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Molecular characterization of S-locus expression in *Petunia hybrida*

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The Ohio State University, 1992
MOLECULAR CHARACTERIZATION OF
S-LOCUS EXPRESSION IN Petunia hybrida

DISSERTATION

Presented in Partial Fulfillment of the requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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The Ohio State University
1992

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To my mother Patricia and my wife Kathleen
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1.1 Introduction

Self-incompatibility is a genetic barrier to inbreeding found in more than 250 genera and at least 71 families of flowering plants, comprising over half the species of angiosperms (East, 1940, Brewbaker, 1959). Research on self-incompatibility had an auspicious beginning when Charles Darwin, working with petunia, observed in 1876 that “protected flowers, which had their own pollen placed in the stigmas, never yielded nearly a full complement of seeds; whilst those left uncovered produced fine capsules, showing that pollen from other plants must have been brought to them”. Extending the observations of Darwin, the phenomenon of self-incompatibility can be more precisely defined as the inability of a fertile hermaphrodite seed-plant to produce zygotes after self-fertilization. Self-incompatibility acts as a pre-zygotic obstacle to self-fertilization, and should not be confused with fertilization barriers operating post-zygotically; such as embryonic lethalties or interspecific embryo abortion.

1.1.1 Self-Incompatibility and Angiosperm Evolution

The evolutionary success of flowering plants is attributable in large part to genetic mechanisms that efficiently promote outbreeding and thus attenuate the deleterious effects of inbreeding depression. This results in increased overall plant fitness and fecundity (Whitehouse, 1950). A species with both male and female
reproductive organs in the same flower or on the same plant (monoecious) might benefit from a genetic process to promote outcrossing and genetic variability. Plants possess many structural attributes for efficient self-fertilization, and from this derives the rationale for the evolutionary importance of self-incompatibility (de Nettancourt, 1977, Bernatsky et al., 1987, Cornish, Pettitt, and Clarke, 1988, Ebert et al., 1989, and Haring et al., 1990, for reviews). The proliferation of the angiosperms in the mid-Cretaceous is thought to be due in large part to the establishment of mechanisms that promoted outcrossing at the expense of self-fertilization processes (Whitehouse 1950, Stebbins 1957, and de Nettancourt 1977). Several researchers have proposed that self-incompatibility systems originated early in angiosperm evolution, and the early establishment of a system to promote out-crossing (self-incompatibility) was responsible for the extreme divergence and evolutionary success of the early angiosperms (Whitehouse, 1950; Stebbins, 1957). Support for this contention comes from the observation that outbreeding mechanisms of dichogamy and dioecism (separate sexes) operate in both angiosperms and the more primitive gymnosperms, but self-incompatibility is found only in angiosperm species. Whitehouse (1950) states that 70% of the angiosperm genera contain only hermaphroditic species, while 5% of the genera contain only dioecious species. He argues that such a high proportion of hermaphrodite species would not have out-competed angiosperm species possessing dichogamy or dioecism unless the process of self-incompatibility had established itself early in angiosperm evolution.
Furthermore, the hypothesis for an early origin of self-incompatibility systems in angiosperm evolution is supported by the observation that self-incompatibility is widely distributed among the angiosperm orders. Additionally, it has been observed that self-incompatible species of several genera (Bromus, Hordeum, Secale, and Limonium) predominate in central areas of origin, while in peripheral zones, homomorphic self-compatible species predominate (Stebbins 1957). The closing of the carpel (resulting in the formation of a primitive stigma/style structure) early in angiosperm evolution may have provided the proper conditions for the selection of breeding systems that promoted outcrossing, resulting in the early appearance of incompatibility systems.

1.1.2 Flowering and Fertilization  Self-incompatibility is a process that promotes outbreeding by interfering with the ability of self-pollen to participate in fertilization. The response occurs within the larger biological context of the complex reproductive processes of pollination and fertilization. The process of self-incompatibility occurs within an angiosperm’s reproductive structure; the flower.

The components of an idealized flower are diagramed in figure 1. The flower is generally viewed as a specialized structure made up of modified leaves, that are positioned in four concentric whorls. The sepals (outermost whorl) protect the other floral parts during the bud stage. Internal to the sepals are the petals; the corolla is a term used to describe the complete array of petals present on the flower. Inside the petals are the stamens, the male reproductive structures. They are composed of two parts; a thin stalk-like structure called the filament and a terminal ovoid pollen producing structure.
Figure 1. Anatomy of a complete flower.

The figure illustrates the components of a complete flower, showing the male (stamen) and female (pistil) reproductive structures. Pictured within the ovary is the process of double fertilization, were one sperm nucleus fuses with the central egg cell and the other sperm nucleus fuses with the two polar nuclei, yielding triploid endosperm (labeled as the triple fusion).
the anther. In the center of the flower is the female reproductive organ called the pistil, and it is shown divided into three components: (1) the stigma, which is an enlarged apical tip that functions to provide a receptacle for the pollen, (2) the style positioned below the stigma, is a slender stalk-like structure possessing a specialized tissue called the transmitting tract, through which the male gametophyte grows, and (3) at the base of the pistil is a bulbous structure referred to as the ovary. The pistil is derived from carpels (modified leaves) that fuse early in development. Within the ovary there are one or more (usually hundreds) sporangia termed ovules, which are attached to the ovary wall via a small stalk. Within an ovule, the megasporocyte undergoes meiosis to yield four haploid megaspores three of which rapidly degenerate. The remaining megaspore divides mitotically several times to produce the egg sac, which is generally composed of seven cells pictured in figure 1. The egg sac is composed of three distal cells called antipodals, and a large central cell possessing two haploid nuclei called polar nuclei. Positioned near the micropyle (opening where pollen tube enters the ovule) is the egg cell surrounded on each side by accessory cells termed synergids. The synergids are metabolically active cells which are in close contact with the egg cell, often with plasma membrane contact between the egg and synergid.

The anther possesses four sporangia, each of which are composed of tapetal cells which are accessory anther cells that surround the microsporocytes, and the microsporocytes themselves which produce thousands of haploid microspores. Within the sporangia, the microsporocyte (pollen mother cells) undergo meiosis to yield four haploid microspores. Each microspore then undergoes mitosis to produce a generative and
vegetative (tube) cell. The resulting bi-cellular structure is the male gametophyte (pollen grain).

Following pollination, the germinating pollen grain begins to grow downward through the stigmatic surface and between the transmitting tract cells of the style, during which the generative cell divides to produce two sperm cells.

Prior to fertilization, the growing pollen tube tip enters one of the synergids and discharges its sperm nuclei and cytoplasm into the synergid. Following pollen tube discharge, the recipient synergid rapidly degenerates, and the process of double fertilization ensues. One of the two sperm nuclei fuses with the egg to form the diploid zygote. The other sperm nucleus fuses with the two polar nuclei within the central cell, generating a triploid nutritive tissue called endosperm.

1.1.3 Self-Incompatibility Reaction Mechanistically, self-incompatibility is the capacity of a plant to reject self-pollen and allow fertilization with pollen that possesses a distinct compatibility phenotype. The plant has the ability to discriminate between self and non-self pollen; specifically inhibiting the growth of self-pollen, thus ensuring that cross-fertilization ensues. The self-incompatibility reaction can be divided into two components: (1) a recognition phenomenon between pollen and pistil cells, and (2) a molecular mechanism that selectively arrests self-pollen germination or tube elongation.

In most systems under investigation, the genetic basis of this unique form of cellular recognition is governed by a single, complex, multi-allelic locus termed the S-locus, but bi-factorial systems have been identified, most
notably in the grasses (Gramineae).

Traditionally it has been thought that an incompatibility response is elicited following the interaction between identical S-allelic gene products present in pollen and pistil tissue. At its simplest, the genetic rules of pollen/pistil interaction can be stated as follows; pollinations resulting in the juxtaposition of like S-alleles expressed in pollen and pistil result in an incompatibility reaction, and pollinations bringing together unlike pollen and pistil S-allele gene products result in seed formation (compatible pollination).

1.2 Classification of Self-Incompatibility Systems
1.2.1 Heteromorphic Self-incompatibility Self-incompatibility is divided into two broad classifications based on differences in flower morphology. The first type is referred to as heteromorphic self-incompatibility. Plants possessing this system are characterized by a floral architecture that acts as a topological barrier to self-fertilization, for example, plants with elongated pistils and relatively short anthers. Such architecture reinforces the outbreeding potential of the plant, and augments the sporophytic incompatibility reaction (reviewed by Gibbs, 1986). This type of incompatibility has been observed in 24 families consisting of over 160 genera, (*Primula, Oxalis, Fagopyrum, Linum* etc.). The tight linkage between loci affecting floral morphology and self-incompatibility was noted by several researchers. Ernst (1936) observed by recombinatorial analysis 3 complementation groups controlling pollen incompatibility (S), anther height, and style length. Using radiation induced mutational analysis in buckwheat, Sharma and Boyes (1961) developed a
model based on a complex S supergene, where the locus consisted of 5 recombinatorial units: $I^p$, $I^g$, $G$, $P$, and $A$. The first two designations represent loci which direct incompatibility responses within the pollen and style respectively. The other three loci are involved with floral morphology, including; style length ($G$), pollen size ($P$), and anther length ($A$). An interesting variant of heteromorphic incompatibility was described by Lewis (1949) in *Linum grandiflorum*. In this species there are two plant subtypes that differ in the length (long vs short) of the styles that each possesses. Plants with long styles are termed pin and short style plants possess the thrum phenotype. This heteromorphic self-incompatibility system is governed by a single genetic locus $S$, with two alleles, $S$ and $s$. The thrum genotype is $Ss$ and the pin genotype is $ss$. The breeding behavior of thrum and pin plants implies that the population consists of two classes of self-incompatible individuals, analogous to the XY system of sex determination operating in animals. The mechanism by which out-crossing is enforced is based on the ability of pin and thrum plants to produce pollen that possesses different turgor pressures. Fertilization only occurs under pollination conditions that approximate a 4:1 ratio of osmotic pressure between pollen and style; a condition met only as a result of outcrossing between pin pollen on thrum stigma or thrum pollen on pin stigma.

1.2.2 Homomorphic Self-Incompatibility Homomorphic self-incompatibility is estimated to occur in over half the families of flowering plants (East, 1940). Plants exhibiting this widespread form of self-incompatibility do not show any differences in floral morphology, and the rejection of self pollen occurs
solely through the S-locus genetic system. Within this class there are two subtypes, termed sporophytic and gametophytic incompatibility. The two subclasses of homomorphic incompatibility derive their names from differences in the timing of S-allele expression in the male gametophyte. In sporophytic incompatibility, the incompatibility phenotype in the pollen is determined by the diploid genotype of the pollen producing plant (sporophytic generation). In contrast, species exhibiting gametophytic incompatibility express the phenotype of the individual haploid pollen microspore. Thus, in gametophytic self-incompatibility the pollen component of the S-allele is expressed after the microsporocyte has undergone meiosis, and therefore the pollen phenotype must reflect the single S-allele present. A diagram comparing the two types of incompatibility, and illustrating the ramifications of pre- vs post-meiotic S-allele expression is shown in figure 2.

1.2.3 Sporophytic Self-Incompatibility Sporophytic self-incompatibility systems have been described for 6 families, with the Cruciferae family being the most intensely studied (genus Brassica). Most species possess a highly polymorphic, one locus (S) genetic system, where multiple alleles may show various dominance relationships (dominance, co-dominance, and mutual weakening). In sporophytic incompatibility, the S-alleles present in the genotype of the parent plant determine the pollen phenotype. For example, a plant that is heterozygous for the co-dominant S-alleles S₁ and S₂, will produce pollen with S₁ or S₂ genotype, but the genotypic classes are phenotypically identical (S₁S₂). An incompatibility reaction will occur if either S₁ or S₂ pollen (both phenotypically S₁S₂) pollinates a plant expressing either the S₁ or S₂ pistil self-incompatibility gene product.
Gametophytic pollen/pistil interactions are shown in the leftside panel. The plant is genotypically $S_{1,2}$ and expresses both $S$-alleles in the diploid stylar transmitting tract tissue. This plant would produce either $S_1$ or $S_2$ pollen, with an identical genotype and phenotype. Pollination with $S_1$ pollen triggers a self-recognition response within the style tissue, resulting in the cessation of pollen tube growth approximately one-third the way through the style. When pollen and style $S$-alleles do not share allelic identity, as in the case of $S_3$ pollen pollinating a $S_{1,2}$ plant, normal pollen tube elongation and fertilization occurs. The sporophytic system is illustrated in the right hand side of the figure. The plant has a $S_{1,2}$ genotype, with both $S$-alleles expressed in the diploid stigma cells. The pollen depicted is derived from a $S_{1,3}$ plant, and is either genotypically $S_1$ or $S_3$, but because of the pre-meiotic timing of pollen $S$-allele expression, the pollen is phenotypically $S_1/S_3$. Thus, neither pollen grain germinates due to the recognition response between $S_1$ allelic determinants present in the stigma and pollen exine.

Figure 2. Genetic interaction of pollen/pistil components in gametophytic and sporophytic self-incompatibility systems.
In sporophytic self-incompatibility the inhibition reaction is localized to the stigmatic surface of the pistil. In most species the pollen fails to germinate, or if the pollen grain does germinate the pollen tube does not penetrate the stigmatic papillar cells. Species exhibiting sporophytic incompatibility are also characterized as having "dry" stigmatic surfaces, which are thought to increase one-to-one interaction between individual pollen grains and individual stigmatic papillar cells. The source of the pollen S-locus determinants has been debated, and is either derived from pre-meiotic pollen mother cells (Pandey, 1970), or from tapetal cells that secrete the pollen incompatibility determinants unto the exine layer of the pollen wall (Heslop-Harrison, 1973).

Recent molecular evidence indicates that sporophytic and gametophytic incompatibility systems share very little in common at the physiological and molecular level (Nasrallah et al., 1987, Takayama et al., 1987, and Haring et al., 1990). Thus, I will discuss sporophytic incompatibility systems for contrast purposes only.

1.2.4 Gametophytic Self-Incompatibility The vast majority of the species which have this type of homomorphic self-incompatibility possess a multi-allelic, mono-factorial system (S-locus). Species with 2 (grass species) and 4 (Ranunculus) segregating loci, however, have been documented. Gametophytic self-incompatibility has been most extensively investigated in the solanaceous genera: Petunia, Lycospericon, Nicotiana, and Solanum. In this incompatibility system, the haploid male gametophyte determines its own compatibility phenotype, and thus expresses only one S-allelic determinant. Elongating incompatible pollen tubes are inhibited
approximately one-third the way down the style so that the response is localized in the transmitting tract tissue of the style. Species expressing gametophytic incompatibility generally have stigmas that are covered by a mucilaginous stigmatic exudate, characterized as a “wet” surface.

Gametophytic self-incompatibility has been shown to be under strict developmental control. Early evidence for this came from Yasuda (1934), who discovered that the self-incompatibility reaction could be circumvented by the pollination of immature floral buds (2 to 4 days prior to anthesis) with mature pollen (bud-pollination studies). His observations were later confirmed in *Petunia, Nicotiana, and Brassica*, by Attia (1950), Pandey (1958), and Shivanna and Rangaswamy, (1969) respectively. The simplest inference from this data is that the ability of the pistil to elicit the incompatibility reaction is a function of developmental maturity. Bud-pollination studies in our laboratory have demonstrated that the petunia lines in our laboratory have the ability to elicit the incompatibility response 1 to 2 days prior to anthesis (dpa). Before this time, the plant is functionally bud-compatible at stages -3 to -4 dpa. From a practical standpoint, this technique is extremely useful for the generation of homozygous tester lines, which are used to identify S-allele compatibility groupings. As I will detail, my research on the temporal pattern of S-mRNA expression has established a direct correlation between the onset of the incompatibility phenotype and the developmental accumulation of stylar S-mRNAs.

To summarize the information discussed above, I would like to present a classification diagram (figure 3) designed by and adapted from de Nettancourt (1977). The diagram centers on the observation that the vast
Figure 3. Classification diagram of Self-Incompatibility Characteristics.

This diagram illustrates the observed frequency of self-incompatibility characteristics and interactions between various classification schemes, encompassing the complete range of self-incompatibility systems (see text for explanation). Adapted from de Nettancourt, 1977.
majority of species expressing an incompatibility system are mono- or bifactorial in nature. Working outward we can see graphically how the various characterizations overlap. For example, the inner-most concentric ring diagrams the site of pollen inhibition, occurring in some species on the stigma or in the style, and in a few documented cases of sporophytic incompatibility, localized to the ovary (Theobroma cacao, Linum, and Hemerocallis). The second and third concentric rings point out a curious correlation. Species with tricellular pollen possess sporophytic incompatibility and species demonstrating gametophytic incompatibility almost always have pollen that is bicellular. The fourth ring separates the reactions into hetero-(sporophytic; tricellular pollen) vs homomorphic (gametophytic or sporophytic; bicellular or tricellular pollen) characteristics. The outermost ring diagrams the distribution of multi-allelic and di-allelic systems (note that homomorphic incompatibility systems are in almost all cases poly-allelic).

1.3 S-Locus Structure

I would like to briefly discuss two principle models of S-locus structure that have been used by researchers in the field to provide a conceptual framework for understanding pollen/pistil interactions as they pertain to S-locus function.

The first model, based on classical genetic data and early biochemical characterization of the self-incompatibility response, is referred to as the tripartite S-locus model. It was proposed by Lewis (1960), who postulated that the locus possessed a tripartite structure: a component responsible for the physical manifestations of the response, and two associated activity
components, functional in pollen and pistil cells, that were responsible for S-locus expression in the appropriate organ. The activity elements are analogous to tissue specific promoters that cause S-locus gene expression in the male or female reproductive tissue. An important tenet of this model is that identical S-locus gene products are produced in the pollen and style, and that following self-pollination, they interact in an unknown manner (polymerization, oligomeric formation etc.) to bring about pollen growth inhibition.

The basis for much of this model came from the analysis of radiogenic derived unilateral S-locus mutations that affected either pollen or pistil breeding behavior (Lewis, 1949; 1960). Pollen-part mutations were characterized as loss of function mutations, and thus acted as universal pollinators. Unilateral mutations affecting the style component (stylar-part) of the self-incompatibility reaction were also identified, whereby the plant no longer had the ability to discriminate between self and non-self pollen and always produced seed capsules following self-pollination. The failure to observe recombinant progeny between these two classes of mutations, after assessment of a few hundred progeny was taken as evidence that a single genetic locus was responsible for the expression of both the pollen and pistil S-components (Lewis, 1960). Lewis argued that since they derived from the same locus, some type of synergistic or additive interaction was occurring between identical gene products to elicit the incompatibility response. Other evidence for a single locus model came from serological studies on Oenothera (Lewis, 1952), in which it was shown that antibodies raised against pollen extracts elicited an S-locus specific precipitin reaction when combined
with style homogenates. The serological results imply that a common protein is present in pollen and style extracts. A diagram illustrating Lewis' tripartite model is shown in figure 4.

A second model for S-locus function is based on the hypothesis that two separate but tightly linked incompatibility loci interact to elicit the incompatibility response. The model has grown in acceptance, primarily due to the advent of supporting molecular data on gametophytic incompatibility in the Solanaceae, (Clark et al., 1990, Haring et al., 1990, and McClure et al., 1990). In addition, Dana and Ascher (1986) have observed recombinant progeny classes in crosses between pollen-part and stylar-part mutations in *P hybrida*. One possible interpretation of the data implies that a pollen-specific factor that is not identical to the stylar S-locus, but rather linked to S (Dana, 1982) is functioning to yield the observed progeny classes. Alternatively, the possible existence of modifying loci that alter S-locus expression may be responsible for pollen-part or stylar-part phenotypes, whereby, a linked modifier locus may be operating to cause the observed progeny classes.

The molecular model hypothesizes that two tightly linked loci exist at the S locus, with one gene product expressed in the male gametophyte, and another gene product expressed in pistil tissue. The model proposes that the interaction between these two gene products is responsible for eliciting the incompatibility reaction (complementary interaction).

At the molecular level it is thought that one component of the reaction is a style expressed ribonuclease (S-RNase), which is encoded by the S-locus. The second factor is hypothesized to be either a pollen expressed receptor, which may provide a means for the specific uptake of S-RNase by growing
Evidence suggesting the presence of one specificity part common to pollen and style

Specificity segment

- Pollen activity part
- Stylar activity part

Evidence suggesting the presence of two activity parts for pollen and style which are distinct from one another and from the common specificity segment

Negative mutations, i.e. self-compatibility mutations, can occur which affect only the pollen phenotype, the style phenotype, or both (Lewis, 1949; Brewbaker, 1954; Pandey, 1967; de Nettancourt et al; 1971) and which demonstrate independence between the genetic units governing pollen and style reactions.

In diploid pollen, a pollen part mutation can be restored and the original S-specificity reinstalled if the second allele present carries a functional activity part for the pollen reaction (Lewis, 1960).

A change in specificity (constructive mutation), when it occurs spontaneously, does result from the generation of a new S-allele which operates in both pollen and style (de Nettancourt et al., 1971).

Mutations toward new specificities would have led to a breakdown of the incompatibility system if different specificity parts operated independently in pollen and style (Lewis, 1960).

Evidence suggesting the presence of two activity parts for pollen and style which are distinct from one another and from the common specificity segment

Negative mutations, i.e. self-compatibility mutations, can occur which affect only the pollen phenotype, the style phenotype, or both (Lewis, 1949; Brewbaker, 1954; Pandey, 1967; de Nettancourt et al; 1971) and which demonstrate independence between the genetic units governing pollen and style reactions.

In diploid pollen, a pollen part mutation can be restored and the original S-specificity reinstalled if the second allele present carries a functional activity part for the pollen reaction (Lewis, 1960).

Figure 4. Tripartite model for S-locus function.

The diagram is based on a genetic model of S-locus function proposed by Lewis (1960). The tripartite structure is graphically represented in the central portion of the figure, where a specificity segment (S-locus recognition) is controlled in the appropriate organ by either a pollen or style activity component. Supporting genetic data is presented for reference. Adapted from de Nettancourt, 1977.
self-incompatible pollen tubes, or a pollen expressed inhibitor of the S-RNase (figure 5). Recent molecular data (McClure et al., 1990) has illuminated a possible mechanism of incompatible (self) pollen inhibition in *Nicotiana alata*. McClure et al., (1990) have correlated S-allele specific *in vivo* degradation of ribosomal RNA in elongating self-pollen tubes, presumably mediated by the abundant S-RNase protein. The researchers hypothesized that RNA degradation causes the cessation of pollen protein synthesis and consequently, halts pollen tube elongation. I will discuss in detail more of the supporting molecular data later in the discussion section (chapter VIII).

1.4 Physiological and Ultrastructural Characterization of the Incompatibility Response

Several early physiological and biochemical studies attempted to describe and understand the morphological differences between compatible and incompatible pollinations. From these early studies much descriptive information was presented in the self-incompatibility literature. I will discuss and highlight some of the more potentially illuminating physiological aspects of the incompatibility reaction.

1.4.1 Pollen Tube Morphology In gametophytic incompatibility, the reaction is extremely site specific, occurring within the stylar transmitting tract tissue. Under natural conditions compatible and incompatible pollen tubes are growing in close proximity and undergo the self-incompatibility reaction independent of each other. Herrero and Dickenson (1981) found that petunia pollen growth rates do not differ between compatible and incompatible pollen during the first 8 hours of growth, but after 12 hours, markedly reduced
Figure 5 Molecular model of S-locus action.

This diagram pictures a pollen tube tip growing through the extracellular mucilage between the columnar transmitting tract cells of the style. The S-proteins are secreted from the transmitting tract cells into the extracellular matrix, and are therefore, in direct contact with the cell surface of the growing pollen tube. The figure depicts a concentration of cellular organelles: mitochondria, endoplasmic reticulum, vesicles, and golgi bodies, at the pollen tube tip. The model proposes that an allele specific pollen receptor (membrane bound?) interacts with its cognate style S-RNase to achieve introduction of the style protein into the pollen tube in an allele specific manner. Pollen growth inhibition presumably occurs through the degradation of ribosomal RNA (and possibly mRNA), which results in a cessation of protein synthesis. Taken from Haring et al. (1990).
incompatible pollen growth rates were observed. Pollen tube elongation, in incompatible crosses, stopped approximately one-third the way down the style, while compatible pollen reached the ovary ~ 48 hours after pollination.

Light microscopy studies by Schlosser (1961) and van der Pluijm and Linskens (1966), along with electron microscopy by Herrero and Dickinson (1981), revealed an increase in callose plug deposition in incompatible pollen tubes compared to compatible pollen tubes. Callose plugs are located within the inner wall of the elongating pollen tube, and act to restrict the tube cytoplasm to the growing tip of the pollen tube. As a consequence of increased callose plug deposition in incompatible pollen tubes, pollen tube cytoplasm is frequently trapped and degraded behind these plugs. As pollen tube growth ceases, large amounts of callose are deposited at the end of the pollen tube tip, with the eventual bursting of the tip itself. de Nettancourt (1974) used electron microscopy to document the existence of peculiar particles (0.2 \( \mu \)m in diameter), often polyhedral in shape within the degenerating incompatible pollen tubes of Lycopersicon peruvianum. The author noted that these particles were not evident in compatible pollen tubes, and that the particles resemble spheres released by compatible pollen tubes within the ovule of the degenerating synergid at the time of generative sperm nuclei discharge.

1.4.2 Style Morphology Traditionally the role of the pistil in fertilization was thought to be primarily passive and nutritive in nature. Research in the laboratory of E. Lord has indicated this simplistic view may not be entirely correct. Sanders et al., (1989) demonstrated that small latex beads (similar in diameter to pollen) are translocated downward toward the ovary, regardless of
the direction of the gravitational field. The researchers have proposed that the style facilitates pollen tube elongation through a recognition/adhesion system. Recently, Sanders et al., (1991) have identified plant homologs to the substrate adhesion molecules (SAM) present in the extracellular matrix of animal cells. SAM molecules are thought to play a role in cell migration and cell movement in animal systems (Dufour et al., 1988). The investigators report cross hybridization of a human vitronectin cDNA probe to plant genomic DNA, and localize high levels of vitronectin-like protein to the transmitting tract tissue of *Phaseolus*.

In the Solanaceae, maturation of the stigma involves cellular degeneration with the corresponding release of stigmatic exudate (Herrero and Dickinson, 1979). Solanaceous species possess solid styles with a central core of specialized cells that form the transmitting tract, between which the elongating pollen tubes grow (Konar and Linskens, 1966). The transmitting tract cells are large cylindrical, metabolically active secretory cells positioned in a semi-solid intercellular matrix. Pollen tubes grow downward through the intercellular matrix and do not penetrate the transmitting tract cells (van der Pluijm and Linskens, 1966). There is an alteration in cell morphology of transmitting tract cells following the passage of compatible pollen tubes, which resembles some characteristics of tissue senescence. The style cells become vacuolate, with few visible organelles, and possess a thin peripheral cytoplasm. Pistils from incompatible pollinations show no senescence characteristics and resemble un-pollinated pistils (Herrero and Dickinson, 1979). Using electron microscopy de Nettancourt (1974) observed in *Lycopersicon peruvianum* that following incompatible tube growth through
the upper third of the style, the rough endoplasmic reticulum (RER) in the transmitting tract cells appears as a concentric whorl of parallel membranes, which the author notes, could be an indication of protein synthesis inhibition.

Changes in pistil gene expression patterns have also been documented following incompatible pollinations. Kovaleva et al., (1978) have shown that the injection of RNA synthesis inhibitors into maturing styles of Lilium and Petunia resulted in the growth of otherwise incompatible pollen tubes. van der Donk (1974) observed qualitative differences in the fractionation patterns of radiolabeled RNAs from incompatible, compatible and un-pollinated styles. Similar differences were again seen by van der Donk using Xenopus translation products of stylar RNAs from compatible, incompatible, and un-pollinated pistils. In summary these results indicate that profound and substantial differences in gene expression patterns are evident as a result of incompatible vs compatible pollinations, and that these differences extend to the ultrastructural and physiological levels.

1.5 Biochemical Characterization of Gametophytic Self-Incompatibility

1.5.1 Introduction Much of the early biochemical work in self-incompatibility attempted to identify specific proteins (either present in pollen or style) involved in the inhibition of incompatible pollen tube growth. These studies utilized SDS-PAGE and isoelectric focusing techniques to identify specific proteins based on their presence in compatible vs incompatible crosses. Researchers focused on style derived isozyme assays of proteins such as: cytochrome oxidase (Schlosser, 1961), peroxidases (Pandey,
1967, and Bredemeyer and Blaas 1975), and glycan hydrolases, (Linskens et al 1969). Pollen derived proteins such as catalases (Makinen and Brewbaker, 1967), acid phosphatases (Poddubnaya-Arnoldi et al., 1961), esterases and leucine aminopeptidases (Makinen and Brewbaker, 1967), were found to be released from the pollen wall and were hypothesized to play a role in pollen recognition. However consistent and repeatable segregation between S-allele breeding behavior and protein isozymes were never demonstrated for the above proteins, therefore, these proteins are thought to play at best a secondary role in pollen recognition and/or pollen rejection.

