutant S_{11} with four amino acid substitutions, the GGGG construct, was generated in several steps. First, one pair of primers 5'-AAATCGGAACGCGAATCCTCCACCCAAACCA-3' (C4-A) and 5'-GAGACCATGGTTAAATCAGGCTTACAT-3' (NcoI-Met) was used to amplify

a 0.5 kb mutated fragment corresponding to the sequence from the start codon to the C4 region (the mutated sequences are underlined and restriction sites are boxed). A second pair of primers 5'-GGAGGTGGGTTTGGTCTTCTGAG AACTCTCC-3' (C4-B) and 5'-CTCTGAATTCAAGGACATACATTTGATAG-3' (EcoRI-stop) was then used to amplify a 1.2 kb fragment corresponding to the sequence from the C4 to the end of the 3'-UTR. Lastly, the primers NcoI-Met and EcoRI-stop were used to amplify the 1.7 kb full length mutated S_{11} which was then cloned into pBluescript SK$^+$ (Stratagene). A 1.4 kb HindIII-NcoI promoter fragment from the style-specific tomato chitinase gene (Harikrishna et al., 1996) cloned separately into pBluescript, was merged with the mutated S_{11} at the NcoI site. Following digestion with SalI and EcoRI, the 3.1-Kb chimeric fragment containing chitinase promoter and the mutated S_{11} was cloned in the binary transformation vector pBIN19 (Clontech, Palo Alto.CA). The construct was introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. Plants were transformed by the leaf disc method using the *S.chacoense* G4 genotype as described (Matton et al., 1997)

For the additional constructs, a previous construct containing the chitinase promoter and wild type S_{11} in pBluescript SK$^+$ was used as template for PCR-based mutagenesis using a kit (QuikChangeXLsite-Directed Mutagenesis Kit, Stratagene). In the R115G construct, arginine was substituted with glycine using the primers 5'-TTGCGCTTAAAAGATGGT TTTGATCTTCTGAGA-3'and 5'-AACGCGAATTTTCTACCAAAACTAGAAG ACTCT-3'. In the K113R construct, lysine was substituted with arginine using the primers 5'-TTGCGCTTAAGAGATAGGTTTGATCTTCTGAGA-3' and 5'-AACGCGAAT TCT CTATC CAAACTAGAAGACTCT-3'. All mutant constructs were cloned into vector pBIN19 as described above. All constructs were

sequenced before and after transformation into *Agrobacterium tumefaciens* to confirm the sequence at the mutated site.

The presence of a transgene in DNA extracted from regenerated plants was verified by PCR using primers NcoI-Met and EcoRI-stop. These primers specifically amplify the S_{11} gene from the S_{12} S_{14} background of the transformed host G4.

RNA and protein analysis

For determination of the amount of transgene accumulation, total RNA was extracted from the styles of transformed plants as described (Matton et al., 1997). The RNA was electrophoresed on agarose gels, transferred to nylon membranes and hybridized to a radiolabeled probe prepared from the authentic S_{11} genomic DNA. For measurement of accumulated S_{11}-RNase, total protein of freshly collected styles was extracted and electrophoresed on standard SDS gels. After transfer to nitrocellulose membranes, Western blot analysis was performed with an anti- S_{11} antibody raised against a 15 amino acid peptide corresponding to the HVa region (Matton et al., 1999).

Genetic crosses

Genetic crosses were performed using fresh pollen from plants of known S-allele constitution and were monitored by fruit set. They were classified as fully incompatible if they resulted in no fruit formation after pollination, and compatible if fruits were formed after almost every pollination. Plants were classified as partially compatible when fruit set was between these two extremes. Where appropriate, pollen tube growth inside styles collected about 48 h after pollination, was monitored by epifluorescence microscopy using aniline blue staining (Matton et al., 1997).

Results

The predicted three-dimensional structure of the S_{11}-RNase (Fig.III.1) indicates that the C4 region (amino acid sequence: LKDRFDLL) has 4 charged amino acids directed outwards. The architecture of the C4 region is striking and suggests the possibility that it may bind with high affinity to a protein partner either on the membrane or inside the cytoplasm of the pollen tube. To investigate the role of this highly conserved region, the sequence of the C4 region in the wild type S_{11}-RNase was modified by site directed mutagenesis to produce a series of various constructs (Fig.III.1b) that were introduced into the host genotype G4 ($S_{12}S_{14}$).

In construct GGGG, all four charged amino acids in C4 (KDRD) were substituted with glycine (Fig.III.1b). A total of 27 transgenic plants harboring the GGGG construct were crossed with S_{11} and S_{12} pollen from either 314 or L25 individuals (producing both S_{11} and S_{12} pollen). All crosses resulted in fruit formation. Pistils, examined by fluorescence microscopy, revealed the presence of numerous incompatible tubes arrested in the upper third of the style, together with numerous tubes entering the ovary. This semi-compatible response was interpreted as full rejection of S_{12} pollen, an expected behavior of the G4 host (Qin et al., 2001), and full acceptance of S_{11} pollen.

The acceptance of S_{11} pollen, and the resultant compatible phenotype, could have resulted either from inability of the GGGG-S-RNase to enter pollen tubes or from below threshold levels of S_{11}-RNase accumulation. The molecular analyses clearly distinguished between these two possibilities: no S_{11}-RNase could be detected in any transgenic plant with an anti- S_{11} antibody (Fig.III.2a). This failure to express the RNase at the protein level was not due to lack of the transgene, as PCR analysis revealed its presence in all regenerants (not shown), or to lack of transcription of the transgene, as Northern blot analysis showed high levels of transgene transcripts in about

one third of the plants (Fig.III.2b). Our previous transformation studies also show transgene expression in about one third of the plants examined (Matton et al., 1997; Matton et al., 1999). Taken together, these results suggest that the mutated protein might be unable to fold properly and, as a result, is rapidly degraded soon after synthesis.

To avoid this potential problem, a less drastic mutagenesis was performed in the C4 region. In the R115G construct, only the arginine in position 115 was substituted with glycine (LKDGFDLL). In this experiment, a total of 35 transgenic plants were submitted to genetic analysis. The crosses revealed that 19 of them were fully or partially compatible with S_{11} pollen, whereas 16 fully rejected S_{11} pollen (TableIII. 1). The rejection of S_{11} pollen excluded the possibility that the modification in the C4 domain affected entry of the R115G S-RNase into the pollen tubes. Furthermore, all these transgenics accepted S_{13} pollen (the normal compatible response), excluding the possibility that this modification to the C4 region could prevent binding to a general S-RNase inhibitor (Luu et al., 2001). An impairment of the S-RNase to bind to the S-RNase inhibitor should lead, in fact, to full rejection of all pollen types, since the mutant S-RNase would remain fully active.

The western blots confirmed that plants fully incompatible with S_{11} pollen also displayed levels of transgenic S-RNase accumulation close to the values found in wild type plants (Fig.III.3a), whereas individuals partially compatible with S_{11} pollen had lower levels of transgenic S-RNase in the styles. This is most evident if the ability of the styles to reject S_{11} pollen is plotted directly against the levels S_{11}-RNase in the styles as determined by densitometric scans (Fig.III.3b). To directly address the potential role of the C4 region in targeting the S-RNase for degradation by ubiquitin-mediated proteolysis, the conserved lysine was substituted with arginine in the K113R construct. A total of 35 transgenic plants were analyzed, of which 17 were fully

or partially compatible with S_{11} pollen and nine fully rejected S_{11} pollen (Table III.2). The rejection of S_{11} pollen indicates that the modified S-RNase can enter the pollen tubes. Once again, densitometric scans of Western blots confirm the expected relationship between S-RNase levels and the pollen rejection phenotype (Fig. III.4). However, crosses with S_{13} pollen revealed full compatibility (Table III.2). This compatible response clearly rules out a role of the modified lysine in targeting the S-RNase to the proteasome.

Discussion

The analyses described here were undertaken to provide insight into the role played by the C4 domain in S-RNases. The C4 region is highly conserved in the solanaceous S-RNases (Richman et al., 1996; Sassa et al., 1996) and the crystal structure reveals an unusual and rather unique organization (Ida et al., 2001b). The four charged amino acids extend outwards from the surface of the protein much like fingers from one hand, while the hydrophobic residues in this region are buried deep in the heart of the protein and in fact constitute an important element defining the RNA-binding fold at the active site of the protein. This organization is observed in the published structure of the *Nicotiana* S-RNase (Ida et al., 2001b) and in the predicted structure of our S_{11}-RNase (Fig. III.1).

One interpretation of this structure is that the necessity of keeping the charged amino acids in the aqueous phase provides a mechanism for pushing the adjacent hydrophobic amino acids into the protein core. However, while this would explain the conserved pattern of charged and non-polar residues, it does not account for the conserved pattern of positively and negatively

charged residues. This characteristic suggests instead an alternative role for the C4 region as a high-affinity binding site for other proteins.

The possibility that this region might bind other proteins was intriguing, as there are several candidates potentially binding to a region common to different S-RNases. One candidate is an RNase inhibitor similar to that proposed by the various inhibitor models (Kao and McCubbin, 1996; Luu et al., 2001). Alternatively, the C4 (or more specifically a conserved lysine in this region) might be involved in directing the formation of an ubiquitinated intermediate. Lastly, the region might be involved in assuring S-RNase entry into the pollen tubes either alone or as part of a complex. While it is still unclear how many different stylar proteins enter pollen tubes (Wu et al., 1995; Lind et al., 1996) a mechanism clearly exists for non *S*-haplotype-specific uptake of S-RNases (Luu et al., 2000).

The constructs used here were designed to assess all of these possibilities. First, if the charged residues are involved in maintaining the hydrophobic residues of the C4 region in the core of the protein, the GGGG construct should lack this stabilizing force. The position of the hydrophobic residues suggests they are likely to be important for the structure of the protein, so that this mutation might well produce a dramatic change in the three dimensional structure of the protein. Indeed, our results are consistent with this, as none of the transgenics accumulated detectable levels of the S-RNase despite an apparently normal range of mRNA accumulation. It is possible that the modified protein misfolds inside the ER lumen and thus becomes targeted for degradation (Kostova and Wolf, 2003). However, we cannot exclude the possibility that the choice of glycine itself may have influenced the stability of the protein structure. This issue could perhaps be explored by examining protein stability after other alterations (such as to alanines, for example).

The effects of the R115G mutation are expected to be much less drastic than the GGGG mutation, at least with regard to the three dimensional

structure of the protein. However, thermodynamic considerations suggest that this single amino acid substitution might have a large effect on the binding affinity with any potential partners. The formation of single ionic bond provides a standard free energy change of about -3 kcal/mol, and thus replacement of this arginine with glycine would be expected to change the equilibrium constant by a factor of 100. Our data indicates, however, that the levels of S-RNase needed for pollen rejection are not different from those observed in wild type or the K113R mutant (compare figures III.3 and III.4). This result provides no indication that additional substitutions in the C4 might provoke an effect on the phenotype, and thus suggests that this region is not directly involved in protein-protein interactions. We also note that a single glycine substitution can easily be accommodated without adverse effect on protein stability and RNase activity.

Ubiquitination can occur at specific lysine residues within a polypeptide chain (Galluzzi et al., 2001; Batonnet et al., 2004) presumably by recognition of a particular amino acid context around the targeted lysine. Amino acid sequence alignments of solanaceous S-RNases reveal that in almost all cases two lysines are present in the C4 region (Sassa et al., 1996). In contrast, our S_{11}-RNase has only one, thus allowing a direct test of the potential role of the C4 in protein degradation. In the K113R mutant, lysine was replaced by arginine so that the charge of the protein would remain unchanged, and to thus minimize any effect of the mutation on the three dimensional structure of the protein. However, assuming that ubiquitination is responsible for degradation of S-RNase in Solanum as it is in Anthirrhinum (Qiao et al., 2004a) and that the C4 lysine is used in targeting the protein for ubiquitination, the mutant S-RNase would be expected to reject all pollen types as it could no longer be degraded. Our results clearly demonstrate that the phenotype of K113R transgenics expressing wild type amounts of the mutant RNase are indistinguishable from wild type S_{11}-RNase with respect to its breeding

behaviour with both S_{11} and S_{13} pollen. It is important to note that our results do not rule out ubiquitination of the S-RNases in mediating compatibility. However, they do clearly eliminate the possibility that the C4 lysine alone is the target. It is still possible that the other conserved lysine (in the C3 region) might play such a role or, alternatively, that other lysines in the protein can be used when the C4 lysine is lost.

Acknowledgements

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Table III. 1. Phenotypes of transgenic R115G plants

	Transgenic Plant Phenotype			
	x $S_{11}S_{12}$ Pollen Donor			x $S_{13}S_{14}$ Pollen Donor
Pistil Genotype $S_{12}S_{14}$	Comp[a]	Partial Comp	Incomp	Comp
Number of plants	16	3	16	35
Number of fruits per pollinated flowers	61/63	13/21	0/51	68/69

[a] Comp, compatible ; Partial Comp, partially compatible ; Incomp, incompatible

Table III.2. Phenotypes of transgenic K113R plants

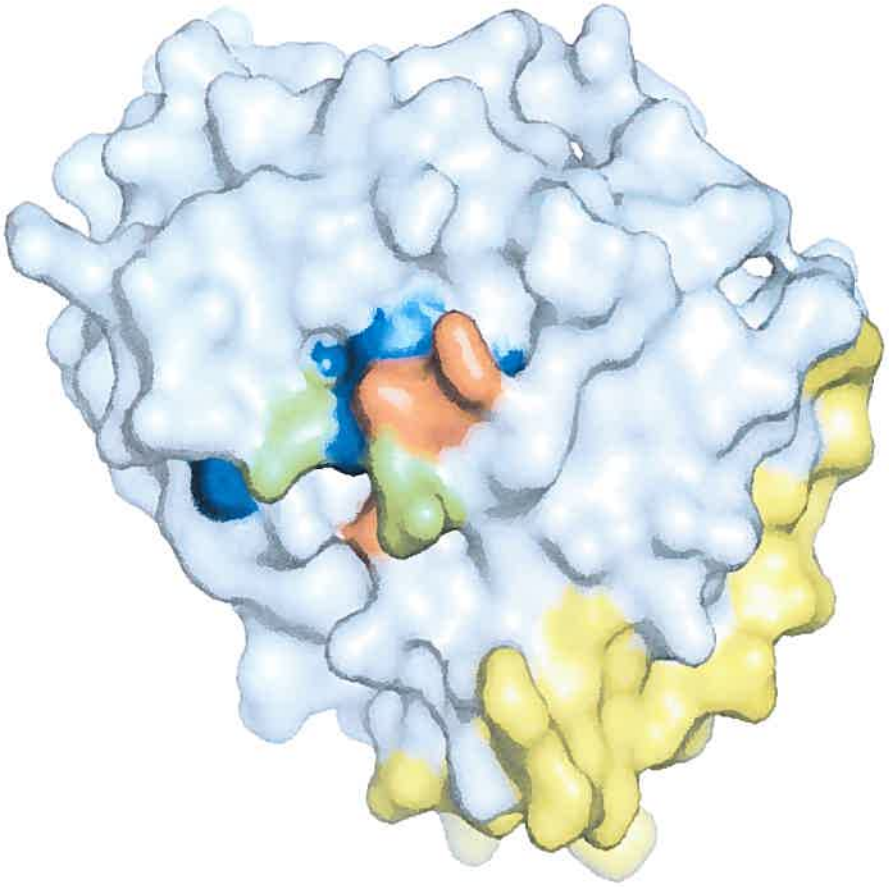
	Transgenic Plant Phenotype			
	x $S_{11}S_{12}$ Pollen Donor			x $S_{13}S_{14}$ Pollen Donor
Pistil Genotype $S_{12}S_{14}$	Comp[a]	Partial Comp	Incomp	Comp
Number of plants	17	9	9	35
Number of fruits per pollinated flowers	84/86	35/106	0/52	132/136

[a] Comp, compatible ; Partial Comp, partially compatible ; Incomp, incompatible

Figure Legends.

