TRANSGENIC APPROACHES TO CONTROL SELF-INCOMPATIBILITY IN *PETUNIA HYBRIDA*

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ABSTRACT

Petunia hybrida belongs to the family Solanaceae and is an important horticultural crop grown worldwide. *Petunia hybrida* and one of its parental species, *Petunia inflata*, are also established model organisms for the study of gametophytic self-incompatibility (SI). All members of the Solanaceae, including *Petunia*, share the *S-ribonuclease* mechanism of self-incompatibility. The pistil phenotype is determined by an extracellular stylar ribonuclease or "*S-RNase*". The pollen phenotype is determined by several *S*-linked F-box genes (*SLFs*). All these genes are tightly linked at the *S*-locus to form an "*S*-haplotype".

Most cultivated Solanaceae exhibit self-compatibility (SC) as this greatly facilitates the production of seed for annual crops. SC can arise from the breakdown of a functional SI system during domestication and breeding. In this PhD project, we have attempted to develop approaches to control SI in *Petunia.* Several stocks of *Petunia* that exhibit SI are available at the University of Nottingham. These carry five distinct *S*-haplotypes with corresponding *S-RNase* sequence data, three in *P. inflata* (*PiS*$_3$, *PiS*$_{k1}$, *PiS*$_d$) and two in *P. hybrida* (*PhS*$_{3L}$, *PhS*$_v$).

Our approach to engineer SC into lines with SI involved the introduction of a heterologous *SLF* gene from *Antirrhinum hispanicum*. It has previously been shown that transformation of *P. hybrida* with the *AhSLF-S2* gene under the control of a pollen-specific promoter (LAT52) causes SI to breakdown (Qiao *et al*, 2004b). This is explained as a "competitive interaction". We have obtained the LAT52: AhSLF-S2 construct used by the group of Prof. Yongbiao Xue (Chinese Academy of

Sciences, Beijing). In addition, we have obtained three constructs containing other *SLF* family members (*AhSLF-S2C*, *AhSLF-S4D* and *AhSLF-S1E*) all expressed specifically in pollen (Zhou *et al*, 2003). The aim of this project is to extend the analysis of these heterologous *SLF* genes to test whether they offer a general approach to overcome SI in *P. hybrida*. This involved using the wider range of *S*-haplotype stocks available (5) and the full range of constructs available (4).

Initial experiments confirmed the genotype and phenotype of the *P. inflata* stocks. Crosses were made between *P.hybrida* and *P.inflata* and the resulting F_1 progeny were tested for SI. It was noticed that some progeny inheriting the PiS_d allele of *P. inflata* have a tendency towards SC, but others have a stable SI phenotype. This observation was further analysed in the F_2 generation and based on reciprocal crosses it was determined that the pollen part of the PiS_d allele had lost its function resulting in compatibility.

Prior to transformation the constructs were checked by sequencing plasmid DNA and colony PCR products using specific primers. The expected fusions of *SLF* gene and LAT52 promoter were confirmed. An established protocol was used to transform the S_3/S_v genotype of *P. hybrida.*

Different numbers of transgenic plants have been identified for each construct (6, 17, 3 and 11 for *AhSLF-S2*, *AhSLF-S2C*, *AhSLF-S1E* and *AhSLF-S4D* respectively). However, in the T_0 generation competitive interaction was not observed. Transgenic plants were crossed with *Petunia inflata* but F_1 hybrid plants remained SI. Transgenic plants obtained for *AhSLF-S2* and *AhSLF-S1E* were analysed in the T_1

generation. In spite of the fact that all plants derived from *PhSLF-S2* remained SI, one plant derived from *AhSLF-S1E* became SC. It was predicted that the compatibilty in this particular plant arises as a result of homozygosity. Based on this observation a hypothesis was proposed for a relationship between compatibility and homozygosity and several techniques were used to test this hypothesis. It has been concluded that there is a relationship between homozygosity and transgene behaviour in specific epigenetic situations.

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LIST OF ABBREVIATIONS

ARC	Armadillo Repeat Containing protein
C1-C5	Conserved domains (1 to 5) of solanaceous S-RNases
GSI	Gametophytic Self-Incompatibility
HVa & HVb	Hypervariable domains (a & b) of solanaceous S-RNases
LAT 52	Late Anther Tomato 52
PCR	Polymerase Chain Reaction
QRT-PCR	Quantitative Real Time-Polymerase Chain Reaction
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
RNAi	RNA interference
SBP	S-RNase binding protein
SC	Self-Compatibility
SCR	S-locus Cysteine Rich
SFB	S-haplotype-specific F-Box
SI	Self-Incompatibility
SLF	S-locus F-box
SLG	S-locus Glycoprotein
SP11	S-locus Protein 11
S-RNase	S-locus Ribonuclease
SRK	S-locus Receptor Kinase
SSI	Sporophytic Self-Incompatibility
SSK	SLF-interacting SKP1-like
THL	Thioredoxin-h proteins

CHAPTER 1: GENERAL INTRODUCTION

1.1 THE GENUS PETUNIA

Petunia belongs to the family Solanaceae and is a widely cultivated genus of flowering plants of South American origin. The name of this horticultural specimen was derived from the word "petun" meaning "tobacco" in the Tupi-Guarani language (Sink, 1984). The genus of *Petunia* has 30 species with one synthetic garden species *Petunia hybrida* Hort Vilm. The origin of *Petunia hybrida* is believed to be hybridization between *Petunia axillaris* (a large white flowered and night-scented petunia) and *Petunia integrifolia* (a violet flowered petunia). *Petunia hybrida* is of considerable commercial interest as it is an important floriculture crop. Consequently, its seeds have become an economically significant ornamental resource for several countries (Gerats and Vandenbussche, 2005; Stehmann *et al*, 2009).

Petunias grow as bedding plants in addition to being displayed in hanging basket containers and window boxes. Moreover, species in this genus have an important role as an experimental model for horticultural and biological research (Stehmann *et al*, 2009). For instance, in 1944, large numbers of bedding plants died in the United States, as a result of severe summer drought, but Petunias became national news as they had drought resistance (Haughton, 1978). Consequently, this drought tolerance resulted in a major effort to develop new cultivars which led to improved garden performance and produced novel flower colours. As a result of these improvements, in 1960, *Petunia* became the number one bedding plant in the United States in terms of both sales and number of plants produced (Griesbach, 2007).

Currently, a wide range of flower colours and patterns are produced by flower breeders as a result of domestication and hybrid seed production (Ganga *et al.*, 2011).

Petunia has also been the subject of many molecular and biochemical studies. For example, *Petunia hybrida* has been used as a model of choice to study the floral volatile biosynthesis and regulation (Boatright *et al*., 2004). As floral scent attracts pollinators to the sexual organs, it is very important for plant reproduction. Consequently, volatile emission is usually tuned to the foraging activity of the pollinators. In *Petunia hybrida*, the products of the floral volatile benzenoid/phenylpropanoid (FVBP) metabolic pathway dominate the volatile emissions. The pathways for benzenoid biosynthesis have been characterized. Verdonk *et al* (2005) identified a candidate for the regulation of volatile benzenoids in *Petunia hybrida* CV W115 (Mitchell), *ODORANT1* (*ODO1*), a member of the R2R3-type MYB family of genes. The flowers of *Petunia hybrida* cv W115 (Mitchell) are fragrant only in the evening and at night, and there is a direct correlation between levels of *ODO1* and volatile emission. In addition, the downregulation of *ODO1* in transgenic plants strongly reduced volatile benzenoid levels through decreased synthesis of precursors from the shikimate pathway.

Recently Fenske *et al* (2015) found the circadian clock gene for *Petunia hybrida LATE ELONGATED HYPOCOTYL (PhLHY)*, which regulates the daily expression patterns of the floral volatile benzenoid/phenylpropanoid (FVBP) pathway genes for floral volatile production, and plays a crucial role in controlling the timing of volatile synthesis and emission in Petunia. They concluded that, in the beginning of the day, *PhLHY* represses the expression of many enzyme-encoding genes in the FVBP

biosynthesis pathway, such as *Petunia hybrida* GIGANTEA (PhGI), the master scent regulator ODORANT1 (ODO1), and many other evening-expressed *FVBP* genes. In addition, in the Petunia lines, the timing of peak expression of *PhGI*, *ODO1*, and the *FVBP* pathway genes advanced after reducing *PhLHY* expression. This work can provide key insights into the complex yet relatively unexplored transcriptional regulation of scent production. In addition, it can illustrate how the circadian clock can regulate the timing of large metabolic pathways (Fenske *et al* 2015).

Another example of the contribution of Petunia to our understanding of floral traits is vacuolarPh. Recently, Cna'ani *et al* (2015) identified the role of the MYB-R2R3 transcription factor, *PH4* (previously studied for its role in vacuolar acidification) in the later steps of floral scent production. The expression of *PH4* was knocked down using virus-induced gene silencing (VIGS). Consequently, the emission of phenylpropanoid scent compounds was decreased, but volatiles, with the exception of benzaldehyde and eugenol, were increased simultaneously. It was concluded that two essential floral traits, colour and scent, are interconnected by integrated volatile production and emission processes, which are regulated by a key floral gene, PH4. Previously the role of PH4 in petal pigmentation had been illustrated to involve the direct activation of ATPases (Verweij *et al*., 2008; Faraco *et al*., 2014). However, this does not mean that modifications of vacuolar pH affected scent because scent emission or internal pool accumulation did not change as a result of silencing of a tonoplast-localized H^+-ATPase (*PH5)* that maintains vacuolar pH homeostasis.

In terms of pigmentation, *Petunia* offers one of the best genetic systems for the molecular analysis of flower color (Gerats and Vandenbussche, 2005). In *Petunia*,

the pigments responsible for flower colour are flavonoids, which can be classified into two groups: anthocyanins and co-pigments. Five different anthocyanidins (cyanidin, peonidin, delphinidin, petunidin and malvidin) are found in *Petunia* in addition to three different acyl moieties (coumaric, ferulic and caffeic acid) and two different sugars (glucose and rhamnose) (Ando *et al*., 1999; Gonzalez *et al*., 2001)

Meyer *et al* (1987) transformed *Petunia hybrida* mutant RL01 white with the maize A1 cDNA (encoding DFR) driven by CaMV 35S RNA promoter which led to the production of pelargonidin derivatives, resulting in a brick red flower phenotype. In addition, recently Chu *et al* (2015) transformed *Petunia hybrida* cultivar 9702 with Dihydroflavonol 4-reductase (*DFR*) genes from *Rosa chinensis* (Asn type) and *Calibrachoa hybrida* (Asp type), driven by a CaMV 35S promoter. In both types of *DFR* transgene a high expression level in petals was observed just before opening buds, which led to change in flower colour, and affected anthocyanin concentration. These results could be interpreted as DFR transgenes can change DFR enzyme activity. Consequently, anthocyanin concentrations can lead to changes in flower colour and this result was illustrated by anthocyanin ultra-performance liquid chromatography (Chu *et al*, 2015).

Another area of intense research in *Petunia hybrida* is the molecular and cytological study of self-incompatibility. Gametophytic Self-incompatibility (GSI) in the genus *Petunia* was demonstrated by Darwin in the 19th century. He described the essential features of self-sterility in *Petunia*. At the present time, there is much research at the molecular level to provide more information about this phenomenon. In addition, in

terms of horticulture this phenomenon could be exploited to produce hybrid seed as it has been done in the Brassica family.