The most fruitful research from this period came from the biochemical investigation of pollen recognition factors by Lewis (1952). Using immunodiffusion techniques in *Oenothera*, Lewis demonstrated an S-allele specific precipitin response between a style homogenate and agar medium containing an antiserum made against a pollen extract of the same S genotype. The inference Lewis made from the work was that pollen derived antiserum was cross-reacting with similar epitopes (presumably the S-protein) present in pollen and style tissue in an S-allele specific manner. The early serological observations by Lewis were confirmed by Makinen and Lewis, (1962), and Nasrallah et al., (1967). Nasrallah, also demonstrated that an antiserum raised against pollen extracts from *Brassica oleracea* (sporophytic incompatibility) would react in a S-allele specific manner to stylar homogenates using immunodiffusion agar techniques. The *Oenothera* and *B. oleracea* serological results support the hypothesis that in systems demonstrating stigmatic pollen inhibition (usually sporophytic; *Oenothera* possesses gametophytic incompatibility), the pollen recognition
factors are already present within the walls (exine and/or intine) of the mature pollen grain (Heslop-Harrison et al., 1973). This is in contrast to gametophytic systems (stylar pollen inhibition), where the exact timing of the production of the pollen recognition factors in the male gametophyte is not known, except that it occurs post-meiotically.

1.5.2 Biochemical Characterization of Stylar S-Allelic Determinants

An important advance in the characterization of self-incompatibility came from the work of Hinata and Nishio (1978), who demonstrated the co-segregation of a stylar protein from *Brassica campestris* (sporophytic incompatibility) with S-allele breeding behavior. Subsequently, in species expressing gametophytic incompatibility, an abundant stylar protein class was identified that co-segregated with S-genotype, indicative that these proteins were either the product of the S-locus or tightly linked. S-associated style proteins were identified in *Nicotiana alata*, (Bredemeijer and Blaas, 1981), *Prunus avium*, (Mau et al., 1982), *Lycopersicon peruvianum*, (Mau et al., 1986), *Petunia hybrida* (Kamboj and Jackson, 1986), and *Solanum tuberosum* (Kirch et al., 1989). All isolated S-linked stylar proteins share many biochemical properties. Among these: the proteins are present at high levels (1-5% of the total stylar protein), are glycosylated, possess a high pI (7.5-8.7) and thus, are basic in nature, and are of low molecular weight (26,000 - 33,000 daltons). Mau et al., (1986) showed that the *Lycopersicon peruvianum* putative S-allele proteins were present in a spatial and temporal pattern consistent with a role in the incompatibility reaction. The proteins were most abundant in the stigma and upper one-fourth of the style, and present in increasing amounts throughout development, reaching maximal levels at flower maturity. Mau
et al., (1986) performed Edman degradation analysis on the N-terminal end of purified S-proteins for several alleles from *Lycopersicon peruvianum* and *Nicotiana alata*. These S-proteins possessed a highly conserved N-terminal domain. The identification of this highly conserved N-terminal region, which is illustrated in figure 6, was an important first step in the isolation of the genes encoding these stylar S-linked proteins (Anderson et al., 1986; Clark et al., 1990).

1.6 Modifications of the Self-Incompatibility Response

Changes in the capacity of a plant to express the self-incompatibility reaction are known to occur through a variety of environmental, mutational and genetic effects. Genetic breakdown of the incompatibility reaction is referred to as pseudo-self-compatibility (PSC), the expression of which varies greatly depending on the species being analyzed.

An example of a non-genetic process that can circumvent the incompatibility response is green bud-pollination experiments, where the incompatibility response is by-passed as a result of the developmental immaturity of the response (Yasuda, 1934). Irradiation of petunia flowers immediately prior to self pollination was also shown to be an effective agent for obtaining partial (50%) seed set (Linskens, 1960), presumably through the action of S-locus gene inactivation. Previously mentioned radiogenic unilateral mutations generated by Lewis (1949; 1960) also demonstrated the ability of mutational S-locus impairment as a means to circumvent the incompatibility reaction.
Figure 6. Conserved N-terminal portion of *Nicotiana alata* and *Lycopersicon peruvianum* S-proteins.

Pictured is the amino acid sequence of the N-terminal end of four *Nicotiana alata* S-alleles (NAS 2, 6, f, and z), along with the N-terminal protein sequence from two *Lycopersicon peruvianum* S-alleles (LPS1 and LPS6). The boxed regions represent areas of sequence conservation among the six S-alleles. The high degree of amino acid conservation led our laboratory to synthesize a 45-mer oligonucleotide that encodes the N-terminal portion of the *N. alata* S2 allele. The NAS2 45-mer was used as a heterologous probe to isolate *P. hybrida* S-alleles. The top line in the figure shows the nucleotide sequence of petunia S-allele (PS1B), with the sequence of the NAS2 45-mer on the second line. Lower case letters represent base pair mismatches between the two sequences. The central region of the NAS2 45-mer (nucleotides 15-35) possessed the highest degree of cross-homology with the three petunia S-alleles isolated in our laboratory (PS1B, PS2A, and PS3A).
1.6.1 Temperature Effects  Temperature is another well documented self-incompatibility modifying agent, were elevated greenhouse temperatures (30°-50°C) can cause breakdown of the incompatibility system. This phenomenon has been documented with our petunia lines which demonstrated partial breakdown of the self-incompatibility reaction at ~32°C. The breakdown of self-incompatibility due to elevated temperature has been described in several species displaying gametophytic incompatibility, including; *Oenothera*, (Hecht, 1964), *Prunus*, (Lewis, 1942), and *Trifolium*, (Townsend, 1965). It appears that the site of breakdown is in the style, where the effect of elevated temperatures on self-incompatibility breakdown (PSC) results in the inability of the style to discriminate between self and non-self pollen. Elevated temperatures presumably affect S-locus function, or related modifying loci. Working with a tetraploid clover species, Townsend (1968) identified a temperature sensitive plant line, that under normal greenhouse conditions (25°C) was self-incompatible, but breeding was pseudo-self-compatible after raising the temperature to 32°C. Elevated temperatures apparently resulted in complete breakdown of the self-incompatible system. The inheritance of this temperature sensitive phenotype corresponded to a simple mendelian dominant/recessive genetic locus (termed T), that showed no linkage to S.

1.6.2 Pseudo-Self-Compatibility  The existence of loci unlinked or weakly linked to S that modulate the expressivity of the self-incompatibility phenotype has been hypothesized as the primary cause of genetic PSC (Takahashi, 1972, 1974, Flaschenriem and Ascher, 1979a, 1979b and Dana and
The ability to select for high levels of PSC, using selective breeding schemes over several generations, indicated that a quantitative genetic trait caused some forms of PSC (Takahashi, 1974). Progeny seed set data between petunia parental crosses expressing high and low PSC respectively produced progeny with a PSC distribution suggesting additive inheritance with multiple loci operating (Takahashi, 1974). Polygenic pseudo-self compatibility, tended to decrease S-allele activity rather than alter S specificity (Takahashi, 1974, Henny and Ascher, 1976, and Flaschenriem and Ascher, 1978). Presently, little is known about the nature of these modifying loci. Flaschenriem and Ascher, (1979b) have reported a map distance of approximately 28 map units between S and a modifying locus affecting S expression in the style of petunia. Not all PSC seems to be polygenic in nature. Takahashi (1974), working in *Petunia hybrida*, reported self-seed set which was affected by a factor segregating as a null-specificity allele termed Sf (East, 1929). In theory Sf would act as the equivalent of an S-allele in a self-compatible species, and individuals homozygous for Sf would be fully self-compatible.

### 1.6.3 Allelic Diversity

The existence of extremely high numbers of segregating S-alleles operating in wild plant populations (Lewis, 1949; Ockendon, 1974), raises the question of the mechanism by which allelic diversity is generated. Radiogenic treatment results in the generation of null mutation phenotypes, in which the S-locus is inactivated, but radiogenic treatment has never been observed to produce new S-allele specificities. Working with *Lycopericon peruvianum*, de Nettancourt *et al.*, (1971) demonstrated that under conditions of forced inbreeding, new S-allele specificities can be generated.
Forced inbreeding mechanisms for the generation of new S-alleles suggest recombinatorial mechanisms may be operating at the S-locus to cause the extreme S-allelic diversity observed.

Examples of biological systems that utilize recombinatorial mechanisms for the formation of allelic diversity include the immunoglobulin gene superfamily, and the production of antigenic variation in *Trpanosoma brucei*. The expression of the variable surface glycoprotein (VSG) gene in *Trpanosoma brucei* is determined by the positioning of the expressed VSG gene in an active transcriptional context termed the expression site. Different VSG proteins are synthesized as a result of intragenic chromosomal rearrangements that substitute one VSG gene for another at this active site (for review see Borst, 1986).

1.6.4 Self-Compatible Species The apparent paradox of the existence of large numbers of self-compatible species that are well adapted and established is contrary to the expectations of a system (self-incompatibility) that functions as an effective barrier to inbreeding. This observation can be explained by several lines of reasoning; the first, proposed by Lewis and Crowe (1958), is that self-incompatibility may be required (and provide a selective advantage) only during the time that a species is in direct competition for a particular evolutionary niche, and that once established the species may undergo a transition to self-compatibility (presumably by mutational events).

A second point is that many self-incompatible species set seed (environmental PSC) under adverse environmental conditions, and this is well documented in the Solanaceae. Environmental PSC may provide a method for the establishment of self-compatible colonizing plant
populations. Baker (1955) notes that the long-distance dispersal of a single seed to a receptive environment, in conjunction with environmental PSC or mutational breakdown of the S-locus, could provide conditions for the establishment of a self-fertilizing colony population. The distribution of related species (same genus), some of which are self-compatible and others are self-incompatible, suggests that self-compatible species proliferate around the peripheral regions of origin, consistent with the scenario that Baker (1955) postulates.

Finally, the existence of many well adapted self-compatible plant species is probably aided by the plasticity and flexibility of the plant genome; a point that should be taken into consideration when discussing the deleterious effects due to inbreeding. The ability of plant genomes to undergo alterations and rearrangements in response to environmental conditions has been documented, as well as the existence of high levels of genetic diversity within some inbred plant populations (Allard et al, 1968). Couple this with the existence of extremely large genome sizes (some possessing active transposon elements), and mechanisms for intragenic recombination, the generation of genetic diversity in the absence of sexual reproduction seems probable.

1.7 Eukaryotic Transcription Control Elements

A portion of my doctoral research focused on the analysis of cis-acting 5' DNA sequences that affect S-locus gene expression. Therefore, I would like to discuss transcriptional control mechanisms broadly operating in eukaryotes, and more specifically, functioning in the higher plants.
Gene regulation is a fundamental aspect of many biological processes. Differences in gene expression are intrinsically involved in development and biological responses to environmental stimuli. The cell employs multiple strategies to exert precise control over its genetic repertoire. Primary control points for gene regulation and production of the gene encoded protein product are: (1) the initiation of transcription (2) processing control, affecting steps involved with the maturation of the hnRNA (e.g., capping, splicing, and export into the cytoplasm, (3) translational control, (4) turnover rates that affect message stability and (5) protein stability/turnover. Thus, it is important to bear in mind that regulation of gene expression can occur at several points besides the initiation of transcription, which will be focused on in this section of the introduction.

Transcription occurs as a result of the formation of an open transcription complex. Ancillary DNA binding proteins (trans-activating factors) target upstream DNA promoter elements (cis-sequences) in specific RNA polymerase II/DNA interactions. Therefore, transcriptional activation is the result of multiple cis-acting regulatory sequences acting in concert with multiple protein transcription factors, to define the final expression properties of a particular gene. Eukaryotic transcriptional control appears to be based on a modular design, where combinations of structurally distinct 5' flanking DNA control elements interact with different combinations of trans-activating factors that bind to them. The principle of modularity also introduces the concept of synergistic combinatorial interaction (Benfey and Chua 1989; 1990). Unique experimental combinations of cis-elements can often result in novel gene expression patterns, distinct from simple additive
expression effects. The ability to dissect, re-align, and manipulate DNA elements in vitro, and then re-introduce the altered promoter sequences back into the organism, has resulted in a wealth of information on the manner in which organisms regulate transcription.

1.7.1 Yeast Transcription Control The analysis of yeast (Saccharomyces cerevisiae) transcriptional control mechanisms has provided a paradigm for eukaryotic gene control, which has subsequently been shown to be conserved in higher eukaryotic organisms. Yeast promoters typically possess two types of regulatory DNA sequence elements, both of which interact with DNA binding proteins to regulate the rate of transcription. The first element is the TATA box. It is approximately 7 bp long, and is positioned between 40-120 bp from the yeast transcriptional start site. The TATA box is thought to play a general role within the transcriptional machinery by binding the general transcription factor TFIID. Its binding interacts with and facilitates the binding of RNA polymerase II, in the presence of additional TFIII protein factors.

The second type of regulatory elements in yeast promoters are the upstream activating sequences (UAS). UAS elements are usually 10-30 bp in length and are located between 100- 1500 bp upstream of the start site. Genes demonstrating coordinately regulated expression contain similar UAS sequences, and these sequences are usually the major determinants of the regulatory properties of a particular promoter.

The analysis of mutations that affected the expression of specific sets of genes, led to the isolation and cloning of yeast transcriptional activating genes. Perhaps the most well-known example of a yeast transcriptional
activating factor is the Gal4 protein (881 amino acids). This protein is necessary for the expression of structural genes involved in the metabolism of galactose. Gal4 binds to UASG, which is positioned upstream of two structural galactose metabolizing genes, Gal1 and Gal10 (the UASG is positioned bi-directionally between the two genes). In vivo footprinting experiments revealed that Gal4 was binding to a 17 bp sequence within the UASG. Keegan et. al., (1986) demonstrated that the N-terminal 73 amino acids of Gal4 were responsible for DNA binding, and that the protein DNA binding domain could be separated from the transcription activating domain. The requirement for high levels of zinc in several Gal4 mutant lines, suggested a role for the mineral in transcriptional activation. Miller et al., (1987) first reported on the role that the zinc cation played in tertiary protein formation of the TFIIIA protein. A DNA binding motif with a consensus structure of Cys-X$_{2-4}$-Cys-X$_3$-Phe-X$_5$-Leu-X$_2$-His-X$_3$-His (Cys$_2$/His$_2$) was identified (a zinc finger), where the zinc cation is coordinately complexed between two cystines and two histidines. This generates a two-stranded $\beta$-sheet and an $\alpha$-helix, where the side chains of the $\alpha$ helix interact with the major groove of the DNA. Protein sequence analysis revealed that the Gal4 protein was a member of this class of DNA binding proteins. Deletion analysis of the Gal4 protein located two transcriptional activating domains, one in the C-terminal region (amino acids 768-881) and another positioned at residues 147-196 (Ma and Ptashne, 1987).

Brent and Ptashne (1985) provided strong evidence for the modular organization of promoter sequences with domain swap experiments, in
repressor DNA binding domain. The resulting hybrid protein activated transcription of a reporter gene positioned downstream of a LexA binding site. Experiments with deletion constructs of Gal4, possessing only the DNA binding domain, were shown to repress transcription when the binding recognition sequences were placed between a TATA element and an upstream UAS. Thus, the Gal4 DNA binding domain, when separated from the activating domains could act as a transcriptional repressor by presumably interfering with protein/protein interactions between proteins positioned at the upstream UAS and the transcriptional machinery located downstream near the TATA sequence.

Another well characterized yeast trans-activating factor is the GCN4 (281 amino acids) regulatory protein. It binds to UAS sequences of several genes involved in amino acid biosynthetic pathways. GCN4 recognizes a 9 bp sequence motif that possesses dyad symmetry (e.g. the HIS3 regulatory site), which suggested that GCN4 binds as a dimer. Deletion analysis revealed that the 60 C-terminal amino acids were responsible for the DNA binding and dimerization properties.

The dimerization of transcriptional regulatory monomers (e.g. GCN4, jun/fos heterodimers) illustrates, that in addition to protein/DNA binding, protein/protein interactions are critical in constructing an active transcriptional complex. Landschulz et al., (1988) have proposed that an amphipathic α-helical amino acid motif (leucine zipper) is involved in assisting the interaction of protein subunits to form dimers. The leucine zipper motif results from the positioning of a leucine residue every 7 amino acids along the primary sequence. The leucine residues would be positioned
on one side of the α-helix, where it was theorized that the leucines could interdigitate between two zipper motifs to bring about dimer formation. Recently, the structure of the GCN4 leucine zipper dimerization domain (33 amino acids) has been shown to exist as a two-stranded, parallel α-helical coiled coil.

The transcriptional activating domain of GCN4 was located by deletional analysis to residues 107-125. A comparison of the activating regions from Gal4 and GCN4 revealed no particular sequence homology, except both regions possessed a highly acidic character. Ma and Ptashne (1987) discovered that transcriptional activation regions could be randomly generated by synthesizing small, negatively charged amino acid sequences (12-81 residues), and then fusing these regions to the DNA binding domain of Gal4. The resulting fusion proteins activated the transcription of a downstream reporter gene. The activating domains showed no homology among any of the various constructs synthesized, except that they all possessed an overall negative charge. Deletional analysis also determined that for these constructs the distance between the DNA binding domain and activating regions was functionally unimportant. These and other experiments suggested that activating domains are relatively unstructured protein elements, in which the negative charges facilitate an interaction with another component of the transcriptional complex. Recently, several researchers implicated TFIIB (Lin and Green 1991), or TFIID (Stringer et al., 1990) as the proteins interacting with the negatively charged activating domains of yeast trans-factors.
1.7.2 Higher Eukaryotic Transcription Control  The modular organization of promoter elements is also evident in higher eukaryotes, where multiple cis-acting sequences have been identified (TATA box, CAAT box, GC box, and enhancers etc.), and shown to bind proteins in vivo. The TATA box (consensus of TATAAA) is located between 25-30 bp upstream of the start site, and provides the binding site for TFIID (around which the transcription initiation complex is organized). Enhancers, which are analogous to the UAS elements in yeast promoters, act as potent transcriptional activators, often in a developmental or tissue-specific manner. Enhancers by definition function when positioned either 5' or 3' to the gene, in either orientation, and are functional at distances of several kilobases distal to the start site. The method of transcription enhancement is not known, but enhancers may function by interacting with the general transcription complex (via a looping out of intervening DNA) and increasing the affinity of binding of RNA polymerase II or required accessory factors. Enhancers could also act by altering the chromatin structure, creating a "nucleosome-free" environment for the establishment of an open promoter complex.

Analysis of Drosophila developmental mutations revealed the existence of a unique family of DNA binding/activating regulatory proteins. The original mutations were referred to as homeotic, because changes in these gene loci often caused the replacement of one body organ for another (e.g. antennepedia, which resulted in legs growing in place of the insect's antenna). The proteins for these regulatory loci shared a 61 amino acid DNA binding domain called the homeobox. Kissinger et al., (1990) have shown that the homeobox domain forms three α-helices and interacts with DNA in
a similar fashion to the helix turn-helix motif of the bacteriophage λ repressor DNA binding proteins. Homeobox containing regulatory loci (mice possess 10 to 20 different homeobox containing loci) exert differential gene expression on the target structural loci by recognizing often related cis sequences at differing affinities.

1.7.3 Plant Transcription Control The detailed dissection of plant promoters (e.g. Benfey and Chua, 1989; Gilmartin and Chua, 1990; Singh, 1989) has shown that the organization is based on a modular design. This allows for combinatorial relationships between multiple cis and trans elements, that act via differential protein/DNA binding affinities and trans-factor concentration gradients, to define the expression characteristics of a particular gene. Two broad classes of DNA sequence elements have currently been characterized: those necessary for qualitative expression of a gene in a specific cell type or tissue, and sequences that confer quantitative modulation of transcriptional levels.

Light is responsible for stimulating numerous developmental and metabolic processes, and has been one of the most intensively studied plant stimuli. Several genes (e.g. ribulose- 1,5-bisphosphate carboxylase-oxygenase and the chlorophyll a/b binding proteins) that are transcriptionally regulated by light have been shown to contain multiple cis elements that bind trans-activating proteins in a tissue specific and developmental manner.

Based on deletional analysis, 5' flanking sequences of the pea rbcS-3A gene have been shown to possess 3 upstream regions (LRE, light regulated elements) that confer responsivity to white light. Gel retardation assays and DNA footprint analysis have shown that the upstream region (-410 bp)
contains 12 separate DNA binding sites for nuclear factors. Kuhlmeier et al., (1987), Green et al. (1988), and Gilmartin and Chua (1990) have collectively defined five individual consensus binding elements within the rbcS-3A promoter, two of which are repeated multiple times. The observed redundancy of some of the elements (GT-boxes) may represent a mechanism whereby small differences in binding affinities, may allow differential gene expression at various developmental stages and/or different wavelengths of light. Proteins binding the five cis-elements have been isolated, and their characterization should enable researchers to identify the terminal components of the light transducing pathway.

The ability to synthesize promoter/reporter gene (β-glucuronidase) fusions, and then transform plant cells (either stably or transiently) has enabled researchers to study the tissue-specific and developmental expression of dissected plant promoter elements. A powerful example of the ability to use in vitro promoter mutagenesis (chimeric promoter/β-glucuronidase constructions) in combination with Agrobacterium transformation was provided by Benfey and Chua (1989, 1990). They analyzed the 35S cauliflower mosaic virus (CaMV) promoter region using chimeric GUS constructions (expression detected with histochemical and fluorescence techniques). Deletion analysis revealed that a region possessing -90 to +8 bp (element A) of the 35S promoter could direct high levels of root specific GUS expression. Another 35S deletion construct that contained 5' sequences -343 to -90 (element B) directed high levels of GUS expression in the cotyledons of seedlings and vascular tissue, with moderate levels being observed in most
CaMV 35S promoter was known to act as a constitutive promoter in most plant tissues, and their research provided an explanation for constitutive gene expression based on the combinatorial action of multiple cis and trans elements. Further deletion analysis of the B element (−343 to −90) subdivided this region into 5 sections (B1-5), with four of these regions able to direct cell-specific expression. Reconstitution of the 35S promoter in various permutations revealed expression patterns suggestive of synergistic interactions that were not merely additive. For example, when the the minimal TATA element was combined with subdomains B4 and B5 expression was observed in the vascular tissue of seedlings, a pattern not seen when either B4 or B5 was assayed alone. Species specific differences in control element interactions were also observed for the 35S promoter in tobacco and petunia. 35S expression is weak in petal cells of tobacco (expression seen only in trichome and epidermal cells) and only when the entire promoter was assayed (−346 to +8). In petunia, strong 35S promoter expression was observed in all cells of the petal, with expression being attributed to a synergistic interaction between elements A, B1, and B3.

To analyze the trans-factors that were interacting with the cis-elements of the 35S CaMV promoter, the authors utilized DNase footprint analysis. They determined that a nuclear transcriptional activating factor (ASF-1) was binding to TGACG upstream sequence motifs, and that the ASF-1 protein sequence implicated it as possessing a leucine zipper dimerization motif.

With the identification of 5' cis-elements in many plant experimental systems there has been a recent increase in the isolation and characterization of several plant trans-activating factors. Lam et al., (1990) have isolated the
gene encoding a tobacco DNA binding factor (3AF1), that binds to an AT rich motif of the rbcS-3A pea promoter. 3AF1 contains a zinc finger consensus sequence and shows transcriptional sensitivity in the presence of a metal chelator.

Singh et al., (1990) have reported the isolation of a gene encoding a protein (OCSBF-1) that binds to the octopine synthase enhancer (ocs), which is found in the 5' flanking region of several Agrobacterium T-DNA genes. OCSBF-1 possesses a consensus leucine zipper motif with homology to AP1 and CREB (OCSBF-1 has been shown to form heterodimers in vitro with AP1 and CREB).

The recent genetic identification and cloning of three homeotic-like mutants (the knotted locus in maize, deficiens in Antirrhinum majus, and agamous in Arabidopsis) points to the identification of three more potential transcription factors that should yield important information on the genetic control of organ pattern formation in plants.

In summary, transcriptional control in eukaryotes possess a modular organization, with cis and trans elements interacting in a combinatorial and often synergistic manner to bring about differential gene expression. The synergistic effects between two transcription elements could involve the concept of binding cooperativity. Binding cooperativity could act for example, between two transcription elements, that when present individually caused gene expression in one particular cell type, but when they were combined conferred gene expression in a third cell type. Thus, trans-factor binding (and accordingly gene expression or repression) is determined by multiple factors such as; DNA binding affinity for the particular site, cellular trans-factor
concentration, and the extent of binding cooperativity between multiple transcriptional activating proteins.

1.8 Transient Expression Assays

Transient expression assays provide an alternative system to stable transformation techniques for the analysis of plant gene expression. The transient transformation of cultured animal cells and plant protoplasts have been successfully used to define transcription control regions in many biological systems. For example, transient expression assays were utilized to analyze gene regulation by plant hormones (Marcotte et al., 1988), UV light (Lipphardt et al., 1988), heat shock (Callis et al., 1988), and anaerobic stress (Walker et al., 1987). Electroporation, polyethylene glycol treatments, and CaPO_4 precipitation have been used to transiently introduce genes into cultured animal cells and plant protoplasts. The principle advantage of transient expression assays is the rapid and facile manner in which gene activity can be measured after DNA introduction. Another advantage is that because the majority of the transferred DNA is not integrated into the chromosome (Werr and Lorz, 1986), the analysis is not subject to the influence of sequences adjacent to the integration site (position effects). Stable transformation experiments can be confounded by these position effects at the site of gene integration, which can affect the level and specificity of expression of the introduced gene (Jones et al., 1985; Poulsen et al., 1986).

However, genes introduced into plant protoplasts do not always respond to factors that regulate their expression in whole plants, and it is not clear whether regulated gene expression in cells without walls and separated
from the original tissue compares to \textit{in vivo} expression. (Callis \textit{et al.}, 1987; Ebert \textit{et al.}, 1987). Several studies have shown the limitations of transient expression assays in plant protoplasts. Vernet \textit{et al.}, (1982), working with protoplasts isolated from tobacco leaf mesophyll, demonstrated that these protoplasts do not synthesize the small subunit of ribulose-1, 5-bisphosphate carboxylase. Loveys and Robinson (1987) showed that barley leaf protoplasts do not synthesize abscisic acid in response to osmotic stress, even though leaves do. Sheen (1991) further illustrates the potential problems encountered with transient expression assays using plant protoplasts by the demonstration of metabolic repression of photosynthetic gene expression in maize protoplasts. The inhibition of photosynthetic gene expression was likely due to the presence of sucrose and/or glucose in the protoplast osmoticum. Glucose and sucrose are commonly used osmotica in tobacco protoplast culture (Nagy and Maliga, 1976).

After protoplast isolation, the cells immediately begin to resynthesize new cell walls, divide in culture, and phenotypically de-differentiate, becoming homogeneous in appearance regardless of tissue origin. The loss of the differentiated state quite probably is reflected in the loss of control elements unique to that particular tissue, thus hindering the analysis of tissue-specific or developmental gene regulatory elements.

The advent of microparticle bombardment technology has allowed the analysis of transient gene expression in intact plant tissues, enabling the researcher to circumvent some of the limitations inherent to transient expression assays utilizing plant protoplasts.
McCormick et al., (1991) utilized particle bombardment of tobacco pollen to study the expression of a pollen specific tomato promoter (LAT59). The authors also compare transient LAT59/GUS expression data with the LAT59 promoter activity in stable Agrobacterium-mediated transformation experiments. Transient assay analysis of LAT59 promoter deletions fused to the β-glucuronidase reporter gene revealed that a construct containing 115 bp of 5′ sequence was sufficient for pollen specific expression, and the -115 construct was expressed at 25% of the level observed using a -418 bp LAT59 deletion construction. A construct possessing 1.3 kb of upstream sequence did not increase GUS expression levels above the level seen with the -418 bp construction in the transient expression assays. Analysis of these same LAT59 constructions (-115, -418, and -1.3 kb) in stably transformed plants were largely in accord with the transient expression results, except that the 1.3 kb containing construct demonstrated an approximately two-fold increase in GUS expression levels as compared to the -418 LAT59 deletion construct. Thus, the transgenic plants may indicate that a quantitative expression element is located between -418 bp and -1.3 kb in the 5′ flanking region of the LAT59 promoter.

As mentioned, the utility of quantitative transient expression assays in plants is that the functionality of promoter deletion constructs can be rapidly assayed to define putative promoter regions involved in gene control. Once the promoter deletion constructions are defined as functionally important (through transient assays), their expression can be further analyzed via stable transformation experiments.
To analyze the 5' cis-sequences involved in S-locus regulation, I have utilized transient expression assays of chimeric *P. hybrida* $S_1$ promoter/GUS fusions. By using particle bombardment technology, I was able to obtain information on 5' flanking sequences that modulate the expression of the $S_1$ allele as detected in intact style tissue.

1.9 Proposal

Self-incompatibility is an attractive experimental system for studying cell/cell intercommunication, but it is also suited for carrying out molecular studies on tissue-specific and developmental gene expression. The response is under precise temporal and spatial gene control, as evidenced by the developmental potentiation of the incompatibility response 1 to 2 days prior to anthesis (dpa). The incompatibility response is also extremely localized, occurring either on the stigmatic surface of the pistil or in the transmitting tract tissue of the style, depending on the species. It was reasoned that a locus intimately involved in the self-incompatibility reaction (S-locus) would also likely reflect the expression characteristics of the self-incompatibility process.