Fig.III.1 Three-dimensional structure of the C4 domain in the S_{11}-RNase. (**a**) Swiss-model structure prediction for the S_{11}-RNase sequence based on the structure of *Nicotiana alata* SF_{11}-RNase. The basic and acidic residues in the C4 domain are in red and green, respectively, while the hydrophobic residues are blue. The HV recognition domains in yellow are well separated from the C4 region. Alternate templates for threading, such as S3 from *Pyrus pyrifolia* (Matsuura et al., 2001b) produce only slight variations in the predicted structure. (**b**) Amino acid sequences of the C4 regions in the wild type and mutant sequences used in the present study.

a



b

Genotype	C4 region sequence
S_{11} RNase	LKDRFDLL
GGGG	L**GGG**F**G**LL
R115G	LKD**G**FDLL
K113R	L**R**DRFDLL

Fig.III. 2 Expression of the GGGG transgene at the protein and RNA levels.(**a**) Western blot analysis of stylar protein extracts from six transgenic plants (lane 1, T16; lane 2, T21; lane 3, T24; lane 4, T25; lane 5, T26; lane 6, T34) using the anti- S_{11}-RNase. The plants L25 (S_{11} S_{12}, lane 7) and VF60 (S_{12} S_{12}, lane 8) are shown for comparison. No protein is detectable in the transgenic plant extracts even at long exposure times. Bottom panel shows Ponceau-stained membranes as control for protein load. (**b**) Northern blot analysis of total RNA extracted from styles of five transgenic plants (lane 1, T21; lane 2, T24; lane 3, T25; lane 4, T26; lane 5, T34) using a probe prepared from S_{11} genomic sequence. The plant L25 (S_{11} S_{12}, lane 6) is shown for comparison. Bottom panel shows the ethidium bromide-stained gel as control for RNA load.

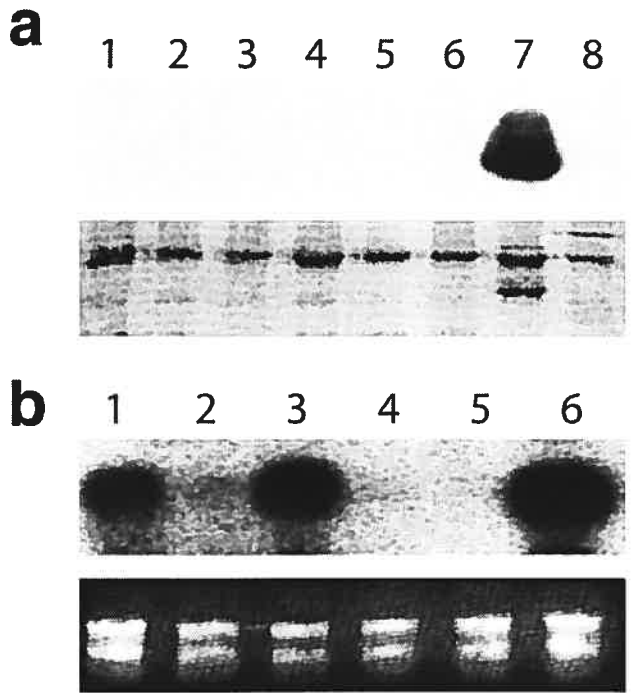

a
1 2 3 4 5 6 7 8
b
1 2 3 4 5 6

Fig.III.3 Expression of the R115G transgene and correlation of phenotype with protein levels. (**a**) Western blot analysis of stylar protein extracts from seven transgenic plants (lane 1, T1; lane 2, T18; lane 3, T38; lane 4, T39; lane 5, T24; lane 6, T36; lane 7, T44) using the anti-S_{11}-RNase. The plants L25 ($S_{11}S_{12}$, lane 8) and VF60 (S_{12} S_{12}, lane 9) are shown for comparison. Bottom panel shows Ponceau-stained membranes as control for protein load. (**b**) The number of incompatible pollinations (calculated as percent of wild type) is plotted as a function of transgene S-RNase levels (shown as percent of L25 wild type levels). RNase levels were quantitated from densitometric scans of the Western blots.

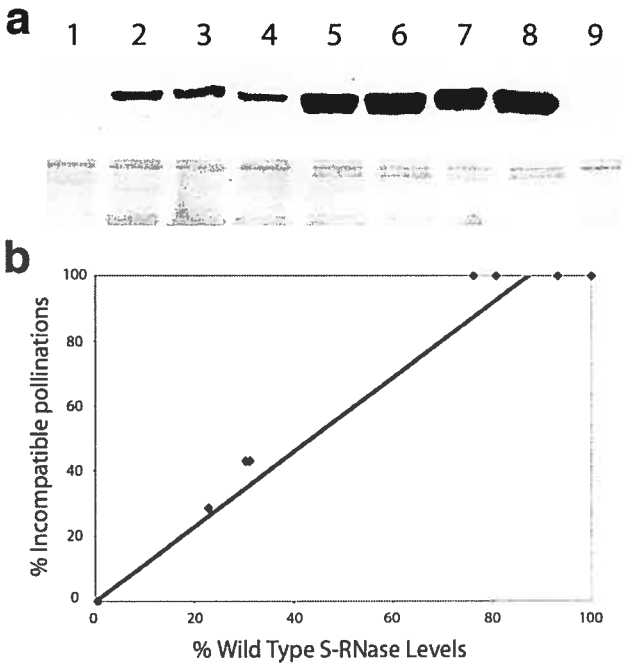
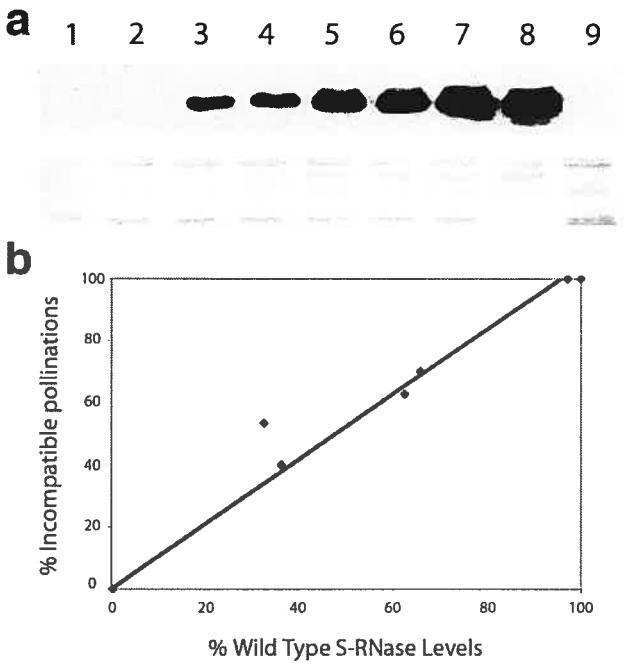

a
1 2 3 4 5 6 7 8 9
b
% Incompatible pollinations
100
80
60
40
20
0
0 20 40 60 80 100
% Wild Type S-RNase Levels

Fig.III.4 Expression of the K113R transgene and correlation of phenotype with protein levels. (**a**) Western blot analysis of stylar protein extracts from seven transgenic plants (lane 1, T6; lane 2, T28; lane 3, T2; lane 4, T38; lane 5, T27; lane 6, T32; lane 7, T56) using the anti-S11-RNase. The plants L25 (S_{11} S_{12}, lane 8) and VF60 (S_{12} S_{12}, lane 9) are shown for comparison. Bottom panel shows Ponceau-stained membranes as control for protein load. (**b**) The number of incompatible pollinations (calculated as percent of wild type) is plotted as a function of transgene S-RNase levels (shown as percent of L25 wild type levels). RNase levels were quantitated from densitometric scans of the Western blots.


a
1 2 3 4 5 6 7 8 9
b
% Incompatible pollinations
100
80
60
40
20
0
0 20 40 60 80 100
% Wild Type S-RNase Levels

Chapter IV. Style-by-style analysis of two sporadic self-compatible *Solanum chacoense* lines supports a primary role for S-RNases in determining pollen restriction thresholds

Objectives

1) **To determine experimentally the S-RNase threshold in *Solanum chacoense***
2) **To evaluate the factors that influence S-RNase threshold**

Publication of chapter IV: This manuscript has been accepted by J Exp Bot and is presently published on line

I did most of the experimental procedures, except the some production and purification of the S-RNases (B.Liu), the some analysis of the progeny (J.Soulard). Dr Morse helped with the interpretation of the results and discussions.

Style-by-style analysis of two sporadic self-compatible *Solanum chacoense* lines supports a primary role for S-RNases in determining pollen rejection thresholds

Running title: S-RNase threshold in *Solanum chacoense*

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Abstract

A method for quantification of S-RNase levels in single styles of self-incompatible *Solanum chacoense* was developed and applied toward an experimental determination of the S-RNase threshold required for pollen rejection. We find that when single style values are averaged, accumulated levels of the S_{11}- and S_{12}-RNases can differ up to ten-fold within a genotype, while accumulated levels of the S_{12}-RNase can differ by over three-fold when different genotypes are compared. Surprisingly, the amount the S_{12}-RNase accumulated in different styles of the same plant can differ by over twenty-fold. We have measured a low level of 160 ng S-RNase in individual styles of fully incompatible plants, and a high value of 68 ng in a sporadic self-compatible (SSC) line during a bout of complete compatibility, suggesting these values bracket the threshold level of S-RNase needed for pollen rejection. Remarkably, correlations of S-RNase values to average fruit sets in different plant lines displaying sporadic self-compatibility (SSC) to different extents as well as to fruit set in immature flowers, are all consistent with a threshold value of 80 ng S_{12}-RNase. Taken together, these results suggest that S-RNase levels alone are the principal determinant of the incompatibility phenotype. Interestingly, while the S-RNase threshold required for rejection of S_{12}-pollen from a given genetic background is the same in styles of different genetic backgrounds, it is different when pollen donors of different genetic backgrounds are used. These results reveal a previously unsuspected level of complexity in the incompatibility reaction.

Key words: Gametophytic self-incompatibility, single style analysis, S-RNase, Solanum chacoense, threshold

Introduction

Self-incompatibility (SI) is a widespread genetic mechanism used by many species of flowering plants to prevent inbreeding by promoting outcrossing. This prezygotic barrier is based on recognition of the gene products expressed in specialized cells of the pistil by those expressed in the pollen, which results in rejection of self- but acceptance of non-self pollen (de Nettancourt, 1977; de Nettancourt, 2001). The Solanaceae, Rosaceae and Scrophulariaceae are characterized by gametophytic SI, or GSI, where the incompatibility phenotype of the haploid pollen is determined by its own genotype. In these families the male and female determinants to SI are both encoded at a highly complex and multiallelic *S*-locus. Pollen rejection occurs when the S-haplotype of the haploid pollen matches either of the two S-haplotypes of the diploid pistil, and it takes place inside the upper part of the style. The pistillar gene product to SI is a highly polymorphic ribonuclease termed S-RNase (McClure et al., 1989) that is synthesized by the cells of the transmitting tissue of the style and secreted into the surrounding extracellular matrix where the pollen tubes grow. The pollen determinant to SI (pollen-S gene product) has been recently identified as a polymorphic F-box protein, termed either SLF (S-locus F-box) or SFB (for S haplotype-specific F-box) by the various authors (for details see (Kao and Tsukamoto, 2004b; McClure, 2004).

The cytotoxic action of the S-RNases mediates rejection of incompatible pollen by degrading pollen tube RNA in S-haplotype-specific manner, although the minimal amount of S-RNase required for pollen rejection has not been determined. S-RNases have been shown to enter and accumulate inside the pollen tubes in haplotype-independent manner (Luu et al., 2000), suggesting that the pollen contains proteins able to inhibit or destroy S-RNases. The mechanism whereby S-RNases penetrate inside the pollen tubes, however, is unknown. It has been suggested that this may occur either by endocytosis, via

inclusion into a membrane-bound compartment (McClure 2004) or through a receptor (or a receptor complex) that recognizes a conserved domain of the S-RNase (Kao and Tsukamoto, 2004b). In this regard, we have recently ruled out the involvement of one of the most attractive possibilities (the conserved C4 region in S-RNases) in uptake (Qin et al., 2005).

Permanent self-compatibility (SC) has been reported several times among SI species (for review, see (de Nettancourt, 1977). In most cases, it can be attributed to mutations directly affecting either the pistillar or the pollen determinants to SI (de Nettancourt, 2001). Examples of the former include mutations at the S-RNase gene causing loss of the RNase activity as reported in *Lycopersicon* (Kowyama et al., 1994; Royo et al., 1994) and *Petunia* (Huang et al., 1994; McCubbin et al., 1997), or deletion of the S-RNase gene itself (Sassa et al., 1997). With regards to self-compatibility resulting from pollen-part mutations, it is most often associated with the so-called competition effect that takes place when two distinct pollen-S genes are expressed in a same pollen grain. An extra pollen-S gene introduced into a host plant by transgenesis (Sijacic et al., 2004; Qiao et al., 2004b) produces the same effect. In some instances, pollen compatibility has been shown to result from the loss of pollen function (Tsukamoto et al., 2003), or from mutations affecting the pollen S-gene (Ushijima et al., 2004; Sonneveld et al., 2005), or from deletion of the pollen S-gene itself (Sonneveld et al., 2005). This last case is particularly important as it suggests S-RNases are inactive in pollen tubes without their cognate pollen-S, as predicted by the two-component inhibitor model (Luu et al., 2000; Luu et al., 2001). Lastly, other cases of SC have been shown to depend on so-called modifier genes, located outside of the S-locus, that appear to be required for proper manifestation of the SI response, such as HT-B (O'Brien et al., 2002) or a stylar 120 kDa glycoprotein in *Nicotiana* (Hancock et al., 2005) (for a further discussion see (Kao and Tsukamoto, 2004b).

A special category of partial incompatibility is represented by pseudo-self-compatibility, (i.e. formation of fruits containing variable amounts of seeds

observed after crosses expected to be incompatible) (Clark et al., 1990) and sporadic self-compatibility (i.e. occasional fruit formation after crosses expected to be incompatible) (de Nettancourt et al., 1971) (Qin et al., 2001). In particular, sporadic self-compatibility has been observed in some but not all S_{12}-containing genotypes of *Solanum chacoense*, and is characterized by occasional bouts of self-compatibility with S_{12} pollen that can affect from 10 to 60 % of the styles on a given plant. We have analyzed expression of the S_{12} allele in several plant lines and found genotype-specific differences in the amount of S_{12}-RNase and S_{12} –mRNA. As sporadic self-compatibility occurred only in those genotypes with the lowest average S_{12}-RNase levels (Qin et al., 2001), we proposed that there may be a variation between flowers that could result in levels of S-RNase in some individual styles too low to reject otherwise incompatible pollen. Indeed, style-to-style variations in S-RNase levels could explain both pseudo and sporadic compatibility but has not been previously demonstrated.

Weakening of the SI response, associated with a reduced level of S-RNases present in the pistil, has led to the hypothesis that a threshold level of the RNase is required for full expression of the SI phenotype (Clark et al., 1990). Support for this idea has been provided by studies on partially compatible Japanese pear cultivars displaying low levels of S-RNase expression (Zhang and Hiratsuka, 1999, , 2000; Hiratsuka et al., 2001; Hiratsuka and Zhang, 2002), and by the partial incompatibility of plants expressing an S-RNase transgene at levels significantly below those produced by the endogenous alleles (Lee et al., 1994; Murfett et al., 1994; Matton et al., 1997; Matton et al., 1999; Qin et al., 2005). Finally, accumulation of the S-RNases in the style during flower development is temporally regulated and the increase in S-RNase levels correlates with the acquisition of the incompatibility phenotype (Xu et al., 1990) (Clark et al., 1990) (Zhang and Hiratsuka, 2000). All these examples are consistent with the hypothesis that a threshold level of S-RNase is required to inhibit the growth of incompatible pollen tubes.

However, the threshold itself has never been measured, and the factors that potentially influence it (S-RNase haplotype, pollen genotype, environmental conditions) have not been assessed.

The aim of the present study was the experimental determination of an S-RNase threshold and a preliminary evaluation of the factors that may influence it. To do so, we took advantage of the sporadic self-compatible phenotype of some of our S_{12}-RNase containing plant lines (Qin et al., 2001). We developed a technique for measurement of the S-RNase levels in single styles, and found a definite S-RNase threshold for a particular pollen haplotype in a particular genetic background. However, we also found that the S-RNase threshold required for rejection of this pollen haplotype can vary depending on the genetic background of the pollen donor. We also note that the S-RNase threshold differs when incompatibility is defined by either fruit formation or by the lack of pollen tubes entering the ovarian region.