1.2 AN OVERVIEW OF SELF-INCOMPATIBILITY (SI)

Self-incompatibility can be defined as the phenomenon in which a pollen grain of a fertile plant is unable to achieve fertilization after self-pollination (De Nettancourt, 1997). However, the inability of pollen to fertilise is not the sole system used by plants to prevent inbreeding; plant and flower morphology may play a crucial role as well. Plant morphology could involve the presence of only one type of reproductive organ (male and female reproductive structures on separate plants (dioecy), such as in Pistachio and date Palm. Flower morphology could take two forms: First, stigma and anther mature at different times (dichogamy), which has two types: anther mature before stigma (protandry), as in carrot , walnut and *Salvia* species, or stigma matures before anther (protogyny),such as in arum lilies (see Figure 1.1). Secondly there are differences in style and anther length (Heterostyly), which will be explained in detail in section 1.3.1.

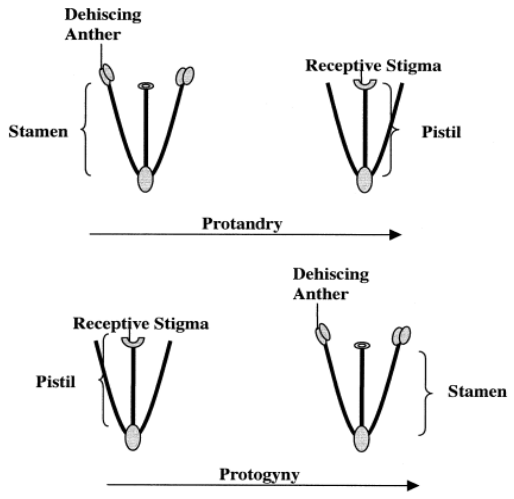


Figure 1.1 The order of anther dehiscence and stigma receptivity for aprotandrous (top) and protogynous (bottom) species (Sargent *et al*, 2006)

The vast majority of flowering plants are hermaphroditic in nature and reproductive organs are located in proximity to each other and mature at similar times, which gives the opportunity for pollen grains to land on the stigma of the same flower, resulting in self-fertilization. Self- incompatibility (SI) is by far the most important mechanism used to prevent self-fertilization. The importance of SI is that it ensures outbreeding, which is beneficial for providing variation within a species. The evolution of this mechanism has helped flowering plants to become the most successful groups of plants on earth (Silva and Goring, 2001).

The SI response consists of a self / non-self recognition process between pollen and pistil that is followed by selective inhibition of the self-pollen development. Conventional genetic studies have revealed that a single multiallelic locus (*S-locus*) controls self-/nonself-recognition in most species and pollen inhibition occurs when the same "*S-allele*" specificity is expressed by both pollen and pistil (Takayama and Isogai, 2005). Approximately a century ago, the genetic basis of GSI was established and the polymorphic *S-locus* that specifies the genetic identity of pollen and the pistil was revealed (Wang and Kao, 2012).

The *S-locus* is comprised of at least two transcriptional units organized in tight genetic linkage, with one functioning as the female and the other as the male determinant. This multigene complex at the *S-locus* is inherited as one individual unit. Consequently, the variants of the gene complex are called "*S-haplotypes*" (Takayama and Isogai, 2005). In *Petunia hybrida* at least 10 distinct *S alleles* have been described (Ai *et al*.1991; Robbins *et al*. 2000). Two independent loci, which are called S and Z are found in some grasses (de Nettancourt, 2001), such as *Secale*

cereale (Lundqvist, 1954), *Briza media* (Murray, 1974), and *Festuca pratensis* (Lundqvist, 1955).

At the present time, three distinct SI mechanisms have been identified at the molecular level in three families: the Solanaceae, Brassicaceae and Papaveraceae. In this chapter these different SI systems will be described and contrasted.

1.3 CLASSIFICATION OF SELF-INCOMPATIBILITY

1.3.1 Heteromorphic Self-incompatibility.

Heteromorphic self-incompatibility can be due to differences in flower morphology notable differences in style length and stamen length. This system is called Heterostylous SI and can be divided into two types: distylous and tristylous. Distylous SI has two flower forms (short style and long anthers and long style with short anthers). Style and anther positioning in these types are genetically controlled by dominance between alleles at the single *S-locus* (Lewis, 1947). Tristylous SI has three flower forms (short style with middle and high stamen, middle style with low and high stamen and long style with low and middle stamen) (see Figure 1.2) (de Nettancourt, 2001). In tristylous SI, style and stamen positioning are genetically controlled by dominance relationships between alleles at two loci, S and M (Mather, 1943). The molecular genetics of both these systems remains unclear despite the biology and genetics being described in detail in some plant species, such as *Primula* (Barrett, 1998; McCubbin, 2008).

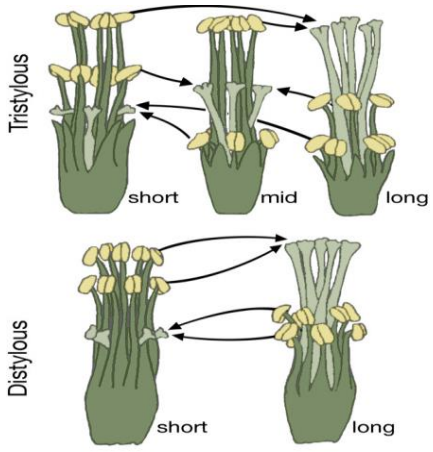


Figure 1.2 Heterostyly in the American bulb-bearing Oxalis. Only pollination indicated by the arrows, "legitimate", leads to fertilization of ovules. (Modified from Gardner *et al*., (2012)).

1.3.2 Homomorphic Self-incompatibility .

Homomorphic Self-incompatibility is found in plants that have morphologically identical flowers (Bernatzky *et al*, 1988; Kao and McCubbin, 1996). Classification of Homomorphic Self-incompatibility in flowering plants is based on the mode of genetic control of the pollen phenotype which can be derived sporophytically or gametophytically. Consequently, Homomorphic Self-incompatibility systems can be classified into two types: gametophytic (GSI) and sporophytic (SSI). These types of Self-incompatibility were revealed by classical genetic studies in the early 1950s and are reviewed by McCubbin and Kao (2000), Silva and Goring (2001) and de Nettancourt (2001).

1.4 SPOROPHYTIC SELF-INCOMPATIBILITY (SSI)

The genetics of SSI was first revealed in two species from the Asteraceae: *Crepis foetida* (Hughes and Babcock, 1950) and *Parthenium argentium* (Gerstel, 1950). After that, it was described in species from the Brassicaceae (Bateman, 1955). Subsequently, it has been found in the Convolvulaceae, Betulaceae,

Caryophyllaceae, Sterculiaceae and Polemoniaceae. However, this system has only been characterized at the molecular level in detail in the Brassicaceae family (Hiscock and McInnis, 2003; Kachroo, *et al*, 2002; Takayama and Isogai,2003; Watanabe *et al* , 2003).

In spite of the fact that the SSI system is present in the Convolvulaceae and Asteraceae families, the molecular mechanisms are different from the Brassicaceae family. This significant point was highlighted in cellular and molecular studies in *Senecio squalidus* (Oxford ragwort). The different mechanisms may arise as a result of the 'semi-dry' stigma found in Asteraceae, rather than the 'dry' stigma typical of the Brassicaceae (Allen *et al*, 2011). Moreover, style-S and pollen-S are not yet defined at the molecular level in the Asteraceae.

In sporophytic self-incompatibility, the pollen SI phenotype is determined by the parents' diploid genome (Figure 1.3). Consequently, the pollen grain carries the product of two different alleles, instead of one allele. Furthermore, dominance relationships between pairs of alleles exist in this type of SI, so complicated patterns of compatibility and incompatibility give either male or female opportunity for homozygotes holding the recessive S allele (Hiscock and Tabah, 2003). In this system of self-incompatibility, the proteins in the outer coat of the pollen grain are recognized by the stigma surface as it is the site of recognition.

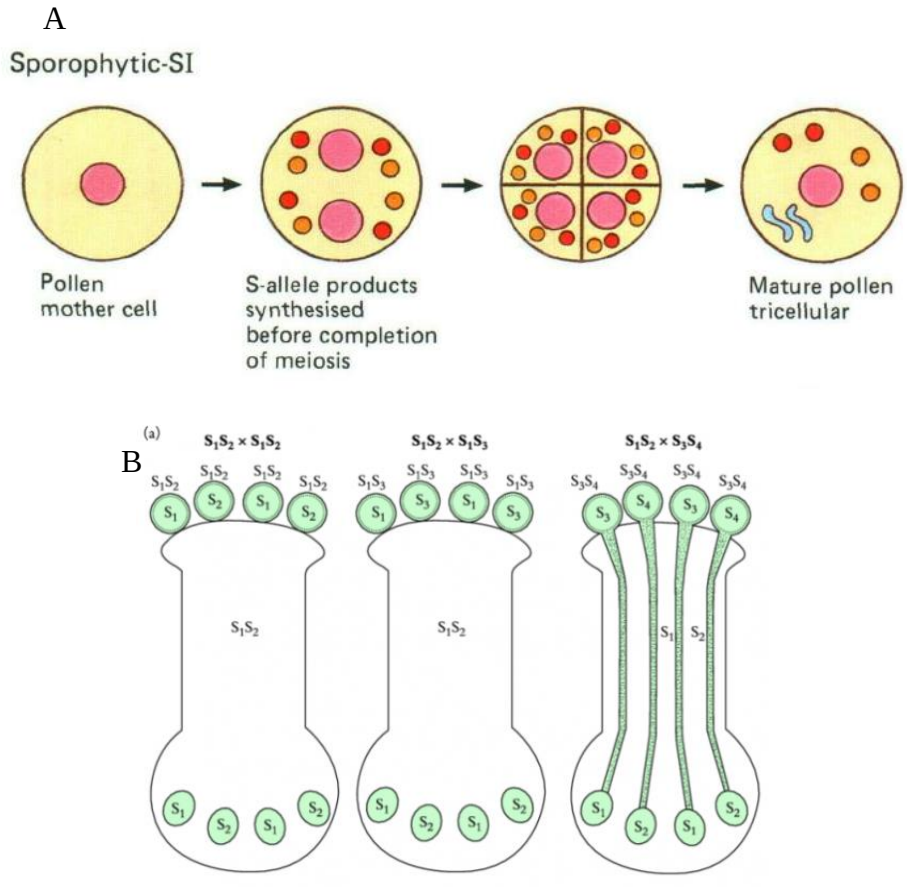


Figure 1.3 Genetic Control of SI in Sporophytic system. A: Possible explanation for SSI which illustrates how early expression of S-allele products in pollen could lead to genetic control by the pollen parent's diploid genotype (Newbigin, *et al* 1993). B: Illustration of how genetic control occurs in the sporophytic SI system for accepting and rejecting S-alleles. Pollen grains that came from parents which hold the same S-alleles as the pistil are rejected while those carrying different S-alleles are accepted. (Based on Sedgley and Griffin (1989)).