My long-term research goal focused on delineating DNA control elements that impart precise tissue-specific and developmental regulation of the S-locus. As a prelude to analyzing the S-locus 5' flanking gene sequences, I first had to isolate and characterize putative petunia S-locus genes. To accomplish this more immediate objective, I constructed two stylar cDNA libraries. I determined DNA blot hybridization conditions to detect petunia $S_2$ and $S_3$ alleles using a heterologous probe, and then isolated the petunia $S_2$ and $S_3$
alleles. To determine whether the isolated cDNA sequences demonstrated an expression pattern consistent with S-locus function, I characterized the temporal and spatial expression patterns of the three petunia S-alleles present in our laboratory. To investigate the structure of the S-locus DNA sequences, I provided partial cDNA sequence information and determined the nucleotide sequence of 3.2 kb of S\textsubscript{1} genomic DNA. I subsequently performed RNase protection experiments to map the S\textsubscript{1} allele 5' transcriptional start(s) sites.

Following the construction of multiple S\textsubscript{1} promoter/GUS translational fusion genes, I began an analysis of the tissue specific distribution of S\textsubscript{1} gene expression using GUS histochemical localization experiments. I determined that the petunia S\textsubscript{1} allele is not transcribed in petunia pollen tissue, but is transcriptionally active in petunia style, ovary, and petal tissue.

To obtain a quantitative estimate of S\textsubscript{1} promoter regions that modulate S-locus gene expression, I developed a transient expression assay system, using biolistic microparticle bombardment technology, to introduce S\textsubscript{1} promoter/GUS gene fusions into intact petunia style cells. I determined that 68 bp of S\textsubscript{1} 5' flanking sequence is sufficient to impart correct spatial gene expression. It also appears that an element is present between bp -223 and -69 that is responsible for modulating the quantitative expression of the S\textsubscript{1} allele.
CHAPTER II
MATERIALS AND METHODS

2.1 Plant Material

*Petunia hybrida* lines characterized with respect to S-allele breeding behavior and having genotypes S$_{1.1}$, S$_{1.2}$, and S$_{3.3}$ (Flaschenriem and Ascher, 1979a, 1979b) were obtained from Dr. Peter Ascher (University of Minnesota). MSU1093, a self-compatible *Petunia hybrida* line, was obtained from Dr. Kenneth Sink (Michigan State University). A listing of all petunia lines currently in use in the laboratory is given for reference in table 1. Seeds were germinated in growth chambers on a commercial soilless seed starter mix and transferred to a soilless mixture (Fafard’s 3B) at approximately 2 weeks old and fertilized weekly (Bloom Builder; Miracle Grow). Plants were maintained under a 16 hour photoperiod in growth chambers or greenhouse at temperatures as close as possible to 20°C day/15°C night. Individual lines were maintained by vegetative cuttings and scored for self-incompatibility by hand pollinations under various environmental conditions.

2.2 Staging of Petunia Floral Development

Individual floral buds were tagged as early as they could be identified (9 days prior to anthesis, with a floral bud length = 5 mm). The length of the floral bud measured on successive days until corolla opening and anthesis.
Table 1. *Petunia hybrida* genetic lines.

Below is a description of the genotypic lines that currently exist in our laboratory, with the S\(_{1.3}\) line being isolated only recently. My research has focused on self-incompatible lines S\(_{1.1}\), S\(_{1.2}\), and S\(_{3.3}\). Two lines (MSU1093 and 80-15-5) show partial breakdown of the incompatibility response. Both lines seem to possess unilateral mutations blocking the function of either the style or pollen component of the self-incompatibility reaction. MSU1093 plants do not discriminate between self vs non-self pollen, and 80-15-5 plants produce pollen that acts as a universal pollinator.

**Petunia hybrida** Genetic Lines

<table>
<thead>
<tr>
<th>Self-Incompatible</th>
<th>S-alleles</th>
<th>Stock</th>
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<tbody>
<tr>
<td>S(_{1.1})</td>
<td>S(_1) allele, homozygous</td>
<td>87F1</td>
</tr>
<tr>
<td>S(_{1.2})</td>
<td>S(_1), S(_2) alleles, heterozygous</td>
<td>87G1</td>
</tr>
<tr>
<td>S(_{3.3})</td>
<td>S(_3) homozygous</td>
<td>87L1</td>
</tr>
<tr>
<td>S(_{1.3})</td>
<td>S(_1), S(_3) alleles Heterozygous</td>
<td>87F1X87L1</td>
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</tbody>
</table>

**Pseudo-Self Compatible**

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<table>
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<tbody>
<tr>
<td>MSU1093</td>
<td>S(_1) allele or related (stylar-part mutation)</td>
<td></td>
</tr>
<tr>
<td>80-15-5</td>
<td>S(_1) allele or related (pollen-part mutation)</td>
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</tbody>
</table>
A minimum of 20 measurements were made for each floral stage and genotype, standard deviations calculated, and cut-off values for tissue collection determined to be within 10% of the mean.

2.3 Standard Molecular Cloning Techniques

2.3.1 Molecular Biology Reagents  Unless otherwise noted, reagents commonly used in the laboratory; buffers, detergents, and salts etc. were purchased from Sigma (St. Louis, MO) or Baker (Phillipsburg, NJ) chemical companies. Gel electrophoresis reagents, phenol, and formamide were purchased from Bethesda Research Labs (Gaithersburg, MD). Guanidine thiocyanate for RNA isolations was purchased from Fluka Biochemical (Ronkonkoma, NY).

Standard recombinant DNA cloning techniques were used according to the methods of Sambrook et al., (1989). Plasmid DNA was isolated by the alkaline lysis method (Birnboim and Doly, 1979) and purified by isopycnic CsCl₂ gradient centrifugation. Enzymatic manipulations involving routine cloning, DNA restriction digestions, and DNA ligations were carried out according to the methods specified by Sambrook et al., (1989) or by the manufacturer (BRL).

2.3.2 Bacterial Transformation  To obtain maximum transformation efficiencies with the E. coli strains used (HB101, BB4, and XL1-Blue), a transformation protocol was adapted from Sambrook et al. (1989). Plasmid DNA (50-200 ng) was mixed with 100-200 µl of rapidly thawed competent E. coli cells (>10⁶ transformants/µg of supercoiled DNA) that were previously prepared by a CaCl₂ suspension method (Mandel and Higa, 1970) and frozen
at 80°C. The DNA/cell mixture was placed on ice for 30 minutes, heat shocked at 42°C for 90 seconds, and quickly transferred to ice for an additional 2 minutes. The cells were added to 1-3 ml of LB broth (10 gm, NaCl, 5 gm yeast extract, and 10 gm tryptone per liter) and agitated on a rotary shaker (200 rpm) for 1 hour at 37°C to provide time for expression of the antibiotic resistance gene. The cells were pelleted in a clinical centrifuge at 3000 x g, resuspended in 50-100 µl of LB broth, and 20-100 µl of the cells were plated onto the appropriate antibiotic.

2.3.3 Oligonucleotide Synthesis The NAS2 oligonucleotide (5'AAAAGTGATT GGCCATGTAAACACGAGTTGCATATACTCGAAAAGC3') homologous to a highly conserved region of the Nicotiana alata S2 allele (Anderson et al., 1986), and two oligonucleotides used as sequencing primers for the petunia S1 allele were synthesized on an Applied Biosystems DNA synthesizer and purified using HPLC, or in the case of the sequencing primers, by ethanol precipitation.

2.3.4 DNA Random Prime Radiolabeling Radiolabeled DNA (specific activity of > 10^9 cpm/µg) for use in DNA:DNA and DNA:RNA blot hybridization experiments was synthesized using a random hexamer protocol (Feinberg and Vogelstein, 1983). DNA fragments used for hybridization probes were isolated by agarose gel banding (Gene Clean, Bio 101). The DNA (100-200 ng in 14 µl final volume) was heat denatured and rapidly cooled to room temperature. Denatured DNA was mixed with 23 µl of LS reagent, 2 µl of BSA (225 µg/ml), 10 µl of α-32P-dCTP (6000 Ci/mmol), and 1 µl of Klenow fragment, and the reaction was incubated at room temperature for > 4 hours. The LS reagent
consists of a 25:25:7 ratio of the following 3 components; (1) 1 M Hepes (pH 6.6), (2) DTM reagent (250 mM Tris; pH 8.0, 25 mM MgCl₂, 50 mM β-mercaptoethanol, 100 mM each of dATP, dTTP, dGTP), and (3) random hexamers (Pharmacia) at a concentration of 90 O.D. units/ml. Unincorporated nucleotides were removed by chromatography through a Sephadex G-50 spun column and an aliquot counted to determine hybridization probe specific activity.

2.3.5 RNA Gel Electrophoresis  Formaldehyde gel electrophoresis was carried out according to the protocol outlined by Rave et al., (1979). Following electrophoresis, RNA was transferred by capillary blot (Thomas 1980) for >8 hours to uncharged nylon membrane (Genetran). Formaldehyde agarose gels consisted of 1% agarose, 50 mM Hepes, pH 7.8, and 1 mM EDTA, 6% formaldehyde. To prevent the volatilization of the formaldehyde, the liquefied agarose was allowed to equilibrate to 60°C prior to addition of formaldehyde. RNA samples were suspended in 2.37 µl of buffered H₂O (0.15 M Hepes, pH 7.8), and to this was added 4.63 µl of a mixture of 25% formaldehyde and 75% formamide. The RNA samples were heated at 70°C for 10 minutes and quickly transferred to ice to inhibit secondary structure formation. After heat denaturation, 1.5 µl of sample loading buffer (50 mM Hepes, pH 7.8, 3 mM EDTA, 40% sucrose, 0.5% xylene cyanol, 0.5% bromocresol green, 16.2% formaldehyde, and 50% of formamide) was added and the sample loaded onto a 1% formaldehyde gel. The gel was electrophoresed (running buffer; 50 mM Hepes, pH 7.8, 1 mM EDTA, 6% formaldehyde) at 60 volts for 4-5 hours. RNA was visualized with ethidium
bromide staining (0.25 μg/ml) for 3 minutes, followed by 1-2 hours of dH2O destaining. Size standards consisted of either a RNA (2 μg) ladder or a 1 kb DNA ladder (BRL). To visualize the DNA size standards following formaldehyde gel electrophoresis, 8 μg of the DNA ladder was dessicated and resuspended in 3 μl of 0.15 M Hepes, pH 7.8, 3 mM EDTA. Eight μl of sample loading buffer was added and the DNA heated to 70°C for 10 minutes and loaded directly onto the formaldehyde gel.

2.3.6 Dideoxy DNA Sequencing Double stranded dideoxy DNA sequencing was performed using a genetically modified T7 DNA polymerase (Sequenase) according to the vendor’s instructions (United States Biochemical, Cleveland), except that the protocol for the initial denaturing of the template DNA was modified for optimal chain elongation. The template DNA (cloned into either Bluescript or pUC 18/19 -Janisch-Perron, Vieira, and Messing, 1985) was either CsCl2 gradient purified or isolated by the Birnboim and Doly alkaline lysis method from small scale (1.5 ml mini-prep) DNA preparations. Small scale DNA isolations were further purified by selective binding to silica glass beads and subsequent elution in 10 mM Tris and 1 mM EDTA (Gene Clean, Bio 101). Three to five micrograms of template DNA was denatured in 0.2 N NaOH (20 μl final volume) for 5 minutes. The solution was then neutralized by the addition of 3 μl of 1M sodium acetate (pH 4.8), and ethanol precipitated with three volumes of 100% ethanol. Following a 70% ethanol rinse, the DNA pellet was lyophilized, and resuspended in sequencing buffer. After the addition of oligonucleotide primer, the sequencing reactions were heated (65°C) for 5 minutes and allowed to cool slowly to room temperature. Sequencing reactions were performed according to the suppliers protocol.
2.4 RNA Isolation

2.4.1 Total RNA Isolation RNA was isolated via a modification (Sims and Hague, 1981) of the method of Chirgwin et al., (1979). All RNA manipulations were performed using diethylpyrocarbonate (DEPC) treated solutions and baked glassware (> 12 hours at 270°C). Large scale total RNA isolations involving 20 to 50 gm of frozen petunia leaf or petal tissue were homogenized for 3 to 5 minutes in a Waring blender in the presence of 4-5 volumes of guanidinium thiocynate (GSCN) extraction buffer (5 M GSCN, 5% β-mercaptoethanol, 2 mM EDTA, and 50 mM Tris, pH 7.5). RNA isolations from small (200 mg to 2 gm) to intermediate amounts of tissue (5-10 gm) were extracted using a Tissumizer (Tekmar Industries). After ~2 minutes of homogenization, sodium lauryl sarcosinate (0.5% final concentration) was added and stirred at room temperature for 15 minutes. The homogenate was transferred to a DEPC treated 50 ml polypropylene centrifuge tube and centrifuged at 25,000 x g for 30 minutes at 10°C in order to pellet cellular debris. The supernatant was filtered through two layers of DEPC treated Nitex gauze (100 micron mesh) to remove flocculent matter. The supernatant was gently layered onto a 5.7 M CsCl₂ pad and centrifuged at 36,000 rpm for 18 hours at 20°C in a Beckman 70Ti rotor. Following the removal of the GSCN supernatant, the RNA pellet was resuspended in 0.5 ml 5% phenol solution (10 mM Tris, pH 7.5, 1 mM EDTA, 5% sodium lauryl sarcosinate, and 5% buffer saturated phenol). After resuspension (30 minutes at room temperature with occasional mixing), the RNA solution was extracted with an equal volume of phenol for 5 minutes. A phenol equivalent amount of
chloroform was added and the RNA was extracted for another 5 minutes. Following centrifugation, the aqueous upper phase was then removed, and 2.5 volumes of 100% ethanol added to precipitate the RNA.

2.4.2 Poly A+ RNA Isolation Poly A+ RNA was isolated by affinity chromatography over oligo dT cellulose according to the method of Aviv and Leder (1972). Typical poly A+ RNA yields were found to be between 0.6-1.5% of the total RNA passed over the column, yields higher than these values were indicative of ribosomal RNA contamination as evidenced by distinct 28S and 18S ribosomal bands on 1% formaldehyde agarose gels.

2.4.3 Petunia Germinated Pollen RNA Isolation Petunia pollen was germinated according to the method of Brewbaker et al. (19??). Petunia pollen (0.5 - 2.0 gm) was germinated by first hydrating the pollen for 1 hour at 100% humidity. This was accomplished by placing the pollen on a sheet of weighing paper positioned on a support stand, carefully placing the stand and pollen into a Nalgene dessication chamber partially filled with warm water, and then covering the chamber to allow it time to humidify. The hydrated pollen was transferred to a flask containing 30 ml of pollen germination media (10% sucrose, 0.1% boric acid, and 10 mM CaNO₃) and agitated on a rotary shaker at 120 rpm for 2-3 hours at 37°C. The pollen germination rate was 20-50% as visualized under low power on a compound microscope. Germinated pollen was collected by gently pelleting (2000 x g) the pollen in a 50 ml polypropylene tube, freezing the pellet with liquid nitrogen, and transferring the frozen pollen pellet to 10 ml of GSCN extraction buffer. The sample was homogenized for 2-3 minutes and then treated as discussed above. Non-germinated pollen was extracted and treated in the same manner.
as the other petunia tissues (style, leaf, ovary, and petal).

Optical density measurements at 260 nm were used to determine RNA concentrations with an extinction coefficient of 40 µg/ml = 1A<sub>260</sub> being used as a conversion factor.

2.5 Nucleic Acid Blot Hybridization Conditions

Blot hybridization conditions were determined from theoretical calculations outlined for RNA:DNA (Casey and Davidson, 1977) and DNA:DNA hybrids (Britten and Davidson, 1985). Low stringency (criterion) hybridizations were carried out at 30<sup>o</sup>-40<sup>o</sup>C below T<sub>m</sub>, which corresponded to typical conditions of 25<sup>o</sup>C, 1M Na<sup>+</sup>, and 50% formamide. Moderate stringency conditions consisted of hybridization temperatures approximately 20<sup>o</sup> -25<sup>o</sup>C below T<sub>m</sub>, which translated into blot conditions of 42<sup>o</sup>C, 1M Na<sup>+</sup>, and 50% formamide. RNA blot hybridizations were routinely performed in 10-20 ml of northern hybridization solution (50% formamide, 5X SSPE, 0.2% sodium dodecyl sulfate -SDS, 1X Denhardtts solution, 0.1 mg/ml poly rUTP, and 0.2 mg/ml sheared salmon sperm DNA) for > 16 hours and the filter washed multiple times (3X for 5 minutes, 2X for 1 hour) in 50% formamide, 5X SSPE, and 0.2% SDS at the identical hybridization temperature. Southern blot hybridization conditions were similar except that the poly U was omitted and the SDS concentration was increased to 1%. 
2.6 RNA Slot Hybridization

To estimate $S_1$ and $S_2$ allele message abundance in petunia lines $S_{1,1}$ and $S_{1,2}$, RNA slot hybridization experiments were carried out according to the method in Sambrook et al., (1989) with minor alterations. RNA samples containing between 1-20 μg of total RNA were dissolved in 25 μl of DEPC treated TE. Following the addition of 75 μl of a slot loading buffer (0.5 ml formamide, 0.2 ml formaldehyde, and 50 μl 20X SSPE -174 gm/l NaCl, 27.6 gm/l NaH$_2$PO$_4$:H$_2$O, 7.4 gm/l EDTA, pH 7.4), the RNA samples were heated (68°C for 15 minutes) to remove secondary structure. RNA samples were placed on ice for 2 minutes, and 0.2 ml of 20X SSPE was added to each sample. Sample wells (Sarstedt vacuum slot blot apparatus) were washed 2-3 times with 0.4 ml of 10X SSPE. During the washes, the vacuum pressure was adjusted so that it took approximately 3 minutes for each well to clear, the suction pressure is critical for even distribution of the RNA sample to the membrane surface. High vacuum pressures often resulted in halo-like ring images on autoradiographic film indicative of uneven RNA deposition, with the majority of the signal evident along the sides of the wells. RNA samples were pipetted into individual wells, and following well clearing, an additional 0.4 ml of 10X SSPE was added to thoroughly wash the sides of the wells. Following air drying the membrane was baked in a vacuum oven for 2 hours at 80°C.
2.7 Plasmid rescue

Plasmid rescue ("zapping") is a term used to describe the process for the \textit{in vivo} excision and re-circularization of cloned inserts from recombinant \(\lambda\) Zap phage molecules into the a recombinant Bluescript phagemid cloning vector. The process of \textit{in vivo} excision is precipitated by the super-infection of a f1 helper bacteriophage (filamentous phage R408). R408 derived replication proteins act on the f1 origin of replication for positive strand synthesis. Sequences within the \textit{ori} can be divided into two overlapping elements; (1) an initiation sequence, and (2) termination sequences, that signal the end of positive strand synthesis. In the \(\lambda\) Zap cloning vector these origin of replication sequences (initiation and termination) have been separated, with polylinker sequences cloned between them. Through the process of simultaneous infection of \textit{E. coli} cells with \(\lambda\) Zap and R408, helper replication proteins (R408 derived) act at the initiation sequences within \(\lambda\) Zap, and cause a single stranded endonuclease nick. Subsequently, replication of all DNA sequences downstream from the initiation element occurs until the termination element is contacted. The recombinant single stranded replication product is circularized by the gene II product, supplied by the helper bacteriophage, and all sequences of the Bluescript phagemid are replicated along with the recombinant insert. Signals for "packaging" of the phagemid are located within the termination sequences, which results in the secretion of the single stranded phagemid into the supernatant. After isolation of the single stranded phagemid, double stranded Bluescript can be generated by re-infection of new \textit{E. coli} host cells.
The actual methodology involved coring from an agar plate the plaque of interest, suspending in 0.5 ml of SM buffer (5.8 gm NaCl, 2.0 gm MgSO₄, 50 mM Tris; pH 7.5, and 5 ml of 2% gelatin per liter), and 20 μl of chloroform. In a 50 ml tube, 0.2 ml of the plaque plug supernatant was added to 0.2 ml of OD₆₀₀=1 XL1-Blue or BB4 E. coli cells, and 10 μl of R408 phage stock (>1X10⁶ pfu/ml). The co-infection solution was incubated at 37°C for 15 minutes. Five ml of 2X YT media (10 gm NaCl, 10 gm yeast extract, and 16 gm bacto-tryptone per liter) was added to the cells and allowed to incubate at 37°C for 4 hours. The tube was then heated at 70°C for 20 minutes to kill the E. coli cells, and the cellular debris was pelleted by centrifugation at 4000 x g for 5 minutes. The supernatant was removed (contained the single stranded phagemid) and an aliquot was used to reinfect new E. coli cells. A mixture of 0.2 ml of OD₆₀₀=1 XL1-Blue (BB4) with 20 μl of a 10⁻² dilution of the phagemid supernatant was combined, and incubated at 37°C for 15 minutes. Finally 5 μl of this solution was spread on LB/ampicillin plates.

2.8 Exonuclease III DNA Deletion Construction

Two unidirectional S₁ promoter/GUS deletion series were synthesized by using the exonuclease III unidirectional deletion method of Henikoff (1987), according to the protocol supplied by the commercial vendor (Promega, Erase-a-Base deletion kit). Exonuclease III specifically digests DNA from a 5’ protruding end (overhang) or blunt end and will not digest DNA that possesses a four base 3’ overhang. Thus to efficiently generate a deletion series, there should exist properly positioned flanking restriction sites that
yield a 3' overhang to protect the "vector end" from digestion, and a 5'
protruding end to act as the substrate for exonuclease III digestion. The
protocol used to generate a large scale deletion series (200-300 bp increments)
was as follows; after restriction digestion with enzymes (ClaI and Kpnl) that
produce the respective 5' and 3' restriction site overhangs, the enzymes were
removed by phenol extraction and ethanol precipitation. Lyophilized DNA
was dissolved in 60 µl of exonuclease III digestion buffer (66 mM Tris; pH 8.0,
0.66 mM MgCl2), concurrently, individual time point reactions (10-20) were
prepared, with each reaction containing 7.5 µl of a S1 nuclease mixture (172 µl
dH2O, 60 units S1 nuclease, and 27 µl of 7.4X S1 buffer - 0.3 M potassium
acetate, pH 4.6, 2.5 M NaCl, 10 mM ZnSO4, and 50% glycerol) that was then
placed on ice. S1 nuclease possesses a single-stranded nuclease activity that
degraded the DNA strand not affected by the single strand exonuclease activity
of exonuclease III. An approximate rate of digestion of 400 bp per minute was
observed under the reaction conditions used (300 units of exonuclease III at
35°C). After the addition of the exonuclease III enzyme, 2.5 µl of the digestion
were removed at 30 second intervals and mixed with the iced S1 nuclease
mix. The individual S1 nuclease digestion tubes were placed at 23°C for 30
minutes and then 1 µl of S1 stop buffer (0.3 M Tris, and 50 mM EDTA) was
added. The reactions were then heated to 70°C for 10 minutes to inactivate
the S1 nuclease. Two microliters from each time point were transferred to a
1% agarose gel to verify processive deletion of the DNA. Time point reactions
were transferred to 37°C and 1 µl of Klenow fragment was added and
incubated for 3 minutes. Then 1 µl of 10 mM dNTP's was added to each
reaction and the incubation continued for an additional 5 minutes, this was
done to ensure the complete synthesis of flush ends which are required for
efficient intramolecular ligation. Ten µl of a ligation mix (20 units of T4 DNA
ligase, 25% PEG, 10 mM DTT, 50 mM Tris, pH 7.5, 10 mM MgCl₂ and 1 mM
ATP in a final volume of 200 µl) was then added to each sample, and the
ligation reactions were incubated for 1 hour at 23°C, followed by
transformation of competent E. coli cells. A fine scale (increments of ~50 bp)
deletion construction was also synthesized, except that exonuclease III
deletion conditions were altered slightly so that fewer base pairs would be
deleted per time point (20 second time points at 30°C).

2.9 S₁ Allele 5' End Mapping Using RNase Protection

2.9.1 In Vitro RNA Synthesis To identify the S₁ transcriptional start using
RNase protection experiments, in vitro synthesized RNA possessing a high
specific activity was required. The RNA probe used to protect the S₁ 5'
transcription start site was synthesized from a 554 bp PstI restriction fragment
(S₁ genomic fragment) that was thought to span the presumptive start site.
The S₁ 554 bp PstI fragment was subcloned into the PstI site of Bluescript, to
yield the subclone termed S₁ PstI 600 (derivation diagramed in figure 33). S₁
PstI 600 was linearized internally at the DraI restriction site, and used to
generate a radioactive (α-³²P rGTP, 800 Ci/mM) antisense transcript (358 nt)
from the T3 promoter according to the suppliers instructions (Stratagene).
The template DNA was prepared for RNA synthesis as follows:, 10 µg of DraI
digested S₁ PstI 600 was treated with proteinase K (50 µg/ml final
concentration) for 30 minutes at 37°C, phenol/chloroform extracted (1:1), and ethanol precipitated. The actual synthesis reaction contained 1 µg of Dral digested S1 PstI 600 DNA template, 40 mM Tris, pH 8.0, 8 mM MgCl₂, 2 mM spermidine, 50 mM NaCl, 10 mM each of UTP, rATP, rCTP, 15 µl of α-32P rGTP, 800 Ci/mM (7 mM), 10 units of RNase-Block (ribonuclease inhibitor - Stratagene), 1 µl 0.75 M DTT, 10 units of T3 polymerase in a final volume of 25 µl. The reaction was incubated at 37°C for 30 minutes (an additional spiking with 10 mM non-radioactive rNTP's after 15 minutes is thought to increase the proportion of full length transcripts, V. Walbot personal communication). To degrade the DNA template, the sample was digested with 10 units of RNase-free DNase for 15 minutes at 37°C. Template degradation is necessary because if not removed it could hybridize to the RNA transcript, possibly producing artifactual protected fragments. The RNA run-off was phenol/chloroform extracted (1:1) and passed directly over a DEPC treated G-25 Sephedex (Pharmacia) spun column and the elutant ethanol precipitated. The RNA probe was resuspended in 10-100 µl of TE and 1 µl was counted in a scintillation counter to calculate the specific activity and concentration of the radiolabeled RNA. Typical specific activities obtained were routinely 1 x 10⁹ cpm/µg of RNA probe. RNA protection probes were used in hybridization experiments within 2 days of synthesis.

In addition to the synthesis of radiolabeled RNA, non-labeled RNA was synthesized for use in RNA reconstruction experiments to estimate the message prevalence of the S₁ and S₂ alleles in developmental RNA slot hybridization experiments (chapter 4), and differed only in the substitution of
non-labeled rGTP.

2.9.2 RNase Protection Experiments Experiments to map the $S_1$ transcriptional start site were carried out, with slight modifications, according to the method of Sambrook et al. (1989). Hybridization reactions between radiolabeled RNA run-off probes (S1 PstI 600 derived) and total RNA isolated from S$_1$, style and petal tissue were performed under moderately stringent conditions (53°C, 0.4 M Na$^+$, 50% formamide), which was 25°C below the calculated T$_m$. This was followed by a RNase digestion (RNase A and T1) to degrade non-hybridized single stranded RNA. The protection reaction was ethanol precipitated and run on a 6% denaturing acrylamide gel and the size of the protected fragments were estimated from a comparison with size standards.

To achieve the strongest signal to noise ratio between the full length protected fragments versus partial hybrids or non-full length in vitro synthesized RNA, many parameters were defined on an empirical basis. The hybridization conditions used consisted of between 1-10 µg of total style RNA or 40-100 µg of total petal RNA hybridized to a 3-5 fold molar excess of in vitro synthesized RNA probe over that of the target sequence. Approximately $3 \times 10^6$ cpm of radiolabeled RNA (specific activity of $1 \times 10^9$/µg = 3 ng of probe) was hybridized to varying amounts of style or petal total RNA. The hybridization reaction consisted of 50% formamide, 40 mM PIPES; pH 6.4, 1 mM EDTA, 0.4 M NaCl, in a final volume of 30 µl. The hybridization mixture was incubated at 85°C for 10 minutes to denature the RNAs and then was transferred to a 53°C water bath and allowed to anneal for 2-3 hours.
After cooling the reaction to 23°C, the nonhybridized RNA was digested by the addition of 0.3 ml digestion buffer (0.3 M NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA, 2 μg/ml RNase T1, and 100-200 μg/ml RNase A) at 35°C for 1 hour. After digestion, 20 μl of 10% SDS and 10 μl of proteinase K (10 mg/ml) was added and the sample incubated for 30 minutes 65°C to inactivate the ribonucleases. The sample was phenol/chloroform extracted and ethanol precipitated after the addition of 20 μg of carrier tRNA. The sample was re-hydrated in 10 μl of formamide gel loading buffer (80% formamide, 10 mM EDTA, 0.5% xylene cyanol and 0.5% bromophenol blue) and heat denatured at 95°C for 5 minutes and then placed on ice until being loaded (5 μl) onto a 6% polyacrylamide/7 M urea gel. DNA dideoxy sequencing reactions were run concurrently as size standards.