Materials and methods

Plant genotypes

The plant material used in these experiments includes the fully self-incompatible G4 (S_{12} S_{14}), VF60 ($S_{12}S_{12}$), 582 ($S_{13}S_{14}$) genetic lines, as well as the two sporadically self-compatible L25 ($S_{11}S_{12}$) and 314 ($S_{11}S_{12}$) lines of *Solanum chacoense* (2n=2x=24) described previously (Qin et al., 2001). In addition, an individual called TP48 ($S_{12}S_{12}$) issued from the selfed 314 line (Qin et al., 2001), and a plant named 2548 ($S_{12}S_{12}$) produced by crossing L25 as pistillate parent with TP48 as staminate parent, and selected for its high vigor, pollen fertility and high *in vitro* regenerability, were also used.

Genetic crosses

Genetic crosses on recently open flowers were always made with fresh pollen collected from plants of known S-haplotype constitution grown in the Montreal Botanical Garden greenhouses at 23 ± 2°C under natural light conditions. Pollen viability was estimated by staining with aceto-carmine. Bud pollinations were performed on flower buds at 3, 2, 1 days before anthesis (DBA). Crosses were classified as fully incompatible if there was no fruit formation after pollination, and compatible when fruits were formed after almost every pollination. Where appropriate, pollen tube growth was monitored by staining the styles with aniline blue about 48 hours after pollination, followed by observations at fluorescence microscopy as previously described (Matton et al., 1997). In some cases, the styles were observed 72 hours after pollination. In other cases, flowers were gently shaken (touched) 4-5 days post pollination, and the styles of fallen flowers observed by fluorescence microscopy to determine if the lack of fruit set could be attributed to self-incompatibility.

Progeny analysis

Seeds obtained from bud pollination of L25 line selfed at 3 DBA were germinated in vitro as described previously (Van Sint Jan et al., 1996), and the S-constitution of the resulting plantlets assessed by PCR. For each genotype, five leaf discs of 2 mm diameter were crushed with a plastic mortar in 20 µL 0.25N NaOH, and incubated for 5 min at 95°C. The mixture was then neutralized with 20 µL 0.25N HCl, 20 µL TrisHCl 1 M pH 8.0 and 0.5% w/v Igepal CA-630 (Sigma). The tubes were incubated for 5 additional min. at 95 °C, centrifuged one min. at 5,000 RPM, the supernatant collected and immediately employed for the PCR reactions (40 cycles of 94°C 30 sec, 55°C 30 sec, and 70°C 1 min) using a commercial PCR Buffer (Promega) and Taq polymerase (Promega). The primers used for analysis of the S_{11} allele were 5'-CTATTTCAGTGTAAGCAGC-3' and 5'-ATTTCTAGAGGACGAAAAAATATT

TTC-3', while primers 5'-TAACTTGACCACCACCG-3' and 5'-GTCATGGAAATGTAACCC-3' were used for the S_{12} allele.

Expression of S_{11}- and S_{12}-RNases in E. coli

The cDNA clones encoding S_{11}- and S_{12}-RNases were first mutated at the active site to avoid possible RNase activity toxic to the *E. coli* host cells, then cloned into an expression vector pQE30 (Qiagen, Valencia, CA). For the S_{11}-RNase, the histidine encoded by CAC in the conserved region C2 was substituted with arginine (CGT). The three primers used for site-directed mutagenesis were S_{11}HisSmaI (at the C-terminal end of the coding sequence), 5'-CTCTCTCTCTCCCGGGCAAGGACGAAAAAATATTTCC-3'; S_{11}HisSacI (corresponding to the N-terminal end of the mature coding sequence), 5'-GAGAGAGAGAGAGCTCAAATTGCAACTGGTATTA-3'; and S_{11}cmc2 (containing the substituted codon), 5'-CCTTATCCGGCC AAAGACCACGAATCGTAAAGTTTTTTG-3'. For the S_{12}-RNase, the histidine encoded by CAT in C2 conserved region was substituted with arginine (CGT). Two pairs of primers, S_{12} HindIII (at the C-terminal end of the coding sequence), 5'-CTCTCTCTCTAAGCTTGGAAATGTAACC CCGGTA-3'; MuS_{12}-HisA, 5'-AACTTTACAATCCGTGGGCTTTGGCCC-3' and S_{12} BamHI (corresponding to the N-terminal end of the mature coding sequence), 5'-GAGAGAGAGAGGATCC GAGCAGTTGCAACTGGT-3',MuS12-HisB, 5'-GGGCCAAAGCCCACGGATTGTAAAGTT-3' were used to amplify the mutant clone. The mutated cDNA fragments were cloned into pQE30 between the SmaI and SacI sites for S11 and between the BamHI and HindIII sites for S12. The sequences of both mutated clones were confirmed by sequencing and named pQE30-S11-hisΔ-C2 and pQE30-S12-hisΔ-C2.

These two plasmids were transformed separately into competent M15 cells, and protein expression was induced by adding IPTG to a final

concentration of 1 mM. The cultures were typically grown for 4-5 hr before the cells were harvested. The target proteins were purified with Ni-NTA resin (QIAGEN, Maryland, USA), electrophoresed on SDS-PAGE, then eluted from gel slices using an Electro-Elutor (Bio-Rad, California, USA) following the manufacturer's protocol. The purity of both proteins was confirmed by Coomassie blue staining after SDS PAGE and Western blot analysis.

Quantification of the standard S_{11} and S_{12} proteins

The concentration of purified S_{11} and S_{12} proteins was determined both by **OD^{280}** and by the Micro BCA Protein Assay Kit (PIERCE Inc., Illinois, USA) following manufacture's protocol. The two measures gave similar results and were thus used to calibrate S-RNases measurements in styles of *S. chacoense*. One batch of each purified protein was used as the standard for all gels.

Western blots and quantitative analysis of S_{11} and S_{12} RNases in Individual styles

For Western blots, plant styles were collected and frozen immediately in liquid nitrogen. The term "style" as used here comprises both the style *per se* as well as the stigmatic region. Proteins were extracted from individual styles using 50 µl extraction buffer (0.05M Tris pH 8.5, 1 mM DTT, 1 mM EDTA, 0.05M $CaCl_2$, 1 mM PMSF). Typically, 5X SDS sample buffer was added to 25 µl crude extract from each individual style and electrophoresed on SDS-PAGE. Immunological detection of the S-RNases is linear over the range used, so only one aliquot containing 400 ng of each purified S-RNase was run on each gel for standardization. The proteins were transferred to nitrocellulose membranes and stained with 2%(w/v) Ponceau red as a control for a uniform protein load; S-RNase measurements from samples with visibly different

protein loads were excluded from our analyses. The membranes were blocked by an overnight incubation with TBS-T (Tris-buffered saline containing 1.5 % w/v BSA fraction V (Sigma) and 0.05% w/v Tween 80), incubated for 2 hours at room temperature with a 1:1000 dilution of either polyclonal anti-S_{11} (Matton et al., 1999) or anti-S_{12} (Qin et al., 2001) antibodies, rinsed with TBS-T 3 times, and incubated with 4 µl of 0.5 µCi/mmol I^{125}-protein A (Perkin-Elmer) in 5 mL TBS-T for 2 hr. After washing 3 times with TBS-T, the membranes were exposed with a Phosphor screen for 12-76 hr at room temperature and the screen imaged using a PhosphorImager scanner (Amersham Bioscience). The data on the scanned images were quantified using the software supplied by the manufacturer. Both antibodies used are specific for their respective substrates.

Results

S-RNase levels can be reproducibly assayed in single styles

The threshold hypothesis for S-RNase-mediated pollen rejection posits that an incompatible phenotype requires a minimum level of S-RNase within the styles. To determine this level experimentally, it was first necessary to develop a technique that would permit the absolute levels of S-RNase to be accurately and reproducibly measured in extracts from single styles. This assay thus requires two elements, a sensitive detection system and a calibration method to calculate the amount of S-RNase at ng level. For the latter, S-RNase protein standards were prepared by expressing an inactive S-RNase as a His-tagged construct in bacteria. Two tagged constructs, an S_{11}-RNase and an S_{12}-RNase, were purified by Ni-affinity chromatography and SDS-PAGE elution, and were homogenous by the criteria of Coomassie blue staining (Fig.IV.1).

For development of a sensitive and easily quantifiable assay, we coupled Western blotting using an I^{125}-labeled protein A, with detection using a PhosphorImager. To characterize the response to the antibodies, standard curves using different amounts of both S-RNases were prepared. The immunological response is linear under our conditions (Fig.IV.1 C). To characterize the precision of the measurements, S_{11}-RNase levels in each of four equal aliquots of an individual style extract were compared (Fig.IV.2). These S_{11}-RNase measurements have a coefficient of variation of only 5 % and show that the method can faithfully assess the RNase levels in individual styles.

Since we were interested in measuring levels of both S_{11}- and S_{12}-RNases in single style extracts, we tested if single transfers could be analyzed using both anti-S_{11}- and anti-S_{12}-RNases. In one experiment, two membranes containing half the extracts from 18 styles of plant 314 ($S_{11}S_{12}$ genotype) were prepared. One membrane was treated first with the anti-S_{11}-RNase and the S-RNase levels quantitated using the PhosphorImager. This membrane was then stripped and treated with the anti-S_{12}-RNase (Fig.IV.3, panel A). A second membrane, containing the other half of the same samples was similarly treated except that the order of the two antibodies on the membrane was reversed (Fig.IV.3B). An additional 18 styles from the plant L25 (also $S_{11}S_{12}$) were analyzed in parallel using the same protocol (Fig.IV.3 C and D). All samples show a substantial decrease in calculated S_{11}-RNase (Fig.IV.3E) and S_{12}-RNase (Fig.IV.3F) levels when measured on previously used membranes. We conclude that our technique does not accommodate multiple analyses from the same membrane. However, as reliable measurements were obtained from one-half the stylar extracts, both S-RNases can be measured in a single style extract by simply preparing two membranes from each sample. We also note from this preliminary study that a substantial difference in the average levels of S_{11}- and S_{12}-RNases can be observed in the styles of both plant lines.

We have previously shown that average S_{12}-RNase levels in pooled stylar extracts were dependent on the plant genotype used (Qin et al 2001). Here we confirm these observations using the single style measurements of S_{12}-RNase in sporadically self-compatible $S_{11}S_{12}$ lines L25 and 314 (Fig.IV.4 A, B), the strictly incompatible S_{12} homozygote line VF60 (Fig.IV.4 C) and the $S_{12}S_{14}$ G4 line (Fig.IV.4 D). As expected, average S_{12}-RNase levels are low (86 ± 55 ng) in styles from L25 plants, intermediate (136 ± 86 ng) in those from 314 plants, and high in VF60 (269 ± 55 ng) and G4 (301 ± 93 ng) plant styles (Fig.IV.4 E). More important, however, are the style-to-style variations observed within each plant genotype which here can vary up to three-fold (Fig.IV.4 F). Interestingly, as S-RNase levels in the fully incompatible lines VF60 and G4 can be as low as 160 ng (Fig.IV.4 F), this level of S-RNase must lie above the minimum required for pollen rejection. The S_{12} –RNase appears as a doublet in L25 and 314 but not in G4 or VF60 due to genotype dependent glycosylation differences (Qin et al., 2001). Samples with markedly different Ponceau staining (for example, panel B lane 5) were excluded from further analysis.

Experimental determination of S_{12}-RNase thresholds for pollen rejection

In contrast to the fully incompatible VF60 and G4 lines, L25 and 314 plant lines have been previously demonstrated to display a sporadic self-compatibility (SSC) phenotype (Qin et al., 2001). Although the SSC phenotype has not yet been traced to a specific environmental or physiological cause, the fact that only a fraction of the L25 or 314 styles pollinated set fruit (Qin et al., 2001), coupled with a large standard deviation in measured S_{12}-RNase levels, suggested that a style-by-style comparison of S_{12}-RNase levels and fruit set might allow the threshold levels required for pollen rejection to be determined.

We were fortunate, during the course of our experiments, to have collected fourteen styles from L25 plants during a bout of almost complete

compatibility with S_{12} pollen (31 fruits set from 33 flowers pollinated with S_{12} pollen from line 2548). The maximum amount of S_{12}-RNase in the styles sampled was 68 ng (Fig.IV.5 A, B), and the complete compatibility phenotype during this period suggests that this amount of S-RNase must lie below the minimum level required for pollen rejection.

The S-RNase levels in individual styles described above appear to define the upper limit for pollen acceptance and the lower limit for pollen rejection, yet two problems are associated with this conclusion. First, in these cases the phenotype is complete (i,e. no pollinations produce fruits), and to determine more precisely the threshold, additional data must of necessity use incomplete phenotypes (i.e. where some pollinations are observed to set fruits). Since S-RNase levels and the incompatibility phenotype cannot both be measured in the same style, correlative techniques are thus necessary. Second, it is possible that other factors might contribute to pollen rejection. It is therefore necessary to repeat these correlative experiments under several different conditions to ensure that the effect observed can indeed be ascribed the S-RNase itself. To address these issues, we thus examined S-RNase levels in the SSC plant line L25 under several developmental stages. In one series of experiments, S-RNase levels were measured in individual styles of L25 plants at the time of anthesis (Fig.IV.5 C). The average fruit set in these plants with S_{12} pollen from line 2548 is 55%, and if the S-RNase level was the principal contributor to the SI phenotype, then a threshold value of 80 ng S_{12}-RNase would leave this proportion of individual data points below the line. We also note the levels of S_{11}-RNase are far above this in agreement with a full S_{11} pollen rejection phenotype if the threshold was similar (Fig.IV.5 H).

In a second series of experiments, styles were taken between one and three days before anthesis (DBA), a timing based on the size and morphology of the flower buds (Fig.IV.5 D-F). Again, the predicted threshold value of 80 ng S-RNase is able to account for the incompatibility phenotype observed with S_{12} pollen from line 2548 (Table IV.1). It must be noted that complete compatibility

with flowers pollinated at 3 DBA is not obtained, even with completely compatible pollen. This is due to the fragility of the buds, the tendency of the stylar tissues to dry after opening of the buds, and the fact the stigma is apparently only partially receptive, as assessed by microscopic observations of fewer pollen grains that adhered and germinated.

Next, a similar series of experiments was performed with the styles taken from 314 plants. Once again, styles were analyzed at anthesis (Fig.IV. 6A) as well as between one and three days prior to flower opening (Fig.IV.6 B-D). The general pattern of S_{11}- and S_{12}-RNase values is similar to that observed in L25, with S_{11}-RNase high at anthesis and at two days prior to flower opening (Fig.IV.6E) while S_{12}-RNase levels were generally lower at anthesis and substantially lower two days prior (Fig.IV.6 F). A predicted pollen rejection phenotype based on the number of individual styles with S_{12}-RNase levels less than 80 ng (32%) agrees well with the observed results using S_{12} pollen from line 2548 (25%) (Table IV.1) suggesting that the threshold S_{12}-RNase level is similar in styles from 314 and L25 plants. Taken together, therefore, our data support the idea that a threshold of 80 ng S_{12}-RNase is sufficient to block fruit set after pollination with S_{12}-pollen.

Progeny analysis

Bud pollination of plant L25, selfed at 3 DBA, resulted in fruit formation. Plants were raised *in vitro* from the seeds and analyzed by PCR using S-allele-specific oligos to assess whether the three possible genotypes ($S_{11}S_{11}$, $S_{11}S_{12}$ and $S_{12}S_{12}$) were present in the expected ratios. Of the 202 F1 progeny, 90 were $S_{11}S_{12}$, 112 were $S_{12}S_{12}$, and none were $S_{11}S_{11}$ (X^2 =59.17, $P< 0.001$). We conclude that S_{11} pollen is fully rejected even at 3 DBA, despite our observation of low (53 ng) S_{11}-RNase levels measured in one out of six styles analyzed (Fig. 5). It is possible that the threshold for S_{11}-RNase is lower that the 80 ng threshold estimated for S_{12}-RNase. Alternatively, the steady increases in S_{11}-RNase observed between 3 and 2 DBA may be sufficient to block S_{11} pollen tubes before they reach the ovary.