To provide more evidence to comprehend the evolution and complexity of SSI systems, more studies relating to other species will be needed at the molecular level particularly of those families that have previously received little attention.

1.4.1 SSI in Brassicaceae Family.

The genetics of SI in the family Brassicaceae were interpreted in the early 1950s by Bateman (1955) as a single Mendelian locus, the *S* (Sterility) locus with multiple alleles or variants, each of which encodes a distinct mating specificity. The number of *S-locus* alleles is usually large, in *Brassica rapa* (syn. *campestris*) and in *B.*

oleracea, more than 30 and 50 *S-haplotypes* have been identified respectively (Nou *et al*, 1993;Brace *et al*. 1994 and Ockendon, 2000). Until 2014, the *S-locus* responsible for SI was only investigated in distinct species in Brassica and *Arabidopsis capsella*, but recently, Leducq *et al* (2014) revealed SI in a third deep-branching lineage of Brassicaceae: the tribe Biscutelleae.

In self-incompatible plants of this family, the stigma that expresses the same *S-haplotype* as the pollen's parent prevents pollen germination. Self-pollen rejection is caused by an inhibition of pollen hydration, or an immediate arrest of the pollen tube growth at the stigma surface (Hiscock and Tabah, 2003).

According to their phenotypic effect on self-incompatibility and through classical genetic analysis, the Brassica *S* alleles have been classified into two groups. The first category of alleles (high-activity) demonstrate a strong self-incompatible phenotype which are placed relatively high on the dominance scale with an average of 0 to 10 pollen tubes developing per self-pollinated stigma. The second group of alleles (low-activity) exhibit a weak or leaky self-incompatible phenotypic effect in which 10 to 30 pollen tubes develop per self-pollinated stigma and they are postulated to be recessive (Nasrallah *et al.* 1991).

Molecular analysis of the *S-locus* region has revealed that this locus is a complex spanning many kilobases, which contains several physically linked transcriptional units that cosegregate perfectly with SI phenotype (Boyes *et al*. 1991; Casselman *et al.* 2000).

1.4.1.1 Female determinant of SSI in Brassica

The exploration of the female determinant was initiated with the immunological identification of an *S*-haplotype specific antigen in the stigma and then by the biochemical identification of stigma glycoproteins called *S*-locus glycoproteins (SLGs) that cosegregate with *S*-haplotypes. Subsequently, two closely linked genes were identified at the S-locus, *SLG* (for S-locus glycoprotein) and *SRK* (for S-locus receptor kinase) and are considered to participate in the perception of self-pollen by the stigma (Nasrallah *et al*. 1985, Stein *et al*. 1991). These two genes are highly polymorphic, and both are expressed specifically at the surface of mature stigmas. The important difference between SRK and SLG is that only SRK has an intracellular domain. Mutations in SRK (Goring *et al*. 1993; Nasrallah *et al*. 1994) and loss of SLG expression (Toriyama *et al.* 1991; Nasrallah *et al.* 1992; Shiba *et al.* 1995) have been related to loss of SI.

The *SLG* gene encodes a 55 kDa glycoprotein secreted into the papillar cell wall. Although the deduced amino acid sequence of SLGs are highly polymorphic, 12 conserved cysteine residues are revealed to be conserved. This feature suggests a common function for the cysteine residues of the glycoprotein (Nasrallah *et al.* 1992; Shiba *et al.* 1995). In spite of the fact that SLGs have several potential N-linked glycosylation sites, there are only two conserved sites (Kusaba *et al*. 1997).

There are several pieces of evidence that cause doubt about the role played by SLG in the SI response. Notably the characterization of self-incompatible plants which express a very low level of *SLG*, but also the identification of naturally self-compatible haplotypes which express a high level of *SLG* (Gaude *et al.* 1995).

Conversely, it has been shown that the *SRK* gene is a much more reliable candidate for the female determinant than *SLG*. The principal evidence for this is the gain of function experiment using transgenic plants of *Brassica rapa* reported by Takasaki *et al* (2000). According to this study only *SRK* determined the *S-haplotype* specifity of the stigma and *SLG* only enhanced the activity of *SRK* in the SI recognition response. Further confirmation of the precise role of *SRK* was obtained from another gain of function experiment, using *Brassica napus* (Silva *et al*, 2001). In contrast, there was no evidence regarding the enhancing role of *SLG*. Different researchers have concluded various possibilities related to the role of *SLG* (Silva and Goring, 2001). There may be variability among different *S-haplotypes* in the *SLG* requirement during SI response in the Brassicaceae (Silva and Goring, 2001; Takayama and Isogai, 2005).

1.4.1.2 Male Determinant of SSI in Brassica

Through extensive sequence analysis of the *S-Iocus* region between the SRK and SLG genes of *Brassica rapa,* the male determinant of SSI was identified. It was found to encode a small cysteine-rich pollen specific protein designated SP11 (S-Iocus protein 11) (Suzuki *et al.,* 1999) or SCR *(S-Iocus* cysteine rich) (Schopfer *et al.,* 1999). Moreover, functional analysis in transgenic plants has confirmed that this gene (*SP11/SCR*) encodes the Brassica pollen S determinant (Schopfer *et al*., 1999; Suzuki *et al*., 1999; Takayama *et al*., 2000). Furthermore, Jung *et al*. (2012) illustrated the self-compatible phenotype in a transgenic line *of Brassica rapa* after silencing of the *SP11* gene. Moreover, in subsequent generations, this trait was stable, even after crossing with other commercial lines. These results also proposed

that the consequent self-compatibility could be transferred to commercial cultivars with the required performance in *Brassica rapa*.

Recently Gao *et al* (2016) found that *SP11* in *B. napus* had lost function after insertion of 3.6 kb of a non-autonomous Helitron transposon into the promoter. Comparison between *B. napus* lines which contain the *S-haplotype* BnS-1 revealed that the Helitron transposon is widely distributed. This insertion might lead to a change of the mating system and facilitate the speciation of *B. napus.*

1.4.1.3 Interaction of Male and Female Determinants in Brassica

As mentioned before three polymorphic SI genes have been identified at the S-locus of Brassica species. However, the most reliable information is that the *SRK* gene determines the S-specificity of the stigma (Takasaki *et al*, 2000) and the *SCR/SP11* gene is the pollen determinant (Schopfer *et al*. 1999; Shiba *et al*. 2002; Suzuki *et al.* 1999; Takayama *et al,* 2000). The role of the *SLG* gene in female determinant function is to enhance the SI recognition reaction (Takasaki *et al*, 2000) possibly by stabilizing *SRK* (Dixit *et al,* 2000).

In Brassica stigmatic papillae, it has been revealed that *SRK* is localized in both endosomes and the plasma membrane (Ivanov and Gaude 2009). SI occurs as a result of *S-haplotype* specific ligand-receptor interaction between the *SCR*/*SP11* protein present in the pollen coat of the self-incompatible pollen and *SRK* (Kachroo *et al.* 2001; Takayama *et al*. 2001; Ivanov and Gaude 2009).It is thought that, the *SCR*/*SP11* acts as a pollen borne ligand, consequently, activating the stigmatic *SRK*

receptor by binding to its extracellular domain in an *S-haplotype* specific manner during a self-incompatible pollination. The activation of *SRK* then causes the initiation of a signalling cascade in the stigmatic papilla, thereby leading to the eventual rejection of the incompatible pollen as illustrated in Figure 1.4. However, when the *SCR*/*SP11* ligand is absent, *SRK* forms ligand independent dimers (Giranton *et al.* 2000; Shimosato *et al.* 2007).

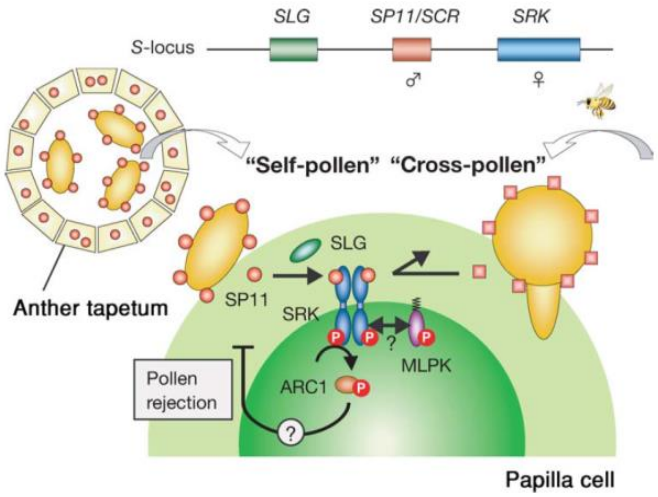


Figure 1.4 Molecular model of the self-incompatibility (SI) response in the Brassicaceae. The *S*-locus consists of three genes, *SRK*, *SP11/SCR*, and *SLG*. The receptor kinase (SRK) and SP11 represent female and male determinant respectively. During pollen maturation, Sp11/*SCR* is expressed mainly in the anther tapetum and accumulates in the pollen coat and penetrates the papilla cell wall binding with SRK in an *S*-haplotype-specific manner. Self-pollen is rejected as a consequence of the binding which increases the autophosphorylation of SRK, triggering a signalling cascade. In some *S*-haplotypes the SI reaction is enhanced by SLG as it is localized in the papilla cell wall but it is not essential for the self-/nonself-recognition. The signalling cascade downstream of SRK has not yet been characterized. However, MLPK and ARC1 are essential positive effectors. A signalling complex may form between MLPK which localizes in the papilla cell membrane and SRK in a phosphorylation-dependent manner which may target unknown substrates for ubiquitination,ARC1, an E3 ubiquitin ligase, binds to the kinase domain of SRK, pollen rejection can occur as a result of the proteasomal degradation of these substrates (Takayama and Isogai, 2005).

Another feature of activation of SRK is that it may cause the initiation of signalling pathways which can finally be responsible for the blocking of vital steps, such as the release of water for pollen hydration and release of stigmatic papilla cell wall-degrading enzymes which are required for the growth of the pollen tube (Silva and

Goring, 2001). In addition to the role of both SCR/SP11 and SRK in the events that initiate the allele-specific rejection of self-incompatible pollen, other components are required to encourage cellular responses leading to pollen rejection. Yeast two-hybrid screens have been used to identify proteins which interact with the SRK kinase domain as one approach to search for these downstream signalling molecules (Goring, 2000). Three proteins THL1, THL2 and ARC1 were identified by screening of a *B. napus* pistil cDNA library. Using this approach THL1 and THL2 are two stigmatic thioredoxin-h proteins identified as binding partners with SRK. The interaction between SRK and THL1/2 was revealed to be independent of phosphorylation. Moreover, they required a conserved cysteine residue in the SRK transmembrane domain (Bower *et al*., 1996). There is a good evidence that THL1/2 act as negative regulators of the kinase activity of SRK in vitro (Cabrillac *et al*, 2001). Firstly, Haffani *et al* (2004) analysed antisense THL1 and THL2 transgenic plants of *Brassica napus*. The suppression of TH1/2 transcripts in stigmatic tissues leads to weak constitutive rejection of the pollen grains of *Brassica napus* which are self-compatible. The function of TH1/2 is to keep SRK in an inactive state by interacting with SRK upstream of the SCR-SRK complex as illustrated in Figure 1.5 (Kaothien –Nakayama *et al*., 2010).