2.10 Tissue and DNA Preparation for Biolistic Bombardment

Cesium chloride purified DNA (1-10 μg) was precipitated onto gold or tungsten (1-3 micron in diameter) microcarrier particles according to the method of Klein et al., (1988). Particles were suspended > 4 hours in 100% ethanol at a concentration of 45 mg/ml. Following occasional vortexing to disperse the particles, the microcarriers were pelleted in a tabletop microfuge for 5 minutes at 12,000 x g. After removal of the ethanol supernatant, the microcarriers were resuspended in an equal volume of dH₂O and pelleted as above. This process was repeated two more times to remove all traces of ethanol. Finally, the particles were suspended in 50% glycerol to ameliorate the problem of particle conglomerization when DNA was added.
Absorption of DNA to the gold or tungsten particles was done as follows; 1-10 µg of DNA (volume of 3-6 µl) was added to 25 µl of the vortexed particle/glycerol suspension, CaCl₂ (25 µl of 2.5 M) and spermidine-free base (10 µl of 100 mM) were rapidly added and the mixture briefly vortexed and placed on ice for 10 minutes with intermittent agitation to ensure complete mixing. The particles were pelleted by a 1 second pulse in a microfuge, a portion of the supernatant was removed so that only 12 µl remained. Particles were kept on ice and were used within 1 hour of preparation.

Immediately prior to bombardment a tube containing the particle preparation was vortexed and then quickly scraped against a microfuge tube holder to mix the particle precipitate, this produced a characteristic splattering of the preparation on the sides of the microfuge tube which was indicative of a properly mixed sample. Immediately after agitation, 2.8 µl of the suspension was placed on the surface of a cylindrical polyethylene macrocarrier. The macrocarrier (containing the DNA/particle droplet) was loaded into the barrel of the PDS-1000 biolistic particle gun (DuPont) and a 22 caliber blank ammunitions charge was placed directly behind the macrocarrier. The sample chamber containing the tissue of interest (positioned 10 cm below the end of the barrel) was evacuated to 20-22 millitorr and the device discharged. Nylon mesh (100 µm pore size) was positioned below the barrel aperature to promote particle dispersal.

Tissue sample preparation for particle bombardment consisted primarily of excising the appropriate organ (leaf, petal, pistil, pollen and anther) from the plant and placing it on MSO nutritive agar (Horsch et al., 1988). Petal, leaf, (axial side up) and anther tissue were placed directly onto
the agar surface. Excised pistils (22-25 per plate) were placed in a circular pattern so that the basal style portion of the pistil (nearest the ovary) would penetrate the agar and thus the stigmatic surface was positioned parallel to the agar surface. Pollen (germinated or ungerminated) was pipetted unto the surface of nitrocellulose membranes, that were positioned to rest on top of a sterile piece of 3MM Whatman filter paper already placed on the agar surface. The nitrocellulose filter provided structural support and facilitated the examination of the pollen after histochemical staining.

2.11 Histochemical Localization of Transient GUS Activity

Following particle bombardment, tissue samples were allowed to express the introduced β-glucuronidase (GUS) reporter gene constructs for 24-36 hours under ambient light conditions (except germinated pollen was allowed to transiently express the introduced gene construct for 4 hours). GUS enzyme activity was localized to individual petunia cells according to the method of Jefferson (1987b). Histochemical localization of GUS activity is a result of the reaction catalyzed by GUS on the substrate 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc), which is visualized as the formation of an indigo dye precipitate at the site of enzymatic activity. The direct product of the reaction between GUS and X-Gluc is colorless, and the indoxyl derivative that is produced must undergo an oxidative dimerization to yield the insoluble dye. The addition of oxidizing agents (potassium ferricyanide and potassium ferrocyanide) are commonly used to stimulate the reaction and have been included in the histochemical assay buffer (1 mg/ml X-Gluc, 100 mM NaPO₄, pH 7.5, 10 mM EDTA, 5 mM each of potassium ferricyanide and
potassium ferrocyanide, and 0.1% Triton). Whole pistils, anthers and pollen were placed in 1.5 ml microfuge tubes containing 0.5-1 ml of X-Gluc assay buffer and incubated at 37°C for 4-24 hours. Petal and leaf tissue were cut up into 1 cm squares and stained as above. Samples were analyzed under a dissecting microscope for indigo colored expression units (individual cells or small aggregates) and photomicrographs were taken using Zeiss Photomicroscope III (compound) or a Wild M54 dissecting microscope.

2.12 GUS Fluorometric Determination

To obtain quantitative GUS measurements in the transiently expressing petunia tissue, fluorometric analysis was performed essentially as described by Jefferson (1987a). Assay protocols were modified slightly due to the necessity of co-bombardment experiments involving two reporter gene constructs (GUS and beetle luciferase). A CaMV 35S/luciferase gene (luc) construct (pDO432, Ow et al., 1986 was co-bombarded with various S1 GUS constructs. The purpose of the luciferase construct was to provide an internal control which could be used to normalize plate-to-plate fluctuations in bombardment transformation efficiencies. After 24-36 hours of transient gene expression, the tissue (weighed on a per plate basis) was frozen in liquid nitrogen, ground to a fine powder, and transferred to a sterile 15 ml Corning polypropylene tube. Two milliliters of extraction buffer (100 mM NaPO4, pH 8.0, 10 mM DTT, 1 mM EDTA, and 0.1% Triton) were added and homogenized using the Tissumizer for 2 minutes. Particulate matter from the crude extracts was pelleted in a clinical centrifuge at 3000 x g for 5 minutes. The supernatant (1.5 ml) was transferred to a 1.5 ml microfuge tube and spun at
12,000 x g for 10 minutes in a refrigerated microfuge. The clarified crude protein extract was split into two aliquots and independently assayed for GUS and luciferase activity. Due to the stability of β-glucuronidase, the protein extract used for GUS enzyme quantification was routinely stored at -70°C and assayed at a later time (within 1 week), with only a slight decrease in activity (~5%) as compared to fresh extract assays.

Fluorogenic GUS assays consisted of mixing the protein extract with an assay solution containing the fluorogenic substrate 4-methyl umbelliferyl glucuronide (MUG) and measuring the concentration of product (4-methyl umbelliferone; MU) produced, by measuring fluorescence at 455 nm (excitation 365 nm) on a Perkin-Elmer LS-5 fluorescence spectrophotometer. The reaction contained 0.55 ml of prewarmed (37°C) GUS assay buffer (22 mg/ml MUG dissolved in 100 mM NaPO₄, pH 8.0, 10 mM DTT, 10 mM EDTA, and 0.1% Triton) To this was added 0.4 ml of protein extract and the reaction allowed to proceed at 37°C. Timed aliquots (t = 0, 1 hour, and 2 hour) of 0.3 ml were removed and added to 1.7 ml of 0.2M NaCO₃, the NaCO₃ serves to ensure that the hydroxyl group on the product (MU) is ionized, which is necessary for maximal fluorescence. Calibration of the spectrophotometer was done as directed by Jefferson (1987a), and raw fluorescence units converted to pmol MU hr⁻¹ mg⁻¹ protein using a 10 nM concentration of MU as being equal to 1000 fluorescence units.
2.13 Luciferase Enzyme Assay

Luciferase activity was measured in clarified crude protein extracts by using a luciferase assay buffer (20 mM Tricine, pH 7.8, 10 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A, 470 μM luciferin, 530 μM ATP) essentially as described by Wood (1990). The procedure involved introducing 100 μl of the crude protein extract (assayed within 1 hour after extraction) into glass tubes containing 0.5 ml of the assay buffer and immediately assaying for luminescence in a Monolight 350 luminometer. An integration mode on the machine conveniently allowed for a single digital reading over the first 10 seconds of photon production. Two or three replicate readings were taken for each sample with an average luciferase value being reported.
3.1 Overview

The isolation of several S-alleles from *Petunia hybrida* was facilitated by a cloning strategy that took advantage of the existence of a conserved N-terminal region in S-allele proteins from *Nicotiana alata* and *Lycopersicon peruvianum* (Mau et al., 1986; figure 6). Our laboratory had a 45-mer oligonucleotide (NAS2; methods section 2.3.3) synthesized, which was homologous to the S2 allele from *Nicotiana alata* (Anderson et al., 1986), and used the 45-mer as a heterologous probe to identify related sequences in *Petunia hybrida*. As shown in figure 7, radiolabeled NAS2 oligonucleotide was used as a probe, under conditions of low stringency (24°C, 1M Na+, 50% formamide), for a nylon filter containing 2 µg of poly A+ RNA from S1.2 style, S1.2 leaf, S3.3 style, S3.3 leaf, S3.3 petal and 10 µg of poly (A-) S1.2 leaf RNA. NAS2 specifically hybridized to a 900 nt transcript found in S1.2 and S3.3 style poly A+ RNA, with the strongest hybridization detected in S1.2 style RNA. Two additional higher molecular weight bands are also evident. Based on their size and strong presence in leaf A- RNA, they were identified as 18S and 28S ribosomal RNAs, visible because of non-specific hybridization due to the reduced stringency conditions. Once the empirical hybridization conditions were determined, I used these conditions to screen two style cDNA libraries.
Figure 7. Hybridization of NAS2 oligonucleotide to *Petunia hybrida* RNA.

Polyadenylated RNA (2 μg from each organ indicated) and poly A- RNA (10 μg from *S*₁₂ leaves) was electrophoresed on a 1% agarose-formaldehyde gel, blotted to a nylon membrane. The filter was hybridized with the NAS2 45-mer oligonucleotide, under conditions of low stringency (24°C, 1M Na⁺, and 50% formamide). The weak background bands are due to non-specific hybridization with ribosomal RNA.
3.2 Style cDNA Library Construction

3.2.1 Introduction  S1.2 and S3.3 petunia style cDNA libraries were synthesized from poly A+ RNA isolated from mature pistil tissue as diagrammed in figure 8. Synthesis of double stranded cDNA was accomplished using the protocols of Okayama and Berg (1982), and modified by Gubler and Hoffman (1983). Construction of the cDNA library entailed a series of enzymatic reactions to convert mRNA into complementary copy double stranded DNA (cDNA). The steps performed in the synthesis are outlined for reference in figure 8.

Polyadenylated transcripts were copied using one of two different viral reverse transcriptases (avian myeloblastosis virus; AMV or mouse Moloney leukemia virus; MMLV), into complementary first strand DNA. Second strand DNA synthesis was facilitated by the action of RNase H (specifically degrades RNA in RNA:DNA hybrids creating nicks and gaps) on the RNA template strand, which provides 3' OH priming sites for DNA synthesis by E. coli DNA polymerase I.

An adaptation of the methods of Neve et al., (1986) and Gubler (1987) were utilized for the efficient creation of blunt ended cDNA molecules using a T4 DNA polymerase end fill in reaction.

To prepare the flush ended cDNA for ligation into a λ phage vector, an adapter ligation step was performed using the conditions outlined by Wu et al., (1987) to provide the blunt ended cDNA with EcoRI restriction site ends. Excess unligated adapters were removed using chromatography over Sepharose CL-4B (Esschenfeldt and Berger, 1987). The cDNA was then ligated
Stylar cDNA Library Construction Flow Chart

1st strand cDNA synthesis (20-50% yield)

2nd strand cDNA synthesis (75-95% yield)

Blunt end polishing protocol (50-95% recovery)

Adapter construction:
Radiolabeling 6-mer
NENsorb purification
Anneal with 10-mer

Adapter ligation

Removal of un-ligated adapters using CL-4B column

Phosphorylation of Adapter/cDNA

λ Zap EcoRI arm ligation

λZap packaging and library titer

Plasmid rescue and DNA insert analysis

Library amplification

Figure 8. Flow chart for stylar cDNA library construction.

Outlined above are the sequential enzymatic reactions performed for the synthesis of two full-length style cDNA libraries from the S1,2 and S3,3 Petunia hybrida lines (as discussed in section 3.2).
with EcoRI predigested arms and packaged into λ Zap vector according to the specifications of the manufacturer (Stratagene).

3.2.2 First Strand cDNA Synthesis First strand cDNA was synthesized from 5-10 μg of S1.2 or S3.3 style poly A+ RNA. The reaction consisted of poly A+ RNA (final concentration of 50 μg/ml), 5 mM DTT, 100 units/ml RNase Block (Stratagene), 50 mM Tris; pH 7.5, 6 mM MgCl2, 100 μg/ml oligo dT, 40 mM KCl, 500 μg M dATP, dCTP, and dTTP, 200 μM dCTP, 6.25 μM 3H-dCTP, 4 mM sodium pyrophosphate in a final volume of 200 μl. The reaction was pre-incubated at 42°C for 5 minutes, and then avian myeloblastosis virus (AMV) reverse transcriptase (400 units/ml; Seikagaku America) was added and the reaction allowed to proceed for 1 hour at 42°C. The synthesis was stopped by the addition of 100 mM EDTA, and phenol/chloroform (1:1) extraction. After ethanol precipitation and resuspension in 100 μl of TE, the sample was passed over a Sephadex G-50 spun column to remove unincorporated nucleotides. A aliquot of the synthesis (spotted on DEAE-81 paper and washed) was counted (tritium channel) to calculate percentage yield and specific activity. First strand cDNA was visualized by fluorography (50,000 cpm), to estimate the overall size distribution of the first strand cDNA and to confirm that it reflected the size distribution of the template mRNA population.

3.2.3 Second Strand Synthesis Template cDNA (1-2 μg first strand cDNA) for second strand synthesis was used at a concentration of 2.5 μg/ml. The reaction consisted of 20 mM Tris, pH 7.6, 5 mM MgCl2, 10 mM NH4SO4, 100 mM KCl, 1 mM DTT, 150 μM β-NAD, 50 μg/ml BSA, 40 μM dNTP's, 32P-dCTP
(40 µCi/ml). The reaction was pre-incubated at 12°C for 3 minutes and then 8.5 units/ml RNase H, 230 units/ml E. coli DNA polymerase I, and 10 units/ml E. coli DNA ligase were added and the reaction incubated for 1 hour at 12°C. The incubation temperature was then raised to 20°C and the reaction allowed to continue for another hour. Second strand synthesis was stopped by the addition of 50 mM EDTA and was followed by a phenol/chloroform extraction. After ethanol precipitation, the cDNA was resuspended in 100 µl of TE and passed over a Sephadex G-50 spun column. The sample was counted in both ³H and ³²P channels and estimates on yield and specific activity were obtained. The length of second strand cDNA was visualized using standard autoradiography (10,000 cpm) techniques (Sambrook et al. 1989). This allowed for a comparison between the size distribution of the mRNA, first strand cDNA, and second strand cDNA.

The cDNA ends were made flush by utilizing an end fill-in reaction, this reaction consisted of double stranded cDNA (0.1-1.0 µg), 310 µM of each dNTP, 40 mM Tris-acetate; pH 7.8, 80 mM KC₂H₃O₂, 12 mM Mg(C₂H₃O₂)₂, 0.6 mM DTT, 100 µg/ml BSA, 62 µM β-NAD, 100 ng/ml RNase A, 50 units/ml RNase H, 250 units/ml E. coli DNA ligase, 100 units/ml T4 DNA polymerase. The components were added in the order listed and incubated at 37°C for 45 minutes. The cDNA was phenol/chloroform extracted and ethanol precipitated. After dessication, the cDNA was resuspended in 100 µl of TE, the sample was passed over a Sephadex G-50 spun column, percent recovery was determined by scintillation counting.
3.2.4 cDNA Adapter Construction and Ligation  EcoRI adapters were constructed by annealing a Smal 6-mer (5'OH-CCCGGG3' with a EcoRI 10-mer (5'OH-AATTCCCGGG3'), which generates a molecule possessing an EcoRI overhang on one end with the opposing end having a blunt end. Due to initial difficulties in obtaining high adapter/cDNA ligation efficiencies, an alternative radiolabeling strategy was employed to more closely monitor the effectiveness of the adapter/cDNA ligation reaction. After standard first strand cDNA synthesis, two second strand cDNA reactions were performed. The first was a "tracer reaction" where a small amount of radiolabeled second strand cDNA was synthesized so that I could visualize the extent of synthesis by autoradiography. The second and principle reaction omitted $^{32}$P-dCTP and thus a non-labeled second strand was synthesized, recovery and percentage yield could still be calculated based on tritium counts. Radiolabeled ($^{32}$P) adapters were synthesized and subsequently used to monitor the ligation reaction by analyzing the amount of $^{32}$P that was incorporated into the cDNA fraction ($^{3}$H).

A Smal 6-mer possessing a 5' OH group was radioactively end labeled with $\gamma$-$^{32}$P ATP (Cobianchi and Wilson, 1987). Briefly, 1 $\mu$g of Smal 6-mer was combined with 12.8 $\mu$l $\gamma$-$^{32}$P ATP (6000 Ci/mmole), 50 mM Tris; pH 7.5, 10 mM MgCl$_2$, 5 mM DTT, 100 $\mu$M spermidine, 100 $\mu$M EDTA, 20 units of T4 kinase in a final volume of 40 $\mu$l and incubated for 30 minutes at 37°C. An additional 1 unit of T4 kinase, 1 $\mu$l kinase buffer, and 100 mM ATP in a final reaction volume of 50 $\mu$l was added to ensure all 6-mer molecules had phosphorylated 5' ends. The reaction was incubated an additional 30 minutes
at 37°C. Unincorporated nucleotides were removed by passing the reaction over a NENsorb column (DuPont) and bound 6-mers were eluted off of the column in 50% ethanol according to the suppliers instructions (DuPont). The sample was dessicated in a Savant speed-vac and annealed with an equimolar amount of 10-mer (assuming a 50% recovery off the NENsorb column) to produce the EcoRI adapter.

The radiolabeled EcoRI adapter was ligated to the blunt ended cDNA according to the protocol of Wu et al., (1987). Based on theoretical considerations for blunt end ligations (Wu et al., 1987), it is advisable to have a 50-100 fold molar excess of adapters over cDNA ends to increase the efficiency of ligation. The reaction consisted of 200 ng of blunt ended cDNA, ~50 fold molar excess (175 ng) of the 16 nucleotide adapter, 66 mM Tris; pH 7.6, 10 mM MgCl₂, 3 mM ATP, 200 μg/ml BSA, 1 mM hexamine cobalt chloride, 1 mM spermidine, 10 mM DTT, 150 units/ml of T4 DNA ligase in a final volume of 20 μl. The stoichiometry of the above reaction is as follows; the 200 ng of cDNA possesses 0.672 pmoles of ligatable ends assuming there are 1.68 pmoles of cDNA/μg (calculations assume a modal cDNA length of 900 bp). The 16 nt EcoRI adapter possesses ~190 pmoles of ligatable ends/μg, thus in the reaction there are ~33 pmoles of adapter and 0.672 pmoles of cDNA ends. The ligation reaction was incubated at 15°C for 6-10 hours and then passed over a Sepharose CL-4B column to remove the unligated adapters. Following size fractionation, the ³²P/³H peak was collected and ethanol precipitated.
Prior to λ Zap arm ligation, the 5'-OH group on the EcoRI overhang (present as a result of the adapter construction strategy) was phosphorylated, this was accomplished using the kinasing protocol outlined above (Cobianchi and Wilson, 1987) except 10 mM ATP was substituted for the radiolabeled ATP. Following heat inactivation of the T4 kinase (65°C for 10 minutes), the phosphorylated cDNA was recovered using a NENsorb column. The bound cDNA was eluted in 50% ethanol, and quantitated by scintillation counting.

3.2.5 λ Zap Vector Ligation  λ Zap EcoRI restricted arms (500 ng) were ligated to an equimolar amount (12.5 ng) of adapter/style cDNA. The conditions used for the ligation reaction were: 50 mM Tris; pH 8.0, 7 mM MgCl₂, 1 mM DTT, 1 mM ATP, and 3 units of T4 DNA ligase (high specific activity; New England Biolabs) in a final volume of 5 µl. The ligation was incubated at 12°C for 12 hours. Packaging of the recombinant λ Zap DNA was carried out according to the instructions supplied by the manufacturer (Gigapack, Stratagene). Both cDNA libraries were amplified to titers of 3x10¹⁰ pfu/ml and stored over chloroform at 4°C.

3.3 Oligonucleotide Hybridization Parameters  The successful isolation of the petunia S-alleles relied on the empirical identification of the proper hybridization conditions between the NAS2 45-mer oligonucleotide and the cognate sequence in the three S-alleles. I performed RNA blot hybridization experiments using the NAS2 45-mer with petunia RNA immobilized on filter membranes, and did not detect any hybridization signal under
conditions of 30°-35°C, 1M Na+, and 50% formamide. When the stringency conditions were lowered to 24°C, 1M Na+, 50% formamide, I detected strong hybridization to a style-specific mRNA. A calculation of duplex stability to estimate the melting temperature (T_m) was performed based on the formula derived by Meinkoth and Wahl (1984). A T_m of 62.2°C was calculated for the NAS2 oligonucleotide under the conditions of 1M Na+, 40% G+C content, 50% formamide, and a length of 45 nucleotides. The maximal rate of hybridization has been empirically determined to occur at 25° below T_m. Thus, the maximal rate of hybridization for a perfectly matched NAS2 hybrid would be at 37.2°C. Another parameter affecting the correct hybridization conditions is the fact that base pair mismatches lower the T_m of the duplex. For long hybrids (>150 bp) the T_m is reduced 1°C for every 1% base mismatch, while the T_m for duplexes <20 bp in length decrease approximately 5 degrees for every 1% mismatch. If one assumes 10% mismatch with the NAS2 heterologous probe, this would reduce the T_m 10° to 15°C resulting in a optimal hybridization temperature of between 22°-27°C. This exercise in hybridization kinetics points to the utility of performing T_m and rate calculations, both of which can provide useful information in determining successful hybridization conditions.

A comparison of the NAS2 45-mer with the hybridizing S-allele sequences revealed that the PS1B clone was 67% (30/45) homologous over the entire 45 bases, and the PS2A and PS3A shared 71% (32/45) nucleotide identity. A comparison of gene sequences that have been isolated through the
use of long (>20 bp) oligonucleotide probes has revealed that to clone the corresponding gene sequences it is the number of contiguous base pair matches rather than overall homology that is the critical factor for hybrid formation (Wood, 1987). Empirically it seems necessary to have a minimum of 11 continuous bp matches. Several examples illustrate this sequence homology requirement: (1) a trypsin inhibitor gene was isolated by a 5X11X8 match (X is a mismatch), (2) an insulin-like growth factor -5X11X2X5X8X5, (3) tumor necrosis factor α -8X17, and (4) a β-insulin receptor -5X12. The PS1B sequence hybridized to the NAS2 45-mer in an 8X11 pattern, and the PS2A and PS3A clones possessed a 2X7X12 match pattern.

3.4 $S_{3,3}$ cDNA Library Screen

The $S_{3,3}$ library possessed ~ 2x10^5 recombinant phage (47% of total phage were recombinant) with a mean insert size of 1.8 kb, as determined by DNA restriction analysis on 18-20 randomly chosen phage plaques. Based on observed NAS2 hybridization to a $S_{3,3}$ style transcript, I screened the $S_{3,3}$ amplified library (total of 10,000 recombinant phage) with the NAS2 oligonucleotide under conditions of reduced stringency (24°C, 1M Na+, and 50% formamide), and was able to detect several positive plaque hybridization signals. Four recombinant phage were purified by plaque purification techniques (Benton and Davis, 1977), and our laboratory focused on the largest recombinant insert (PS3A, 860 bp) for further study.
3.5 $S_{1.2}$ cDNA Library Screen

The $S_{1.2}$ library consisted of $\sim 1 \times 10^5$ recombinant phage (65% recombinant), with a mean insert size of 1.5 kb. These values are based on DNA restriction analysis of 18-20 randomly chosen plaques. Prior to the determination of the correct oligonucleotide hybridization conditions, I screened the non-amplified $S_{1.2}$ style library twice (20,000 total recombinant phage screened) with the NAS2 oligonucleotide (Benton and Davis, 1977) at 30°C, 1M Na+, 50% formamide and did not observe any positive plaque hybridization. However, a soybean chlorophyll a/b cDNA clone (Walling et al., 1986) that was used as a positive control did detect numerous positive plaque signals, indicating perhaps that the hybridization conditions were not optimized. J. Okuley subsequently rescreened the $S_{1.2}$ cDNA library at the reduced stringency conditions (24°C, 1M Na+, and 50% formamide) and identified a cDNA sequence (PS1B) corresponding to the $S_1$ allele. To isolate the $S_2$ allele, the $S_{1.2}$ style cDNA library was re-screened with the PS3A cDNA. The rational for this approach was based on RNA blot hybridization data (figure 9) which indicated strong cross homology between the $S_2$ and $S_3$ alleles. Following plaque filter hybridization at moderate stringency conditions (40°C, 1M Na+, and 50% formamide), several positive plaque signals were detected; all putative $S_2$ recombinants were of similar size and possessed identical restriction maps, the largest cDNA insert (858 bp) was chosen (PS2A) for further analysis.
CHAPTER IV
CHARACTERIZATION OF S-LOCUS SPATIAL
AND DEVELOPMENTAL EXPRESSION

4.1 Overview

The process of self-incompatibility is of interest to the plant biologist for several primary reasons, first the system is accessible to the study of cell-cell recognition. Secondly, the extreme localization (within the transmitting tract tissue) and strictly defined period of developmental activation (1 to 2 days prior to anthesis) that characterize self-incompatibility make this system ideally suited for studying tissue specific and temporal gene regulation.

As detailed earlier, the self-incompatibility response in the Solanaceae is under the control of a single, multi-allelic locus, termed the S-locus. The mechanism of pollen tube growth inhibition and pollen recognition is thought to reside within this "master" locus. It is logical to assume that a locus so strongly implicated in the process of self-incompatibility should demonstrate an expression pattern consistent with that role (ie. expressed late in floral development and expressed in a style specific manner).

Elucidating the molecular signals responsible for this unique form of intercellular communication is a primary goal of self-incompatibility research, and coupled with an understanding of the factors that affect S-locus gene expression should lead to a better overall comprehension of the self-incompatibility phenomenon.
A research goal of mine was to obtain information concerning the importance of cis-acting regulatory elements, as assayed by deletion analysis, on the modulation of S-locus gene expression. Prior to asking specific questions about the 5' flanking regions that affect S-locus transcriptional activity, my research necessarily focused on; the (1) isolation of petunia S-alleles and (2) a characterization of the organ specific and developmental expression patterns of three Petunia hybrida S-alleles.

4.2 Spatial Characterization of S-Allele Gene Expression

4.2.1 Homology Relationships Between PS1B, PS2A, and PS3A Sequences To ask whether the isolated cDNA sequences PS1B, PS2A, and PS3A demonstrate any cross-homology, and more importantly whether these sequences correspond to a style mRNA transcript, RNA blot hybridization experiments were performed using poly A+ RNA (1 μg) isolated from mature S_{1,1}, S_{1,2}, and S_{3,3} styles and ovaries. This petunia style/ovary filter was hybridized to radiolabeled PS1B, PS2A, and PS3A cDNA's under conditions of moderate stringency (42°C, 1M Na+, 50% formamide), and the results are shown in figure 9. PS1B strongly hybridized to a 900 nt transcript from S_{1,1} and S_{1,2} styles, but weakly to an mRNA species from S_{3,3} styles. PS2A and PS3A strongly hybridized to mRNA from S_{1,2} and S_{3,3} styles, but only weakly to a message from S_{1,1} styles. The pattern of cross hybridization clearly indicates that the cDNA clones PS2A and PS3A correspond to closely related messages, while the PS1B cDNA strongly detects a mRNA only in S_{1,1} style RNA. As a control for loading effects, the RNA blot was re-probed with a Zea maize actin cDNA clone (Shah et al., 1983), which detected a 1.2 kb transcript in all RNA
Figure 9. Hybridization of cDNA clones (PS1B, PS2A, and PS3A) to Petunia hybrida stylar mRNA.

Polyadenylated RNA (1 μg) was isolated from mature styles and ovaries of S_{1,1}, S_{1,2} and S_{3,3} lines, electrophoresed on a 1% agarose-formaldehyde gel, and capillary blotted to a nylon membrane. The lane assignments from left to right were as follows; S_{1,1} style (hybridization evident), S_{1,1} ovary, S_{3,3} style (hybridization evident), S_{3,3} ovary, S_{1,2} style (hybridization evident), and S_{1,2} ovary. The filter was hybridized under conditions of moderate stringency (42°C, 1M Na^+, and 50% formamide). The particular cDNA clone that was used as a hybridization probe is labeled at the top of the figure. All cDNA clones detect a 900 nt transcript in style mRNA.
lanes. Densitometry scannings of the actin filter hybridization revealed a 3-fold difference in RNA loading of style RNA (figure 10), with the S_{1,2} RNA being the least prevalent (present at 1/3 the level of the S_{1,1} RNA and 1/2 the RNA level of the S_{3,3} style RNA). Loading effects are the most likely explanation for the markedly reduced hybridization signal in the style S_{1,2} RNA lane when hybridized with the PS3A cDNA clone. These results indicated that the S_{1,2} style mRNA possessing sequence similarity to the PS1B cDNA is the S_1 mRNA, whereas the mRNA having sequence similarity to the PS2A cDNA is the S_2 mRNA.

4.2.2 S-Allele Organ Specific RNA Hybridization To determine whether the accumulation of the mRNA species homologous to the cloned cDNAs was organ specific, I isolated RNA from styles, leaves, petals, ovaries, anthers, and in vitro germinated pollen from all three S-locus genotypes (S_{1,1}, S_{1,2} and S_{3,3}) and hybridized RNA slot blot filters containing the various organ RNA's (10 µg total RNA/slot) to each individual radiolabeled cDNA. The data is shown in figure 11, and indicates that all three of the respective cDNA's hybridize strongly and preferentially to mRNA from styles, with S_2 and S_3 cross-homology particularly evident.