S_{12}-RNase thresholds differ for different pollen types

The S_{12}-RNase threshold estimated from pollen rejection phenotypes using pollen from plant 2548 is similar when styles of L25 and 314 plants are compared (Table IV.1). However, this experiment does not address the potential influence of the pollen itself on the estimated thresholds. Thus, in another experiment, we compared the pollination efficiency of four types of pollen from different genetic background on L25 styles. All these pollen show similar viability (based on their appearance after staining with acetocarmine) and good germination. Despite this, major differences are observed in pollination efficiency (Table IV.2), and this suggests that the S-RNase thresholds may be different for the different types of pollen. Indeed, if thresholds are estimated as before by placing an arbitrary threshold line in Fig.IV. 5 G at a value where the proportion of styles with lower S-RNase levels corresponds to the percent fruit set, the estimated S_{12}-RNase threshold differs by up to five fold (Table IV.2). We conclude from this that the S-RNase threshold must be defined for each particular pollen type.

Microscopic observations

To complement the fruit set measurements on genotypes L25 and 314, microscopic observations were also made to assess the behavior of pollen tubes inside the styles 2-3 days after pollination. Following self-pollination, S_{12} pollen tubes were typically observed in the middle or lower third of the style, unlike S_{11} pollen tubes, which all arrested in the upper third of the style. Following pollination with S_{12} pollen from VF60 or plant 2548, however, a large variation was found in the number of pollen tubes observed at the stylar basis. In some cases, numerous pollen tubes were observed to have entered the ovary, and the appearance of the stylar bases were undistinguishable from

compatible pollinations (usually yielding about 120 seeds) (Fig.IV.7A). We interpret this as consistent with a low level of S_{12}-RNase in these styles. In other cases, no tubes were observed at the stylar base, which we interpret as consistent with a high level of S-RNase (Fig.IV.7B). In other instances, a reduced number of pollen tubes (from one up to ten, but most often one or two) can be seen to have reached the base of the style and to have entered the ovarian region. Some of these pollen tubes may arrest just after their entrance into the ovarian region, while others can be observed to penetrate inside the ovules (Fig.IV.7 C, D). Curiously, however, fruits containing only one or two seeds were never observed. Indeed, the smallest numbers we have ever observed were 4 and 5 seeds in two different fruits, and in both cases they were accompanied by numerous aborted seeds and swollen ovules.

These observations suggested that a single pollen tube reaching the ovary may be insufficient to allow fruit set, and that assessment of SI either by number of pollen tubes at the stylar base or by fruit set may differ. To test this, we used the simple expedient of collecting, 4-5 days after pollination of L25 or 314 plants with S_{12} pollen, flowers that would fall when gently shaken. These incompatible crosses were then examined microscopically to determine the number of pollen tubes at the stylar base. Although in many cases the pollen tubes were all arrested in the middle or lower third of the style, we also found instances where up to ten tubes could be found entering the ovarian region (Fig.IV.7 E, F). We deduce from this that fruit formation may require more than 10 pollen tubes entering the ovarian region, although we note as a caveat that this value may include some slow growing tubes that reach the ovary after the process leading to flower abscission has already been initiated. As a complement to these studies, we have also examined at 72 hours post pollination 24 styles of L25 plants pollinated during its bout of almost complete self-compatibility. While 21 styles had 20 or more (in most cases, uncountable) pollen tubes in the ovarian region, in the remaining cases only 14 (two cases) or 15 (one case) pollen tubes were found in the ovarian region. Since the

ovarian regions contained fourteen pollen tubes in these compatible crosses, and ten in the incompatible crosses described above, these results suggest that a number of pollen tubes in excess of this 10-14 tubes threshold must enter the ovary to insure fruit formation.

Discussion

Style to style variations in S-RNase levels can be used to estimate the S-RNase threshold

We have found a considerable natural variability in the amount of S-RNases present in individual styles. The extent of this variation was surprising, as individual styles can differ in S_{12}-RNase levels by over twenty fold. This phenomenon has not previously been reported, and we have observed it here only because S-RNase levels have been measured for individual styles. Interestingly, these variations neatly account for the observations reported here in which fruit set for an individual style is all-or-none phenomenon but where different styles on the same plant may behave differently. We do not believe that these variations represent technical problems with the assay since our standard curves are linear (Fig.IV.1) and replicates from the same biological samples have a low coefficient of variation (Fig.IV.2).

These variations in individuals can be exploited to estimate the threshold below which S-RNases are ineffective in pollen rejection. For example, when an S-RNase expressing plant is completely compatible (Fig.IV.5 B), the highest stylar level found (68 ng) defines the highest point still below threshold. Similarly, when the plant is completely incompatible (Fig.IV.4 F), the lowest value of S-RNase obtained (160 ng) defines the lowest point still fully capable of pollen rejection.

Do these values truly reflect the existence of an S-RNase threshold? This question is not trivial, as other factors such as HT-B (O'Brien et al., 2002)

or a stylar 120 kDa glycoprotein in *Nicotiana* (Hancock et al., 2005) have been shown to be required for pollen rejection. However, if a factor other than the S-RNase were to contribute substantially to pollen rejection, a repeated correlation between incompatibility and S-RNase levels would be unlikely. It is thus significant that the thresholds estimated from two different plants lines (L25 and 314) under all our conditions are so similar. Furthermore, the same threshold accounts for the acquisition of the incompatibility phenotype in developing flowers (Fig.s IV.5G, 6E), long cited as support for the threshold hypothesis itself. Taken together, we propose that the many times the same correlation is observed provides strong support for the idea that S-RNases play the primary role in determining the SI phenotype. If so, this in turn suggests that the S_{12}-RNase threshold required for pollen rejection is indeed around our value of 80 ng per style.

S-RNase concentrations inhibiting pollen tube growth are remarkably similar in different plants

The value proposed here for the threshold of the S_{12}-RNase in *S. chacoense* should be considered in the context of the average amounts of S-RNase present in the styles of other fully self-incompatible Solanaceae genotypes. Average S-RNase levels have appeared in a number of different reports, and it must first be noted that these values can differ markedly depending on the method used. For example, calculations of the RNase levels in S_2S_2 homozygous self-incompatible *N. alata* based on the amount of S-RNases purified from styles yielded estimates of 10 µg (Jahnen et al., 1989b; Jahnen et al., 1989a) or ~20 µg (Gray et al., 1991) S_2-RNase per style. In contrast, measurements based on comparisons of staining intensity of an S_2-RNase band after electrophoresis of crude extracts with that of the purified S-protein, yielded estimates of 90 µg S_2-RNase per style in the same plants (Harris et al., 1989). The lower levels calculated after protein purification may perhaps be

attributable to losses during purification. Alternatively, the higher levels found after electrophoresis may have been due to the presence of contaminating proteins with the same electrophoretic mobility as the S_2-RNase. Our protocol, which compares immunoreactivity in crude extracts with those of a pure standard S-RNase, appears less susceptible to experimental errors of either type. Our values about 1 µg S_{11}-RNase and 0.1 µg S_{12}-RNase in the styles of L25 plants (Fig.IV.3) appear lower than what is found in *Nicotiana*, but it must be borne in mind that *Nicotiana* styles (~15 mg) weigh roughly ten times more than *S. chacoense* styles (~1.3 mg). Indeed, the calculated concentrations of S-RNases in styles of *S. chacoense* (0.25 – 1 mg/mL) and *N. alata* (0.5 – 5 mg/mL) are quite similar.

The calculation of S-RNase thresholds *in vivo* is difficult, as generally the genotypes studied are fully incompatible and the degree to which their S-RNase levels surpass the threshold can thus not be ascertained. Even in plants where some degree of compatibility is observed, measurements of S-RNase levels and incompatibility phenotype in an individual style are mutually exclusive. Methods employing correlations in populations of styles are thus required. One such approach has been taken with the Japanese pear (Rosaceae), where some cultivars can be up to 15 % compatible (Hiratsuka and Zhang, 2002). Since the S-RNase values vary two-fold between different plant genotypes, and the fully incompatible S_3-RNase is present at roughly 0.2 mg/mL in styles of the fully incompatible cultivar "Choiuro" (Matsuura et al., 2001a), this suggests that the threshold may lie close to 0.1 mg/mL (Hiratsuka and Zhang, 2002). This value is remarkably similar to the 80 ng (0.06 mg/mL) per style estimated from the studies reported here.

Genotypic differences in expression of style and pollen components of SI

The observation that different S-RNases are expressed to different levels in styles of a given plant (Fig.IV.3) leaves open the important question of the

expression of the same S-RNase in different plant genotypes. In general, an effect of the genotype of the donor plant on the amounts of the S-RNase it produces, i.e. the level of expression of the same S-RNase in different genetic backgrounds, has only rarely been considered. In one study, the expression of S_{A2}-RNase constructs in different *Nicotiana* species was found to depend on the genetic background of the host (Murfett and McClure, 1998). In another study using Japanese pear, the same S-allele produced different amounts of S-RNase depending upon the cultivars (Zhang and Hiratsuka, 1999). In *S. chacoense*, a comparison of the levels of S_{11}-RNase in genotypes L25 and 314 suggests that only slight differences may be observed (Fig.IV.3E). However, in the case of the S_{12}-RNase, up to three-fold differences were observed among the four genotypes tested (Fig.IV.4). For the S_{12}-homozygote line VF60, at least part of the increase may be related to the number of S_{12} genes present. However, this explanation cannot account for the high levels in the $S_{12}S_{14}$ heterozygote G4 line. In this regard it is interesting to note, in various cultivars of Japanese pear, the systematic (almost exclusive) association of S-RNases (S_1, S_3, S_5) that are both more abundant in the styles and yield strong SI phenotypes, with those (S_2, S_4, S_6 and S_7) with weaker SI phenotypes and whose abundance in the styles is reduced (Zhang and Hiratsuka, 1999; Hiratsuka and Zhang, 2002). It was concluded that the strength of the SI system in the various cultivars depends on the total S-RNase content rather than the levels of individual S-RNases (Zhang and Hiratsuka, 1999).

The genetic background of the pollen may influence the S-RNase threshold

In addition to genotype-dependent differences in expression of the stylar component to SI, our analyses also suggest that differences may be found in expression of the pollen component. For example, when pollen of different staminate genotypes was tested on the same pistillate parent, the proportion of

fruits set and the calculated S_{12}-RNase thresholds were considerably different (Table IV.2). We calculate a roughly four-fold difference in the S_{12}-RNase thresholds for pollen from the three S_{12}- homozygous individuals tested, similar to the over three-fold differences observed for S_{12}-RNase expression in different genotypes (Fig.IV.4). Although the difference in the calculated threshold of L25 pollen appears even lower, it must be noted that only half the pollen derived from the L25 pollen has the S_{12} haplotype. It is tempting to speculate that expression levels of the pollen component to SI may also vary according to the genetic background, and that these variations may be responsible for the differential sensitivity of the pollen to its cognate S-RNase. Alternatively, it is possible that the different pollen types tested here differ in their S_{12}-RNase uptake efficiency. These factors can be scrutinized when the pollen component is finally identified in *S. chacoense*.

Consequences of an S-RNase threshold for conceptualizing SI

The determination of what appears to be a relatively sharp S-RNase threshold has some important implications that have not previously been made explicit in studies on self-incompatibility. The reason for this is that several molecular mechanisms have previously been reported to give rise to threshold phenomena, also termed ultrasensitive or switch-like. For example, ultrasensitive responses can be due to cooperative interactions between subunits, to a requirement for multiple phosphorylation events, or to covalent modifications carried out by modifying enzymes working at saturating substrate levels (zero-order kinetics) (Ferrell, 1998). The mechanism leading to the threshold phenomenon in GSI is presently unknown, but to date the only covalent modification suspected is ubiquitination, and as S-RNases levels must rise in the pollen after import from the style they will not be initially saturating. However, as we have previously proposed that pollen S may function as a multimer (Luu et al., 2001), one attractive possibility is that cooperative

interactions might also exist between S-RNase subunits. Indeed, it would be of interest to examine the kinetics of purified S-RNases *in vitro* for evidence of cooperative behavior. A second intriguing possibility is that the threshold might reflect the presence of stoichiometric levels of a factor that binds with high affinity to the RNase. If true, this suggests that biochemical approaches might be successful in isolating the factor responsible.

Fruit formation requires a minimum number of fertilization events

Our results suggest that more than ten pollen tubes entering the ovary and accomplishing fertilization may be necessary to trigger fruit formation and sustain its subsequent development. This facet of the SI phenotype has been revealed by observations at fluorescence microscopy of styles from flowers that drop, after gentle shaking, 4 to 5 days after pollination. Since in SI studies a cross is generally considered incompatible when pollinated flowers drop, the presence of up to 10 pollen tubes entering the ovary seems surprising. It is known that a minimum number of fertilization events is required for fruit set and its subsequent growth (Gillaspy et al., 1993; Hiratsuka and Zhang, 2002), and this is consistent with our finding that when fruits contained only a few well developed seeds, numerous aborted seeds or swollen ovules were always present. This is similar to what was observed in other species, for example in *Lycopersicon* (de Nettancourt et al., 1974; Gradziel and Robinson, 1989). However, it is difficult to assess if this may be more generally true, as reports of fruits with only a single seed (de Nettancourt and Ecochard, 1968; de Nettancourt et al., 1971) made no mention of either the presence or absence of aborted seeds, nor were microscopic observations performed in those studies. In addition, it is important to recognize that entrance of pollen tubes into the ovarian region represents only an estimation of the outcome of the cross, as the number of pollen tubes actually accomplishing fertilization is the determinant factor for fruit set. It is known that environmental factors such as

temperature, heat, humidity etc. that can affect self-incompatibility, although it seems unlikely that these factors would selectively affect only some styles on a plant as we observe here.

The number of fertilization events required for fruit formation has a particularly interesting implication for the evolution of the SI system. It has long been recognized that mutations of S-alleles occur in nature, yet attempts to produce new S-alleles by mutagenic treatments have not been successful (de Nettancourt, 1977; de Nettancourt, 2001), and have only been observed after site-directed mutagenesis (Matton et al., 1997; Matton et al., 1999). Our results suggest that even if pollen mutants were generated experimentally, these mutations could not be transmitted to the progeny upon experimental selfing if a single mutant pollen grain is insufficient to allow fruit formation, unless hormone treatments were applied to the pollinated flowers in order to prevent premature abscission (de Nettancourt et al., 1971; Golz et al., 1999). By contrast, under open pollination conditions such as occur in nature, the concomitant pollination with a mutant pollen and abundant compatible pollen from neighbor plants would allow fruit set to occur normally, in turn allowing the mutant zygote to develop and begin to spread the mutant genotype within the population.

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Figure Legends.

Figure IV.1. S_{11} and S_{12}-RNase standards are pure by Coomassie blue staining.

(A) and **(B)** Bacterial cells transformed with either an S_{11}-RNase or an S_{12}-RNase cDNA modified to remove the N-terminal signal peptide and to add a C-terminal His tag, and protein extracts were analyzed by SDS-PAGE (upper panels) and Western blots with the indicated antibodies (lower panels). Samples are from uninduced cells (lane 1), cells induced by IPTG (lane 2), induced cells after Ni affinity chromatography (lane 3) and an extract from the $S_{11}S_{12}$ containing L25 styles. The authentic S_{12}-RNase (arrow in B) is heavily glycosylated and migrates as a doublet above the bacterially produced protein, while the authentic S_{11}-RNase runs slightly faster (arrow in A). The apparent molecular weight of protein standards (lane 5) is shown at right. **(C)** The amount of radiolabel bound to anti-S_{11}- and anti-S_{12}-RNase on Western blots was quantitated using a PhosphorImager for a range of both S_{11}- (closed circles) and S_{12}-RNases (open squares). S-RNase amounts shown cover the range of values measured in styles.