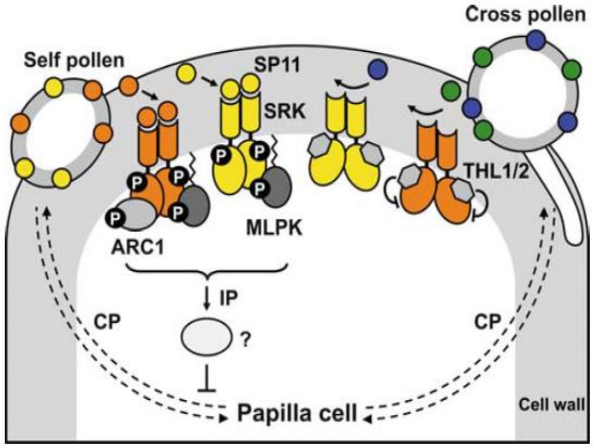


Figure 1.5 Hypothetical model for molecular events leading to the sporophytic self-incompatibility (SSI) response in the Brassicaceae. The SI response occurs as a result of the **S**-haplotype-specific interaction between (SRK) on the membrane of stigma papilla cell and its small, cysteine-rich ligand from the pollen coat, SP11/SCR. This phenomenon includes trans-autophosphorylation of the SRKs, and phosphorylation of the two *SRK* cytoplasmic interactors, the membrane-anchored protein kinase MLPK, and the E3 ubiquitin ligase ARC1. MLPK function is crucial for the SI response, although the event downstream from MLPK is still unknown. The protein component of compatible pathway (CP) might be targeted by ARC1 in order to promote degradation in the proteasome. Lack of SP11/SCR, the thioredoxin-h proteins, THL1 and THL2 keep the *SRK* in an inactive state which results in the accuracy and polarity of the SI response. Different colors represent different *S-haplotypes* of the SP11s/SCRs and the SRK*s*. IP, incompatible pathway (Kaothien –Nakayama *et al*., 2010).

Regarding genes involved in SI response in stigma, Gu *et al* (1998) identified ARC1 (Armadillo (Arm) repeat-containing protein) a member of the plant U-box (PUB) family which interacts with the kinase domain of Brassica napus SRK in a yeast two-hybrid screen. Later, it was noticed that, *ARC1*, in Brassica stigmas expressed specifically and its down regulation using antisense constructs led to partial breakdown of SI in the stigmas of *Brassica napus* (Stone *et al.* 1999).

In contrast to THL1/2 it was revealed that ARC1 is regulated by phosphorylation. The phosphorylation of ARC1 by SRK occurs as a result of specific binding of ARC1 to the phosphorylated kinase domain of SRK. ARC1 protein with its mRNA have a tissue specific pattern of expression localized in the stigma. This demonstrates

a specialized role of ARC1 in pollen-pistil interactions (Gu *et al*., 1998). Furthermore, the analysis of ARC1 antisense transgenic plants confirms its role as a positive regulator of Brassicaceae SI signalling pathway. Transgenic plants partial breakdown in SI as a result of suppressing this protein (Stone *et al*, 1999).

Arabidopsis thaliana, which belongs to the Brassicaceae family, is considered to be a model of choice for analysis of a large variety of physiological, developmental, and evolutionary processes. However, some biological phenomena such as self-incompatibility are not observed in this model species. Consequently, self-incompatible plants were obtained using transgenic approaches by transferring *SRK–SCR* gene pairs isolated from the self-incompatible crucifers *A. lyrata* or *Capsella grandiflora* (Nasrallah *et al*., 2002, 2004; Boggs *et al*., 2009 a, b).

However, there is a doubt about the exact role of ARC1, because of the different points of view and interpretation by teams involved. For instance, Indriolo *et al* (2014) transformed *Arabidopsis thaliana* [Col-0 ecotype (CS22625) and Sha (CS22652)] with *A. lyrata SCRb* and *SRKb* genes driven under their native promoters. They noticed that *ARC1* is required for self-incompatibility in *Arabidopsis thaliana* as it promotes a stable and robust pollen rejection after expression with two other *A. lyrata* self-incompatibility factors. However, these results were criticized by Nasrallah and Nasrallah (2014) because they realised that in all *A. thaliana* accessions which have been analysed to date the *ARC1* gene was deleted (Kitashiba *et al*, 2011; Indriolo *et al*, 2012) including those exhibiting strong incompatibility after transforming with SRKb-SCRb (Kitashiba *et al*, 2011).

1.5 GAMETOPHYTIC SELF- INCOMPATIBILITY SYSTEM (GSI)

In the gametophytic system the phenotype of pollen incompatibility is determined by its own haploid genotype see Figure (1.6-A). Self pollen is rejected during pollen tube growth in the style Figure (1.6-B). This type of SI is by far the most widespread and it exists in more than 60 flowering plant families (Weller *et al*, 1995). Only two forms have been described in detail at the molecular level revealing two different stigmatic *S-genes* so far, to achieve self-pollen recognition. The first form is the stylar ribonuclease or S-RNase system that was originally investigated and is broadly characterized in several members of the Solanaceae Anderson *et al* (1986) and following this was described in the Rosaceae Sassa *et al*., (1996) and Plantaginaceae (Xue *et al.,* 1996).

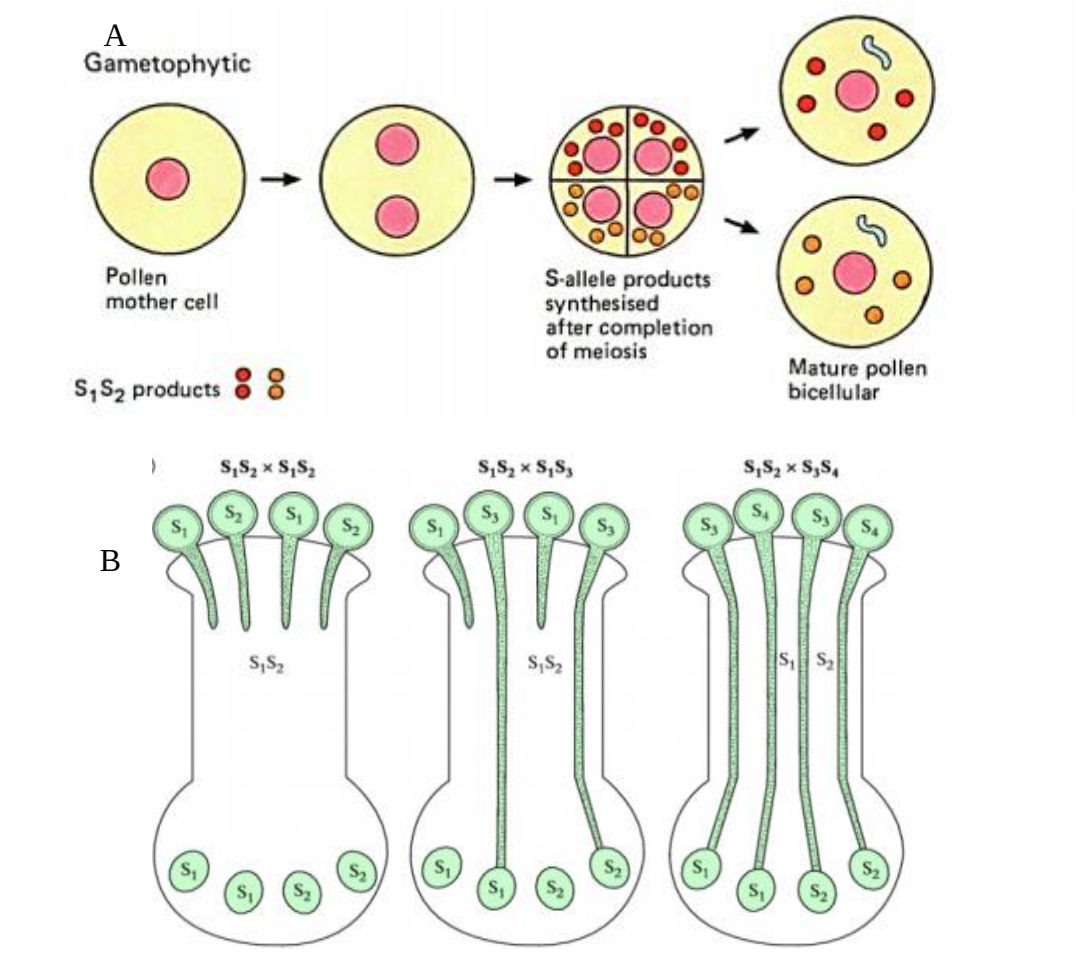


Figure 1.6 Genetic Control of SI in gametophytic system. A: Possible explanation for GSI which illustrates how late expression of S-allele products in pollen could lead to genetic control by the haploid pollen genotype. Modified from Newbigin *et al* (1993). B: Illustration of how genetic control occurs in the gametophytic SI system for accepting and rejecting S-alleles. (Modified from Sedgley and Griffin (1989)).

The second form is found in the Papaveraceae and more specifically in *Papaver rhoeas* (Hiscock and McInnis 2003; Franklin-Tong and Franklin, 2003b).

1.5.1 Self-incompatibility in Papaveraceae.

In spite of the fact that self-incompatibility in both the Papaveraceae and Solanaceae is gametophytic, they are totally different at the molecular and biochemical level. In both families, the phenotype of the pollen is determined by the pollen genotype. However, in poppy, the self-incompatible pollen is rejected on the surface of stigma

and not in the style as observed with the Solanaceae (Franklin-Tong *et al*., 1992). This SI system has been extensively studied in a single member of the Papaveraceae, *Papaver rhoeas* (wild poppy).

GSI in *Papaver rhoeas* is genetically controlled by a single multi-allelic *S-locus*. Molecular studies were expected to identify the existence of two tightly linked, multi-allelic *S-genes* encoding a stigmatic S protein and a pollen S protein (Lawrence *et al*., 1978).

1.5.1.1 Female determinant in Papaveraceae

As both Solanaceae and Papaveraceae share gametophytic self-incompatibility, initially the stigmatic S proteins of *Papaver rhoeas* were predicted to be RNases. However, this idea was in doubt after several experiments failed to give any evidence of RNase activity associated with the *Papaver rhoeas* self-incompatibility response (Franklin-Tong *et al*, 1991).

When the first stigmatic S_1 *gene* was cloned and sequenced, it gave evidence that it encodes something other than an RNase. Franklin-Tong *et al* (1988, 1989, 1991), Foote *et al* (1994) and Kurup *et al* (1998) have isolated several other stigmatic S proteins corresponding to different *S* alleles such as (S_3, S_7, S_8) and (Sn_1) from *Papaver rhoeas* and its close relative, *Papaver nudicaule* respectively. These stigmatic *S* genes were expressed in *Escherichia coli* and recombinant proteins were illustrated in vitro to have the ability to inhibit pollen tube growth in an S-allele-specific manner (Foote *et al*., 1994; Walker *et al*., 1996). Consequently, the multi-

allelic stigmatic *S* gene represents the pistil component of the Papaver SI system (Foote et al., 1994).