I also screened a self compatible line (MSU1093) for cross homology to the three petunia S-alleles, to see whether the MSU1093 line shows detectable levels of a S-locus transcripts. Figure 12 shows that the MSU1093 line possesses a S-allele that is highly homologous to the S_1 allele (based on hybridization intensity), and the message accumulates to similar levels seen for the S_1 allele in the S_{1,1} genotypic line. Based on self- and reciprocal outcrossing data, the MSU1093 line behaves as a unilateral stylar-part mutation;
Figure 10. *Zea mays* actin control hybridization.

The RNA filter blot (1 μg of poly A+ RNA loaded per lane) pictured in figure 9 was rehybridized with a *Zea mays* actin clone, as a control for RNA loading differences. The tissue and S-allele genotype of the tissue used to isolate the RNA is listed at the top of the figure. The filter was hybridized under conditions of moderate stringency (42°C, 1M Na+, and 50% formamide). Evident is the 1.2 kb actin transcript in all six RNA lanes, along with a lower molecular weight (900 nt) hybridization signal in the S_{3,3} and S_{1,2} style RNA, these were due to residual S-locus hybridization from a prior hybridization (when the PS3A cDNA clone was used as a hybridization probe).
Figure 11. Organ specific hybridization of petunia $S_1$, $S_2$ and $S_3$ alleles.

Total RNA (10 µg) was isolated from petal, style, leaf, ovary, and anther tissue, and in vitro germinated pollen from the $S_{1,1}$, $S_{1,2}$, and $S_{3,3}$ petunia lines. The RNA was applied to a nylon membrane using a slot blot apparatus, and hybridized under conditions of moderate stringency ($42^\circ$C, 1M Na+, and 50% formamide). The particular petunia $S$-allele cDNA clone that was used as the hybridization probe is labeled at the top of the figure.
Figure 12. Hybridization of homologous sequences in pseudo-self-compatible *Petunia hybrida*.

Total RNA (10 μg) from mature styles of the *S*₁₁, *S*₁₂, *S*₃₃, and MSU1093 petunia lines was applied to membranes using a slot blot apparatus, followed by hybridization (42°C, 1M Na⁺, 50% formamide) with the individual S-allele cDNA clones. S.I. style: RNA from *S*₁₁ (PS1B), *S*₁₂ (PS2A), or *S*₃₃ (PS3A) styles. PSC style: RNA from MSU1093 styles. No hybridization to RNA from MSU1093 styles was seen with either the *S*₂ or *S*₃ alleles, but equal levels of hybridization were seen between RNA derived from *S*₁₁ and MSU1093 styles, when the PS1B clone was used as a hybridization probe.
whereby the styles from this line do not discriminate between self and non-self pollen.

Utilizing a more sensitive approach, RNA blot hybridization experiments indicated that the style specific slot hybridization pattern was incomplete in detail. A tissue specific RNA blot containing 2 μg of poly A+ RNA from S₁₂ petal, style, leaf, ovary, anther, and germinated pollen was hybridized under conditions of moderate stringency (42°C, 1M Na+, 50% formamide) to random hexamer labeled PS2A (illustrated in figure 13). In addition to the strongly expressed style transcript, longer exposures revealed a ~900 nt message present in S₁₂ ovary poly A+ RNA. A 900 nt ovary transcript was also detected in all three P. hybrida lines, when any of the three S-allele cDNA clones were used as probes, (unpublished observations, K. R. Clark).

Transient expression experiments using histochemical detection of S₁ promoter/GUS expression (detailed in chapter VII) demonstrated S-locus transcription in petals. Thus, a high sensitivity RNA blot hybridization experiment was performed to determine whether the S₁ allele was being expressed at low levels in petunia petal tissue. A filter containing 10 μg of poly A+ RNA isolated from S₁₁ petal, leaf, and ovary tissue, along with 2 μg of S₁₁ style total RNA was hybridized, under conditions of moderate stringency (42°C, 1M Na+, 50% formamide), to radiolabeled PS1B (figure 14). A 900 nt transcript was detected in petal, style, and ovary RNA, while no hybridization was detected in the leaf RNA containing lanes. Based on densitometry scannings and taking into consideration the differences in
Figure 13. Hybridization with PS2A detects a transcript in *Petunia hybrida* ovary RNA.

Polyadenylated RNA (2 μg) was isolated from the various *S.12* petunia organs as listed at the top of the figure. The RNA samples were electrophoresed on a 1% agarose-formaldehyde gel, and capillary blotted to a nylon filter membrane. The RNA was hybridized with radiolabeled PS2A cDNA probe under conditions of moderate stringency (42°C, 1M Na+, and 50% formamide, after extended autoradiographic exposure (72 hr) a 900 nt ovary transcript is clearly evident.
Figure 14. Petal transcript identification in S\textsubscript{1,1} isolated RNA following hybridization with the PS1B clone.

Polyadenylated RNA (10 μg) or total RNA (2 μg; style) was isolated from S\textsubscript{1,1} styles (S), S\textsubscript{1,1} ovaries (O), S\textsubscript{1,1} petals (P), and S\textsubscript{1,1} leaves (L). The RNA was electrophoresed on a 1% agarose-formaldehyde gel, and transferred to a filter membrane. The filter was hybridized with PS1B cDNA clone (S.A. = 1 X 10\textsuperscript{9} cpm/μg) at 42°C, 1M Na\textsuperscript{+}, and 50% formamide. Following extended exposure (72 hr), a 900 nt transcript was observed in petal and ovary RNA.
hybridizable RNA present (2 μg of total style RNA vs 10 μg of petal and ovary poly A+ RNA -500-fold difference assuming 1% of total is mRNA), I estimated that the ovary \( S_1 \) message is present at \( 5 \times 10^{-4}\% \) of the mRNA mass (~1.5 \( \times 10^3 \) fold less than the level found in the style), and the petal transcript accumulates to approximately \( 3 \times 10^{-4}\% \) of the mRNA mass (~2.5 \( \times 10^3 \) fold less than the level found in the style), a level equivalent to one to two transcripts per cell (Goldberg et al., 1978 and Clark et al., 1990).

The significance, if any, of the weak ovary and petal tissue expression is not known, but based on the RNA blot hybridization (figure 14) and ribonuclease protection assays (figure 34, chapter VI) the data strongly suggest that the style and petal transcripts are identical. If the transcripts are identical, it seems probable that the S-RNase expressed in ovaries and petals, like the S-RNase present in styles (Anderson et al., 1989) is secreted into the extracellular spaces. One possible activity for petal S-RNases is a role in petal senescence. Baumgartner et al., (1975) has reported that ribonuclease activity is associated with petal senescence in morning glory. I have measured \( S_1 \) mRNA expression levels in senescing petal tissue using a quantitative RNA slot hybridization assay (figure 15). I observed a approximately equal levels of \( S_1 \) message accumulation (1.5-fold increase) in senescing petal RNA (harvested +3 to +4 days after flowering) as compared to the level found in non-senescing petals (day 0).

In summary, tissue specific RNA blot hybridization experiments indicated; (1) that the style cDNA clones isolated (PS1B, PS2A, and PS3A) all detect a highly expressed 900 nt mRNA message in style poly A+ RNA and (2)
Figure 15. Slot hybridization of petunia $S_{1.1}$ petal RNA with PS1B cDNA.

RNA was isolated from $S_{1.1}$ petal tissue, that was harvested at anthesis (0 dpa) or 3 to 4 days after flowering (+3 to +4 dpa). $S_{1.1}$ petal (4 µg poly A+) and $S_{1.1}$ style RNA (0.25 µg to 4 µg of total RNA) was electrophoresed on a 1% agarose-formaldehyde gel and blotted to a filter membrane. The filter was hybridized with the PS1B cDNA under conditions of moderate stringency ($42^\circ$C, 1M Na+, and 50% formamide). Total style RNA was loaded in a serial dilution fashion to estimate $S_1$ petal mRNA prevalence.
these cDNA sequences also detect a similarly sized transcript (900 nt), albeit at lower levels, in ovary and petal mRNA. No detectable hybridization to leaf, anther, or germinated pollen RNA has been observed with any of the three S-alleles (PS1B, PS2A, and PS3A) under hybridization conditions of high sensitivity (10 μg of pollen poly A+ RNA probed; Clark et al., 1990).

4.3 Floral Development in *Petunia hybrida* Lines S\textsubscript{1,1}, S\textsubscript{1,2}, and S\textsubscript{3,3}  

To determine if the putative S-locus cDNA sequences possessed a temporal pattern of mRNA message accumulation that was consistent with the late developmental onset of self-incompatibility (versus immature bud-compatibility), I undertook a descriptive study of petunia floral development.

Defining discrete floral developmental stages based on bud length, and expressed as a function of days prior to anthesis (dpa), enabled me to collect sufficient quantities of immature floral tissue at all developmental stages (pooled collections, stored at -80°C) for RNA isolation. Pam Collins and I measured sepal and floral bud lengths of the S\textsubscript{1,1}, S\textsubscript{1,2}, and S\textsubscript{3,3} petunia lines, and determined the mean length values for each stage. Cut-off values within 10% of the mean were used to assign a specific stage of developmental maturity (as a function of dpa) for individually measured floral buds. The mean length determinations for the three petunia genotypes analyzed (S\textsubscript{1,1}, S\textsubscript{1,2}, and S\textsubscript{3,3}) are shown graphically in figure 16.

4.4 Characterization of Temporal Expression for the $S_1$ and $S_2$ Alleles

4.4.1 Developmental Accumulation of the $S_1$ Allele in the $S_{1,1}$ Petunia Line  

To investigate whether the accumulation of $S_1$ mRNA paralleled the
Petunia Floral Development

Comparison of S$_{1.1}$, S$_{1.2}$, S$_{3.3}$

Figure 16. Mean floral bud length determinations in *Petunia hybrida* development.

Mean bud length determinations for the S$_{1.1}$, S$_{1.2}$ and S$_{3.3}$ genotypic lines are shown graphically. Mean developmental values were based on a minimum of 50 independent floral bud measurements. These mean developmental values were then used to harvest (only buds within 10% of the mean were collected) floral tissue of defined developmental stages.
acquisition of self-incompatibility, I used a quantitative RNA slot-blot assay to measure the relative level of $S_1$ mRNA at different stages of floral development. The experiments relied on the isolation of style RNA from all developmental stages (-9 to 0 dpa) of the two petunia lines assayed ($S_{1.1}$ and $S_{1.2}$). PS1B and PS2A cDNA clones were used as templates for the production of \textit{in vitro} synthesized RNA, which were used in reconstruction experiments to provide estimates of RNA message prevalence.

RNA slot-blot experiments were performed by applying a serial dilution of \textit{in vitro} transcribed PS1B cDNA to membrane slots in amounts corresponding to 2.0%, 1.0%, 0.5%, 0.25%, 0.125%, 0.062%, and 0.031% of the mRNA mass, assuming that mRNA comprised 1% of the total RNA (lane A; figure 17). Message prevalence estimates obtained in the reconstruction lane were compared to the $S_1$ message abundance in the style $S_{1.1}$ developmental slot hybridization (lane B; 1 µg total RNA loaded) to arrive at estimates for $S_1$ mRNA levels. Message abundance levels were calculated from $^{32}$P filter counts that were obtained by cutting out each slot individually. Filter count data were normalized using an actin probe, which served as a control for variations in loading effects.

As graphically shown (figure 17), PS1B mRNA accumulated to high levels (maximum level of 0.68% of the message mass at day 0) over the course of floral development, with no detectable expression present prior to dpa -5. The largest rate of message accumulation occurred between -3 dpa and -2 dpa (2.5 fold increase).

Bud pollination experiments on $S_{1.1}$ flowers demonstrated that in addition to mature flowers, floral buds at stages -1 dpa and -2 dpa were capable
Figure 17. Developmental expression of the \( S_1 \) allele in *Petunia hybrida* line \( S_{1.1} \).

Total RNA was isolated from styles of staged floral buds, or RNA transcribed *in vitro* from the PS1B clone, and was applied to a filter membrane using a slot blot apparatus. The filter bound RNA was hybridized (42°C, 1M Na+, and 50% formamide) with radiolabeled PS1B cDNA (S.A. = 1 \( \times \) 10\(^9\) cpm/\( \mu \)g). Following autoradiography, individual slot-bands were counted in a liquid scintillation counter. Lane A is a reconstruction experiment with RNA transcribed *in vitro* from the PS1B clone. Transcribed RNA was serially diluted and applied to membrane slots in amounts corresponding to 2.0%, 1.0%, 0.5%, 0.25%, 0.125%, 0.062%, and 0.031% of the mRNA mass, assuming that mRNA comprised 1.0% of the total RNA. Lane B contains 1 \( \mu \)g of total style RNA, isolated from developmentally staged floral buds. Numbers along the X-axis represent days prior to anthesis (dpa), with the -9 dpa stage not being shown. The graph diagrams \( S_1 \) allele mRNA developmental accumulation (as a percent of the message mass), along with increases in style and floral bud length (given in centimeters).
of expressing the incompatibility response (prevented self-fertilization). Bud-pollinations of floral buds at stage -3 dpa however, resulted in capsule formation and seed set. Thus, the greatest accumulation of S₁ mRNA occurs during the precise period when the style undergoes the transition from bud self-compatibility to self-incompatibility.

4.4.2 S-Allele Developmental Accumulation in the S₁₂ Petunia Line To compare the developmental accumulation of different S-alleles transcripts in a heterozygous line, I hybridized either the PS₁B or PS₂A cDNAs to slot blots containing 1 µg of total RNA isolated from S₁₂ styles at multiple developmental stages (0 through -8 dpa). RNA transcribed in vitro from the PS₂A cDNA clone was serial diluted and applied to a slot membrane in the same manner and amount as described above for the S₁₁ developmental slot blot (figure 18, lane A).

S₁ message accumulation in the heterozygous background (S₁₂) was very similar in level and form to that for the S₁ allele in the S₁₁ line (lane B). The maximal S₁ message level was 0.64% of the message mass, and this was seen at stage -1 dpa, with day 0 also showing high levels of expression (0.58%). Stage transitions -4 dpa to -3 dpa, and -3 dpa to -2 dpa possessed similar rates of increase (both 3-fold increases) in S₁ transcript accumulation.

The temporal pattern of expression was similar for both the S₁ and S₂ alleles, but the accumulation of S₂ mRNA was substantially greater over all developmental stages assayed (accumulating to a maximal level of 1.63% of the mRNA mass at -1 dpa; lane C). At stage -3 dpa, during which bud pollination of the S₁₁ plant resulted in seed set, the level of S₂ mRNA in the S₁₂ line was approximately eightfold greater than the level of S₁ mRNA.
Figure 18. Developmental S-locus expression in the S\textsubscript{1,2} Petunia \textit{hybrida} line.

Total RNA was isolated from styles of staged floral buds, or RNA transcribed \textit{in vitro} from the PS1B and PS2A cDNA clones. The RNA was applied to a filter membrane using a slot blot apparatus. The filter bound RNA was hybridized (42°C, 1M Na+, and 50% formamide) with radiolabeled PS1B or PS2A clone, and following autoradiography, individual slot-bands were counted in a liquid scintillation counter. Lane A of the figure is a reconstruction experiment with RNA transcribed \textit{in vitro} from the PS2A clone (the S\textsubscript{1} RNA reconstruction is not shown). Transcribed RNA was serially diluted and applied to membrane slots in amounts corresponding to 2.0%, 1.0%, 0.5%, 0.25%, 0.125%, 0.062%, and 0.031% of the mRNA mass, assuming that mRNA comprised 1.0% of the total RNA. Lane B contains 1 μg of total S\textsubscript{1,2} style RNA, isolated from developmentally staged floral buds, and hybridized to the PS1B probe. Lane C contains S\textsubscript{1,2} style RNA (1 μg total RNA loaded) isolated from staged floral buds, and hybridized to the PS2A probe. Numbers along the X-axis represent days prior to anthesis (dpa), with the -7 and -8 dpa stages not being shown. The graph diagrams S\textsubscript{1} and S\textsubscript{2} allele mRNA developmental accumulation (as a percent of the message mass), along with increases in style and floral bud length (given in centimeters).
(0.95% S\textsubscript{2} vs 0.12% S\textsubscript{1}).

This observation is particularly notable because in a separate experiment, S\textsubscript{1.2} plants were bud-pollinated at stage -3 dpa to try and circumvent the self-incompatibility response and produce homozygous S\textsubscript{2.2} tester line progeny. When the F\textsubscript{1} progeny were analyzed (24 plants) by DNA blot hybridization (J. Okuley, unpublished observations) to determine S-allele genotype, only S\textsubscript{1.2} and S\textsubscript{1.1} plants were identified with no S\textsubscript{2.2} plants being recovered. ($\chi^2 = 7.82$, $P< 0.03$). The simplest interpretation of the above breeding data is that the inability to recover F\textsubscript{1} S\textsubscript{2.2} homozygotes was due to elevated S\textsubscript{2} mRNA levels at stage -3 dpa. Thus, if the elevated mRNA levels translate into "prematurely" elevated levels of S\textsubscript{2} protein, there would exist a mechanism to specifically inhibit S\textsubscript{2} pollen from fertilizing S\textsubscript{1} or S\textsubscript{2} ovules.

Taken together these data demonstrate that (1) S-locus mRNAs accumulate in a precise organ specific manner in reproductive tissue, (2) there is a direct correlation between the accumulation of S-allele mRNA sequences and the ability to reject self-pollen tubes, (3) the level of S\textsubscript{2} mRNA at stage -3 is apparently sufficient to render the S\textsubscript{1.2} plants incompatible to S\textsubscript{2} pollen tubes, and (4) based on S\textsubscript{1} and S\textsubscript{2} mRNA levels at stage -3 dpa (transitional stage) in two separate lines (S\textsubscript{1.1} and S\textsubscript{1.2}) it appears that a threshold level of message accumulation (-0.2%) is sufficient to elicit a self-incompatibility response.
CHAPTER V
ISOLATION AND CHARACTERIZATION OF FIVE
PETUNIA FLORAL ENHANCED cDNA CLONES

5.1 Overview

In addition to the isolation of several petunia S-alleles, our laboratory was interested in the identification of other DNA sequences that may play a role in self-incompatibility, pollination, and/or fertilization. Complex biological processes often possess many individual components that are coordinately regulated (e.g. cell cycle proteins, ribosomal RNA and protein synthesis, the hypersensitive response to plant pathogens, proteins that make up the photosynthetic machinery, heat shock proteins, etc.) and which interact to bring about the desired biological function. We reasoned that other genes might exhibit a similar spatial or temporal expression pattern as that of the S-alleles, and if identified, could yield insights into the mechanism of style specific gene expression. To this end, I carried out a differential hybridization screening strategy to isolate style-specific cDNA sequences and then subsequently characterized the expression of these floral enhanced cDNA's.

5.2 Isolation of Floral Enhanced cDNAs

To isolate sequences that may demonstrate similar spatial and temporal expression patterns as that of the cloned S-alleles, I performed a differential
plaque hybridization screen of the $S_{1.2}$ cDNA library. Using random primed first strand radiolabeled cDNA synthesized from mature style (day 0) or leaf poly A+ RNA. I screened ~5,000 recombinant phage, and isolated 10 putative style-specific (leaf negative) cDNA inserts. After hybridization experiments determined that the cDNAs did not share detectable cross-homology to the petunia S-alleles or each other (data not shown), I subsequently performed RNA blot analysis to characterize the organ-specific distribution of message accumulation for the individual cDNA clones. Five cDNA clones, referred to as petunia floral enhanced clones (PFE) were isolated and labeled as follows with the insert size in parentheses: PFE#3 (2.3 kb), PFE#4 (0.9 kb), PFE#8 (1.0 kb), PFE#13 (1.2 kb), and PFE#14 (2.7 kb).

5.2.1 Characterization of Organ-Specific Hybridization for PFE Sequences RNA blot hybridization experiments were performed on membrane filters containing poly A+ RNA (1 to 2 $\mu$g) isolated from $S_{1.2}$ petal, style, ovary, anther and germinated pollen, under conditions of moderate stringency (42°C, 1M Na+, 50% formamide). The hybridization data revealed three patterns of spatial expression for the five $S_{1.2}$ cDNA clones.

The first class, containing PFE#3, PFE#8, and PFE#13, demonstrated an ovary-enhanced expression pattern. PFE#3 cDNA detected a ~2.3 kb transcript expressed 5-fold higher in the ovary than in style, petal, leaf, and in mature anther RNA (figure 19). PFE#8 hybridized to a 1.75 kb message, predominately in ovary RNA, and at a lower level (4-5 fold) in style, petal and leaf RNA (figure 20). PFE#13 hybridized to a 3.0 kb mRNA, present in ovary at a 10-fold greater level than in style and petal RNA, as shown in figure 21.
**Figure 19.** Hybridization of *Petunia hybrida* S$_{1,2}$ RNA with *Petunia* Floral Enhanced cDNA clone #3.

*P. hybrida* Poly A+ RNA, isolated from the tissues listed at the top of the figure, were hybridized with radiolabeled PFE #3 cDNA clone under conditions of moderate stringency (42°C, 1M Na$^+$, and 50% formamide). Hybridization is evident to a 2.3 kb message, present in petal, style, anther, and ovary RNA.
Figure 20. Hybridization of Petunia hybrida S₁₂ RNA with Petunia Floral Enhanced cDNA clone #8.

P. hybrida Poly A+ RNA, isolated from the tissues listed at the top of the figure, were hybridized with radiolabeled PFE #8 cDNA clone under conditions of moderate stringency (42°C, 1M Na⁺, and 50% formamide). Hybridization is evident to a 1.75 kb message, present in petal, style, and ovary RNA.
Figure 21. Hybridization of *Petunia hybrida* $S_{1.2}$ RNA with *Petunia Floral Enhanced* cDNA clone #13.

*P. hybrida* Poly A+ RNA, isolated from the tissues listed at the top of the figure, were hybridized with radiolabeled PFE #13 cDNA clone under conditions of moderate stringency ($42^\circ$C, 1M Na+, and 50% formamide). Hybridization is evident to a 3.0 kb message, present in petal, style, and ovary RNA. The lower molecular weight hybridization signal (900 nt) in the style and ovary RNA is due to residual signal from S-mRNA hybridization from a previous experiment.
PFE#4 demonstrated a style enhanced pattern of expression, hybridizing strongly to a 1.3 kb style mRNA, and very faintly to a 2.0 kb transcript. PFE#4 also hybridized to the 1.3 kb message in ovary RNA, but to a lesser degree (figure 22). Weak hybridization was seen to the 1.3 kb petal poly A+ RNA message.

The final cDNA clone, PFE#14 hybridized to multiple transcripts and/or RNA processing products (a faint 2.7 kb message, 1.8 kb, 1.2 kb, and 750 nt), present principally in style S₁₂ poly A+ RNA, with weak hybridization being detected in the ovary RNA (60 hr exposure) to a 1.2 kb message (figure 23). PFE#14 was therefore classified as a style specific cDNA sequence.

A control hybridization with a *Zea mays* actin clone is shown in figure 24. Based on the densitometry scannings, it appears that poly A+ petal RNA was present at a two-fold greater concentration than style and ovary poly A+ RNA. Therefore, hybridization intensity levels in the style and ovary RNA lanes were under represented relative to the petal hybridization signal. Actin message levels in anther and germinated pollen RNA were present at a 10-20 fold greater level than style, petal and ovary actin mRNA levels. The most logical inference from this data is that actin mRNA is up-regulated in both mature anther tissue and in *in vitro* germinated pollen as compared to actin message levels in other petunia organs. The actin control blot also shows that anther and germinated pollen RNA was isolated in an intact and non-degraded form. The restriction maps of PFE#3, PFE#4, PFE#13, and PFE#14 are given in figure 25.
Figure 22. Style enhanced hybridization of Petunia hybrida S₁₂ RNA with Petunia Floral Enhanced cDNA clone #4.

*P. hybrida* Poly A+ RNA, isolated from the tissues listed at the top of the figure, were hybridized with radiolabeled PFE #4 cDNA clone under conditions of moderate stringency (42°C, 1M Na⁺, and 50% formamide). Hybridization is evident to a 1.3 kb message, present in petal, style, and ovary RNA, with weak hybridization to a 2.0 kb style transcript also detected.
Figure 23. Style specific hybridization detected in *Petunia hybrida* S$_{1,2}$ RNA with Petunia Floral Enhanced cDNA clone #14.

*P. hybrida* Poly A+ RNA was isolated from the tissues listed at the top of the figure, and were hybridized with radiolabeled PFE #14 cDNA clone under conditions of moderate stringency (42°C, 1M Na$^+$, and 50% formamide). Hybridization is evident to multiple messages:, 1.8 kb, 1.2 kb, 750 nt, and a faint 2.7 kb message, present principally in style S$_{1,2}$ poly A+ RNA, with weak hybridization being detected in the ovary RNA (60 hr exposure) to the 1.2 kb message.
Figure 24. Control hybridization with a maize actin clone.

A *Zea mays* actin clone was used as a control for loading differences. Poly A+ RNA was isolated from the various petunia tissues listed at the top of the figure, and were hybridized under conditions of moderate stringency (42°C, 1M Na+, and 50% formamide). Hybridization is evident to message a 1.2 kb message, present in elevated amounts in *S*1.2 anther and germinated pollen poly A+ RNA. Weak hybridization was detected in petal, style and ovary RNA to the actin mRNA.
Figure 25. Restriction map analysis of PFE cDNA clones #3, #4, #13, and #14.

Diagram showing the approximate location of restriction sites within the various petunia floral enhanced (PFE) cDNA clones. Boxed areas represent the cDNA inserts, and the total length of the cDNA clones is given in boldface type on the right hand side of the figure. The analysis involved over 20 Bluescript polylinker restriction enzymes, with enzyme sites labeled vertically.
5.2.2 Developmental Expression of PFE Sequences To investigate the temporal pattern of message accumulation for these sequences, I utilized a developmental RNA slot blot assay. Total style RNA (10 µg) isolated from 9 developmental stages (-9 to 0 dpa) of the petunia S\textsubscript{1.1} line was applied to slotted nylon membranes and hybridized with the various floral enhanced cDNA clones. The slot hybridization data for PFE#3, PFE#4, PFE#8, PFE#14 clones is shown in figure 26, along with an actin control hybridization to monitor RNA loading. The actin control hybridization shows that there is a reduced level (2-fold) of total RNA loaded at stages -1 and day 0, as compared to stages -2 and -3 dpa. Loading variation is probably the reason that hybridization levels generally decreased slightly at stages -1 and 0 dpa. PFE developmental RNA accumulation plot data were based on radioactive filter counts (\textsuperscript{32}P hybridized), normalized against the actin filter count values.

The style RNA slot hybridization data is graphically illustrated for PFE#3, PFE#4, PFE#8, and PFE#14 in figure 27. The data is plotted as the relative percentage of counts obtained for each individual clone; the data plots were overlayed upon each other and do not represent message level prevalence comparisons against each of the PFE messages. The floral enhanced mRNAs were expressed at very early stages of floral development (-9 dpa), and message levels did not vary more than 5 fold over the entire developmental series, with message levels generally increasing as the flower matured. An ovary developmental RNA assay may reveal additional expression information, especially for the ovary enhanced (PFE #3, #8, and #13) messages. The message accumulation in styles for the floral enhanced cDNA's was dramatically different than what was visualized with the petunia
Figure 26. Hybridization pattern of developmental RNA accumulation of PFE cDNA clones; #3, #4, #8, and #14 in Petunia hybrida line S11.1.

Total S11 style RNA (10 µg) isolated from multiple developmental stages (-9 to 0 dpa) was applied to a nylon filter membrane using a slot blot apparatus. The RNA was hybridized under moderate stringency conditions (42°C, 1M Na+, and 50% formamide) with the various PFE clones listed at the top of the figure. Slot-bands were cut out of the filters and quantitated using liquid scintillation methods, and normalized against actin control hybridization filter counts. Days prior to anthesis (dpa) are shown to the left of the figure, where day 0 represents flower opening (-8 dpa and -9 dpa staged tissue was pooled).
Figure 27. Developmental accumulation of PFE #3, #4, #8, and #14 mRNA.

The graphic illustrates the pattern of developmental message accumulation for four (PFE #3, PFE #4, PFE #8, and PFE #14) of the S₁₂ petunia floral enhanced cDNA clones. Message accumulation was quantified by cutting out each slot-band and counting in liquid scintillation medium. The developmental mRNA accumulation data was plotted as a function of days prior to anthesis (dpa; X-axis). The raw count data were converted into percentages of the total hybridized filter counts obtained for each individual PFE mRNA. The conversion of the data in this manner allows for a comparison of the pattern of message accumulation, but cannot be interpreted as a comparison of relative message abundance among the four PFE mRNAs.
S-allele mRNAs; where S-locus expression is first detectable at -4 dpa and rapidly accumulates up to the time of anthesis. A message prevalence estimate of the PFE #4 clone was obtained by utilizing a similar experimental strategy as that for the S-alleles. Using defined levels of in vitro transcribed PFE #4 cDNA for comparison, I estimate that the PFE #4 mRNA accumulates to a maximal level of approximately 3-4% of the message mass at day 0. Based on hybridization intensity comparisons with the PFE #4 cDNA using RNA blot and RNA developmental slot blots, approximate mRNA prevalence estimates of the other PFE clones were obtained. PFE #14 was also abundantly expressed at stages -3 to 0 dpa, present at ~1-2% of the mRNA mass. The ovary enhanced cDNA sequences (PFE #3, #8, and #13) were expressed at significantly lower levels; none of the three messages accumulating (at maximal levels) to greater than 0.2% of the mRNA mass.