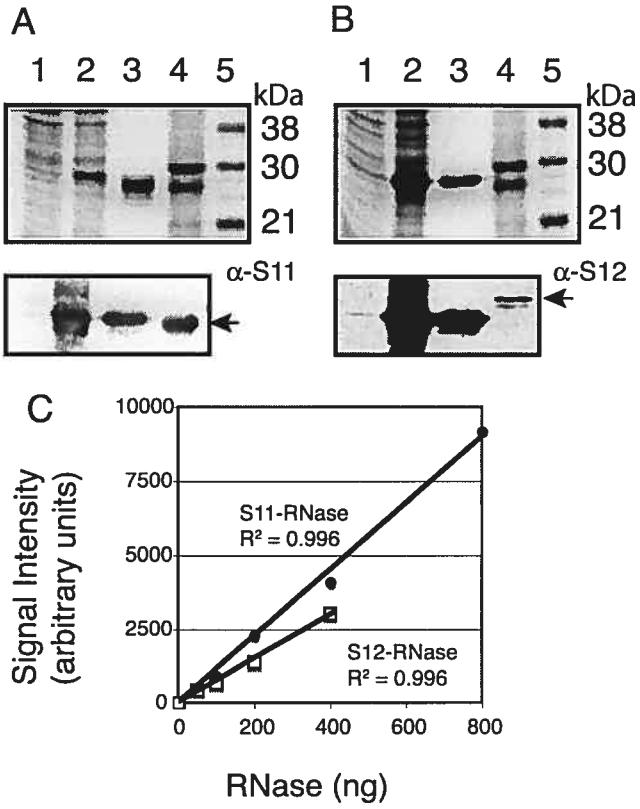

A
1 2 3 4 5
kDa
38
30
21
α-S11
B
1 2 3 4 5
kDa
38
30
21
α-S12
C
Signal Intensity (arbitrary units)
10000
7500
5000
2500
0
S11-RNase
R² = 0.996
S12-RNase
R² = 0.996
0
200
400
600
800
RNase (ng)

Figure IV. 2. Style-by-style RNase measurements are highly reproducible. **(A)** The S_{11}-RNAse levels in quadruplicate analysis of protein extracts from two large styles (style 1, lanes 1-4; style 2, lanes 5-8) were determined by Western blot analysis. An S_{11}-RNase protein standard (400 ng pure protein, lane 9) was included to standardize the measurements between gels. **(B)** The amount of S-RNase in each gel lane of style extract was calculated relative to the standard after quantitation of radiolabel using a PhosphorImager. For each style, the four replicate samples (stippled) and the average ± SD (black) are shown.

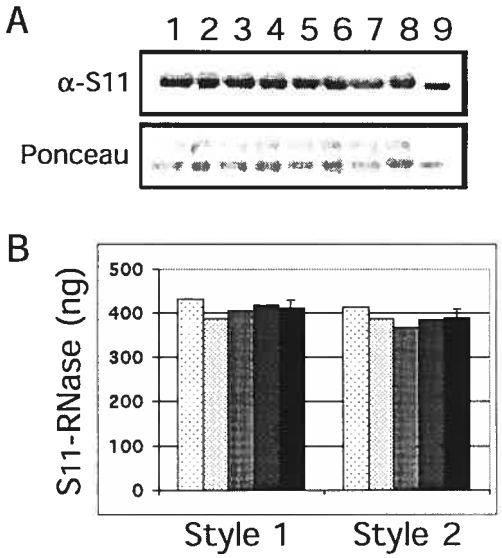

A
1 2 3 4 5 6 7 8 9
α-S11
Ponceau
B
S11-RNase (ng)
500
400
300
200
100
0
Style 1
Style 2

Figure IV.3. Accurate S-RNase quantitation requires single-use Western blots.**(A)** The levels of both S_{11}- and S_{12}-RNAse were measured in one half the protein extracts from single styles of $S_{11}S_{12}$ 314 plants (lanes 2-7) by sequential Western blot analysis using first an anti-S_{11}- RNAse then an anti-S_{12}- RNAse. Standards (400 ng pure protein) for both the S_{11}-RNase (lane 1) and the S_{12}-RNase (lane 8) were included on each gel. The samples shown are a representative sample of three different gels. **(B)** The remaining half of the protein extracts of the 314 plants was treated as in (A) except that the order of the antibodies was reversed. **(C)** Analysis of one half the protein extracts from $S_{11}S_{12}$ containing L25 plants as in (A). **(D)** Analysis of the remaining half the extracts from L25 plants as in (B). **(E)** The average ± SD ($n \geq 16$) S_{11}-RNase levels calculated on a per-style basis after PhosphorImager detection in styles of L25 and 314 plants when the anti- S_{11}-RNase was used first (lightly stippled bars) or second (darkly stippled bars). **(F)** The average ± SD ($n \geq 16$) S_{12}-RNase levels in styles of L25 and 314 plants determined as in **(E)**.

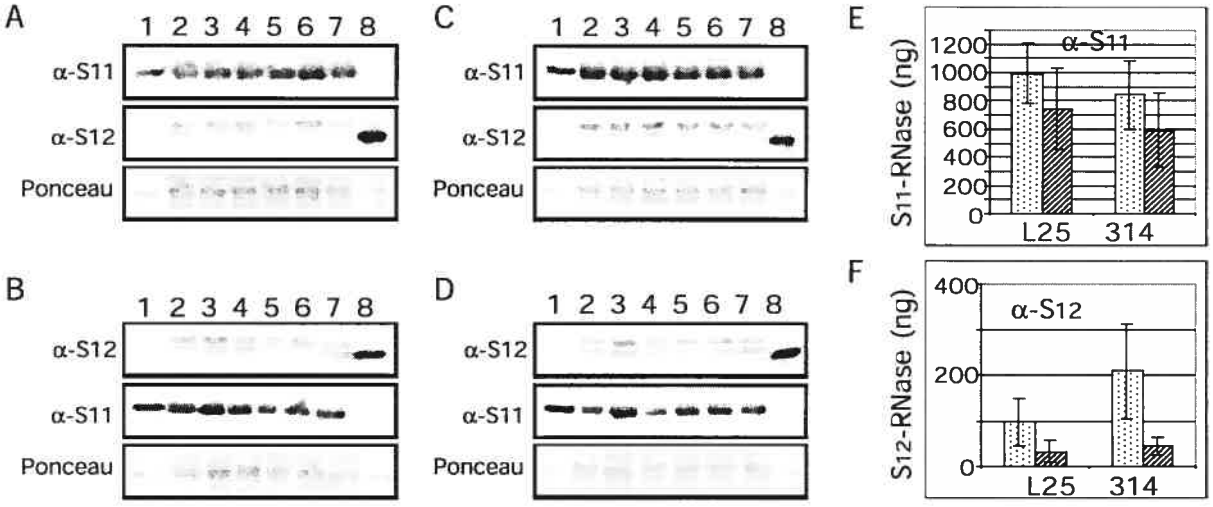

A
1 2 3 4 5 6 7 8
α-S11
α-S12
Ponceau
B
1 2 3 4 5 6 7 8
α-S12
α-S11
Ponceau
C
1 2 3 4 5 6 7 8
α-S11
α-S12
Ponceau
D
1 2 3 4 5 6 7 8
α-S12
α-S11
Ponceau
E
α-S11
S11-RNase (ng)
1200
1000
800
600
400
200
0
L25
314
F
α-S12
S12-RNase (ng)
400
200
0
L25
314

Figure IV.4. S_{12}-RNase levels are genotype-dependant. **(A-D)** Representative Western blot analysis of one-half the protein extracts from single styles (lanes 2-7) of **(A)** $S_{11}S_{12}$ L25 plants (n=29), **(B)**, $S_{11}S_{12}$ 314 plants (n=44), **(C)** $S_{12}S_{12}$ VF60 plants (n=14) and **(D)** $S_{12}S_{14}$ G4 plants (n=13) were determined as described in the legend to Fig. 3. Note that S_{12}-RNase displays genotype-specific glycosylation patterns. **(E)** The average ± SD S_{12}-RNase levels in each of the plant genotypes. **(F)** The individual S_{12}-RNase levels in VF60 (open circles) and G4 (closed circles) styles. Each point represents a different S-RNase measurement, and all points were distributed horizontally so as to allow all individual points to be seen without overlap.

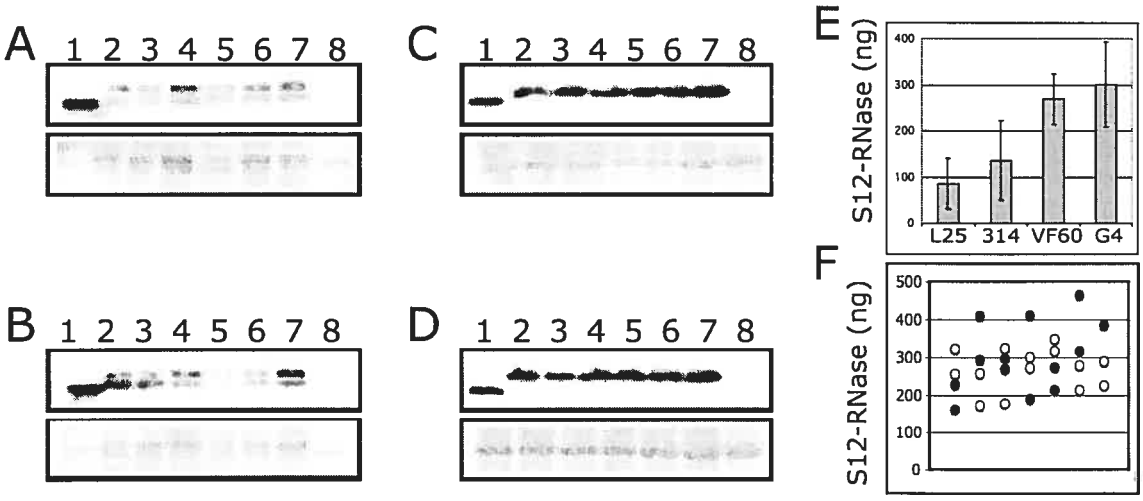

A
1 2 3 4 5 6 7 8
B
1 2 3 4 5 6 7 8
C
1 2 3 4 5 6 7 8
D
1 2 3 4 5 6 7 8
E
S12-RNase (ng)
400
300
200
100
0
L25 314 VF60 G4
F
S12-RNase (ng)
500
400
300
200
100
0

Figure IV.5. S-RNase levels in L25 plants increases during stylar development **(A)** Western blot analysis of one-half the protein extracts from single styles (lanes 1-14) of $S_{11}S_{12}$ L25 plants taken at a time when the plants were fully compatible with S_{12} pollen (see text). **(B)** The individual per-style S_{12}-RNase values of the data in (A) after quantification. The S_{12}-RNase value of lane 9 was not included because of its low protein level. Again, each point represents a single S-RNase measurement, and all points were distributed horizontally so as to allow all individual points to be seen without overlap. **(C-F)** Representative Western blot analysis of one-half the protein extracts from single styles (lanes 1-6) of $S_{11}S_{12}$ L25 plants taken at **(C)** the time of flower opening, **(D)** one day before, **(E)** two days before and **(F)** three days before flower opening using either an anti- S_{11}-RNase (left panels) or the anti- S_{12}-RNase (right panels). Protein standards (400 ng pure protein) are the S_{11}-RNase (left panels) or the S_{12}-RNase (right panels). Note that the contrast with the anti- S_{12}-RNase was increased using Photoshop in (E) and (F) to visualize the signals. **(G)** The individual S_{12}-RNase values (ng per style after PhosphorImager quantitation) are shown for single styles (represented on the X-axis) taken at the time of flower opening (closed circles), one day before (open squares), two days before (open triangles) or three days before (open circles). Individual data points represent those contributing to the average in Fig. 4 E. **(H)** The individual per-style S_{11}-RNase values. Data points include those from Fig. 3 where the anti-S_{11}-RNase was used first.

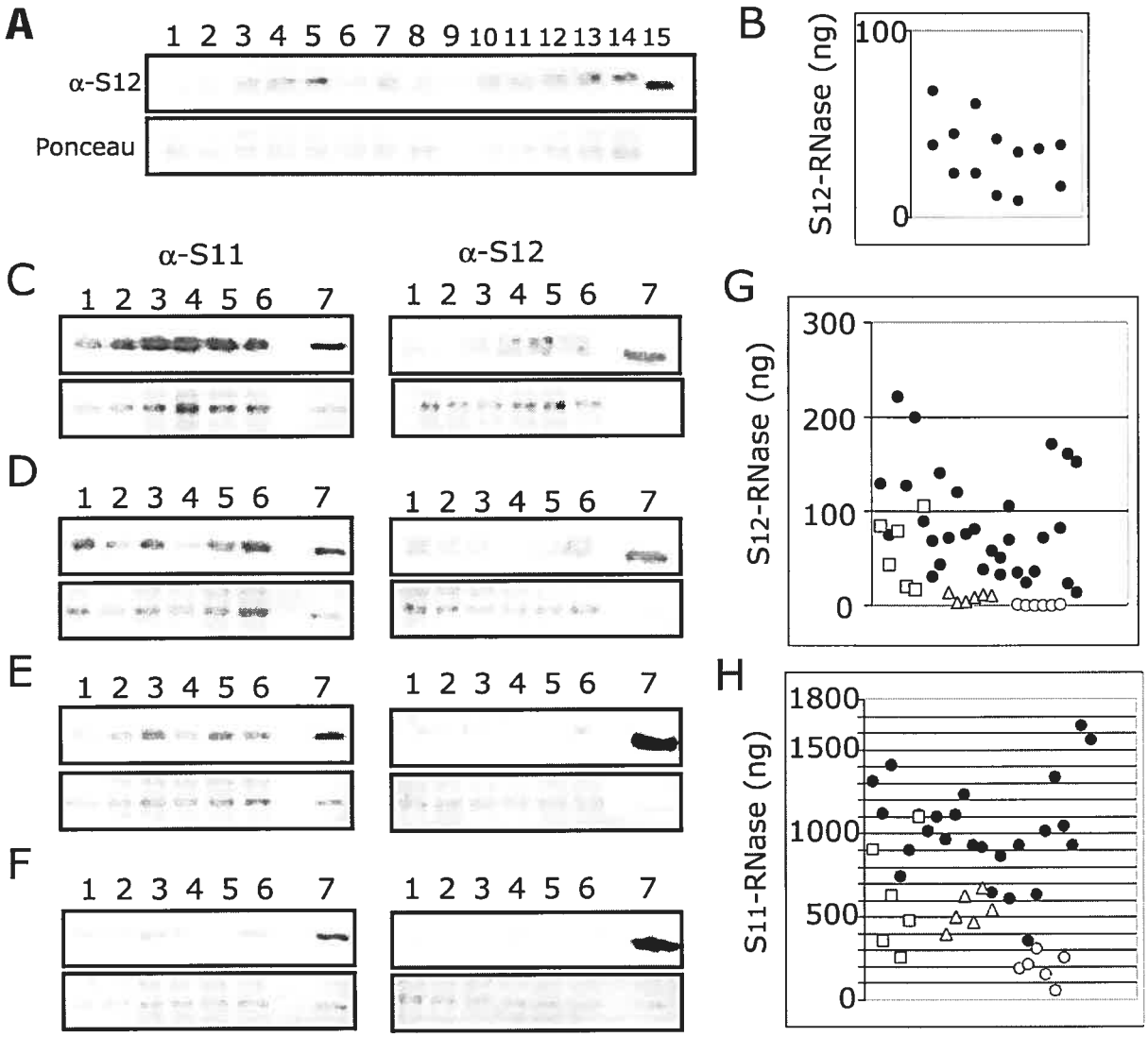

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
α-S12
Ponceau
B
S12-RNase (ng)
100
0
C
α-S11
α-S12
1 2 3 4 5 6 7
D
E
F
G
S12-RNase (ng)
300
200
100
0
H
S11-RNase (ng)
1800
1500
1000
500
0

Figure IV.6. S-RNase levels in 314 plants increases during stylar development **(A-D)** Western blot analysis of one-half the protein extracts from single styles (lanes 1-6) of $S_{11}S_{12}$ containing 314 plants analyzed as in the legend to Fig. 5. **(E)** The individual per style S_{11}-RNase values (ng per style after PhosphorImager quantitation) are shown for plants taken at the time of flower opening (closed circles), one day before (open squares), two days before (open triangles) or three days before (open circles). Data points include the first anti-S_{11}-RNase in Fig. 3. **(F)** The individual per style S_{12}-RNase values presented as in (E). Individual data points represent those contributing to the average in Fig. 4.