The stigmatic S-proteins are small (~15 kDa) extracellular signalling molecules, some of which are modified by N-glycosylation (Franklin-Tong and Franklin, 2003a; McCubbin and Kao, 2000). Although the exact basis of allelic specificity remains to be illustrated, site-directed mutagenesis of the S_1 protein has established that certain residues located in hydrophilic surface loops are crucial for the recognition of S_1 pollen (Kakeda *et al*., 1998; Jordan *et al*., 1999).

1.5.1.2 Male determinant in Papaveraceae

Biochemical studies proposed that SBP (S-Protein binding protein) was a candidate pollen receptor which interacts with the specific stigmatic S-proteins (Hearn *et al.,* 1996). SBP is a pollen plasma membrane glycoprotein of 70–120 kDa. According to in vitro studies SBP binds specifically to stigmatic S-proteins but apparently does so in a non *S*-haplotype-specific manner. Consequently, SBP may be an accessory receptor rather than the pollen S-receptor *per se* (Jordan *et al.* 1999). However, biochemical analysis using site-directed mutagenesis identified that all S-protein mutants that exhibit a decreased ability to inhibit incompatible pollen are also reduced in SBP binding activity; this provided evidence that SBP could be the pollen S-receptor (Jordan *et al.* 1999).

Wheeler *et al* (2009) identified a highly polymorphic pollen-expressed gene named *PrpS* (*Papaver rhoeas* pollen S determinants) which encodes a novel protein of ~20 kDa as the male S-determinant. Consequently, they suggested renaming the pistil *S*-

gene from *S* to *PrsS* (Papaver *rhoeas* stigma S determinant). Both PrpS and PrsS proteins exhibit similar levels of polymorphism. However, PrpS proteins have two conserved domains. One of the domains overlaps with part of a predicted extracellular domain and the other is part of a hydrophobic region surrounding the centre of the protein (Wheeler *et al*., 2009, 2010).

1.5.1.3 Interaction of Male and Female Determinants in Papaveraceae

According to several studies regarding the SI system in poppy, S-specific interaction between the male and female determinants triggers an SI signalling network, which integrates several cellular components in incompatible pollen (see Figure 1.7). There are two categories of these signalling events. Firstly, it is confirmed that, according to Ca^{2+} -imaging studies, $[Ca^{2+}]$ is increased rapidly in the pollen tube encountering incompatible stigmatic S-proteins (Franklin-Tong *et al*., 1993, 1995, 1997). Consequently it is proposed that the SI response in *Papaver* is a receptor-mediated response. Influx of both Ca^{2+} and K^{+} which are part of the signal initiation events cause inhibition of sPPase (soluble inorganic pyrophosphatase) activity, depolymerisation of F-actin and inhibition of incompatible pollen tube growth. Secondly, the activation of caspase-3-like/DEVase activities leads to suicide signalling events which are involved in commitment to PCD (programmed cell death) and involves activation of p56 MARK (mitogen activated protein kinase) , increases in ROS (reactive oxygen species) and the formation of F-actin foci. These produce ‘gateways’ through which incompatible pollen must pass to become irreversibly inhibited by setting up self-pollen ‘suicide’. The incompatible pollen is programmed to die and disassemble directly after cytosolic acidification and caspase

activation occur. These mechanisms together coordinate to prevent self-fertilization (Eaves *et al*, 2014).

Moreover, recently Wilkins *et al* (2015) confirmed the relation between cytosol acidification and self-incompatibility, using manipulation of the cytosolic pH of the pollen tubes in vivo. They revealed that, during 10 min of SI induction, cytosolic pH changed to 6.5. There are several observations which support the relationship between the cytosolic acidity and the key targets of the major physiological alteration. The activity of a soluble inorganic pyrophosphatase which is required for pollen tube growth is inhibited by a small drop in [pH] cyt. With regard to the reactive oxygen species (ROS), which have previously been recognized as toxic metabolic products, recently new roles for the reactive molecules have been identified. Biological processes such as, growth, development, response to biotic and abiotic environmental stimuli, and PCD are regulated by ROS. In addition, nitric oxide (NO) has been recognized as a key regulator of PCD (Serrano *et al*, 2015).

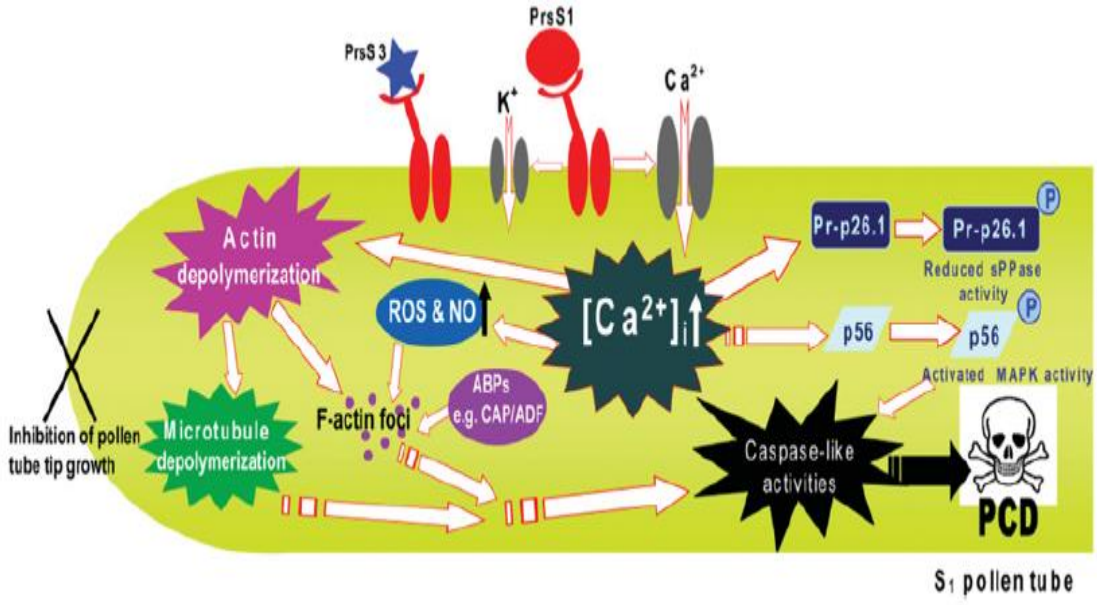


Figure 1.7 Model of the integration of the signals and targets of the poppy SI signalling network. PrsS protein interacts with PrpS proteins in an S-specific manner. This triggers both K^+ and Ca^{2+} influx. The increase of cytosolic Ca^{2+} triggers a signalling network which targets several downstream components (only shown the inhibition of pollen tube growth and PCD of incompatible pollen). The inhibition of pollen tube growth leads to the rapid depolymerisation of F-actin cytoskeleton. Later in the SI response, F-actin forms large punctate foci. The microtubule cytoskeleton is also depolymerised rapidly. These changes to the cytoskeleton signal to PCD, involving caspase-like activities, as does an MAPK, p56, which is activated by phosphorylation. ROS and NO increases are also involved in signalling to PCD. Together, these events ensure that fertilization cannot occur in an incompatible situation. ABP, actin-binding protein; CAP, cyclase-associated protein. Modified from (Eaves *et al*, 2014)

In spite of the fact that key components have been identified in the signalling network of the poppy SI system, many questions remain unanswered. Further studies and efforts are required to illustrate the mechanisms involved in the poppy SI response. In addition, the demonstration of wide transgenic functionality of the poppy SI system in *Arabidopsis thaliana* indicated that the transfer of the poppy SI system to unrelated crop species is possible (Eaves *et al*, 2014). A significant advance towards this goal was obtained when robust self-incompatible *Arabidopsis thaliana* was produced as a result of transferring *PrsS* and *PrpS* from *Papaver rhoeas* (Lin *et al*, 2015). Consequently, this SI system can be exploited at least in principle

to increase efficiency in the production of F_1 hybrids in crop plants (Eaves *et al*, 2014).

1.5.2 S-RNase system or stylar ribonuclease system.

According to Igic and Kohn (2001) this type of SI is by far the most phylogenetically widespread form of self-incompatibility that has been found in angiosperms. In addition to the Solanaceae family, it has been identified in Scrophulariaceae (Plantaginaceae) and Rosaceae. More details about self-incompatibility in the Rosaceae family will be described in section 1.5.3.1.

1.5.2.1 Female determinant in the S-RNase system

The female determinant was initially revealed in self-incompatible *Nicotiana alata* as style glycoproteins of ~30 kDa that are secreted into the extracellular matrix of the style transmitting tract and inner epidermis of the ovary (Anderson *et al*., 1986; Cornish *et al*.,1987; McClure *et al*.,1993). These glycoproteins cosegregate with the S-haplotype in genetic crosses which led to cloning and identification of related proteins from members of the Solanaceae and other families (Takayama and Isogai, 2005). The first cDNAs for one of these proteins was cloned from *Nicotiana alata* by Anderson *et al* (1986). These proteins are extracellular and were revealed to have similarity to fungal ribonucleases (RNases) and later confirmed to have ribonuclease activity (Bredemeijer and Blaas, 1981; Anderson *et al*., 1986). Subsequently, related proteins and their corresponding cDNAs were identified in the style of other self-incompatible Solanaceae plants, for instance, *Petunia inflata* and *Petunia hybrida* (Ai *et al*, 1990; Clark *et al*, 1990) *Solanum chacoense* and *Solanum tuberosum* (Xu *et al*, 1990; Kaufmann *et al*, 1991) in addition to *Lycopersicon peruvianum* (*Solanum peruvianum*) (Tsai *et al*, 1992).

Regarding the Plantaginaceae family, most species of the genus Antirrhinum also possess gametophytic self-incomptibilty which is regulated by a single multiallelic *S-locus* (East, 1940). Xue *et al* (1996) identified three cDNAs encoding polypeptides homologous to S RNases in Antirrhinum, which all are encoded by genes at the S locus. They revealed that the Antirrhinum S RNases are primarily expressed in the style transmitting tissue and assist with arresting the growth of self-pollen tubes.

As both Solanaceae and Rosaceae are phylogenetically distant, it was unexpected that both families employ the same molecule as the GSI pistil determinant (Chase *et al.,* 1993; Igic and Kohn, 2001). In the Solanaceae family molecular analysis of S-RNases identified five conserved domains (C1, C2, C3, C4 and C5) and two hypervariable regions (HVa and HVb) located between the C2 and C3 domains (Ioerger *et al*, 1991). On the other hand, S-RNases in the Rosaceae family do not have the C4 domain, which is found in the Solanaceae; it is replaced with RC4 and it has only one hypervariable region (RHV) (Takayama and Isogai, 2005). The S-RNase structure for Solanaceae and Rosaceae family is illustrated in the Figure 1.8

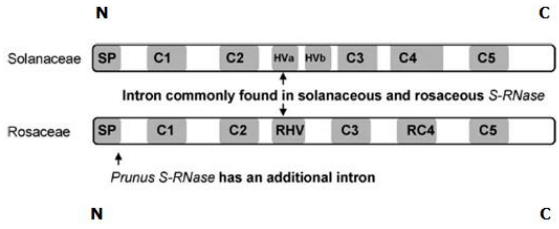


Figure 1.8 Schematic illustration of Solanaceous and Rosaceous S-RNase structure and positions of intron sequences in *S-RNase* DNA sequence. In the central region of the coding sequences, an intron sequence is present for HVa in the Solanaceous and RHV of the Rosaceous *S-RNases*, respectively. In addition to this intron, there is another intron in the 5' region of the *Prunus S-RNase,* but not in the *Malus* or *Pyrus S-RNase*. SP, signal peptide; C1 to C5, conserved regions 1–5; RC4, Rosaceous conserved region 4; HVa and HVb, hypervariable regions and b; RHV, Rosaceous hypervariable region. Modified from Tao and Iezzoni (2010).