Genomic DNA blot hybridization analyses were performed using several of the floral enhanced cDNA clones as probes (figure 29). In contrast to the highly polymorphic genomic organization of the S-alleles (J, Okuley in Clark et al., 1990), the DNA blot hybridizations to PFE#3, PFE#4, PFE#13, and PFE#14 reveal conserved restriction fragments, independent of the S-allele genotype. All PFE clones appear to be single copy genes based on hybridization signal complexity and intensity.

In summary, the S_{1,2} floral enhanced cDNA clones isolated represent two broad classes, both distinct from the expression pattern observed for the S-alleles. The first class, characterized by cDNA clones PFE#3, PFE#8, and PFE#13 possess an ovary-enhanced spatial distribution, and are present at detectable levels early in floral development (-9 to -8 dpa). The second class
Figure 28. DNA blot hybridization of PFE cDNA clones #3, #4, #13, and #14 to *Petunia hybrida* genomic DNA.

The PFE cDNA clones were radiolabeled and hybridized (42°C, 1M Na+, 50% formamide) with filter bound genomic DNA isolated from leaf tissue of petunia lines $S_{1,1}$, $S_{1,2}$, $S_{3,3}$, self compatible MSU1093 (SC), and *Petunia mitchel* (*P.m*). The genomic DNA was restricted with EcoRI or HindIII, and following hybridization, detected similar sized restriction fragments, independent of the petunia line used. All four cDNA clones (#3, #4, #13, and #14) hybridized at single copy intensity.
Figure 28
are style-enhanced sequences. PFE#4 is expressed at high levels in style RNA at all developmental stages assayed (2-4% of mRNA population, K.R. Clark, unpublished observations), and demonstrates moderate to low levels of mRNA expression in ovary and petal tissue. PFE#14 was shown to possess a style specific pattern of gene expression, and was also present at abundant message levels (0.5-1% of message population at maturity) throughout the developmental time course assayed (-9 to 0 dpa).
6.1 Overview

To begin a structural characterization of the isolated petunia S-alleles (PS1B, PS2A, and PS3A), I performed restriction map analysis using multiple restriction enzymes present in the Bluescript cloning vector (Stratagene; shown in figure 29). The maps reveal a large degree of restriction site heterogeneity, with only a single site (XbaI) conserved between the PS2A and PS3A, and no restriction sites conserved with the PS1B clone. The length of the cDNA clones (750 bp, 858 bp, and 860 bp respectively) approximate the length of the observed ~900 nt style specific transcript seen in the oligonucleotide hybridizations and were considered to be close to full-length.

6.2 DNA Sequence Analysis on PS1B, PS2A, and PS3A

To gain insight into the structure and function of the petunia S-alleles, our laboratory determined the nucleotide sequence of the three cDNA clones (PS1B, PS2A, and PS3A) using dideoxy Sanger sequencing (Sanger et al., 1977). I provided approximately 200 bp (~25%) of nucleotide sequence information for the PS1B cDNA sequence, and 200 bp of 5' DNA sequence for the PS3A cDNA clone. The observed homology relationships, based on hybridization experiments, were confirmed upon subsequent sequence analysis. The PS2A cDNA clone corresponding to the $S_2$ allele shared 90.5% nucleotide identity
Figure 29. Restriction maps of cDNA clones PS1B, PS2A, and PS3A.

A restriction map of the three petunia S-allele sequences is pictured. Only one conserved restriction site (XbaI, 190/195) was observed, and is present only in the $S_2$ and $S_3$ alleles. The flanking EcoRI sites derive from EcoRI adapters that were used in the cloning process, and do not reflect an actual restriction site within the cDNA clones. Translational stop (TGA) codons and polyadenylation positions are listed in boldface type.
with the \( S_3 \) allele (PS3A). At the amino acid level the petunia \( S_2 \) and \( S_3 \) alleles shared 89% identity, differing at 22/200 residues, with most (16/22) of the difference occurring in three localized areas. One region, possessing 9 of the amino acid mismatches, corresponds to the carboxy terminus portion of the protein (final 23 amino acids), the other two regions are localized between residues 46-55 (possesses 4 mismatches), and residues 75-85 (contains 3 mismatches). The PS1B cDNA was only \( \sim 60\% \) homologous at the nucleotide level to either the \( S_2 \) or \( S_3 \) cDNA sequences, and the \( S_1 \) protein possesses only 40% amino acid identity with the predicted \( S_2 \) and \( S_3 \) proteins.

The base pair differences between the \( S_1 \) vs \( S_2/S_3 \) alleles was often in the form of insertion or deletion of bases, which dramatically altered the amino acid sequence between the \( P. \) \( hybrida \) \( S \)-alleles in certain regions, while preserving a higher degree of nucleotide sequence homology. Base pair differences between the \( S_2 \) and \( S_3 \) alleles resulting in amino acid substitutions consisted primarily of point mutations, but several instances of the insertion or deletion of bases were observed, which also contributed to amino acid differences between the \( S_2 \) and \( S_3 \) alleles. The derived amino acid sequence for the three \textit{Petunia} \( S \)-alleles is shown in figure 30, as well as a comparison with three \( S \)-locus derived protein sequences identified in \textit{Nicotiana alata} (NaS2, NaS3, and NaS6, Anderson \textit{et al.}, 1986 and 1989).

The derived petunia \( S \)-protein sequences all possess a 22 amino acid signal peptide characteristic of secreted proteins (von Heijne, 1986). Cleavage rules for secreted proteins (von Heijne, 1986) predict that the mature \( S_1 \) protein should begin with a serine residue, and both the \( S_2 \) and \( S_3 \) proteins with an asparagine residue (consistent with N-terminal amino acid
Figure 30. Comparison of protein sequences from *P. hybrida* and *N. alata.*

DNA sequences of the petunia cDNA clones (PS1B, PS2A, and PS3A) were translated to yield the protein sequences. The derived amino acid sequences were aligned with those for the *N. alata* alleles (NaS2, NaS3, and NaS6, Anderson et al., 1986, 1989) and the Rh ribonuclease of *R. niveus* (Rhnv; Horiuchi et al., 1988) using the Gap program (Devereux et al., 1984). Boxed sequences show regions of >80% amino acid residue similarity based on the scoring table of Gribskov and Burgess (1986). *R. niveus* residues showing identity with S-allele amino acids are underlined. Consensus glycosylation sites (NXT/NXS) are designated by darkened triangles. Sequence numbers shown refer to the PS1B protein sequence.
sequence data of mature S-proteins isolated from *N. alata* and *L. peruvianum* (Mau et al., 1986 and Anderson et al., 1989). The presence of a leader sequence is also consistent with the observation that S-proteins are localized in the extracellular matrix of the transmitting tract tissue.

Of particular interest is the existence of 6 conserved regions of protein homology possessing > than 80% residue similarity (boxed) interspersed with regions of divergent protein sequence (< 40% residue similarity). The existence of 9 conserved cysteine residues between the solanaceous S-proteins identified to date (*P. hybrida*, *P. inflata*, *Nicotiana alata*, *Lycopersicon peruvianum*, and *Solanum chacoense*), suggests a high degree of functional conservation and tertiary protein structure. To determine which protein regions (either conserved or variant) were most likely positioned on the exterior of the protein, a hydropathy plot analysis (Kyte and Doolittle, 1982) was performed on the petunia S-protein sequences (T. Sims). The analysis revealed that the N-terminal and C-terminal conserved domains are quite hydrophobic in character, and are possibly buried within the protein core. The second and fourth conserved domains (numbering beginning from N-terminus) possess a hydrophilic character, and are presumably positioned on the outer surface of the protein.

These conserved regions (domain 2; residues 50-60, and domain 4; residues 105-119) were shown in several laboratories (McClure et al., 1989, Clark et al., 1990, and Singh et al., 1991) to possess sequence homology with the catalytic domains (as identified by Horiuchi et al., 1988) of two secreted fungal ribonuclease proteins (RNase Rh from *Rhizopus niveus* and RNase T₂ from *Aspergillus oryzae*). In figure 30 the *Rhizopus niveus* ribonuclease
protein (Rhnv) sequence is also shown for comparison, with underlined amino acids corresponding to conserved protein residues.

The *Petunia hybrida* S-proteins also contain multiple regions that contain divergent sequence (>40% amino acid identity). Several divergent regions possess a hydrophillic character and therefore, are potential candidates for *in vitro* mutagenesis experiments to ascertain what role if any these regions play in the recognition phenomenon.

6.3 Nucleotide Sequence Determination of the *Petunia hybrida* S₁ allele

To more fully analyze the structure of the S-locus, and identify regions of functional importance for S-locus expression, I sequenced a 3.2 kb region of S₁ genomic DNA. The S₁ sequencing strategy utilized is pictured in figure 31. The genomic sequence was obtained by sequencing two deletion series (Erase-a-base; Promega), that were also used in conjunction with a S₁ promoter analysis. The DNA sequence derived from a 3.2 kb HindIII/EcoRI genomic fragment.

The nucleotide sequence of the 3.2 kb of S₁ genomic DNA fragment is shown in figure 32. The sequence consists of 1905 bp of 5' non-coding sequence, 669 bp of coding sequence plus a 118 bp intron, and 504 bp of 3' non-coding sequence. Several features of the DNA sequence are highlighted: two transcriptional start sites, TATA box consensus sequences located approximately 25-30 bp upstream of each start site, polyadenylation consensus sequence, and the single intron. The S₁ intron is conserved in position with a single petunia S₃ intron (J. Okuley), both introns are inserted between residues 57-58 of the proposed N-terminal end of the mature protein.
Figure 31. Sequencing strategy for *P. hybrida* $S_1$ allele sequence determination.

Various $S_1$ deletion subclones used for sequence analysis are schematically shown. The arrows represent specific subclones isolated, length of sequence and direction of synthesis are defined by each individual arrow. Deletion subclones used in $S_1$ promoter analysis are designated by $\Delta \#$, with the numbers corresponding to approximate 5' promoter length. Two sequencing oligonucleotides were synthesized to obtain the final overlapping segments. In addition to DNA sequence determination, the Pst 600 subclone was also utilized for $S_1$ 5' transcription start mapping experiments.
Several features of the genomic nucleotide sequence (3.2 kb) are highlighted for reference, among them; two transcription start adenines (position 1879 bp and 1906 bp) identified in boldface type, the ATG translational start, located (1930 bp) 25 bp downstream of the major transcriptional start site (1906), and a Pvu II restriction site (CAGCTG) located 15 bp 3' of the ATG start that was used for the synthesis of S1 promoter/GUS translational fusions. A 19 bp region (1710-1728) located 223 bp 5' of the first transcription start (1906) that possesses sequence homology to several DNA consensus binding sites identified in other systems is underlined. Further 5' (653-745), is a 82 bp region that contains a stretch of 39 tandem repeats of the dinucleotide TA, the significance of this region is not known, but a similar TA tract was identified in the 3' region of the PS3A cDNA clone. The single 118 bp intron (2168-2185) is also underlined. The TGA stop codon (2715-2717) is identified in boldface type, along with the polyadenylation site (2787). The polyadenylation consensus sequence AAUAAA is located 17 bp (2765-2770) 5' to the polyadenylation site.
Figure 32
(22 from the amino acid leader sequence). The $S_1$ intron is also of similar size to the $S_3$ intron (118 bp vs 101 bp respectively), and possesses a thymidine tract equal to or greater than 6-7 contiguous residues ($S_1$ intron has a 9 residue tract), which is characteristic of many plant introns (V. Walbot personal communication). Also highlighted are partial sequence homologies to 5' upstream cis-acting consensus sequences identified in other systems that are possibly involved in DNA protein binding and transcriptional activation.

The only significant sequence homology between the 5' region of the petunia $S_1$, and $S_3$ alleles, and the Nicotiana alata $S_2$ and $S_6$ alleles was in the TATA box region, as determined using the Seqaid (ref) sequence analysis program.

6.4 Identification of $S_1$ 5' Transcription Start Sites

The $S_1$ allele transcriptional start sites (2) were identified using an RNase protection assay (Sambrook et al., 1990). Figure 33 shows the proximal $S_1$ 5' promoter sequence and the strategy employed to protect a putative transcriptional start site. Briefly, a 554 bp PstI restriction fragment that was thought to span the transcriptional start (based on sequence data and the observed length of the $S_1$ mRNA ~900 nt), was linearized with Dral restriction enzyme, and this molecule was used to generate a 358 nt in vitro synthesized RNA molecule, transcribed from the T3 RNA polymerase promoter. The radiolabeled RNA run-off was hybridized (methods 2.9) with total RNA (1-10 ug) from $S_{1.1}$ style or $S_{1.1}$ petal (40-100 µg) for 2-3 hours, and unhybridized RNA degraded by digestion with RNases A (100 µg/ml) and T1 (2 µg/ml) at 35°C for 1 hour. The results of this protection experiment are shown in figure 34. The $S_1$ radiolabeled RNA protected two style mRNAs, one of 131 nt (major product) and another 158 nt (minor product), indicative
Figure 33. Strategy utilized to map the S₁ mRNA transcription start site.

The S₁ 5' proximal DNA sequence is shown at the top of the figure, along with the region encoding the amino terminus of the S-RNase protein. Bases positioned between -201 to -180 (underlined) represent sequences possessing homology to several cis DNA binding elements; a G-box element present in Arabidopsis, adenovirus major late promoter enhancer, and a region from wheat HMW glutenin related to AP1/GCN4 binding sites. Two TATA box elements (underlined) are located 25-30 bp upstream of two putative transcriptional start sites (each marked by an asterisk). The sequence CAGCTG (positioned at bp 40) is the location where the S₁/GUS translational fusions were constructed (the gene fusions contained the the first 5 amino acids derived from the S₁ allele (MFKL Q). The bottom portion of the figure is a schematic representation of the strategy used to map the S₁ 5' start site. The first line represents the 554 bp S1 PstI genomic clone that was used in the mapping experiments. A 358 nt in vitro transcribed radiolabeled RNA molecule was synthesized from a T3 RNA polymerase promoter (present in the Bluescript vector) after DraI digestion of the S1 PstI genomic subclone. Following hybridization (53°C, 0.4M Na⁺, and 50% formamide) with S₁,₁ style or petal total RNA, the samples were digested with RNase A and T1, and the resulting protection fragments were resolved on a 7M urea/acrylamide sequencing gel.
CATCTAGCTGTTAACACATATATTATTTTAACATATCCAACCTCATGTG

CTCACACACCATCCACGTCTCATATATATAATTATTTAAAAAGGCTGTTAACA

CTATTTAAGTGCCCTAATCCACTTTCTCTAGTACAGATTCTAGCTTGAAA

AAAAAATTAGTTAGTTAGTGAAAGTGATAAGTCTATATTTAAACCATTCC

CACTGAAAAATACGATTATTATATTGCTATATGCAAAGGGAAGGAACCT

AAACATGAGTTGTTCAAACTTTAGTTCAAGTTACAGCTGGCGTCAG

AAACATGAGTTGTTCAAACTTTAGTTCAAGTTACAGCTGGCGTCAG

TTTTATGTGTTTTTCTTTTTGCTTGCTCTCCAATTTCTGGGTCTTTCGAC

PstI Dial PstI

T O

H u

| 358 nt RNA |

| S1 5' mRNA |

| 131 and 158 bp |

protected fragment

Figure 33
of two transcriptional start sites that map to two adenine residues; positioned
-25, and -52 bp upstream of the ATG translational start.
Figure 34. Ribonuclease protection mapping of the 5' end of the S₁ mRNA.

Pictured are the RNase protection products from a hybridization reaction between S₁ style and petal total RNA and a 358 nt *in vitro* transcribed S₁ genomic, DraI restricted subclone (S1PstI600), which was thought to span the presumptive transcriptional start site. Hybridization conditions were of moderate stringency (53°C, 0.4M Na⁺, and 50% formamide). Lane assignments were as follows: lane 1; 5 μg style RNA digested with 120 μg/ml RNase A, lane 2; 5 μg style RNA digested with 200 μg/ml RNase A, lane 3; 40 μg of petal RNA digested with 100 μg/ml RNase A, and lane 4; 80 μg petal RNA digested with 100 μg/ml RNase. Style RNA lanes were exposed 12 hr, and the petal RNA lanes were exposed for 7 days.
CHAPTER VII
AN ANALYSIS OF ORGAN SPECIFIC AND QUANTITATIVE EXPRESSION
OF THE Petunia hybrida S₁ ALLELE USING
CHIMERIC β-GLUCURONIDASE GENE CONSTRUCTIONS

7.1 Overview

To investigate the potential for S-locus expression in pollen, and begin an analysis of S₁ 5′ promoter sequences that affect S-locus transcriptional activity, the E. coli β-glucuronidase reporter gene (GUS) was fused to S₁ 5′ and 3′ flanking sequences. The use of β-glucuronidase reporter genes fusions (Jefferson, 1986, 1987a, and 1987b) has facilitated the analysis of plant gene expression. The marked stability of the β-glucuronidase enzyme allows the detection of gene expression even in cells and tissues where the level of mRNA accumulation is quite low.

The ability to separate the DNA control sequences from the structural region allows specific regulatory elements to be analyzed free from the potentially confounding effects of the structural gene or homologous sequences present within the genome. Chimeric S₁ promoter/GUS genes were introduced into intact plant tissues utilizing biolistic particle bombardment (Klein et al., 1988). Following gene introduction, a transient expression assay system was used to determine those tissues expressing the S-locus, and to identify regions of functional importance for S-locus expression.
7.2 S₁ Promoter Deletion/β-Glucuronidase Gene Fusion Construction

7.2.1 Synthesis of pUCGUS Cloning Vector To construct a series of S₁ promoter/β-glucuronidase fusion genes, an initial GUS cloning vector (pUCGUS) was synthesized. The pUCGUS vector was constructed by ligating the a promoterless GUS vector (pBI 101.3, Clonetech) with pUC119 restricted with HindIII and EcoRI (this digestion conveniently removes the entire pUC119 polylinker), which is shown schematically in figure 35. The derivation of various S₁ genomic subclones used in the construction of S₁ promoter/GUS constructions are shown in figure 36.

7.2.2 Construction of a 2kb S₁ promoter/GUS gene fusion The initial chimeric S₁ promoter/GUS gene was a translational fusion made up of 1945 bp of S₁ 5' genomic sequence fused in frame to the β-glucuronidase reporter gene. The construct was synthesized by ligating 1945 bp of S₁ 5' sequence, (contained on a HindIII/PvuII DNA fragment) to pUCGUS restricted with HindIII and SmaI. The cloning of 2kbGUS was facilitated by the existence of the PvuII restriction site 15 bp downstream of the S₁ translation initiation codon. The resulting fusion gene, encodes a protein that possesses the first five amino acids from the S₁ allele (figure 37). The S₁ 5' upstream sequence was derived from a 4.2 kb HindIII genomic subclone, that was derived from petunia genomic λ clone S1G-E (see figure 36).

7.2.3 Replacement of Nopaline Synthase Termination Sequences with Endogenous S₁ 3' Sequences Nopaline synthase termination sequences (nos) present in the 2kbGUS construct were replaced with 551 bp of S₁ 3' sequence. The synthesis consisted of restricting the 2kbGUS construct with SstI, (figure
Figure 35. Construction of the β-glucuronidase cloning vector pUCGUS.

The GUS Agrobacterium transformation vector, pBI101.3 was restricted with HindIII and EcoRI to release the GUS polylinker, coding, and 3' termination sequences. This DNA fragment was ligated into appropriately restricted pUC119.
Figure 36. $S_1$ genomic subclone derivation.

This diagram identifies the derivation of various subclones utilized in DNA sequence analysis and $S_1$ promoter/reporter gene constructions. The top line of the figure represents approximately 6 kb of $S_1$ genomic DNA, that was restriction mapped by J. Okuley. Two $\lambda S_1$ genomic clones ($S1G-A$ and $S1G-E$) that I used to subclone various DNA fragments for use in chimeric promoter/GUS constructions are shown on the next two lines. I subcloned and restriction mapped a 4.2 kb HindIII subclone, (isolated from $\lambda S1G-E$), the location relative to the genomic $\lambda$ clones is graphically shown on the fourth line. For reference the location of the PS1B cDNA and sequenced $S_1$ genomic DNA is given in the next two lines. Finally, the derivation of two subclones used in $S_1$ promoter/GUS constructions (HindIII/PvuII 2.0 kb and EcoRV-EcoRI 900) are listed on the bottom two lines. An asterisk located above a Bgl II site on line 1 ($S_1$ Locus restriction map) represents the 5' restriction site used for a 8 kb $S_1$/GUS construction.
Figure 37. Construction of the chimeric $S_1$/GUS gene fusion - 2kbGUS.

2kbGUS was synthesized by restricting the pUCGUS polylinker with HindIII and Smal (blunt end), and ligating the vector with band isolated $S_1$ 5' flanking sequence (1945 bp) obtained as a HindIII, PvuII (blunt end) digestion fragment from subclone S1 HindIII 4.2.
Figure 38. Construction of the chimeric $S_1$/GUS fusion - 2kbGUS3'.

This is a schematic showing the ligation components in the synthesis of the 2kbGUS3' construction. The 3' construction was made by restricting 2kbGUS SK- (2kbGUS subcloned into Bluescript SK-) with SstI (restriction site overhang filled in to create a blunt end) and EcoRI which removes the 200 bp nopaline synthase termination sequences. The endogenous $S_1$ 3' sequences were isolated as a 551 bp Rsal (blunt end), EcoRI DNA restriction fragment from a 900 bp $S_1$ genomic subclone; S1 EcoRV-EcoRI 900.
38) filling in the SstI restriction site using Klenow fragment, which created a blunt end for ligation, and then restricting 2kbGUS with EcoRI (which removes the NOS termination sequences). To isolate 3' S1 sequences for ligation into the above prepared 2kbGUS construct, a 551 bp DNA fragment containing S1 3' sequences was gel band isolated from a RsaI (blunt end) partial/EcoRI double digest of a 834 bp S1 genomic subclone (S1 EcoRV-EcoRI 900). S1 EcoRV-EcoRI 900 was subcloned from the 4.2 HindIII S1 genomic clone (refer to figure 36). The S1 3' 551 bp fragment (possesses a blunt RsaI end and an EcoRI end) was ligated to the blunt/EcoRI cut 2kbGUS construction giving rise to 2kbGUS3' (figure 38). This DNA construction was isolated using colony hybridization to random hexamer radiolabeled S1 EcoRV-EcoRI 900 subclone. The 3' construction was confirmed by DNA sequencing from the 3' EcoRI site and from an internal priming site, 86 bp downstream of the RsaI restriction site.

7.2.4 8 kb S1 Promoter/GUS Construction  An approximately 8kb 5' S1 promoter/GUS construction was synthesized by first isolating a S1 Bgl II 8.6 kb genomic DNA fragment containing primarily 5' sequence, (figure 36) and subcloning it into the BamHI site of pUC19. To generate the 8 kb S1 GUS construction, band isolated DNA (~8 kb) derived from the S1 Bgl II 8.6 kb subclone was digested with SphI (in the pUC19 polylinker) and PvuII, and this DNA fragment was ligated with pUCGUS restricted with SphI and SmaI, yielding the DNA construction 8kbGUS (figure 39). This construct was confirmed by comparing the restriction pattern of the S1 Bgl II 8.6 kb subclone versus the digestion pattern for 8kbGUS for various restriction enzymes (Sal I, EcoRI, HindIII, and PvuII).
Figure 39. Construction schematic of chimeric S₁/GUS gene fusion - 8kbGUS.

8kbGUS was synthesized by restricting pUCGUS with GUS polylinker enzymes SphI and Smal (blunt) and ligating the resulting DNA fragment to approximately 8 kb of S₁ 5' flanking sequence. The S₁ 5' sequence was isolated in the following manner; a 8.6 kb Bgl II S₁ genomic fragment known to contain primarily 5' upstream sequence, but also possessing approximately 300 bp of coding sequence (based on restriction mapping data by J. Okuley) was subcloned into pUC 19. I had determined that S1 Bgl II 8.6 subclone did not possess a SphI restriction site, and therefore cloned the 8.6 kb fragment into the BamHI site of pUC 19 (pUC19 has a SphI polylinker site). Thus, by restricting S1 Bgl II 8.6 with SphI and PvuII (positioned 15 bp into the S₁ coding region, generates a blunt end) compatible restriction site overhangs were created for efficient intermolecular ligation.
7.2.5 S1 Promoter Deletion/GUS Fusion Constructions

To begin an analysis of the S1 upstream region, a large scale promoter deletion series was constructed using the S1/GUS construction 2kbGUS and the exonuclease III unidirectional deletion method of Henikoff (1984). The requirement by exonuclease III for a protruding 5' restriction site end, necessitated the subcloning of 2kbGUS into Bluescript SK- to provide the 5' (substrate) and 3' (protected) restriction site ends needed. 2kbGUS was subcloned into Bluescript SK- using the HindIII and EcoRI sites to generate 2kbGUS SK (figure 40). To generate the deletion series, 2kbGUS SK was restricted with ClaI and KpnI. Exonuclease III conditions were used that deleted ~200 bp per time point (35°C, 400 units exonuclease III/ml, 30 second time points, see methods 2.8).

A fine scale promoter deletion series was also constructed for maximal resolution in the proximal 5' region. The approach used took advantage of a PstI restriction site located 454 bases upstream of the transcriptional start. 2kbGUS was restricted with PstI and EcoRI, the 2.65 kb band isolated fragment was ligated into appropriately restricted Bluescript SK- to yield the DNA construction Pst450GUS (figure 41). Pst450GUS was digested with XbaI and SstII to generate 5' and 3' restriction site overhangs, exonuclease III deletion conditions were altered slightly (20 second time points at 30°C), which generated a deletion series differing by ~50 bp per time point. S1/GUS gene fusion junctions for all DNA constructions used in particle bombardment were confirmed by sequence analysis using an internal GUS 20-mer oligonucleotide primer 5'-TCACGCGTTGGGGTCTAC-3' (Clonetech), that hybridizes to bases 30 through 50 in the GUS coding region.
Figure 40. Construction of \( S_1 / \text{GUS} \) gene fusion -2kbGUS SK.

The diagram shows the cloning manipulations performed to synthesize 2kbGUS SK. Bluescript SK- was restricted with HindIII and EcoRI and ligated to a 4.2 kb HindIII, EcoRI DNA fragment obtained from 2kbGUS (contains 2kb \( S_1 \) 5' flanking region, GUS coding sequences, and NOS termination sequences). This subcloning construction was necessary to provide 5' (ClaI) and 3' (KpnI) restriction site overhangs for exonuclease III deletion constructions.
Figure 41. Construction of $S_1$/GUS gene fusion - 450PstGUS.

This diagram illustrates the ligation components involved in the $S_1$/GUS construction called 450PstGUS. Bluescript SK- was restricted with PstI and EcoRI and was ligated to a PstI, EcoRI DNA fragment isolated from 2kbGUS. This 2.65 kb fragment contains 450 bp of $S_1$ 5' flanking sequence, in addition to the GUS coding, and NOS terminator sequences. This gene construction provided a relatively small 5' upstream region to construct a fine scale promoter deletion series using 5' (XbaI) and 3' (SstII) restriction site overhangs.
7.2.6 Characterization of Promoter Deletion/GUS Constructions

DNA sequence analysis (Sanger et al., 1977) was used to determine the deletion endpoints for over 50 independent deletion constructions. Figure 42 diagrams the more notable constructions, and identifies the purpose each construction served in the overall analysis of the petunia S-locus.

7.3 Histochemical localization of GUS Enzyme Activity in Petunia S₁ Tissues

7.3.1 Introduction

To determine if biolistic particle bombardment could be used to deliver chimeric S₁/GUS gene constructs into intact petunia pistil cells for transient expression assays, I bombarded whole petunia flowers or detached stigma/styles (excised above the ovary) and then assayed for GUS expression by histochemical staining (methods section 2.10 and 2.11). The utility of the GUS histochemical assay is based on low levels of endogenous glucuronidase activity in most plant tissues (petunia pollen and pistil tissues do possess differing levels of endogenous GUS activity). Enzymatic cleavage of the substrate (X-gluc; 5-bromo-4-chloro-3-indoyl glucuronide), will generate an intensely blue staining precipitate product at the cellular site of GUS activity. I have found, due to the extreme stability of the E. coli β-glucuronidase protein (Jefferson, thesis), the histochemical assay to be a very sensitive qualitative measure for the ability of specific petunia tissues to express the chimeric S₁/GUS fusions.

Following stigma/style bombardment with the 2kbGUS construction (see figure 43, panel A and B), I observed blue staining cells (note arrows) in areas that correspond to tissue contiguous with the style transmitting tract (stigmatic epidermal cells). Cornish et al., (1987), using in situ hybridization,
Figure 42. Chimeric S₁/GUS gene constructions; a summary diagram.