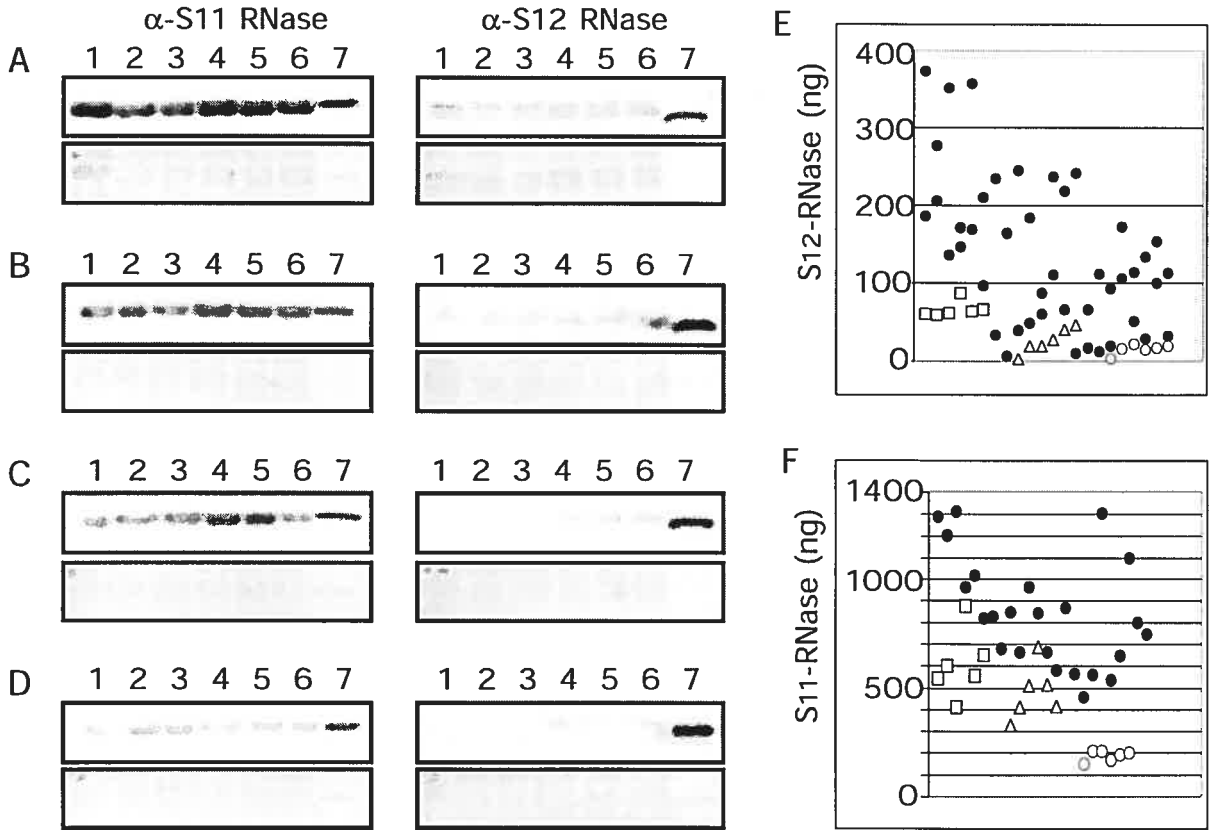
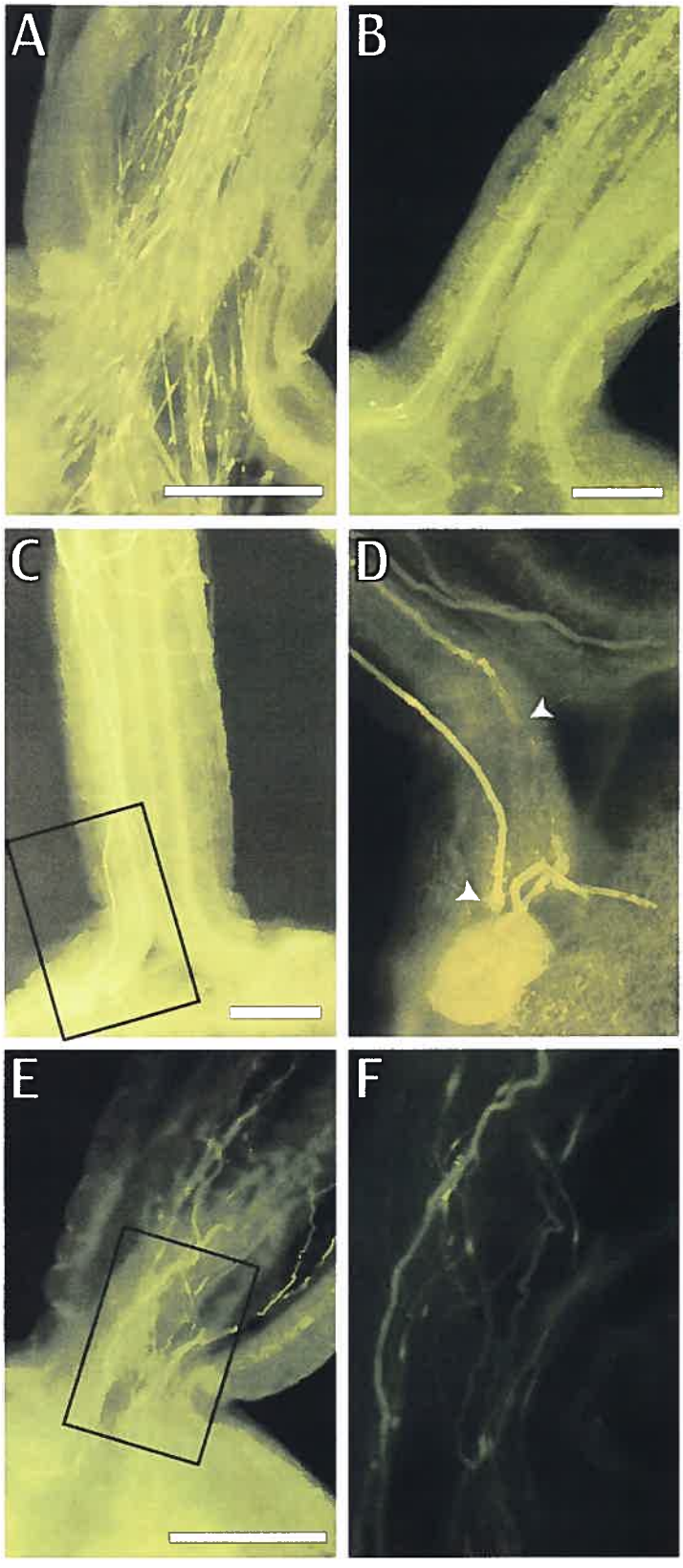

α-S11 RNase
α-S12 RNase
A
B
C
D
1 2 3 4 5 6 7
E
S12-RNase (ng)
400
300
200
100
0
F
S11-RNase (ng)
1400
1000
500
0

Figure IV.7. Fruit set requires a threshold number of pollen tubes entering the ovary to accomplish fertilization. **(A-F)** Pollen tubes in the base of L25 styles visualized by aniline blue staining after pollination with S_{12} pollen from plant 2548. **(A)** A pollination resembling a compatible cross. **(B)** A pollination resembling a fully incompatible cross. **(C, D)** Pollination with a few pollen tubes reaching the ovary. The inset in C has been magnified in D to show both an arrested pollen tube at the ovarian entrance (upper arrow) and a pollen tube entering an ovule (lower arrow). Also note the pollen tubes arrested earlier at the top of the picture, which represents the lower third of the style. **(E, F)** Stylar observation of an incompatible pollination five days post-pollination. The inset in E has been magnified in F to clearly show ten pollen tubes entering the ovarian region. Bars represent 0.5 mm.


A
B
C
D
E
F

IV.Table 1

Comparison of S_{12}-RNase levels and S_{12} pollen rejection phenotype

	Plant line L25			Plant line 314		
	Average ng S_{12}-RNase[1]	No.Styles ≤ 80 ng S_{12}	No.fruits/ No. flowers	Average ng S_{12}-RNase[2]	No. Styles ≤ 80 ng S_{12}	No. fruits / No. flowers
Anthesis	86 ± 55	17/30 (57%)	44/80 (55%)	136 ± 96	14/44 (32%)	25/98 (25%)
1 DBA	58 ± 37	4/6 (66%)	7/14 (50%)	66 ± 10	5/6 (83%)	19/24 (79%)
2 DBA	9 ± 4	6/6 (100%)	16/17 (94%)	26 ± 16	6/6 (100%)	11/12 (92%)
3 DBA	0.4 ± 0.3	6/6 (100%)	8/11 (73%)	16 ± 7	6/6 (100%)	7/10 (70%)
Anthesis[3]	37 ± 18	13/13 (100%)	31/33 (94%)			

[1] Data is the average ± SD of the points in Fig. 5 G.

[2] Data is the average ± SD of the points in Fig. 6 E.

[3] Data is the average ± SD of the points in Fig. 5 B.

IV.Table 2

Threshold S_{12}-RNase levels depend on pollen genetic background

Staminate Parent	Pollen Haplotype	No. fruits / No. flowers[1]	Est. S_{12}RNase Threshold[2]	Pollen Stainability
TP48	S_{12}	32/39 (82 %)	130 ng	>90%
2548[3]	S_{12}	44/80 (55 %)	80 ng	>90%
VF60	S_{12}	10/50 (20%)	35 ng	>90%
L25	S_{11} and S_{12}	5/68 (7%)	25 ng	>90%

[1] All crosses used L25 line as pistillate parent.

[2] Determined from the S_{12}-RNase levels in Fig. 5 G.

[3] Data as in Table 1.

Chapter V. The role of glycosylation in pollen recognition of S-RNases in *Solanum chacoense*

Objectives

1) **To investigate the role of hypervariable domain HVa in pollen recognition.**

2) **To assess the role of the conserved N-glycosylation site in the C2 region of the S_{11}-RNase.**

Publication of chapter V: Article in preparation

I did most of the experimental procedures, except some crosses and western blots (J.Soulard) and the production of transgenic plants (G.Laublin). Dr Morse helped with the interpretation of the results and discussions.

The role of glycosylation in pollen recognition of S-RNases in *Solanum chacoense*

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Key words: Gametophytic self-incompatibility, *Solanum chacoense,* S-RNase, site-directed mutagenesis, allelic recognition, glycosylation,

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Abstract

The stylar determinant of gametophytic self-incompatibility (GSI) in Solanaceae, Rosaceae and Scrophulariaceae stylar is an S-RNase encoded by a multiallelic S-locus. The primary structure of S-RNases shows five conserved (C) and two hypervariable (HV) regions, the latter forming a domain responsible for the S-haplotype-specific recognition of the pollen determinant to SI. All S-RNases in the Solanaceae are glycosylated at a conserved site present in the C2 region, but previous studies have shown that N-linked glycans at this position are not required for S-haplotype-specific recognition leading to pollen rejection. We report here the incompatibility phenotype of constructs derived from the monoglycosylated S_{11}-RNase that were designed to explore in a more generally way the role of the hypervariable domain in determining pollen recognition and the role of the N-linked glycan in the C2 region. In one series of experiments, a second glycosylation site was introduced in the HV region to test for inhibition of pollen specific recognition. This modification does not impede pollen rejection, probably because of incomplete glycosylation at the new site in the HV region. A second construct was designed to permit complete glycosylation at the HV site by suppression of the site in the C2 region. This construct was also able to reject S_{11} pollen. Surprisingly, plants expressing this construct also acquired the ability to reject all different types of pollen, including the otherwise fully compatible diploid heteroallelic pollen. We deduce from this phenotype that the conserved glycosylation site in C2 region plays a pivotal role in SI. We propose that this glycan moiety represents a recognition domain common to all S-RNases that allows pollen to indiscriminately degrade S-RNases not specifically identified as self.

Introduction

Self-incompatibility (SI) is a mechanism widespread among flowering plant species that promotes outbreeding by allowing the pistil of a flower to discriminate between genetically related (self) and unrelated (nonself) pollen (de Nettancourt, 2001). In Solanaceae, Rosaceae and Scrophulariaceae, SI is of the gametophytic type, i.e. the phenotype of the pollen is determined by its haploid genotype and is controlled by a single multigenic S-locus, inherited as a single segregating unit, which includes the highly polymorphic male and female determinants to SI. Variants of the S-locus have been termed S-haplotypes, whereas variants of the polymorphic genes of the S-locus are called alleles (McCubbin and Kao, 2000). In the families mentioned above, the pistil-expressed S-gene product is a glycoprotein with RNase activity termed S-RNase (McClure et al., 1989). S-RNases have a pattern of highly conserved and highly variable regions in the primary sequence whose functions are now reasonably clear. For example, the hypervariable regions are involved in allelic recognition (Matton et al., 1997; Matton et al., 1999) while the constant regions C2 and C3 each contain a histidine residue essential for RNase activity (Green, 1994). The remaining conserved regions (C1, C4 and C5) are now thought to be involved in stabilizing the three dimensional structure of the S-RNase (Ioerger et al., 1991; Qin et al., 2005).

S-RNases must exert their cytotoxic activity inside the incompatible pollen tubes for pollen rejection to occur. Curiously, both self and nonself S-RNases have been shown to enter pollen tubes (Luu et al., 2000), so at least part of the SI mechanism must involve the ability of pollen tubes to block the RNase activity of any nonself S-RNase. Thus, both the indiscriminate entry of S-RNases into pollen tubes and the inhibition of RNase activity for all but the self RNases imply recognition of a region common to all S-RNases (Kao and Tsukamoto, 2004b). To date, however, none of the conserved regions appears able to play this common recognition role, with even the most

attractive possibility (the conserved C4 region with its three charged amino acids) apparently uninvolved (Qin et al., 2005).

The S-locus product expressed in pollen has been recently identified as an F-box protein, termed variously *S*-locus F-box gene (SLF) or *S*-haplotype-specific F-box gene (SFB) by different authors (Sijacic et al., 2004). The role of the F-box sequence motif is to mediate protein-protein interactions. It was first described as the component in SCF ubiquitine ligase (E3) complexes that is directly responsible for binding with selected protein substrates, and thus plays an important role in determining the specificity of protein degradation by the 26S proteasome (Bai et al., 1996). However, F-box proteins are also known to be involved in formation of the kinetochore, transcriptional elongation, spermatogenesis and cyclin F binding to cyclin B (Kipreos and Pagano, 2000). In spite of the fact that both male and female determinants to SI have been identified, how these molecules interact, and more specifically, how incompatible pollen is inhibited in S-haplotype-specific manner remains unclear. According to recent models, SLF/ SFB interacts with both self and nonself S-RNases inside the pollen tubes. The maintenance of self S-RNase enzyme activity is proposed to occur via S-haplotype-specific interactions between their hypervariable regions and those of their cognate SLF/ SFB partners. In contrast, all nonself S-RNases are targeted for ubiquitination and subsequent degradation (Ushijima et al., 2004; Qiao et al., 2004a), which in turn implies that ubiquitination occurs at a conserved region of the S-RNases. The conserved regions C3 and C4 do indeed contain one conserved lysine residue each, although a role in degradation for the conserved lysine in the C4 region of *Solanum chacoense* has recently been ruled out (Qin et al., 2005).

S-RNases are secreted proteins in the styles, and thus enter the secretory pathway by co-translational translocation across the endoplasmic reticulum (ER), where they assume their secondary structure and undergo post-translational modifications. Failure to assume their correct three-

dimensional conformation results in their retention in the ER, followed by degradation mediated by the ubiquitin-proteasome pathway (Nivedita Mitra, 2006). During passage through the ER and the Golgi, secreted proteins can be modified by asparagine-linked glycosylation, a common mechanism for expanding the diversity of the proteome in eukaryotes (Weerapana and Imperiali, 2006). Indeed, glycosylation has been implicated in many cellular processes, including the immune response, host-cell recognition by pathogens and symbiotic associations, intracellular targeting, intercellular recognition and proper protein folding and stability (Nivedita Mitra, 2006). Interestingly, the discovery of new members of the E3 ubiquitin ligase family that specifically recognize proteins attached to N-linked high-mannose oligosaccharides indicates that glycosylation constitutes an additional targeting signal for endoplasmic reticulum-associated degradation (ERAD) pathway (Yoshida et al., 2002; Yoshida Y, 2003). In mice, a specific F-box protein in the SCF^{Fbx2} complex is responsible for recognition of N-glycosylated proteins that have been translocated from the ER to the cytosol for degradation by the ERAD protein quality control system.