There is a good evidence that the S-RNase is a pistil–specific protein with an expression pattern consistent with the role of the female determinant. Firstly, it is produced in transmitting cells of the style and subsequently secreted into the extracellular space of the transmitting tract. Secondly, S-RNase is accumulated mainly in the upper third segment of the style, which is the region of self-pollen tube arrest after incompatible pollination (Ai *et al*, 1990). Thirdly, in immature pistils that do not express SI, S-RNase is present at very low levels. However, it is abundantly accumulated in mature pistils and can account for more than 10% of total pistil protein (Roalson and McCubbin, 2003).

Several experiments have shown that the S-RNase is sufficient and necessary for the pistil to reject and recognize self-pollen. Firstly, gain-of-function and loss-of-function experiments have been conducted in transgenic plants (Lee *at al.*, 1994; Murfett *et al*., 1994). In one experiment the S_3-RNase gene of *Petunia inflata was* introduced into plants of S_1S_2 genotype and it was revealed that the transgenic plants that produce S_3-RNase from the transgene gained the ability to reject S_3 pollen. On the other hand, when an antisense S_3-RNase gene was introduced into plants of S_2S_3 genotype to inhibit the S_3-RNase, the transgenic plants lost the ability to reject S_3 pollen, but retained the ability to reject S_2 pollen (Lee *et al*,1994). Hence, the S_3-RNase gene alone determines pistil specifity in SI (Meng *et al*, 2011).

In an extension of this work a domain-swapping experiment was applied by Matton *et al* (1997) to illustrate the precise role of the HV region in determining S-RNase specificity. In this experiment they used S_{11}- and S_{13}- S-RNase of *Solanum*

chacoense which are two very similar S-RNases that differ in only ten amino acid residues, four of which are located in the HV region. After transformation with S_{11}-RNase chimeric gene constructs having the four different amino acids located in its HV region swapped with those four found in the S_{13}-RNase, the transgenic plants acquired S_{13}-RNase specificity. This provided evidence that the HV region determines the specificity of S-RNase.

However, certain previous studies contradict these ideas, Kao and McCubbin (1996) claim that HV regions are necessary but not sufficient for controlling S-RNase specificity. They revealed that transgenic plants transformed with a chimeric construct of S_3-RNase having the HV region of S_1-RNase, in addition to losing the ability to reject S_3 pollen, failed to gain the ability to reject S_1 pollen. Moreover, Verica *et al* (1998) indicated that regions outside the HV and the conserved regions might play a role in the S-allelic specificity determination of S-RNases.

Although solanaceous plant species have dominated the experimental SI data, it has been confirmed that RNase activity is essential for the pollen rejection response in Rosaceae as well (Sassa *et al*, 1992). Until now all functional S-RNases reported have the two conserved catalytic histidine residues that are located in the C2 and C3 regions (Tao and Iezzoni, 2010).

1.5.2.2 Male determinant in S-RNase system

Initially it was believed that the rejection of pollen may be controlled by the same S-RNase protein studies in the pollen and the style in GSI (de Nettancourt, 1997). Since the 1990s the cloning of the pollen *S* gene was by far the most important

development in SI research (McClure, 2004). The identification of the gene encoding the pollen specificity determinant was much more challenging than the identification of the S-RNase gene (Dowd *et al*, 2000). There was doubt about the identification of the pollen *S*-gene because polymorphism between *S*-haplotypes to determine *S*-specificity was insufficient (McClure and Franklin Tong, 2006).

Initially two possible candidates for the pollen S-factor were reported. The first candidate, which is called *PhSBP1*, was isolated from *Petunia hybrida* (Sims and Ordanic 2001). This protein has a RING-HC finger domain at the C-terminus, which indicated that it may function as an E3 ubiquitin ligase. Since *PhSBP1* does not show allelic variation or haplotype-specific interaction with S-RNases, it cannot be the pollen-S gene product *per se* (Sims and Ordanic 2001). On the other hand, it might be a general RNase inhibitor (Franklin-Tong and Franklin 2003a). The second candidate SLF was isolated from *Antirrhinum hispanicum* (Scrophulariaceae which was renamed Plantaginaceae) as a pollen–expressed gene (Lai *et al*, 2002).

SLF was shown to be the best candidate for the pollen S-gene for several reasons. Firstly, it is located at the S-locus. Secondly, it shows S-haplotype specific polymorphism. Thirdly, it is a pollen specific gene (Sijacic *et al.* 2004). SLFs are predicted to interact with S-RNases to elicit the SI response. On the other hand, in a compatible pollination it attempts to protect pollen tubes from the cytotoxic effect of S-RNases by interaction with S-RNases and targeting them for degradation. In a compatible pollination involving Antirrhinum, an increase in stylar protein ubiquitination and a subsequent decrease in S-RNase was observed (Qiao *et al*.,

2004a). On the other hand, S-RNase amounts were revealed in research involving *Nicotiana* to be stable following a compatible pollination (Goldraij *et al*., 2006).

In spite of the fact that SLF was identified as a pollen-S gene, researchers were surprised that the pistil *S*-gene has higher sequence diversity than the pollen *S*-gene (Newbigin *et al*., 2008). Therefore, it was proposed that the pollen S-gene could possibly be a complex of genes. The presence of multiple SLF genes has indeed been found in sequence analysis of several *S*-locus regions from different families that show S-RNase based self-incompatibility (Hua *et al*., 2007; Wheeler and Newbigin, 2007; Minamikawa *et al*., 2010).

Kubo *et al* (2010) claimed that the pollen *S*-gene of *Petunia* is made up of multiple function a *SLF* genes. Although ten *SLF* genes have been reported in *Petunia inflata* and *Petunia hybrida*, only *SLF1*, *SLF2*, and *SLF3* were initially shown to be involved in pollen specificity by a transgenic functional assay (Sijacic *et al*., 2004; Kubo *et al*., 2010). However, recently, Williams *et al* (2014a) revealed four additional *SLF* genes [*SLF4*, *SLF5*, *SLF6* and *SLF8*] to be involved in pollen specificity, so the total number increased from three to seven. Williams *et al* (2014b) analysed the pollen transcriptomes in S_2 and S_3-haplotypes and the leaf transcriptome of the S_3S_3 genotype. In both haplotypes the same seven *SLF* genes were identified and it was noticed that 17 *SLF* genes determine pollen specificity in each *S*-haplotype. Moreover recently Kubo *et al* (2015) detected 16–20 *SLF* genes in eight S-haplotypes of Petunia. According to an *in vivo* functional assay, it was revealed that eight of them (*SLF1, SLF2, SLF3, SLF4, SLF5, SLF6, SLF8,* and *SLF9*) are involved in pollen specificity.

1.5.2.3 Models for the mechanism of S-RNase Based SI

According to Franklin-Tong and Franklin (2003a) it was not clear how pollen is inhibited in an *S*-haplotype specific manner by the *S-RNase*. However, various models have been postulated in an attempt to explain how male and female determinants interact after the discovery that *S-RNases* are responsible for pollen tube inhibition.

The first hypothesis proposed that pollen tubes take up S-RNase molecules through a pollen S-receptor, which may translocate the S-RNases into the pollen cytosol in an S-haplotype specific manner. There, pollen tube growth can be arrested as a result of degradation of its rRNA or mRNA (McCubbin and Kao, 2000). However, evidence that this model is incorrect was found when immunocytochemical studies showed that S_{12} pollen tubes take up S_{11}-RNase protein (Luu *et al*., 2000; Goldraij *et al*, 2006). Consequently, there is no S-haplotype specific uptake (Franklin-Tong and Franklin, 2003a). An alternative hypothesis is that all S-RNases enter the pollen tube in an S-haplotype independent manner and that interaction between the S-RNase and the pollen S-locus component occurs in the pollen cytosol. This model proposed that the role of pollen S protein is to act as an RNase inhibitor that identifies and inhibits all S-RNases in self-compatible reaction through interaction with a low affinity binding site (Figure 1.9). However, it is proposed that binding with a high-affinity haplotype-specific site occurs when the S-RNase is allelic. This prevents the interaction with the low affinity site which allows the S-RNase to remain active and inhibit growth of the pollen tube (Luu *et al*, 2000).

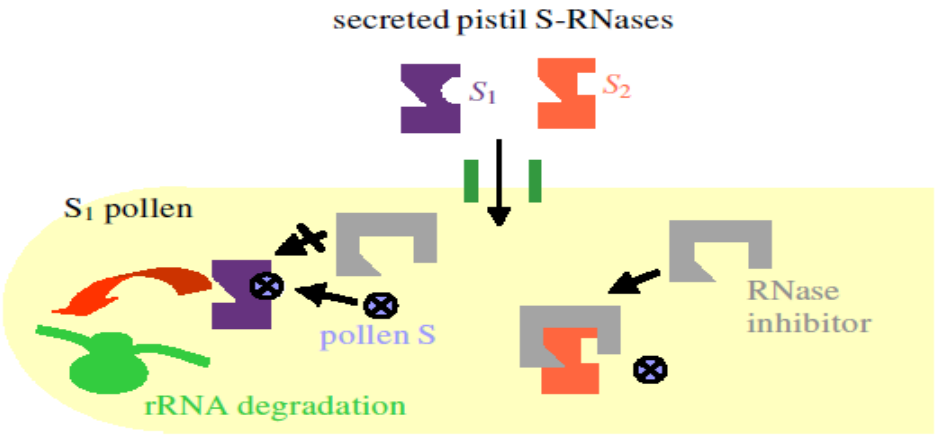


Figure 1.9 A model for the S-RNase mechanism of gametophytic SI found in the Solanaceae, Rosaceae and Scrophulariaceae. The growing pollen tube of genotype S_1 takes up secreted S-RNases, S_1 (purple) and S_2 (red) possibly as part of a protein complex. RNase inhibitor (grey) recognise and binds the S_2 S-RNase result in the inactive S-RNase. However, the S_1 S-RNase is bound at an allele-specific binding site by the S_1 pollen protein (light blue). This interaction between the pollen S-protein and its cognate S-RNase is themodynamically favoured over the interaction with the RNase inhibitor and effectively blocks binding to the inhibitor. Consequently, the S_1 S-RNase remains active and degrades the pollen tube rRNA which leads to the arrest of pollen tube growth. This Figure was modified from Franklin-Tong and Franklin (2003b).

After revealing the *SLF* as a pollen determinant gene of SI reaction is a member of the F-box class of proteins another hypothesis was proposed called the S-RNase degradation model (Hua and Kao, 2006). According to this hypothesis, the growing pollen tubes take up S-RNase into their cytoplasm where they interact with SLF. In a compatible pollination, this interaction will cause ubiquitination and degradation of the non-self S-RNases by the 26S-proteasome and inhibition of the cytotoxic activity of their S-RNases in the growing pollen tubes. In contrast, the interaction does not result in a degradation of S-RNases in an incompatible pollination. Consequently, S-RNases remain active and execute their cytotoxic activity on growing pollen tubes and arrest their growth (Franklin-Tong and Franklin, 2007).