This figure schematically details various S₁ promoter deletion gene constructions that were utilized in the analysis. Most constructs were used for multiple purposes, among these: nucleotide sequence determination, β-glucuronidase (GUS) histochemical localization experiments, and GUS quantitative fluorometric analysis. Identification of the deletion constructs is based on the amount of 5' flanking sequence that each contains (e.g. S1Δ-1.3 kb contains 1.3 kb of S₁ 5' flanking sequence). The small stippled region immediately to the left of the β-glucuronidase coding region represents the translational fusion boundary, defined by a PvuII/SmaI fusion junction. Thus, the first 5 amino acids are encoded by S₁ sequence. Nopaline synthase termination sequences (nos) are present to the right the GUS coding region, except for the 2kbGUS3' construct, where nos sequences were replaced by endogenous S₁ 3' termination sequences (contained on a 551 bp RsaI/EcoRI fragment). The large arrows indicates the position of the major mRNA start site and the small arrow shows the position of the minor mRNA start site. Three control constructs are also shown; pBI221 is a commercially available 35S CaMV GUS construction which was used as a positive control. LAT52-GUS and LAT59-Luciferase are tomato pollen specific promoters fused to the GUS and luciferase reporter genes respectively, and these constructs were used as positive controls for assaying gene expression in petunia pollen tissue.
Figure 42
Figure 43. Histochemical staining of *P. hybrida* organs by chimeric $S_1$ promoter deletion/GUS gene fusion constructions.

This figure shows representative color photomicrographs of the histochemical staining observed in petunia tissues following particle bombardment by $S_1$ promoter/GUS fusions. GUS expression is therefore the result of transient gene expression from the introduced fusion constructs. Arrows indicate the position of stained cells. Panel A is a photograph of the *P. hybrida* stigmatic surface showing GUS expression in stigmatic cells contiguous with the style transmitting tract. Bar=1mm. Panel B is a section through the upper portion of the stigma, that was sectioned on a vibratome in the region of GUS expression. The arrow shows an individual cell expressing the $S_1$/GUS fusion gene, with the stigmatic exudate (brownish upper layer) also being evident. $S_1$/GUS expression in epidermal cells of the ovary is shown in panel C. Intact pistils, including the ovary, were bombarded from the side, allowed to express the introduced gene for 24 hours, and then stained for GUS activity (bar=1mm). The photograph in panel D shows $S_1$/GUS expression in petunia petal cells. Petal tissue was excised from the flower, placed on agar support media, and bombarded. Following a 12-24 hour time period, to allow for transient expression, the petal tissue was stained for GUS activity. Panel E shows a photograph of a control experiment, in which a CaMV 35S/GUS fusion construct was introduced into petunia leaf cells. Arrows point to two individual cells, including a trichome, that stained for GUS activity (bar=1mm). Panel F shows another control experiment, where positive GUS staining was observed in a petunia pollen grain by a LAT52/GUS gene construction. LAT52 is a pollen specific promoter isolated from *L. esculentum*.
demonstrated that these cells express S-mRNAs in vivo. Additionally, numerous S₁/GUS deletion constructions, possessing various amounts of 5’ flanking sequence, also gave rise to style cells expressing GUS. Table 2 summarizes the various S₁/GUS fusion constructions that were used in the histochemical analysis. No stigma/style expression was observed with a promoterless GUS plasmid (pUCGUS), a TATA-less deletion construct possessing 19 bp 5’ to the transcriptional start (S1A-19), or with tungsten particles alone. To determine if GUS expression of the S₁ promoter/GUS constructs was limited to cells contiguous with the style transmitting tract (Satina, 1944), 2kbGUS was introduced into S₁ ovary, petal, leaf, pollen, and germinated pollen tissue via particle bombardment. Positive histochemical GUS expression was observed in ovarian epidermal cells and petal cells (figure 43 C and D respectively), but histochemical staining was never observed in leaf, pollen, or germinated pollen even after multiple replications (> 5 plates bombarded for each tissue).

No detectable S-locus pollen expression was observed in the GUS histochemical assays with any of the S₁ gene fusion constructions. A failure to detect S-locus transcripts using high sensitivity RNA blot hybridizations was also reported (10 μg of poly A+ pollen or germinated pollen RNA; Clark et al., 1990). The inability to detect style S-locus expression in our laboratory is similar to results obtained in other laboratories (Haring et al., 1990). Thus, it seems increasingly likely that the pollen component of self-incompatibility is encoded by a separate gene sequence from that for the style component (S-RNase). I wanted to be confident that the inability to detect pollen GUS expression was not due to a lack of transformation (gene delivery), but was
Table 2. Summary table of GUS histochemical staining experiments.

This table summarizes the GUS histochemical staining results for the $S_1$/GUS constructions shown. 1.8GUS is a $S_1$ promoter deletion/GUS construction having 1.8 kb of 5' upstream sequence, accordingly numbers appearing first in other GUS constructions refer to the amount (in bp) of $S_1$ 5' upstream sequence that each possesses. The construction 19GUS possesses only 19 bp 5' to the transcriptional start, and does not contain either TATA element. LAT52 is a GUS fusion gene, containing a pollen specific promoter isolated from *L. esculentum*. The construct labeled CaMV35 is a commercially available chimeric GUS construction (pBI221) containing the CaMV 35S promoter.

<table>
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<th>Leaf</th>
<th>Pollen</th>
<th>Germ. Pollen</th>
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<td>+++</td>
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<td>+++</td>
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<td>+++</td>
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due to the inability of the petunia stylar $S_1$ promoter sequences to cause GUS expression. Therefore, it was necessary to identify assay conditions (particle bombardment and histochemical staining parameters) for positive histochemical GUS expression in pollen (mature and germinating) and leaf tissues using positive controls. Two independent positive control gene constructions were used, one for leaf expression and the other for pollen expression. For positive GUS expression in leaf cells, a commercially available GUS expression vector called pBI221 was used (Jefferson 1987, Clonetech). pBI221 consists of the GUS coding region fused to 800 bp of the Cauliflower Mosaic Virus 35S promoter (CaMV 35S). The CaMV 35S promoter is highly expressed in leaf tissue (Benfey and Chua, 1990) and upon bombardment consistently yielded multiple ($10-20$ cells/leaf) positive expression units (figure 43 E). To obtain positive histochemical GUS expression in pollen, a GUS gene construction, LAT52-7 (Twell et al., 1989) was obtained from S. McCormick. This pollen promoter/GUS gene fusion possesses a strong pollen specific tomato promoter (LAT52). Following particle bombardment of petunia pollen, the LAT52 construct consistently produced between 20-100 distinctively positive blue staining pollen grains (figure 43 F).

7.3.2 Analysis of Petal and Ovary Expression As mentioned previously, I had detected a low level (0.025% of style message level) of $S_1$ message in ovary tissue (based on RNA blot hybridization analysis), but originally I had not detected an equivalent level of message expression in petal tissue (Clark et al., 1990). Therefore, the initial observation of GUS histochemical staining (presumably transcribed as a result of 1945 bp of $S_1$ 5' flanking sequence) in
petal tissue was surprising. To determine if the petal expression that I observed was possibly the result of foreign 3' flanking sequences (NOS termination sequences) that conferred enhanced mRNA stability, I synthesized the 2kbGUS3' construct, which contains 551 bp of S1 3' flanking sequences. The replacement did not alter the organ specific pattern of histochemical GUS expression as compared to that observed for 2kbGUS. Similarly, increased amounts of 5' flanking sequence (~8 kb of 5' upstream sequence) demonstrated no qualitative change in the pattern of GUS expression, compared to the 2kbGUS construction (Table 2).

As the result of the above GUS expression data, I performed a high sensitivity RNA blot hybridization experiment (section 4.2.2 and figure 14) to identify \textit{in vivo} expression of the S1 allele in S1.1 petal tissue. I detected a ~900 nt transcript at approximately 0.015% of the message level present in stylar tissue. To determine if this ~900 nt petal transcript was transcribed from the same transcription unit as that in styles, I performed RNase protection experiments on total RNA (40-80 |g) from petal tissue using the same strategy as that for mapping the S1 style transcriptional starts (outlined in section 6.4). The petal protection results are shown in figure 34, lanes 3 and 4. Two protected bands of similar size as those protected in style RNA are evident, indicating that the petal and style S-mRNAs are probably identical.

In summary, the various S1 promoter deletion/GUS constructs were assayed for the ability to drive GUS gene expression in a variety of petunia organ systems. The only constructs that did not express GUS in petunia floral tissue were the promoterless GUS construction pUCGUS and S1A-19, which contained the major transcriptional start site, but lacked the associated TATA
elements (Table 2). S-locus transcriptional activity in petunia petal tissue was inferred from positive GUS histochemical staining. Support for the supposition that GUS enzymatic activity reflects in vivo cellular transcriptional conditions, is the identification of a 900 nt transcript in petal tissue by RNA blot hybridization that possesses identical start sites as the S₁ style mRNA.

7.4 Quantitation of S₁ Promoter/GUS Constructions

To begin an analysis of the 5' flanking sequences required for the correct developmental expression of the S-locus, I analyzed several S₁ promoter deletion/GUS constructs in a quantitative manner using fluorescence measurements of GUS activity (methods section 2.12). To analyze the constructions a co-bombardment strategy was utilized so that, in addition to measuring GUS enzymatic activity, each experiment could also provide information as to whether the tissue had been efficiently transformed. GUS constructions were co-bombarded (methods 2.10) with a luciferase reporter gene construct, (pDO432, Ow et al., 1987). The pDO432 construct contains a strong constitutive plant promoter (CaMV 35S) that efficiently expresses the luciferase gene in most petunia plant organs (Benfey and Chua, 1990), and thus serves as an internal control.

The procedure involved the co-precipitation (2 μg each) of the GUS and luciferase constructs onto gold or tungsten particles (1-3 μm), followed by co-bombardment with sufficient time for transient gene expression (6-24 hours) of the introduced reporter gene fusions. The tissue was homogenized and the resulting crude protein extract was divided into three aliquots; (1) the
first was assayed for GUS activity using fluorometric techniques to quantitate GUS activity, (2) a second aliquot was assayed for luciferase activity (methods section 2.12) and (3) the third aliquot was used to determine protein concentration (Bradford, 1976).

7.4.1 Quantitation of $S_1$ Promoter/GUS Expression in Petunia Stylar Tissue

To identify sequences required for the correct developmental expression of the $S$-locus, I analyzed several $S_1$ promoter/GUS gene fusions (listed in figure 42). Seven different $S_1$-GUS fusion genes were quantitatively assayed for the ability to drive GUS expression as detected by fluorometric analysis. Table 3 shows the individual style transient expression data obtained. Mean GUS activity values (pmol MU hr$^{-1}$ mg$^{-1}$) were derived for each of the GUS constructions, and these values are given in table 4, and graphically illustrated in figure 44. GUS Constructs having 8kb, 1.9 kb, or 223 bp of $S_1$ 5' flanking sequence all conferred approximately equal levels of GUS gene expression. Substitution of $S_1$ 3' termination sequences (551 bp) for the nopaline synthase termination sequences had no effect on quantitative levels of GUS activity. The construct having 68 bp of 5' upstream sequence gave levels of GUS activity that were 40% of the levels observed with larger promoter regions. In agreement with the results of the histochemical staining, neither the promoter-less GUS cassette (pUCGUS), nor the construct with 19 bp of 5' sequence ($S_1\Delta$-19) demonstrated any detectable GUS activity. Taken together, these data suggest that an element located in the first 223 bp 5' of the mRNA start is required for normal quantitative expression of the $S$-locus.
Table 3. Transient expression data for $S_{1.1}$ style microparticle bombardment experiments.

Individual construct and replication number are given in the column marked CONSTRUCT. The $S_{1}$ promoter constructs are labeled according to the amount of 5' flanking sequence each contains; for example, .019-2 is the TATA-less 19 bp 5' construct and the 2 signifies the second replicate. [PROT] refers to the protein concentration of the individual plate bombardment in mg/ml. The third column (GUS raw) is the raw fluorescence data obtained after a 2 hr incubation of crude protein extract with the MUG substrate. GUS-bg is a normalization value derived by correcting for endogenous GUS activity. In style tissue this background activity was 10 raw GUS units/mg of protein. For example, the construct .019-2 possessed background activity of equivalent to 254 raw GUS units ($25.4 \times 10$), $246-254 = -8$. The GUS/mg column (5) represents the raw GUS units expressed on a per mg basis, after subtraction of background GUS activity. Column 6 converts the fluorescence units into nanomolar MU (product) formed based on a standard whereby 1000 fluorescence units are equal to a 10 nanomolar solution of MU. Column 7 converts the nanomolar concentration of MU produced per plate into picomoles of MU produced/hr/plate. The standard form for reporting GUS activity is pmol MU/hr/mg (per plate), and the values are reported in the next column (8). The values were derived by noting that the extraction volume was 2 ml, and thus pmol of MU/hr (column 7) was divided by 2 X the protein concentration (column 2). Raw luciferase values are reported in column 9, and converted to LUC/mg in column 10 by taking the raw LUC values, multiplying by 20 (dilution factor) and dividing by the protein concentration. The final data column GUS/LUC (11) is calculated from the GUS activity values in column 8 (pmol MU/hr/mg) divided by LUC/mg (column 10).
Table 3. Transient expression data for S_1.1 style microparticle bombardment experiments.

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Table 4. β-glucuronidase (GUS) activity in S1,1 styles bombarded with multiple S1 promoter deletion/GUS constructions.

Relative β-glucuronidase activity following bombardment of petunia tissues with various gene constructs. β-glucuronidase (GUS) activity units are given in pmol MU hr\(^{-1}\) mg\(^{-1}\). The endogenous background activity of non-bombarded tissue was subtracted from all data points. This results in artificially negative values for some constructs. GUS activity values are an average of the individual bombardments. The standard deviation of the reported GUS activity is given as shown. Values in parenthesis to the far right represent the number of individual replicate experiments performed.

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<td>1.09 ± 0.10 (3)</td>
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<td>S1, -68</td>
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<td>leaf</td>
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<tr>
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<td>S1, 1.9 kb/nos</td>
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<tr>
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<tr>
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<tr>
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Figure 44. Analysis of $S_1$ promoter activity in $S_{1.1}$ styles transformed by chimeric $S_1$/GUS gene fusions.

The graph shows the relative levels of GUS activity in $S_{1.1}$ styles bombarded with $S_1$/GUS deletion constructs containing differing amounts of $S_1$ 5' upstream sequence. The individual gene constructions are depicted in figure 42. Gus activity was expressed as pmol hr$^{-1}$ mg$^{-1}$ of 4-methyl umbelliferone, as measured by a quantitative fluorescence assay (section 2.12). Error bars indicate the standard deviation values for observed expression levels of the different constructs. The gene fusion labeled "pUC" refers to the pUCGUS promoter-less construct. The promoter deletion fusion $S1\Delta -19$ (-19), this construct possesses 19 bp upstream of the major mRNA start site and is lacking both TATA boxes. The -68 construct contains 68 bp upstream of the major transcription start site, and possesses both TATA elements. The deletion construct containing 223 bp of 5' sequence was labeled as -223. The construct labeled 1.9/SI refers to construct 2kbGUS3' to more accurately reflect the amount of 5' sequence. Construct 1.9/nos is 2kbGUS, and construct 8kbGUS is referred to in this figure as 8kb.
7.4.2 Quantitation of Chimeric Gene Constructs in Petunia Leaf Tissue  I analyzed the quantitative expression of several $S_1$/GUS constructions in leaf tissue. CaMV 35S promoter function was also assayed in petunia leaf tissue, as a positive control for the transient expression experiments. I analyzed four different chimeric gene fusions (2kbGUS, 2kbGUS3', S1Δ -1.8, 35S CaMV, and tungsten particles alone) for the ability to direct GUS gene expression in leaf tissue, the fluorescence data for the experimental trials is given in table 5. Shown in figure 45 is a graphic representation of the raw GUS activity for the CaMV 35S and $S_1$ 2kb promoter regions in leaf tissue graphed as a function of luciferase levels. In agreement with the histochemical staining, only the 35S construct gave GUS expression values above background. A minimum of 10-fold greater GUS expression (based on the calculated standard deviation), was observed for CaMV35S/GUS constructs as compared to $S_1$ gene fusion constructs. A comparison of GUS activity vs luciferase levels in leaf tissue, using the CaMV 35S construct, demonstrated a linear relationship between increasing GUS and luciferase levels as shown graphically in figure 45.

A failure to obtain luciferase values > than 0.1 luminescence units always (over 40 independent experimental replications) correlated with a lack of GUS activity, independent of the construct or tissue (style or leaf) being analyze. Therefore, it was reasoned that low levels of luciferase activity represented a failure to obtain a significant level of transformation for that particular bombardment, and were not included in the GUS data analysis. In style tissue I did not detect a direct linear correlation between GUS and luciferase values. This lack of correlation was observed with all constructs used, and no particular pattern was evident (each subset showing unique
Table 5. Biolistic bombardment replicate data for chimeric $S_1$/GUS constructions on $S_{1.1}$ petunia leaf and pollen.

Particle bombardment data for chimeric $S_1$/GUS constructions on $S_{1.1}$ petunia leaf and pollen tissue. Column headings and value determination calculations were identical to those discussed in table 3.

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Figure 45. GUS activity measurements of CaMV 35S/GUS and S₁ 2kbGUS gene constructs in S₁,₁ leaf tissue.

The graph illustrates the pattern of GUS expression that was observed following particle bombardment with 35S/GUS or S₁ promoter/GUS gene fusion constructs in petunia leaf tissue. The GUS activity (raw GUS units) was plotted as a function of luciferase enzyme activity, that resulted from co-bombardment of the pDO432 (CaMV 35S/luciferase) of co-expressing luciferase fusion construct. Consistent with the histochemical staining results, no significant GUS activity above background was detectable with any of the S₁ promoter/GUS fusions assayed (only 2kbGUS shown). A linear relationship is evident between the 35S GUS and 35S luciferase values (triangle) in the leaf tissue.
patterns of relation). However, in all cases where the luciferase values were below 0.1 raw luc units, no GUS expression was detectable. This point is illustrated in figure 46, where raw S1 2kb/GUS values are plotted (including those experiments showing low luciferase activity) as a function of 35S/luciferase values. A distinct separation in GUS activity levels (low vs mid to high) was observed close to the 0.1 raw luc cutoff point, this pattern was observed for all constructs assayed. The cause for the lack of a direct linear relationship in style tissue is not known. A plausible explanation was provided to me by Dr. K. Wood (Promega), he informed me that luciferase extraction yields can be appreciably affected by high concentrations of lipids present in the tissue being analyzed. It appears that the luciferase protein has an affinity for lipids and can be "trapped" within the lipid layer. I have observed that petunia style tissue appears to possess a high lipid content, as evidenced by the presence of a milkly, floating layer present on the surface of the extraction buffer after crude protein extraction procedures. Thus, the differential recovery of the luciferase protein in style tissue could explain the lack of GUS/luciferase correlation. It could also be due to inherent differences in the tissue type being analyzed (stability differences of luc mRNA or protein), or possibly due to an interaction between the different promoters (S1 vs 35S) being co-introduced into style cells, as opposed to leaf experiments where both reporter genes possessed the same promoter (35S CaMV).

By performing multiple replications for each individual construct, empirical data sets were generated, and standard deviations calculated, which verified that only small differences in total GUS activity was observed for a particular GUS construct. Therefore, the utility of the luciferase internal
Figure 46. Transient S₁ 2kbGUS and CaMV 35S/luciferase promoter expression in *P. hybrida* S₁,₁ styles.

Petunia styles were co-transformed with S₁ 2kbGUS and CaMV 35S/luciferase containing plasmids (2 μg each) and the data from 11 independent S₁,₁ style bombardment experiments is graphically shown above. S₁ 2kbGUS vs luciferase raw data values demonstrate that there is a distinct increase in GUS activities when the luciferase level is above 0.1 raw luciferase units.
control in style tissue was to identify those transient assay experiments (luc < 0.1) that were inefficiently transformed (bombarded).

To confirm that the lack of GUS histochemical staining in pollen by chimeric S1/GUS fusion constructs was not due to lack of sensitivity or a failure to appreciably transiently transform pollen tissue, I performed a quantitative analysis on S1/GUS expression in pollen and germinated pollen (2 hours). The 8kbGUS construct (2 μg) was co-bombarded with the LAT59/luciferase construct (2 μg). LAT59 is a strong pollen-specific tomato promoter fused to the luciferase reporter gene, and was provided as a kind gift from S. McCormick. Following pollen plate bombardment (4 replications) GUS activity levels were not detectable above background (0.01 pmol mg⁻¹ hr⁻¹), but high levels (relative to luciferase values measured in style and leaf tissue) of luciferase activity (5.07 raw luc units) were observed, indicative that the pollen tissue was being transformed at an appreciable frequency.
CHAPTER VIII
DISCUSSION

8.1 Overview

My research, to isolate and characterize in detail, S-locus expression in *Petunia hybrida*, has provided strong evidence implicating this gene in an important role in self-incompatibility. The purpose of this section is to analyze and discuss the implications of my expression data as it pertains to the current model of self-incompatibility. Additionally, the structure and function of these cloned S-genes are discussed, which leads to additional questions on how the observed expression and function of the S-locus relates to the biology of self-incompatibility.

8.2 Expression of *Petunia hybrida* S-locus Sequences

My research has demonstrated that the isolated cloned petunia S-mRNAs possess spatial and temporal accumulation profiles consistent with the process of self-incompatibility. S-mRNAs are expressed predominately and at high levels in mature styles, and are present at much lower concentrations (2000-5000 fold) in ovary and petal tissue. Petunia S-allele transcripts are undetectable in leaf, mature anthers, and *in vitro* germinated pollen. The stylar S-genes are expressed at low levels early in petunia floral development (first detectable at -5 dpa), and show a dramatic increase in expression that is correlated in time with the transition from bud self-
compatibility to self-incompatibility. A correlation between S-mRNA expression levels and the onset of self-incompatibility has led to the proposal that a threshold level of message accumulation \(\sim 0.2\%\) (and presumably protein) is necessary for the elicitation of the self-incompatibility reaction. The finding of elevated \(S_2\) mRNA levels relative to \(S_1\) message levels in the heterozygous line \((S_{1.2})\), combined with self-crossing data indicating a failure to identify \(S_{2.2}\) homozygous progeny in the \(S_{1.2}\) line provided a correspondence between genetic and molecular data, relating style S-mRNA prevalence levels with phenotypic expression of self-incompatibility.

8.2.1 Transient Stylar S-Locus Gene Expression I observed transient S-gene expression, via particle bombardment experiments, in whole style, ovary, and petal tissue, but not in leaf or pollen petunia cells. The demonstration that \(P.\) \(hybrida\) \(S_1\) 5' flanking sequences direct GUS expression in transient expression assays provides information concerning minimal sequence requirements for S-locus expression.

As discussed in the introduction, transient expression assays are routinely used to study transcriptional activity, and these assays have provided detailed information on transcriptional gene control elements. The transient expression data, in some cases, has been subsequently confirmed using stable transformation strategies (Twell \textit{et al.}, 1990, McCormick \textit{et al.}, 1991; Schmid \textit{et al.}, 1990). Particle bombardment technology has enabled the use of transient assay strategies on whole tissue, thus circumventing problems associated with their use in plant protoplast assay systems. The fact that petunia petal \(S_1\) expression was seen in transient expression assays using chimeric \(S_1/GUS\) fusions (figure 43, panel D), and was confirmed \textit{in vivo} by
RNA blot hybridization experiments (figure 14) is compelling evidence that whole cell transient assays accurately reflect the in vivo cellular transcription control environment.

The transient style expression data demonstrates that 68 bp of \( S_1 \) upstream sequence is sufficient for tissue specific gene expression, and 223 bp of 5' flanking sequence directs comparable levels of GUS expression as \( S_1 \) promoter constructs containing much larger amounts of 5' sequence (2.0 kb and 8.0 kb). This data indicates that elements required for quantitative levels of S-locus expression reside within 223 bp of the major transcription start site.

8.2.2 Proposed \( S_1 \) Promoter Analysis

Analysis of the DNA sequence in the \( S_1 \) 5' upstream region has revealed sequence elements possessing partial homology to binding sites for transcription factors previously shown to interact with several plant promoters. The region from -199 to -179 (5'-CCACACATCCACGTGTCTCAT-3'), in particular, contains sequences homologous to three different binding sites. These sites are: (1) the G-box sequence (5'-CCACGTGGCA-3') found in \textit{Arabidopsis} Adh, in several rbcS genes, in the adenovirus major late promoter enhancer, and in rat \( \gamma \)-fibrinogen (McKendree \textit{et al.}, 1990); (2) a region from wheat HMW glutenin related to AP1/GCN4 binding sites (5'-ATGAG/CTCAT-3') and shown to bind \textit{fos} and \textit{jun} transcription factors (Hilson \textit{et al.}, 1990); and (3) the soybean CACA sequence (5'-AACACGCA-3'), which may be associated with quantitative regulation and which is protected from DNase I digestion by soybean nuclear extracts (Neilsen \textit{et al.}, 1989). To further analyze the \( S_1 \) promoter region, I would perform mobility shift assays and DNase I footprint analysis on the proximal 5' flanking region (223 bp). Using isolated stylar nuclei, crude nuclear extracts
would be prepared, and radiolabeled restriction fragments (<100 bp) of the S1 promoter sequence would be assayed for the ability to bind nuclear protein factors, using gel retardation assays. DNase I protection experiments would also be used to identify promoter regions that bind nuclear proteins and render that region resistant to nuclease digestion. *In vivo* footprinting techniques by indirect end-labeling methods could also be employed to verify DNA binding sites in the 5' flanking region of the S1 promoter. The long-range goal of determining S-locus transcriptional control involves the eventual isolation of *trans*-activating DNA binding factors by affinity enrichment methods or by screening stylar expression libraries with isolated upstream fragments that have been demonstrated to bind nuclear factors *in vitro* or *in vivo.*

8.2.3 Pollen Expression To date no evidence has been presented demonstrating the expression of the S-locus in the male gametophyte (pollen grain). The failure to observe pollen expression has been noted (Clark et al., 1990) using both high sensitivity RNA blot hybridization experiments (10 μg poly A+ RNA) and pollen histochemical staining with chimeric S1/GUS fusion constructs. Haring et al., (1990) have also reported a lack of S-locus expression in pollen using RNA blot hybridization and S-protein/antibody analysis. Thus, several molecular techniques possessing high sensitivity have failed to identify homologous S-locus sequences in pollen. The possibility remains that the failure to detect pollen S-allele expression is due to a lack of knowledge about temporal expression of the pollen S-locus (e.g. the pollen S-locus may be expressed several hours after germination). Another possibility is that the *in vivo* stylar environment may be critical for
triggering pollen S-locus expression, a condition not met with in vitro pollen germination experiments.

In part, it is the failure to detect pollen expressed S-locus sequences that has led to the current molecular model, proposing that a tightly linked pollen component interacts with the stylar expressed S-RNase. The laboratory's of A. Clarke and R. Thompson both report that they have been unable to amplify pollen homologous S-locus transcript sequences from pollen or germinated pollen (unpublished communication) utilizing PCR. I propose two PCR experiments to further address the issue of whether stylar S-locus homologous sequences are expressed in pollen, which would provide evidence supporting either the classical one-locus genetic model or the current two-locus molecular model.

The high level of detection sensitivity provided by PCR could enable detection of extremely rare class pollen S-mRNAs. The first experiment would entail pollen RNA isolation at multiple developmental stages, pre- and post-meiotic, and in vitro germinated pollen at several timed stages. Reverse PCR using viral reverse transcriptase would be employed, in conjunction with several sets of oligonucleotide primers spanning the coding region (could also include primers overlapping the 5' and 3' flanking regions), in an attempt at amplifying the putative pollen S-mRNA. Another strategy to address the question of whether pollen is transcriptionally capable of expressing the S-locus, would be to introduce, by particle bombardment, S-promoter/GUS constructs into pollen tissue, followed by pollen RNA isolation and reverse PCR of GUS encoding transcripts. As a control for the sensitivity and feasibility of this approach, the same experiments could be
performed on bombarded style tissue, which is known to express the S-locus sequences. These experiments, however, do not address the possibility that the in vivo stylar environment may play a role in male gametophytic S-locus gene expression.

Several experimental strategies could be employed to identify the proposed pollen component. One approach involves the characterization of flanking 5' and 3' sequence to determine if other transcription units are present in the vicinity of the S-locus. J. Okuley, in our laboratory, has transcription mapped a portion of the S1 allele, and has tentatively identified a region weakly expressed in petunia pollen. A second approach would take advantage of the proposed interaction between the style S-RNase and a putative pollen component (receptor?). Expression libraries (λ gt11) would be constructed from RNA isolated from either in vitro germinated pollen or preferably from self-pollinated styles. The libraries would be screened with purified, radiolabeled S-RNase protein. This approach has the advantage of allowing for pollen/pistil interactions, which may be critical for the expression of the proposed pollen component. This expression strategy has been successfully employed to isolate DNA binding protein genes (ref) and the interleukin 1 receptor gene from human T-Lymphocytes (Sims et al., 1989).