The function of glycosylation in plant proteins remains poorly studied, and in particular, the role of some glycoproteins apparently involved in SI is not clear. For example, in *Nicotiana alata,* immunological and biochemical studies have shown that three high molecular weight glycoproteins are expressed in the stylar extracellular matrix (ECM), interact individually with pollen tubes and form a complex with S–RNases, at least *in vitro* (Cruz-Garcia et al., 2005). These glycoproteins include a transmitting-tract-specific protein (NaTTS) deglycosylated by pollen tubes (Wu et al., 1995), a 120kDa protein taken up by growing pollen tubes (Cheung AY, 1995), and a pistil extensin-like protein III (NaPELPIII) that localizes in the pollen tube callose wall and plugs (de Graaf et al., 2003). More importantly, the S-RNases themselves are glycosylated with a variable numbers of potential N-glycosylation sites. For example, S-RNases from *N. alata* contain from one to

five sites (Oxley et al., 1998) while those from *S. chacoense* contain from one to four sites (Qin et al., 2001). The one site conserved in all cases is located in the conserved C2 region in the Solanaceae (Singh and Kao, 1992), whereas in the Rosaceae it is located in the conserved RC4 region (Ishimizu et al., 1998). The biological function of these conserved glycosylation sites is presently unknown, although the enzymatic removal of the glycan side chains has apparently no effect on RNase activity of the native S-RNases (W.Broothaerts et al 1991). Furthermore, when the C2-glycosylation site of the *Petunia hybrida* S_3-RNase was eliminated by site directed mutagenesis, the mutated S_3-RNase retained full RNase activity and was able to completely reject S3 pollen (Karunanandaa et al., 1994). Unfortunately, in these experiments the rejection of other pollen types was not investigated, and therefore, as pointed out by some authors (Oxley et al., 1996), it was formally possible that these transgenic plants could have acquired the ability to reject any pollen type. If this were the case, it would have demonstrated that N-glycosylation is indeed required for the operation of SI. Interestingly, S-like RNases that are not involved in SI are not glycosylated (Green, 1994).

As a complement to the observation that Solanaceous S-RNases contain a conserved glycosylation site, it has been reported that self-compatible *N. sylvestris* expresses a stylar "relic S-RNase" with a new N-glycosylation site inside the hypervariable HVa region. Intriguingly, while levels of S-RNase activity are comparable to those found in self-incompatible *Nicotiana* species (Golz et al., 1998) this particular S-RNase is unable to function in SI. The authors proposed that presence of a bulky polysaccharide moiety to the hypervariable region might prevent the pollen S-gene product from interacting with the hypervariable domains of the S-RNase thus leading to compatibility.

To explore this potential mechanism of steric inhibition of pollen recognition in *S. chacoense*, we added an N-glycosylation site to the HVa region of the S_{11}-RNase, which contains only a single glycosylation site in the

C2 region (Saba-El-Leil et al., 1994). This modification had no effect on pollen recognition, although molecular analyses indicated that glycosylation at the new site was incomplete. We thus also generated an S-RNase where the normal N-glycosylation site in the C2 region was eliminated leaving only the site in the HVa region. Among the transgenic plants harboring this second construct, one was found to reject several different pollen haplotypes. We propose that the conserved glycosylation site in the C2 region constitutes the domain common to all S-RNases that allows non-S-haplotype-specific binding of S-RNases with SLF/SFB and leads to nonself S-RNases degradation. Removal of this site inhibits SLF-SFB binding, thus leaving the nonself S-RNases active and able to reject pollen.

Materials and methods

Plant material

The self-incompatible diploid genotypes (2n=2x=24) of *Solanum chacoense* used in this study include the parental line PI 230582 (called 582), obtained from the Potato Introduction Station at Sturgeon Bay, WI, USA, that carries the S_{13} and S_{14} alleles, plants L25 ($S_{11}S_{12}$) and G4 (S_{12} S_{14}), already described (Matton et al., 1997; Qin et al., 2001) as well as two $S_{12}S_{12}S_{14}S_{14}$ tetraploids.

Genetic crosses of transgenic plants were done using pollen freshly collected from various genotypes and performed as described before (Qin et al., 2001). Where appropriate, styles were stained with aniline blue 48 hours post pollination and examined by fluorescence microscopy to follow pollen tube growth (Matton et al., 1997).

Mutagenesis of the S_{11}-RNase *glycosylation site*

A previous construct (Qin et al., 2005) containing the wild type S_{11}-RNase (here called **S_{11}-Gly^{C2}**) to indicate that it contains only the conserved N-glycosylation site located in the C2 conserved region) under control of the chitinase promoter (Harikrishna et al., 1996) in pBluescript was used as a PCR template for site directed mutagenesis. To create **S_{11}-Gly$^{C2/HVa}$**, with an additional N-glycosylation site added to the protein in the hypervariable HVa region, the lysine (AAA) in the sequence KLTYNFSD was changed into asparagine (Franklin-Tong et al.) using primers **S_{11}-Gly^{C2}A** (5'-AAGTACTGCAAGCCAATCTTACCTATAACTAT-3') and **S_{11}-Gly^{C2}B** (5'-ATAGTTATAGGTAAGATTGGCTTGCAGTACTT-3') and a PCR mutagenesis kit (Stratagene). To create S_{11}-Gly^{HVa}, the amino acid asparagine (AAC) in the endogenous glycosylation site bordering the C2 region was replaced with glutamine (CAA) using two primer sets, first forward-HindIII 5'-(5'-GCGGCGCGTTCAAGCTTTCTAGAAGATCTCT-3') and **S_{11}-Gly$^{C2/HVa}$B** (5'-CCGTGAATCGTAAATTGTTTTGGAACTATTCG-3'), then **S_{11}-Gly$^{C2/HVa}$A** (5'-CGAATAGTTCCA AAACAATTTACGATTCACGG-3') and reverse-EcoR1stop (5'-CTCTGAATTCAAGGACAT ACATTTGATAG-3'). Purified PCR products from the two reactions were mixed and used as template for amplifying a full-length fragment consisting of mutated S_{11} using primers reverse-EcoR1 stop and forward-HindIII. The PCR was performed by annealing at 54 °C for 40 sec, then extending at 70 °C for 3 min (Roche DNA polymerase, Buffer 1X).

All mutated CHIP-S_{11}-RNases were cloned into the transformation vector pBIN19 (Clontech, Palo Alto, CA, USA) and sequenced before and after transformation of *Agrobacterium tumefaciens* LBA4404. Plants were transformed by the leaf disc method, as described previously (Matton et al., 1997), using *S. chacoense* genotype G4 carrying the $S_{12}S_{14}$ alleles. Genetic crosses, using freshly collected pollen from various genotypes, were performed as described before (Qin et al., 2005).

Deglycosylation of S-RNase

Proteins were extracted from both transgenic and non-transgenic plants as described (Qin et al., 2001; Qin et al., 2005). Crude extracts containing 5 µg protein were digested with peptide-N-glycosidase F (PNGase F, Boehringer-Mannheim) according to the manufacturer's instructions in order to remove N-glycan side chains before Western blot analysis.

Western and Northern blot analyses

Stylar proteins were extracted from transgenic plants as described (Qin et al., 2005) and crude proteins from five styles of each plant were analyzed by SDS-PAGE. Western blots were performed as described before (Qin et al., 2005) with a specific antibody raised against a bacterially produced S_{11}-RNase with a 1:1000 dilution and the position of antibody-binding detected with [I^{125}]-protein A. The membranes were incubated with either the first antibody or [I^{125}]-protein A for 2 hours at room temperature. The membranes were rinsed 3 times with TBS-T for 5 minutes after each incubation. The membranes were exposed for 48 hours to a PhosphorImager screen and scanned on a Typhoon 9200 (GE Healthcare). Northern blots were performed with a probe synthesized by PCR using a template from S_{11} genomic DNA.

Results

Styles of **S_{11}-Gly$^{C2/HVa}$** transgenic plants were screened by Western blots to detect expression of the transgene. **S_{11}-Gly$^{C2/HVa}$** expression was detected in the pistils of ten transgenic plants from the 29 plant lines tested, with levels varying from much less to even more than the levels observed with

untransformed L25 plants containing a wild type S_{11}-RNase (Fig. V.1A). To determine the impact of these variable levels of **S_{11}-Gly$^{C2/HVa}$** on the incompatibility phenotype, the plants were crossed with S_{11} pollen (Table V.1). The results indicated that transgenic plants with lower expression levels (lines 16, 17, 31, 35) had an incomplete S_{11}-rejection phenotype, whereas plants with higher expression levels (lines 5,13 26, 27, 29) were found to completely reject S_{11} pollen. Rejection of S_{11} pollen was somewhat surprising, as the S-RNase transgene used for transformation had been engineered to contain a glycosylation site in the HVa allelic recognition domain. To determine if the site was fully occupied, we analyzed the stylar extracts both before and after treatment with PNGase. This analysis revealed that before treatment, two size classes of protein reacting with the anti-S_{11} antibody could be detected. One size class was more abundant and had the same apparent molecular weight as the authentic S_{11}-RNase (26 kDa), and we interpret this band as a monoglycosylated form of the protein. The second had a slightly slower mobility (28 kDa) and we interpret this as a diglycosylated form of the protein. This interpretation is supported by the observation that following treatment of the proteins in the crude extract with PNGase only a single size class of protein with an apparent molecular weight of 24 kDa (Fig. V.1B) was observed. Thus, styles from these plants do not consistently glycosylate both sites in the **S_{11}-Gly$^{C2/HVa}$**-RNase, and the incompatibility phenotype could thus result from an **S_{11}-Gly$^{C2/HVa}$**-RNase glycosylated only in the C2 region as is the wild type S_{11}-RNase.

The inability of the plant to consistently glycosylate both sites in the **S_{11}-Gly$^{C2/HVa}$** may have resulted from the proximity of the two sites. We therefore constructed an additional mutated form of the S_{11}-RNase, termed **S_{11}-Gly^{HVa}**, by removing the endogenous glycosylation site in C2 while leaving intact the newly added glycosylation site in the hypervariable region. Among 100 transgenic plants screened, all except two completely accepted S_{11} pollen. One individual (line 3-16) almost completely rejected S_{11} pollen.

More importantly, this individual also rejected the normally compatible S_{13} pollen as well as diploid heteroallelic pollen from tetraploid plants of allelic constitution $S_{12}S_{12}S_{14}S_{14}$ (Table V.2). Pollen tubes in these styles were seen to display the typical reaction characteristic of an SI response when examined by fluorescence microscopy (Fig. V.2). Most of the pollen tubes were arrested at mid-style, although in rare instances a small number of pollen tubes were found at the stylar basis. Overall, only 10 fruits were obtained in 102 crosses, and these fruits were small (less than 0.8 cm in diameter) and contained between 12 and 20 seeds, many of which were underdeveloped or aborted. The fruits obtained in control pollinations were larger (more than 2.0 cm in diameter) and contained well over 100 seeds. A second individual (line 2-13) was also found to reject both S_{11} and S_{13} pollen, but to a lesser extent. We conclude from the unusual phenotype exhibited by these two plants that the glycan normally located in the C2 region is important for the normal compatibility behavior of pollen.

The level of transgene RNA was low in all transformed styles with the exception of line 3-16 as determined by Northern blot analysis (Fig. V.3A). This low frequency of transgene expression was confirmed by western blots indicating that expression levels of **S_{11}-Gly^{HVa}** in most plants were quite low, except in lines 3-16 and 2-13 (Fig. V.3B). The average level of **S_{11}-Gly^{HVa}** in individual styles of line 3-16 ranged from 203 ng to 644 ng per style (Fig. V.4), whereas L25 styles had an average of 850 ng S_{11}-RNase in agreement with previous measurements (Qin et al., 2006). Levels of **S_{11}-Gly^{HVa}** in plant 2-13 were always lower than 200 ng per each style (not shown). We conclude from this molecular analysis that the degree of pollen rejection is strongly correlated with the level of **S_{11}-Gly^{HVa}** in the styles, indicating that the unusual incompatibility phenotype does indeed result from transgene expression.

Discussion

The crosses with **S_{11}-Gly$^{C2/HVa}$** plants revealed that, as for an authentic S_{11} transgene or other S_{11}-derived constructs (Matton et al., 1997; Matton et al., 1999; Qin et al., 2005), high levels of transgene expression were correlated with full rejection of S_{11} pollen (Table 1, Fig. V.1A). Based on the report of a stylar "relic S-RNase" in *N. sylvestris* (Golz et al., 1998), we had originally anticipated that glycosylation in the middle of the HV region might block pollen recognition, thus leading to compatibility even in styles with high levels of transgene expression. Surprisingly, this was not the case, as five individuals turned out to completely reject S_{11} pollen. However, our western blot data revealed that glycosylation in HVa region was not complete, and that **S_{11}-Gly$^{C2/HVa}$** plants actually express S-RNases with two different apparent molecular weights (Fig. V.1A). The most likely interpretation of this is that the two size classes represent mono- and di-glycosylated forms of the enzyme, with the mono-glycosylated form being more abundant. We propose that the incompatibility phenotype of transgenic **S_{11}-Gly$^{C2/HVa}$** plants results from accumulation of a mono-glycosylated **S_{11}-Gly^{C2}**-RNase indistinguishable from wild type S_{11}-RNase. It is formally possible that the lower band that appears in the westerns might in fact be constituted of a mixture of **S_{11}-Gly^{C2}** and **S_{11}-Gly^{HVa}** mono-glycosylated forms. However, this seems unlikely, given that the wild type S_{11}-RNase is completely glycosylated at the C2 site and that the HV glycosylation site will only become available to the glycosylation machinery after the C2 region has entered the ER.

To overcome the difficulty of ensuring complete glycosylation in the HV region, we prepared **S_{11}-Gly^{HVa}**-RNase whose single glycosylation site was found not in the C2 region but in the HV domain. We anticipated that allelic recognition of this construct would be impossible, thus ensuring complete compatibility of S_{11} pollen. It was thus very surprising to find among the transformed population two lines with an ability to reject S_{11} pollen. The

molecular analysis of these two plants demonstrated a level of transgene expression commensurate with the phenotype, indicating that the phenotype was in fact due to the transgenic S-RNase. Furthermore, the protein is fully glycosylated, as only a single band can be observed by gel analysis, and the size of the band is reduced by treatment with the deglycosylating PNGase.

While it is tempting to conclude that the presence of a bulky glycan in the HV region does not block the recognition domain of the pollen, subsequent experiments suggested a different interpretation. In particular, styles expressing the **S_{11}-Gly^{HVa}**-RNase also reject pollen that would be normally compatible, such as S_{13} pollen or the normally completely compatible heteroallelic pollen obtained from diploid plants. These surprising results indicate that the glycan moiety in the C2 region is a key element involved in recognition of non-self S-RNases. Based on these observations, we propose that recognition of S-RNases via this glycan recognition domain is an essential element for degradation. Compatible pollinations exploit recognition of the sugar moiety in order to target the S-RNase to the proteasome. During incompatible pollinations, interaction between the C2-glycan and the proteasomal targeting system is blocked by high affinity and allele-specific binding to the recognition domain in the HV region. Our modified **S_{11}-Gly^{HVa}**-RNase, which does not contain a glycan moiety in the C2 region, is thus functionally equivalent to an S-RNase where binding to the glycan is impeded by a pollen factor binding to the HV recognition domain.

The conserved glycosylation site in the C2 region of Solanaceous S-RNases has been previously noted (Karunanandaa et al., 1994), and has led to the suggestion that the C2-glycan may play a functional role in SI. Interestingly, a test of this hypothesis in transgenic *P. inflata* used an S-RNase completely lacking any glycan group. When these plants were crossed with self pollen, the rejection phenotype was normal, and it was concluded that the C2 glycan is not required for pollen rejection (Karunanandaa et al., 1994). However, in these studies, control experiments

documenting passage of fully compatible pollen through styles was not reported. It would be of great interest to challenge these plants with fully compatible pollen to determine if our results can be reproduced in Petunia. We note that our results do not conflict with the conclusion that the conserved glycan is not involved in allele specific pollen recognition in Petunia.

It is important to stress that the unusual rejection phenotype of the **S_{11}-Gly^{HVa}** plants that we observe here is not due to overexpression of the transgene, as the S-RNase levels measured in the styles are lower than those found for wild type plants (Fig. V.2B). Furthermore, it is unlikely to result from fortuitous insertion of the transgene in a component of the SI machinery required for allowing pollen to resist the effects of elevated S-RNase activity, as the two transgenic lines with this same phenotype represent independent insertion events (data not shown). Lastly, the phenotype can not be due to female sterility, as some fruits are set by both lines 3-16 and 2-13, and the degree of compatibility shown by the plants is in complete agreement with the levels of transgene expression measured. Clearly then, the unusual phenotype must be directly related to the absence of the C2 glycan.