Efforts have been made to identify the specific features of SLF that allow it to function in the response. These results include the identification of three SLF-specific functional domains (FD) which are called FD1, FD2 and FD3 by Hua *et al* (2007) in biochemical studies of PiSLF (*Petunia inflata* SLF). This led to the modification of the S-RNase degradation model to reflect all identified interacting functional domains (Hua *et al*, 2008).

Goldraij *et al* (2006) proposed a novel model called the S-RNase compartmentalization or sequestration model. By using immunolocalization, it was observed that the vast majority of S-RNase molecules were initially sequestered in a vacuolar compartment of the pollen tube after both incompatible (self) and compatible (cross) pollinations. However, S-RNase was only released into the cytoplasm in a self-incompatibility reaction through breakdown of the compartment in the presence of the HT-B protein (Goldraij *et al* 2006).

Consequently, S-RNase could exhibit its cytotoxic activity on pollen tubes and arrest their growth. On the other hand, in a self-compatible reaction, HT-B degradation was observed and S-RNases remain sequestered in their vacuolar compartment and could not arrest growth of the pollen tube (Goldraij *et al.,* 2006; Kaothien-Nakayama *et al*., 2010).

Recently, Kubo *et al* (2010) reported an experiment which involved *Petunia* and proposed a model called “collaborative non-self recognition”. They showed that in *Petunia* at least three types of divergent SLF protein can function as the pollen determinant. This model proposed that in *Petunia*, each type of pollen-S product

(SLF) within an S-haplotype interacts with a subset of non-self S-RNases. Furthermore, the products of multiple types of SLFs are required for detoxification and recognition of the entire suite of non-self RNases (Kubo *et al* 2010).

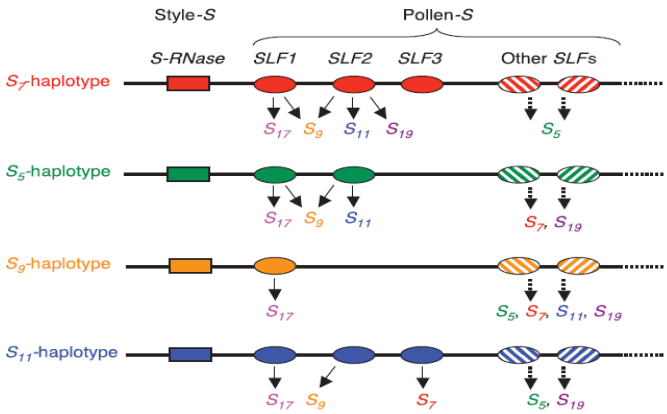


Figure 1.10 Collaborative non-self recognition model for SI in *Petunia*. The single S-RNase gene represents style-S and the multiple SLFs representing pollen-*S* are illustrated as boxes and ovals, respectively. The locations of these SLFs relative to S-RNase are as yet unconfirmed. For each S haplotype, the SLFs whose products are responsible for detoxifying one or more of the five allelic variants of S-RNase examined are connected by solid arrows with their target alleles. For each *S* haplotype, products of one or more other SLFs are predicted to target products of the remaining alleles of S-RNase connected by broken arrows. Figure adapted from Kubo *et al* (2010)

The collaborative non-self recognition model was further supported recently by Entani *et al* (2014), they isolated the components of a SCF^{SLF} complex (SCF = SKP1-CUL1-F-box-RBX1) from Petunia pollen. They revealed that a subset of non-self S-RNases *in vitro* are polyubiquitinated by the SCF^{SLF} and degraded by a proteasome inhibitor. This indicates that in cross-pollination multiple SCF^{SLF} complexes participate in polyubiquitination and degradation of non-self S-RNases by the proteasome.

Recently, the S-RNase degradation mechanism has been supported by approaches using a combination of cellular, biochemical and molecular biology. In *Petunia*

hybrida, immunogold labeling and subcellular fractionation assays illustrated that PhS_{3L}-SLF1 and PhSSK1 (SLF-interacting SKP1-like1), which are two key pollen SI factors, are co-localized in cytosols of both pollen grains and tubes. In addition, after self and cross pollination, it was noticed that PhS_{3L}-RNases are found in the cytosol of pollen tubes, and by yeast two-hybrid and co-immunoprecipitation assays PhS-RNases selectively interact with PhSLFs. Moreover, in compatible pollen tubes S-RNases are specifically degraded by non-self SLF action. Based on this evidence, in the cytosol of pollen tube through the ubiquitin/26S proteasome system SCF^{SLF}-mediated non-self S-RNase degradation occurs, which is considered to be a major mechanism for neutralization of S-RNase cytotoxicity during compatible pollination in *P. hybrida* (Liu *at al*, 2014).

Interestingly, it was recently revealed that the SLF gene itself is subjected to degradation. The degradation of SLF1 in *Petunia inflata* by the ubiquitin–26S proteasome pathway was reported recently by Sun *et al* (2015). In the C-terminal region of S2-SLF1 (SLF1 of S2 haplotype) that contains a degradation motif, 18 amino acid sequence were identified which 7 of them are conserved among all 17 SLF proteins of S_2 and S_3 haplotypes involved in pollen specificity. It was revealed that S_2-SLF1 lost function in self-incompatibility after deleting the 18 amino acid. It is possible that dynamic cycling of SLF proteins has an essential role in self-incompatibility. Sun *et al* (2015).

Increasing evidence about the large number of SLF genes that constitute pollen-S will be useful as it increases the number of potential mating parents, and

consequently, allows pollen to recognize and inactivate more non-self S-RNases. However, increasing the S-RNase diversity can have a negative effect because it gives S-RNases more chance to escape from detoxification by the existing repertoire of SLF proteins (Kubo *et al* 2010).

The cytosolic interactions that determine the recognition specificity between S-RNnase and SLF was demonstrated by previous researchers (Lee *et al*, 1994; Murfett *et al*., 1994, Liu *et al*, 2014). However, the physical force between their interactions remains unclear. Recently, Li *et al* (2016a) revealed that in *Petunia hybrida*, the physical mechanism of 'like charges repel and unlike charges attract' between SLFs and S-RNases resulted from the electrostatic potentials of SLF. Interestingly, it was noticed that the surface electrostatic potentials and pollen S specificity of SLF were reversed after the alteration of a single amino acid at the C-terminus. The identification of the electrostatic potentials can be considered as a major physical force between cytosolic SLFs and S-RNases which is able to illustrate a mechanism in self and non-self recognition between cytosolic proteins in angiosperms.

1.5.3 Self-incompatibility in horticultural crops.

Horticulture is one of the most important aspects of agriculture, consisting of fruits, vegetables and ornamental plants. Both fruits and vegetables are considered to be an essential source of vitamins and minerals are use in the daily diet. Ornamental plants have been used since ancient times as they have attractive shapes and colors, and some of them have beautiful flowers which can have a special scent. As humans became familiar with horticultural crops and domesticated them, they have attempted to understand the problems relating to different crops and overcome them, in order to

survive and improve their quality. It is obvious if the same plant regrows every year it is very easy for pests and diseases to infect them, so it needs a mechanism to overcome this problem. As plants are sessile, they have used self-incompatibility to escape from self-pollination and encourage cross pollination, which gives opportunities to subsequent generation to obtain new characteristics as a result of the segregation of different alleles.

Due to the fact that self-incompatibility prevents inbreeding and encourages outcrossing this can cause a problem especially in commercial orchards. As many fruit crops are self-incompatible, fruit production is dependent on cross-pollination between cultivars and the low efficiency of pollination leads to decreases in fruit production in certain years in commercial orchards (Goldway *et al*. 1999). Consequently, growers have to provide different varieties to use them as a pollen donor, which needs money and takes space. In addition they have to provide honeybees as pollen vectors during blooming. Honeybees play a crucial role in cross pollination, but their activity is reduced by inclement weather, and also prevents the growers from using herbicide and pesticide as they are toxic for honeybees. In addition, some areas are not suitable for honeybees during pollination time.

In many fruit and nut tree crops self-pollination is important as it provides more consistently high production yields compared to cross-pollination. This has been noticed in sweet cherry and almond; in both of them SC cultivars have been derived from dysfunctional SI genes through mutagenesis and interspecific crosses, respectively (Godini *et al*.1998). Consequently, overcoming self-incompatibility can lead to an increase in crop productivity.

In terms of ornamental plants and vegetables, self-incompatibility can have either positive (for plant breeder) or negative consequences (for customers as some plants cannot be propagated vegetatively).

1.5.3.1 Self-incompatibility in fruits

Fruit crop plants are considered to be an important part of horticulture crops and every year can add tens of billions dollars to the global economy. Fruit production occupies over 45 million hectares of land worldwide, which encourages millions of growers to depend on continued global trade. The production of fruit and nut crops in California represents more than one-third of the total farm gate value of the state's agricultural commodities. In spite of the fact that usually conventional vegetative propagation methods, such as cutting and grafting have been used for propagation of the fruit and nut crops, more sophisticated methods such as plant genetic transformation need to be used to produce new cultivars, with better quality, resistance to pests and diseases and drought tolerance (Mou and Scorza, 2011).

Transgenic technologies have been developed in many tree crops in spite of the recalcitrance that these plants display in tissue culture. Transformation of tree fruit using *Agrobacterium tumefaciens* is challenging because success requires high frequencies of both transformation and regeneration. In spite of this, there are many successful examples from fruit tree cultivars (Mou and Scorza, 2011).

There are several important fruit crops which belong to the Rosaceae family such as apple, pears, quinces, apricots, plums, cherries, peaches, raspberries, loquats, strawberries

and almonds and some of them are self-incompatible. For instance, Maita and Sotomayor (2015) reported that in Chile, in commercial almond [*Prunus dulcis* (Mill.) D.A.Webb] orchards, the average fruit set is low and commonly reaches 5–30% because they bloom during a cool period and also suffer from self-incompatibility.

The female determinant in the Rosaceae family is an S-RNase as in the Solanaceae family. This was reported in apple, Japanease pear, and in European pear by Broothaerts *et al*., (1995), Sassa *et al*., (1996) and Zuccherelli *et al*., (2002) respectively. The S-RNase structure in the Rosaceae family is slightly different from the Solanaceae family (see Figure 1.8). Although, the deduced amino acid sequences from the cDNAs for S-RNases of *Pyrus* and *Malus* are very similar and have the active site domain of T2/S-type RNases, differences are obvious between the rosaceous and solanaceous S-RNases (Sassa *et al*., 1996; Ushijima *et al*., 1998).

Regarding the male determinant, in the Malinae a sub-family of Rosaceae, a PCR based approach was used to characterize F-box genes within the S locus,two pollen-expressed F-box genes linked to the apple *S-RNase* were cloned (Cheng *et al*. 2006). Physical characterization of the S-locus structure in this group was reported to provide evidence of multiple and related F-box genes within the S locus of apple and Japanese pear (Sassa *et al.*, 2007).