8.2.4 Stable S-locus Transformation Experiments Because the transient expression data indicate that 5' TATA proximal S1 sequences are sufficient for expression, stable S-locus transformation experiments could be used to directly test whether these cloned S-locus sequences are responsible for determining the breeding phenotype. Stable transformation experiments to alter the incompatibility phenotype, by the introduction of S-locus genomic
sequences, have been attempted in our laboratory (J. Okuley), as well as in the laboratory of A. Clarke. Unfortunately, S-locus expression (mRNA and protein) have not been observed to date in transgenic plants, thus, precluding any analysis of S-locus function. The transient expression data seem to indicate that failure to achieve S-locus expression using stable transformation techniques is most likely due to technical difficulties, because as noted, even small amounts of 5' flanking sequence (223 bp) should be sufficient for S-locus expression. An alternate explanation for the failure to obtain stable S-locus transformants with in vivo expression levels may involve the inadvertent selection for transgenic plants with low levels of S-locus expression (S-RNase). In this scenario, high levels of S-RNase expression may be lethal to the developing organism, the result of a potentially unregulated RNase gene acting early in development. A third possibility for a lack of S-locus expression could be due to a phenomenon similar to that of co-suppression (van der Krol et al., 1990; Napoli et al., 1990). Napoli et al., (1990) and van der Krol et al., (1990) have investigated the role that the over-expression of chalcone synthase (CHS) has on petunia flower pigmentation patterns. The researchers report that by stably introducing multiple copies of the CHS gene into petunia calli, they observed gene expression suppression of the introduced CHS genes in the regenerated transgenic plants, and usually saw an inhibition of endogenous CHS gene expression. Co-suppression was observed in 25-42% of the transgenic plants that Napoli et al., (1990) and van der Krol et al., (1990) analyzed. The mechanism for this inhibition is unknown, but it may act via RNA:DNA or RNA:RNA hybrid intermediates or possibly through methylation silencing mechanisms. While the situation is not directly analogous to that in our petunia transgenic plants (the
endogenous S-genes are not suppressed), the concept of gene interaction/inhibition may be relevant to understanding S-locus expression in transformed plant lines. Finally, chromatin effects (methylation or nucleosome phasing) could potentially affect S-locus expression, whereby S-sequences located outside a specific chromatin environment would fail to be faithfully expressed. Thus, when S-loci are introduced randomly into the plant genome via stable transgenic methods, the expression of the introduced S-genes may be silenced by an unknown chromatin-related mechanism, which does not operate in transient S-locus expression assays, where the majority of the introduced DNA is found extrachromosomally.

In the context of the current molecular model (section 1.3; figure 5) it seems possible that if the pollen component of the gametophytic self-incompatibility reaction is distinct from the stylar expressed S-locus, one would expect that stylar S-locus sequences, by themselves, would alter breeding behavior in a non-reciprocal manner. For example, if the style-expressed \( S_1 \) allele was introduced into a plant that was homozygous for the \( S_2 \) allele, \( S_1 \) pollen would not set seed on this normally compatible plant. Whereas pollen produced from this transgenic plant (\( S_{1.2.2} \)) would produce only \( S_2 \) pollen, due to the \( S_1 \) pollen component not being present in the transformation constructs. This hypothesis could be tested by crossing pollen from the transgenic plant (presumably \( S_2 \) only), with a \( S_{2.2} \) homozygous tester line and observing whether seed set was observed. If the crosses were consistently scored as self-incompatible this would indicate that the pollen derived from the transgenic plant was functionally \( S_2 \), and that pollen of the \( S_1 \) phenotype was not being produced.
To further analyze the pattern of S-locus expression, I would stably integrate \(S_1/GUS\) gene fusions into petunia callus, using \textit{Agrobacterium} mediated techniques. The constructions used should possess both relatively large (>5 kb) and small amounts (-223) of 5' flanking sequence, in the event that upstream silencing sequences are present on the longer 5' \(S_1/GUS\) constructs. Constructions possessing endogenous \(S_1\) allele termination sequences should be analyzed to see what effect \(S_1\) allele 3' sequences have on GUS expression. These experiments would also circumvent potential problems caused by S-locus coding sequences (S-RNase).

8.2.5 Petunia Petal Expression The unexpected observation of petal S-mRNA expression raises the question as to what role, if any, the weak petal expression represents. To pursue this question, protein blot analysis might first be performed to confirm the presence of S-proteins in petal and ovary tissue. As mentioned, S-proteins have been shown to possess ribonuclease activity, and thus potentially could play a role in petal senescence, where nuclease activity is known to be involved. My experiments on the prevalence of \(S_1\) mRNA in senescing petal tissue (+3 to+4 days after anthesis) indicates that the message is present in slightly greater amounts (1.5-fold) in senescing petals versus mature petals. While this data does indicate that the S-mRNA is transcribed in senescing tissue, detailed petal RNA quantitative hybridization experiments should be employed to more accurately follow petal S-mRNA accumulation.

The fact that the smallest \(S_1/GUS\) construct assayed (-68) could direct the same pattern of histochemical GUS expression as \(S_1/GUS\) constructs containing significantly greater amounts of 5' upstream sequence, suggested
two possibilities for the observed GUS expression. The first possibility is that all cis-sequences necessary for correct tissue-specific expression reside in the first 68 bp of the $S_1$ promoter. The second possibility is that because much of the floral tissue (petal, ovary epidermis, stigma, and style) arise from a common cell lineage (Satina, 1944), common binding factors capable of activating lineage specific gene expression could be present in petal cells as well stylar transmitting tract cells (Davidson, 1989). The concept of lineage specific, or in this instance, floral organogenesis specific binding factors could then explain why all of the $S_1$ promoter deletion constructs assayed revealed identical histochemical GUS expression patterns. Only the $S_1$/GUS construct lacking TATA elements ($S_{1A}$-19) failed to direct floral GUS expression. Under this concept of $S_1$ gene expression, the -68 GUS construct directs a default pattern of GUS expression (expression in all sporophytic floral tissues except anthers), possibly the result of common floral organogenesis binding factors causing a constitutive level of S-locus expression in all floral tissues. This implies that the -68 $S_1$/GUS construct possesses a core sequence element (probably the two TATA elements), and that a quantitative expression element is located within the first 5' 223 bp which is responsible for high levels of style S-locus expression.

However, it is unclear whether the vast difference in the accumulation of S-mRNA in styles, relative to that in petals and ovaries, can be explained by quantitative differences in transcription alone. Previously, Walling et al., (1986) demonstrated that a 500-10,000 fold difference in message accumulation in developing soybean cotyledons was correlated with only a 1-40 fold difference in the rate of transcription. Post-transcriptional processes,
including mRNA stability, may be important contributors to the vast difference in S₁-RNase accumulation in different organs. Nuclear run-off experiments performed on isolated style and petal nuclei could be utilized to obtain information on rates of transcription in these tissues, which would provide information as to whether post-transcriptional processes play a major role in determining in vivo expression levels in style and petal cells.

8.2.6 Expression of Homologous S₁ Allele Sequences in a Pseudo-Self-Compatible Line  Pseudo-self-compatibility (PSC) is characterized by variable levels of seed set following crosses that would normally be expected to be incompatible and produce no seed (Ascher, 1984). PSC appears to be present in petunia line MSU1093, a variety that consistently yields high levels of seed set upon selfing of mature flowers (T. Sims, unpublished data). Because the levels of S-allele mRNA sequences in immature floral buds of the self-incompatible varieties appeared to be too low to allow inhibition of incompatible pollen tubes, I analyzed the expression levels of homologous S-allele sequences in the MSU1093 line to determine whether pseudo-self-compatibility in MSU1093 resulted from decreased expression of S-locus mRNA. RNA gel blot analysis demonstrated the presence of a stylar message in MSU1093 that was identical in size to that from the S₁₁ line (K.R. Clark, unpublished data). RNA hybridization experiments with the cloned petunia S-alleles revealed that the MSU1093 PSC line possesses comparable levels of a S₁ allele homologous S-mRNA (figure 12). DNA blot hybridization experiments by J. Okuley demonstrated that the PS1B cDNA hybridized to a HindIII restriction fragment in MSU1093 DNA that was the same size (4.2 kb) as that observed in the S₁₁ and S₁₂ lines. Thus, based on RNA and DNA blot
hybridization, the MSU1093 PSC line expresses the homologous $S_1$ allele, at similar levels as that seen for the $S_1$ allele in the $S_{1,1}$ strain.

8.3 S-RNase Structure and Function

What is the function of the stylar expressed S-genes? Insight into this question came from an analysis of S-locus structure. McClure et al., 1989 identified protein sequence conservation between two solanaceous S-protein domains and two secreted extracellular fungal ribonucleases (Rh ribonuclease of *Rhizopus niveus*; Horiuchi et al., 1988 and the T$_2$ ribonuclease of *Aspergillus oryzae*; Kawata et al., 1988). Haring et al., (1990) compared an S-allele consensus sequence to these two fungal ribonucleases (Rh and T$_2$) and observed that only 16 amino acid residues are completely conserved between S-proteins and fungal ribonucleases, with 13 out of 16 of the invariant sequence conservation being restricted to the two regions involved in catalytic site formation and 4 cysteine residues, which have been shown to form disulfide bridges in the ribonucleases (Kawata et al., 1988; Horiuchi et al., 1988). To date, all style expressed gametophytic S-alleles that have been cloned show homology to the Rh and T$_2$ ribonucleases (McClure et al., 1989; Clark et al., 1990; Singh et al., 1991). Furthermore, purified S-glycoproteins possess ribonuclease activity *in vitro* (McClure et al., 1990; Gray et al., 1991; Singh et al., 1991; Broothaerts et al., 1990), and have been renamed S-RNases to reflect this function.

To further support the contention that S-RNase function is critical to the self-incompatibility response, McClure et al., (1990) showed that incompatible pollen rRNA is degraded in an S-allele specific manner *in vivo*. 
While the observation of S-allele associated rRNA degradation by McClure et al., (1990) is strong evidence implicating a ribonuclease mediated mechanism for pollen growth inhibition, the experiments do not distinguish between cause and effect. Thus, rRNA degradation may be a secondary result of the self-incompatibility reaction, rather than the primary mechanism.

Jahnen et al., (1989), Gray et al., (1991), and Broothaerts et al., (1990) have shown that S-glycoproteins inhibit pollen elongation in a semi-S-allele specific manner in vitro. These experiments are partially confounded by the observation that S-proteins will inhibit elongation of all pollen tubes in vitro, regardless of S-allele phenotype, but pollen growth inhibition is greater in incompatible crosses. Gray et al., (1991) have also shown, by electron microscopy, that style S-proteins are taken up into pollen tubes in vitro. The stylar S-RNase was found in the walls and cytoplasm of the pollen tubes, and acted as an inhibitor of pollen translation as assayed in cell-free extracts and in pollen tubes.

In summary, several lines of evidence implicate the style expressed S-locus as an indispensable component of the self-incompatibility response. Among these: (1) the isolation, from several independent laboratories, of an abundant style glycoprotein (S-protein), that consistently co-segregates with self-incompatibility breeding behavior, (2) demonstration that these style S-locus genes are expressed in a tissue-specific pattern consistent with the observed biology of the self-incompatibility response (3) sequence analysis on these abundant glycoproteins demonstrate that they all are structurally related (S-RNases; figure 47), (4) purified S-proteins possess ribonuclease activity, which suggests a mechanism for pollen tube inhibition (S-RNase),
(5) purified S-RNases inhibit pollen elongation in vitro, (6) demonstration of S-RNase uptake by pollen tubes in vitro, (7) DNA blot experiments (J. Okuley) with the cloned cDNA sequences revealed polymorphic restriction fragments that hybridized in a manner expected for allelic sequences. DNA blot hybridization experiments on PFE cDNA sequences revealed only conserved restriction length patterns in the three petunia lines analyzed, which imply that the polymorphic restriction fragment hybridization pattern seen for the S-alleles was not the result of the differences in genotypic background in the three petunia lines analyzed (S_{1.1}, S_{1.2}, and S_{3.3}). The genetic and molecular data, in total, strongly argue that S-RNase proteins are responsible for the mechanism (the recognition function ?) by which the self-incompatibility reaction is promulgated.

8.4. Model of S-RNase Action

The determination that S-proteins possess ribonuclease activity immediately suggested a mechanism by which incompatible pollen tube elongation could be inhibited, specifically by an arrest of protein synthesis via RNA degradation. McClure et al., (1990) presented in vivo evidence that self-incompatible pollen RNA is degraded, while compatible pollen tubes show no such RNA degradation. This again suggested that a ribonuclease mediated mechanism. It is interesting to note that the growing male gametophyte does not synthesize rRNA, rather the elongating pollen tube relies solely on stored rRNA for protein synthesis (Mascharenis, 1986). Thus, the proposed mechanism of S-allele specific RNA degradation would be a particularly effective means to halt pollen elongation. S-RNase activity coupled with a
failure to identify stylar S-locus homologous sequences in pollen tissue has led to the current cytotoxic, two locus model (Haring et al., 1990). A wealth of correlative and circumstantial evidence involves S-RNase in a mechanism of pollen inhibition; however, nothing is known about the manner in which incompatible pollen tubes are recognized as such and subsequently are specifically inhibited. The two-locus model proposes that a tightly linked pollen component interacts with a recognition domain present on the style S-RNase and this elicits S-allele specific uptake of the S-RNase.

Currently there is extreme interest in identifying the molecular constituents of the recognition reaction. I have discussed the experiments that could be used to isolate a putative pollen receptor. Looking at the other half of the equation, Ioerger et al., (1991) have attempted to identify hypervariable amino acid residues in the solanaceous S-RNases by performing sequence comparisons with the known S-RNases. Amino acid sequences of S-proteins from 12 solanaceous species (7 derived from N. alata, 3 from P. inflata, and 2 isolated from Solanum chacoense) were compared to identify conserved and variable domains within this class of proteins. The authors reason that highly variable S-RNase residues may play a role in the recognition process, partly due to the observation that large numbers of S-alleles are found in natural populations (~200 S-alleles in clover). Ioerger et al., (1991) identified 19 hypervariable amino acid residues, but completed their analysis prior to our P. hybrida sequences being published.

The extreme sequence conservation between our P. hybrida S$_2$ and S$_3$ alleles (89%) implied that if the recognition domain resided within the S-RNase and was due to variable amino acid residues, we could compare the 19
hypervariable residues (Ioerger’s et al., 1991) with the 22 amino acid mismatches between the S₂ and S₃ alleles to locate any overlap. Overlap could be indicate which residues play a role in incompatible pollen recognition. Thus, overlapping amino acids may be particularly good candidates for in vitro mutagenesis experiments involving alterations in S-protein coding sequences and assaying for changes in S-allele specificity. The analysis is shown in figure 47 and reveals that of the 22 mismatches between the P. hybrida S₂ and S₃ alleles 8 overlapped hypervariable sites designated by Ioerger et al., (1991). Two hydrophilic hypervariable domains (HV_a and HV_b) were also identified by Ioerger et al., (1991) based on sequence comparisons. Six of the eight overlapped amino acids were located in these two regions (see figure 47). This analysis should provide a starting point to begin an analysis of S-allele recognition using in vitro mutagenesis combined with stable transformation experiments.

8.5 S-RNase Sequence Homology Among Gametophytic S-Alleles

DNA sequence analysis on PS1B, PS2A, and PS3A confirmed that they belong to a class of genes encoding stylar proteins of similar structure, (Clark et al., 1990; Haring et al., 1990; Ioerger et al., 1991). The P. hybrida S₂ and S₃ genes are closely related at the nucleotide (90.5%), and amino acid level 89%, and are the most closely related S alleles identified to date. In contrast, the S₁ allele is ~58% homologous at the nucleotide level to the S₂ or S₃ allele, and shares only 40% amino acid sequence identity. This extreme divergence in amino acid sequence is characteristic of gametophytic S-alleles. The insertion site of the single intron present in the P. hybrida S₁ and S₃ alleles is
This diagram is an adaptation of the analysis presented in Ioerger et al., (1991). Fifteen solanaceous S-protein sequences are compared, 7 are *N. alata* S-alleles (SF11, Sa, Sz, S1, S2, S3, S6) designated with an "n" after each allele number. Three are derived from the *P. hybrida* cDNA sequences (S1, S2 and S3), which are designated by a "ph" after the allele number, three S-proteins are from *Petunia inflata* (S1, S2, and S3), and are labeled with "pf" after the allele number. Two S-protein sequences are from *Solanum chacoense* (S2 and S3) and are labeled "s" after each allele number. The one letter amino acid code is used, and capital, boldface type letters represent the 41 invariant amino acids. Boldface lower case letters represent positions possessing conserved amino acid substitutions in all 15 S-protein sequences (20 conserved sites). The *P. hybrida* S1 allele possessed 4 amino acid substitutions at the 41 invariant residues, and the *P. hybrida* S3 allele possessed 1 amino acid substitution when compared to the 41 invariant sites. Each of the 5 invariant sites are highlighted by non-boldface, double underlines. Conserved S-protein residues (16) that share amino acid identity with the fungal ribonuclease Rh are labeled with single underlines. Amino acid differences between the *P. hybrida* S2 and S3 alleles are designated by open triangles. Dots represent one of the 19 hypervariable amino acid residues identified by Ioerger et al., (1991). Five boxed regions represent areas of sequence conservation and are labeled C1-C5. These conserved regions were identified in Clark et al., (1990), and further refined by Ioerger et al., (1991). Two hydrophilic hypervariable domains (HVa and HVb) identified by Ioerger et al., (1990) are also shown as boxed.
Figure 47 (continued)
Figure 47 (continued)

190  200
SF11  frCpqsdtaCdktaakvlfr.
S2    fqCphsntCdethgitkilfr.
Sa    itCrrrttnnpinkkeislfnln
S1n   idCprpktckatrn.gitfp..
S2n   idCpnpktCkpt.nkgvmfp..
S3n   idCphpktCkpmnrgikfp..
S6n   idCpnpktCktasnnqimfp..
S1ph  msCpristsCkgtnaritfrq.
S2ph  hdCrhshytCdetdstqtlfr.
S3ph  drCrhshntCdetsstkilfrg.
S1pf  ypChhsytCdetdskmilfr..
S2pf  hdCrhsktCdetdstqtlfr.
S3pf  hdCrhshntCdetdstkilfr..
S2s   ipChrpwiChadentrielvk.
S3s   ihCprpktsCngkgtkgitfp..

ΔΔ    Δ ΔΔ    Δ
conserved, occurring between amino acids 57 and 58 (numbering does not include signal sequence). Additionally, the location of S-allele intron sequences from three *N. alata* S-alleles (Haring *et al.*, 1990) and two *P. inflata* sequences (Ioerger *et al.*, 1991) is conserved in position with the *P. hybrida* S-alleles, suggesting that intron insertion occurred early in S-locus evolution. Interestingly, the intron location corresponds to the HVa domain identified by Ioerger *et al.*, 1991.

To analyze homology relationships between the cloned *P. hybrida* S-locus proteins (derived from the cDNA sequence) and other solanaceous S-proteins, I compared the three *P. hybrida* sequences to *P. inflata* and *N. alata* S-protein consensus sequences, identified by Haring *et al.*, (1990). The *P. inflata* consensus sequence possesses 139 conserved residues out of 200 amino acids. Upon alignment, the *P. hybrida* $S_2$ allele possessed the highest degree of amino acid homology among the three *P. hybrida* alleles, with the *P. inflata* consensus sequence. The *P. hybrida* $S_2$ allele shares 137/139 (98.5%) with the *P. inflata* core consensus. Accordingly, the *P. hybrida* $S_3$ allele also possessed a high degree of homology with the *P. inflata* consensus sequence, sharing 97% amino acid identity (135/139). The most divergent *P. hybrida* S-allele -$S_1$, possessed only 50.3% homology with the *P. inflata* consensus sequence (70/139). When the *P. hybrida* S-protein sequences were compared to the *N. alata* consensus sequence, the $S_3$ allele was the most divergent possessing 62.7% amino acid homology (63/102). The $S_2$ allele was slightly more conserved, possessing 63.7% amino acid identity (64/102) with the *N. alata* core consensus. The $S_1$ allele possessed 68.5% homology with the *N. alata* core consensus sequence, indicating that the $S_1$ *P. hybrida* allele shares more
sequence homology with the \textit{N. alata} S-proteins than with S-proteins within its own genus.

The \textit{P. hybrida} \textit{S}_1 allele is perhaps the most divergent solanaceous S-protein yet isolated. Additional support for this statement comes from a comparison of \textit{P. hybrida} S-alleles with the S-protein structural analysis study recently performed by Ioerger \textit{et al.}, (1991). Their analysis identified 41 invariant S-protein amino acids, implicating these 41 residues in fundamental aspects of tertiary structure formation or S-protein function. As an addendum to Ioerger \textit{et al.}, (1991), I have superimposed the \textit{P. hybrida} deduced S-protein sequence onto the S-protein amino acid alignment, shown in figure 47. A comparison of these invariant sites (diagramed as capital, boldfaced letters) revealed that the \textit{Petunia hybrida} \textit{S}_1 allele differed at 4 (the variant amino acids are double underlined in figure 47) of the 41 invariant amino acid residues. At residue position 32 (the numbering convention is based on the sequence given in Ioerger \textit{et al.}, 1991) the \textit{S}_1 allele contains an aspartate residue in place of an asparagine amino acid, at position 47 is a methionine substitution for a consensus leucine, at position 112 the \textit{S}_1 protein contains a phenylalanine residue in place of a consensus leucine, and finally at position 131 the \textit{P. hybrida} \textit{S}_1 allele possesses a valine instead of the consensus isoleucine residue. Of the four amino acid substitutions two are non-conserved; asparagine to aspartic acid (position 47) and a phenylalanine residue in place of a consensus leucine (112). The \textit{P. hybrida} \textit{S}_3 allele also possesses a conserved amino acid substitution at residue 178, with a tyrosine in place of the consensus phenylalanine. Thus, reducing the number of invariant S-protein residues to 36, and indicating that the \textit{S}_1 allele is in all
probability a highly diverged S-locus protein. Additionally, Haring et al., (1990) identified 16 conserved amino acid residues between the solanaceous S-proteins and the fungal ribonuclease Rh. These conserved residues are represented in figure 47 as capital, boldface, single underlined letters. The *Petunia hybrida* $S_1$ allele contains a valine at position 131, in place of the consensus isoleucine, reducing the number of invariant residues between ribonuclease Rh and the S-proteins to 15.

In summary, it appears that S-locus sequences are highly variable proteins that share conserved regions involved in tertiary protein formation (8 conserved cysteine residues among the solanaceous S-RNases) and ribonuclease function. The two most highly conserved regions correspond to the active catalytic sites of two secreted fungal ribonucleases (Rh and $T_2$), whereas, hydrophilic variable protein regions (HVa and HVb) are promising candidates for S-allele recognition domains. The three *P. hybrida* S-RNases illustrate the extreme sequence divergence that can be present between S sequences within a species (40% homology with $S_1$ vs $S_2/S_3$), but at the same time the *P. hybrida* $S_2$ and $S_3$ alleles are the closest related S-alleles currently identified.

Because these proteins are glycosylated, the possibility that glycosyl moieties contribute to the recognition process can not be overlooked. Glycan chains present on glycoproteins and glycolipids have been shown to be involved with cell/cell interactions in multiple plant, animal and prokaryotic systems (Samson et al., 1987; Wasserman, 1988; Sharon, 1987; Makita and Taniguchi, 1985). Woodward et al., (1989) have shown by glycan hydrolysis that carbohydrate side chains present on *N. alata* S-proteins possess variation
in number, form, and fine structure, and therefore, may be involved in the incompatibility recognition response. The *P. hybrida* S-genes each possess a single consensus glycosylation sequence (NXT/NXS). This sequence occurs in the same position, near the N-terminus, in the S<sub>2</sub> and S<sub>3</sub> proteins, and thus it is unlikely that glycosylation alone determines S-allele specificity.

8.6 Relationship between Gametophytic and Sporophytic Self-Incompatibility

Two viewpoints exist with respect to the relationship between gametophytic and sporophytic incompatibility systems. Traditionally, the ancestral nature of gametophytic self-incompatibility has been espoused by several researchers (Whitehouse 1950, Brewbaker 1959, Crowe 1964, and Pandey 1960), whereby the process of sporophytic incompatibility is thought to have evolved from gametophytic incompatibility. A second viewpoint, based on molecular data of S-locus structure argues that the gametophytic and sporophytic systems are not related and represent an example of convergent evolution.

The rationale for the traditional view is based on the fact that a wide range of species express homomorphic gametophytic self-incompatibility, whereas, in homomorphic sporophytic incompatibility the species distribution range is much narrower. A mechanism for the evolution to a sporophytic incompatibility system can be derived by a shift in the timing of pollen S-locus expression in gametophytic incompatibility systems. By shifting S-locus pollen gene expression from the male gametophyte (gametophytic incompatibility), to pollen mother cells that have not undergone meiosis (or in diploid tapetal cells), the establishment of a
sporophytic self-incompatibility system could be generated.

Moore and Nasrallah (1990) and Kandasamy et al., (1990) demonstrated that sporophytic S-locus sequences derived from B. oleracea are expressed in transgenic tobacco plants, in a pattern that closely resembles the expression pattern described for the gametophytic S-RNases. Recently, Sato et al., (1991), working on sporophytic self-incompatibility in Brassica oleracea, reported the expression of the E. coli β-glucuronidase gene (GUS), fused to a Brassica oleracea S-locus glycoprotein (SLG) promoter. GUS histochemical expression was observed in the tapetal tissue and pollen microsporocyte of transgenic Brassica plants. The authors note that since Brassica species demonstrate the sporophytic type of incompatibility the tapetal expression was expected, but the observed pollen expression was not seen previously using in situ hybridization or RNA blot hybridization techniques. Moore and Nasrallah (1990), Kandasamy et al., (1990, and Sato et al., (1991) suggest that the expression of sporophytic sequences in a gametophytic pattern supports the hypothesis that sporophytic and gametophytic systems are related.

8.6.1 Convergent Evolution Between Gametophytic and Sporophytic Systems

Several lines of evidence refute the contention that the two systems are related. First, differences in S-locus expression patterns between the two systems is evident. The failure to detect pollen expressed S-RNase homologous sequences in gametophytic systems (Clark et al., 1990; Haring et al., 1990) contrasts to the detection of S-locus transcriptional activity in species expressing sporophytic incompatibility (Sato et al.,1991). The finding that sporophytic S-genes are expressed in the style of N. alata, a gametophytic species (Moore and Nasrallah 1990; Kandasamy et al., 1990), could be due the
flexible, and modular nature inherent to eukaryotic promoters, whereby, sporophytic gene expression in transmitting tract cells of *N. alata* results from presence of conserved style-specific transcription factors. Secondly, the lack of any identifiable sequence homology between the gametophytic and sporophytic S-proteins, and the failure of sporophytic S-proteins to exhibit ribonuclease activity, strongly argue, from a functional standpoint, that the two systems are not related.

**8.7 Does the S-Locus Encode S-RNases?**

The cumulative genetic and molecular data strongly associate stylar expressed S-locus activity with a crucial role in self-incompatibility. But because definitive S-locus transformation experiments have not been successful to date, the molecular data is completely compatible with the hypothesis that S-RNases provide only the mechanism, via incompatible pollen RNA degradation, and do not encode the recognition components of the incompatibility response. In this model the S-RNase gene is tightly linked to the actual recognition factors that are responsible for S-allele specific self-incompatibility responses.

There are several inconsistencies that may question whether the gametophytic S-locus and S-RNase gene are one in the same. Among the points of contention: (1) genetic data indicated that the S-locus was expressed in both pollen and style tissue, to date this expression pattern has only been observed in sporophytic systems, (2) the observation that our PSC petunia line MSU1093 contained similar levels of ribonuclease activity as that of the self-incompatible *S*12 (Clark *et al.*, 1990). Ai *et al.*, (1991) report that two *P*.
inflata S-alleles defective in the incompatibility reaction possess ribonuclease activity levels comparable to that of functional P. inflata S-RNases. The data imply that the defect in the incompatibility reaction resides outside the S-RNase, (3) one could also argue from a molecular evolutionary viewpoint that the extreme divergence in S-RNases is due to a lack of selection pressure on non-functional portions of the protein. Thus, only residues involved in catalytic site and tertiary protein structure formation would be expected to be conserved, (4) the demonstration that Arabidopsis possesses sequences that are homologous to both gametophytic S-RNases and sporophytic S-locus sequences (Nasrallah et al., 1988; Taylor and Green, 1991).

Taylor and Green (1991) PCR amplified three S-RNase sequences from the self-compatible crucifer. The sequences (RNS1, RNS2, and RNS3) possess the ribonuclease catalytic domains. The authors observed only ~65% homology among the three RNS sequences, and note that the sequences do not cross hybridize. To my knowledge, experiments to ask whether sequences homologous to the sporophytic S-locus exist in gametophytic species have not been performed or vice versa. To analyze whether S-RNase homologous sequences exist in sporophytic species and also whether multiple S-RNase homologous sequences are present in gametophytic sequences, I would utilize PCR amplification with oligonucleotide primers synthesized from the conserved catalytic domains to attempt to isolate additional S-RNase genes. Due to the sequence divergence of the S-RNases, there is a good probability that hybridization experiments with entire S-RNase coding regions will not hybridize to related sequences under moderate stringency conditions. While sporophytic S-locus genes are more highly conserved, a similar strategy of
PCR amplifying conserved regions first, and then followed by clone isolation with the amplified fragment would be a useful method to identify, if present S-locus genes in the other incompatibility system.

In summary, the mechanism of incompatible pollen recognition remains unknown. The evidence that the S-RNase is involved in the incompatibility reaction is compelling, but its role in the recognition response is based solely on correlative evidence. In this section I have tried to point out the inconsistencies in the correlative data, and have proposed several experiments that may further elucidate the role of the S-RNase protein in self-incompatibility.
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