The general role of glycosylation in protein function is still not clear, although in some cases it may be important for the correct folding and thus the final function of the protein (Mitra et al., 2006). It has been proposed that proteins displaying an exposed hydrophobic surface during the folding process may aggregate, and the presence of glycosylation may protect against aggregation by increasing their soilubility (Mitra et al., 2006). In other cases, removal of the glycan from the folded protein has little effect on enzyme activity but can seriously alter its stability. For example, when the three N-linked glycosylation sites of the cationic peanut peroxidase (cPrx) were removed one by one, the stability and activity of cPrx was affected to different extents (Lige et al., 2001).

The current models for GSI are based on the recent identification of the male determinant to S-RNase-based GSI in Scrophulariaceae (Lai et al.,

2002), Rosaceae (Entani et al., 2003; Ushijima et al., 2003; Yamane et al., 2003b), and Solanaceae (Sijacic et al., 2004) as an F-box protein termed either SLF or SFB. The models predict that SLF/SFB interacts with both self and non-self S-RNases (Ushijima et al., 2004; Qiao et al., 2004a), as S-RNases penetrate into pollen tubes in S-haplotype-independent manner (Luu et al., 2000). Once inside the pollen tubes, SLF/SFB acts as a component to an E3 ubiquitin ligase targeting the non-self S-RNases to the proteasome pathway. However, while allele specific recognition of self pollen occurs via the HV domain, the domain responsible for recognition of non-self S-RNases has not been previously identified. Clearly, this property must reside in a feature common to all S- RNases. We propose that this feature is constituted by the glycan moiety linked to the protein at the conserved C2 glycosylation site. Interestingly, an E3 ubiquitin ligase that uses recognition of N-linked high-mannose oligosaccharides to target a glycoprotein in mice neuronal cells for degradation by the proteasome has previously been reported (Yoshida et al., 2002). It is thus entirely plausible that a pollen F-box may target the S-RNase to the proteasome by binding the N-glycan moiety. It is also interesting to note that in *Antirrhinum*, the SLF-S_2 was found to physically interact with all of the S-RNases in S-haplotype-independent manner (Qiao et al., 2004a).

In conclusion, our results suggest a novel mechanism by which pollen tubes distinguish self and non-self S-RNase during incompatibility reaction. In particular, they suggest that the sugar moiety attached at the conserved glycosylation site in the C2 region of the S-RNases may represent the feature common to all S-RNases that would play a pivotal role in signaling the degradation of all non-self S-RNases.

Acknowledgments

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Legends

Table V.1. Compatibility phenotype of S_{11}-Gly$^{C2/HVa}$ transgenic plants with S11 pollen[1]

Pistil genotype $S_{12}S_{14}$	Transgenic plant phenotype		
	Compatible	Partially compatible	Incompatible
Number of plants	19	5	5
No. fruits / flowers pollinated	73/75	23/43	0/53

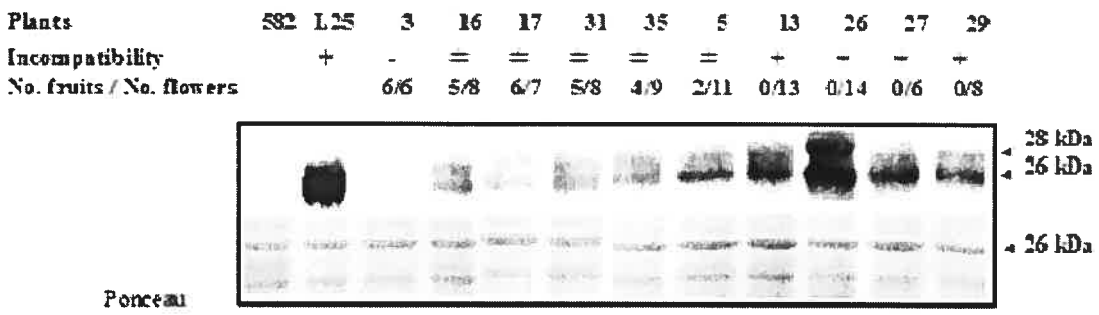
[1] Pollen from $S_{11}S_{12}$ plants

Table V.2. Compatibility phenotype of S_{11}-Gly^{HVa} transgenic plant

Male genotype	Number of fruits/Number of flowers pollinated	
	3-16	**Untransformed Host**
$S_{11}S_{12}$ (L25)	2/29	19/19
$S_{13}S_{14}$ (582)	5/39	23/23
$S_{12}S_{12}S_{14}S_{14}$	3/34	35/35

Fig.V.1. Compatibility phenotype and glycosylation pattern of **S_{11}-Gly$^{C2/HVa}$** transgenic plants. **A.** Western blot analysis of 20 µl of crude protein from 5 styles taken from genotypes 582 ($S_{13}S_{14}$), L25 ($S_{11}S_{12}$) and a selection of different transgenic lines in an $S_{12}S_{14}$ host background. Anti-S_{11} specific antibody detects two bands in the transformed lines but only a single band in the wild type L25. The compatibility phenotype based on number of fruits obtained per number of flowers pollinated is summarized as (-) for a fully compatible phenotype, (±) for a partially incompatible phenotype, and (+) for a fully incompatible phenotype. 28 kDa (**S_{11}-Gly$^{C2/HVa}$**) and 26 kDa (**S_{11}-Gly^{C2}**) designate mutant di-glycosylated and authentic S_{11}-RNase, respectively. Crosses were made with S_{11} pollen. **B.**Same genotypes as Above after deglycosylation with PNGase.

A)


Plants 582 L25 3 16 17 31 35 5 13 26 27 29
Incompatibility + - = = = = = + - - +
No. fruits / No. flowers 6/6 5/8 6/7 5/8 4/9 2/11 0/13 0/14 0/6 0/8
28 kDa
26 kDa
26 kDa
Ponceau

B)

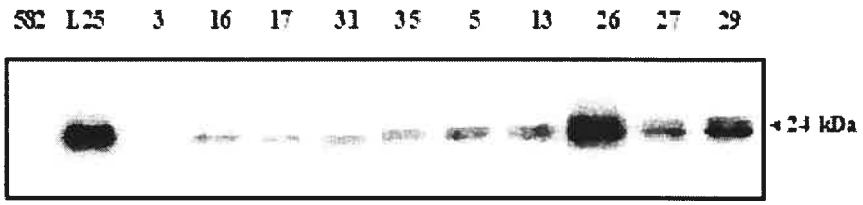

582 L25 3 16 17 31 35 5 13 26 27 29
24 kDa

Figure V.2 Rejection of incompatible pollen tubes at the style of plant (3-16) by construct **S_{11}-Gly^{HVa}** observed by means of fluorescence. Note that pollen tubes display the typical reaction charateristic of an SI response. Bar represents 0.2 mm.

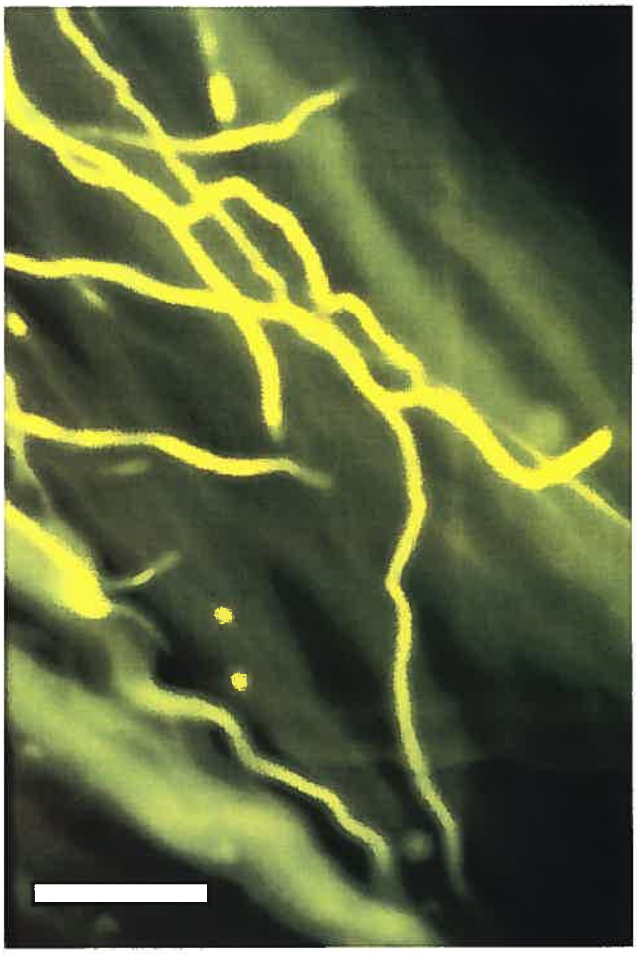
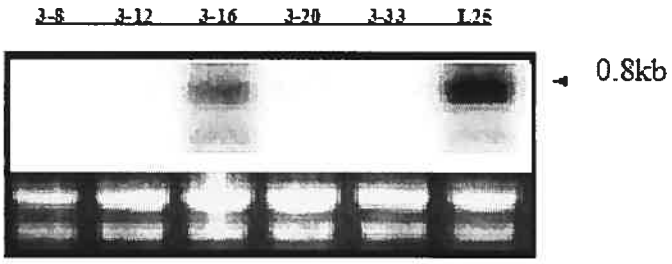


Fig.V.3. Analysis of **S_{11}-Gly^{HVa}** transgene expression (A) Northern blot analysis of RNA extracted from pistils of selected **S_{11}-Gly^{HVa}** transgenic plants. Probe was synthesized from plant genomic DNA by PCR using S_{11} specific primers. (B) Western blots analysis of protein extracted from pistils of selected **S_{11}-Gly^{HVa}** transgenic plants using an anti-S_{11}-RNase.

A)


3-8
3-12
3-16
3-20
3-33
I.25
0.8kb

B)

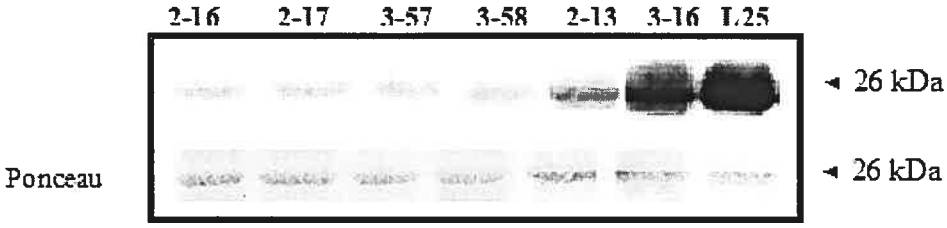
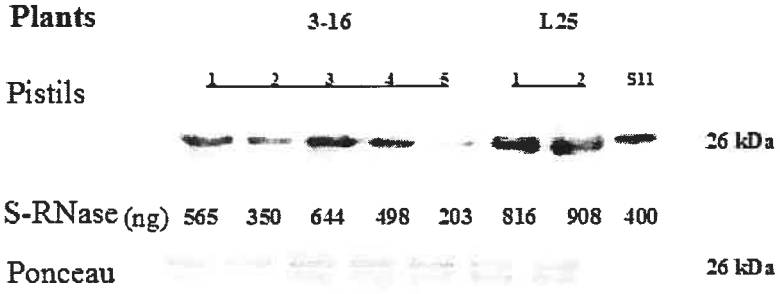

2-16
2-17
3-57
3-58
2-13
3-16
I.25
26 kDa
Ponceau
26 kDa

Fig.V.4. Analysis of **S_{11}-Gly^{HVa}** levels in single styles. Five single pistils of plant 3-16 and two wild type L25 were analyzed by western blot with an anti-S_{11} antibody. The lane at right (S_{11}) contains four hundred µg of an authentic S_{11}-RNase standard. Ponceau staining of the membrane (lower panel) was performed as a control for protein load.


Plants
3-16
L25
Pistils
1 2 3 4 5 1 2 511
26 kDa
S-RNase (ng) 565 350 644 498 203 816 908 400
Ponceau
26 kDa

Chapter VI. Discussion and Perspectives

VI. 1 Discussion

Like animals, most plants avoid mating with close relatives in order to prevent inbreeding depression and to maintain hybrid vigor. How plants achieve that particular goal has long been a mystery in science. For plants, some of the critical mating choices are made through a genetic system called self-incompatibility (SI), which is achieved by an intimate interaction between the pollen (the male) and the pistil (the female part of a flower). As a result, self- (or genetically related) pollen is rejected, whereas non-self pollen is accepted for fertilization. Gametophytic S-RNase-mediated SI (GSI) is the most common type of SI found in flowering plants. Genetic and molecular studies have shown that S-RNase-based GSI is a two-gene system, where the S-RNase constitutes the female determinant and SLF/SFB constitutes the male determinant to SI (Kao and Tsukamoto, 2004b; Takayama and Isogai, 2005). The mechanism of S-haplotype-specific inhibition of pollen tube growth, however, is still not fully understood. In particular, the role of SLF/SFB is unclear, as is the role played by the other F box proteins encoded at S-locus or that of the modifier proteins that are required for full manifestation of the SI response. Although the *S-RNase* gene has been isolated and studied since the early1990s, a number of features remained unknown for a long time, and have been clarified by our work. Although our studies have shed more light on some aspects of the structure/function of S-RNases, they have also raised additional questions. Thus, for example, we have shown that HAP compatibility is not simply due to lack of expression of pollen S-gene. But then, what is the biochemical basis of the "competition effect"? How S-RNases penetrate inside pollen tubes remains unknown, but it is clear from our studies that this process is not mediated by their C4 region. We have shown in the same study that the lysine in C4 is not

the target for ubiquitination: what about the conserved lysine in C3? Our studies show that removing the sugar groups of the S-RNase from the conserved C2 site and moving them into the HV regions generates an unexpected phenotype. Would an S-RNase lacking the carbohydrate moiety reject any type of pollen? Finally, we have developed a method for S-RNase quantification in single styles that other researchers may find helpful. From the evolutionary point of view, the most intriguing questions concern how the specificity *S*-genes expressed in style and pollen coevolve to maintain their functions. Answering these and other questions should not only lead to a greater understanding of how flowering plants discriminate between self and non-self pollen, intra- and inter-specific pollinations, but should also shed more light on the processes used by plants for cell-cell communication.

VI.2 Perspectives: practical applications of SI in agriculture

From a practical point of view, understanding the various and complex events that characterize pollen recognition and its possible rejection, could be transformed into practical applications to improve agricultural economy. In other words, the improved knowledge on plant reproduction opens the possibility of using SI for applications in agriculture by changing the breeding behavior in both SC and SI crop species. Thus, for example, one of the most important potential utilization of self-incompatibility is related to hybrid seed production in self-compatible crop plants (such as corn, wheat and soy bean). Hybrid seed is obtained by crossing two parents with different genetic backgrounds. In many species, the progeny (or at least part of it) produced after such a cross usually shows a substantial gain in hybrid vigor and therefore produces a higher yield. However, hybrid seed production requires manual removal of anthers from female plants to prevent self-fertilization, and this is a very time-consuming and costly process. Since most cultivated crops are self-compatible, if self-incompatibility could be transferred into such crops,

it would facilitate hybrid seed production at lower cost, because all the seeds produced from the resulting self-incompatible crop plants would be hybrid. Although this approach has not yet been applied to S-RNase-based GSI, it has been successfully attempted in SC species using elements of the sporophytic SI system. Thus, the transfer of two S-locus genes (the stigma-expressed receptor kinase SRK, constituting the female determinant to SI, and its cognate pollen-expressed ligand SCR, constituting the male determinant to SI) from the SI species *A. lyrata* into SC *Arabidopsis thaliana* has produced self-incompatible transgenic plants (Nasrallah et al., 2002).

On the other hand, the improved knowledge on self-incompatibility should enable scientists to disrupt SI in fruit trees (such as apple, pear, cherry, olive) to increase fruit yield. Because of the widespread existence of SI in fruit trees, farmers usually have to interplant two different varieties in order to achieve outcrosses and subsequent fruit production. Since a substantial part of the orchard area is used for growing a variety that serves only as a pollen provider, this interplanting practice represents a rather inefficient way in the use of land resources. Generation of self-compatible fruit trees by disrupting SI would eliminate the necessity of interplanting two different varieties and would thus increase reliable fruit production and allow a more efficient use of land. In this regard, the successful acquisition of SC by an SI line of *Petunia hybrida* after the transfer of the SLF male determinant from *Anthirrhinum* appears particularly encouraging (Qiao et al., 2004b).

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