Two F-box genes linked to each of the *S-RNases S3* and *S9* in apple were identified by Sassa *et al*. (2007) and named *MdSFBB* (*Malus domestica* S-locus F-box brothers) -α and -β in a bacterial artificial chromosome (BAC) library of the apple cultivar ‘Florina’. In addition, a PCR-based approach was used to identify three

homologous but distinct genes in Japanese pear, linked to the *S4* and *S5* haplotypes, (*PpSFBB-a*, *-b*, and *-c*, 'Pp' standing for *Pyrus pyrifolia*) (Sassa *et al.*, 2007). *SFBBs* were considered to be good candidates for the pollen S since they exhibited linkage to the *S-RNase*, pollen-specific expression, and S-haplotype-specific polymorphism (Sassa *et al*., 2007). Subsequently, many more examples of such multiple and related *SFBB* genes were identified in apple and shown to have a linkage with *S-RNase* gene, S-haplotype specific polymorphism to be expressed specifically in the pollen (Minamikawa *et al*, 2010).

Recently 40 new apricot cultivars were used from European breeding programmes for testing self-incompatibility. It was revealed that 21 were self-incompatible and 17 were self-compatible (Milatoviü *et al*, 2013).

In addition to the Rosaceae family self-incompatibility is observed in fruit and nut crops belonging to other families of plants such as cashew (*Anacardium occidentale* L.) (Aliyu, 2008), and olive (Seifi *et al*, 2011). However, as the detailed information at the molecular level is not available yet, they may have different SI systems. Moreover, in five mango cultivars various levels of self-incompatibility have been observed by Maklad (2012).

As mentioned above, self-incompatibility can cause problems in a commercial orchard, so it is valuable to overcome SI. Breakdown of self-incompatibility is a significant event in a fruit or nut breeding programme. Consequently, there have been several studies using different methods to understand or to overcome self-incompatibility.

For instance, it was noticed that failure of RNase protein expression or the loss of *RNase* activity of the mature proteins led to self-compatibility in both serotina and *Prunus dulcis* by Sassa *et al* (1992) and Bošković *et al* (1997) respectively. In addition, Broothaerts *et al* (2004) transformed apple shoot cv. Elstar, which displays a strong SI response with a gene silencing construct targeted at knocking out *S-RNase* expression in the pistil. Normal fruits and seeds were observed in two transgenic lines with different genotypes after self-pollination compared to control plants.

In addition to the mutation of the female component (S-RNase), a number of research studies show a breakdown in pollen-S. For instance, Ushijima *et al* (2004) and Sonneveld *et al* (2005) identified that mutations, insertions or deletions in S-haplotype-specific F-box (SFB) genes can lead to loss of pollen-S function in *Prunus* giving rise to self-compatible mutants. In addition, it was noticed that breakdown of self-incompatibility in apricot (*Prunus armeniaca*), Currot (S_CS_C) and Canino (S_2S_C) is associated with a 358-bp insertion in the SFB_C gene. (Vilanova *et al*, 2006).

1.5.3.2 Self-incompatibility in Ornamental Plants

Ornamental plants include herbaceous plants, indoor plants, cut flowers, bulbs, shrubs, trees and vines. Over the last two decades, the monetary value of ornamental plants has increased significantly (Geneve *et al* 1997). In addition, it will be more popular in both local and international markets. It has been noted that nearly half of the major crops and ornamental species are self-incompatible (Brewbaker, 1957). *Nicotiana alata*, *Antirrhinum sp* and *Petunia hybrida* have been used as models for studying self-incompatibility.

The first genetic explanation of self-incompatibility was reported in the ornamental *Nicotiana alata* by East and Mangelsdorf (1925). In self-incompatible *Nicotiana alata* style glycoprotein was identified as the female determinant (Anderson *et al*., 1986; Cornish *et al*., 1987; McClure *et al*., 1993). The first cDNAs for one of these proteins was also cloned from *Nicotiana alata* by Anderson *et al* (1986). These proteins are extracellular and were revealed to be ribonucleases (RNases) and later confirmed to have ribonuclease activity (Bredemeijer and Blaas, 1981; Anderson *et al*., 1986).

Antirrhinum belongs to the Plantaginaceae family, most species of this genus possess gametophytic self-incompatibility which is regulated by a single multiallelic S-locus (East, 1940). The female determinant, *S-RNase* was cloned from *Antirrhinum hispanicum* by Xue *et al.,* (1996). Several factors render the genus *Antirrhinum* to be a good model for studying self-incompatibility. First, although *Antirrhinum* belongs to the the Plantaginaceae family they are closely related to the solanaceae family which shares S-RNase self-incompatibility. Second, species of *Antirrhinum* contain the well-defined families of active transposable elements which can be used to generate mutants in all domains of the S-locus (McCubbin *et al*, 1992).

Five cultivars from *Hydrangea macrophylla* were tested for incompatibility by pollen germination and pollen tube growth after self and cross pollination (Reed, 2005). It was noticed that after 72 hours, the pollen tube length after self-pollination is much shorter than the pollen tube length after cross pollination. This indicates the presence of self-incompatibility in this species (Reed, 2005).

In order to understand floral biology and breeding system of *Metrosideros excels,* which belongs to Myrtaceae family, six trees were tested for compatibility. It was found that three of them were self-incompatible, which indicates that the natural population for *Pohutukawa* may consist of a mix of self-compatible and incompatible individuals (Schmidt-Adam *et al*, 1999).

1.5.3.3 Self-incompatibility in Vegetable Crops

Vegetables are another important part of horticulture and arecrucial for the human daily diet and nearly two-thirds of the world population depends on a vegetarian diet. Vegetables are the source of different types of nutrition. For examples, white potato, dry beans, yam, sweet potato, cassava and taro are sources of carbohydrate. Sweet corn, peas and leafy crucifers are a good sources of protein and Pro-vitamin A can be obtained from carrot, orange/yellow flesh sweet and potato. In addition mature seed of some legumes and cucurbits are sources of fat (Rubatzky and Yamaguchi, 1997).

Plant breeders are always trying to produce new vegetable cultivars with different colour, size, shape in addition to producing resistant cultivars for pests and diseases through F_1 hybrid seeds. Self-incompatibility is one of the methods which could be exploited for this purpose, especially in crops whose flowers (such as tomatoes and lettuce) lack nectaries, because it appears that self-incompatibility is more efficient than male sterility in cross-fertilizing systems (Denna, 1971). In spite of the fact that using CO_2 and NaCl in Brassica are two artificial methods that growers use to control self-incompatibility, they are time consuming and self-compatible plants produced from these methods are not stable and are notinherited by subsequent generations. Consequently, researchers have attempted to understand this

phenomenon at the molecular level in order to provide more information for plant breeders to use this phenomenon in other crops. Vegetable crops either possess sporophytic self-incompatibility as in the Brassica family or gametophytic self-incompatibility as in the solanaceae family. There are several vegetable crops commonly used for food that belong to the Brassica family such as broccoli, cabbage, choy sum, and turnip. In addition, seeds for some of them (such as canola or rape seed) are used for oil production.

Self-incompatibility in the Brassicaceae family is well understood as described in section 1.4.1 and in terms of using self-incompatibility to produce F_1 hybrid, much research has been done. For instance, PCR-RFLP (restriction fragment length polymorphism) was used to identify different S-alleles using primers originating from radish SLG genes (Niikura, 2002). This finding can help plant breeders to recognize the S-genotype before harvesting. However, in the past they could not identify plant phenotypes until pollination tests were done at the reproductive stage. Furthermore, Jung *et al*. (2012) illustrated a self-compatible phenotype in a transgenic line of *Brassica rapa* after silencing of the *SP11* gene. In subsequent generations, this trait was stable, even after crossing with other commercial lines. These findings suggested that the self-compatibility could be transferred to commercial cultivars with the desired performance in *Brassica rapa*.

Regarding gametophytic self-incompatibility, tomato, which belongs to the solanaceae family, has been used as a model. Within the the genus Solanum, *Lycopersicon peruvianum* possesses gametophytic self-incompatibility (Lamm, 1950 ; McGuire and Rick,1954). In several plants belonging to this family, S glycoproteins

and their encoding cDNAs cosegregate with their respective S-alleles in genetic crosses (Anderson *et al.*, 1986; Chung et *al* 1994; Mau *et al*., 1986). Consequently, related proteins and their corresponding cDNA were identified in the style of other self-incompatible Solanaceae plants including *Lycopersicon peruvianum* (*Solanum peruvianum*) (Tsai *et al*, 1992). Later, Chung *et al* (1994) and Kowyama *et al*., (1994) noticed that S Glycoproteins have ribonuclease activity in *Lycopersicon peruvianum.*

It can be concluded that self-incompatibility is an important trait in horticulture. More research is required to identify the genes which cause incompatibility, finding techniques to control this phenomenon and to exploit it effectively. This thesis aims to further understand self-incompatibility in *Petunia* with a view to ultimately transferring this knowledge to a range of horticultural crops that share the same gametophytic system.

1.6 AIMS AND OBJECTIVES OF THE PROJECT

The aim of this project is to use *Petunia hybrida* as a model to improve our understanding of the S-RNase type of self-incompatibility (SI). In particular the aim is to study the phenomenon of "competitive interaction" by introducing heterologous *SLF* genes from *Antirrhinum* into *Petunia hybrida* to test their utility for a general approach to break down SI. Specific aims to be tested are as follows:

1- To characterise the available S-allele/S-haplotype stocks in *Petunia hybrida* and its wild relative *Petunia inflata*. To study the behaviour of different S-haplotypes in different F_1 hybrid backgrounds.

2-To confirm the ability of the AhSLF-S2 construct to induce competitive interaction resulting in self-compatibility using *Petunia hybrida (*PhS_3PhS_V).

3-To extend this analysis to test three additional heterologous SLF genes from *Antirrhinum* (AhSLF-S2C, AhSLF-S4D and AhSLF-S1E) for a similar induction of competitive interaction.

4-To use all four constructs (AhSLF-S2, AhSLF-S2C, AhSLF-S4D and AhSLF-S1E) to test for competitive interaction in the full range of S-haplotypes available in *Petunia hybrida* (*PhS_V, PhS_3*) and in *Petunia inflata* (*PiS_3, PiS_{K1}*).

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 SOWING SEED AND GROWTH CONDITIONS

Petunia hybrida, Petunia inflata, F_1 and F_2 hybrid stocks were grown under controlled glasshouse and growth room conditions (24/22°C, 16/8 hr day/night cycle). The seeds were germinated in small pots using Seed and Modular Compost plus Sand. After two weeks they were transferred to a plant seed tray containing standard Petunia potting mix comprising M3 Levington, John Innes No.3 and perlite in the ratio (3:2:1). After four weeks the plants were transferred to (5 litre) pots. Pesticides and nutrition were used as appropriate.

2.2 PLANT GENETIC STOCKS

Petunia inflata stocks seeds used in this project were obtained from a previous PhD student (Daniel Dzidzienyo). In his study he identified two incompatibility groups. Group I consisted of two plants (P2 and P8) carrying $PiS_{k1}PiS_d$ alleles. Group II consisted of two plants (P5 and P7) carrying PiS_3PiS_d alleles. The incompatibility relationship between these two groups is shown in Table 2.1.

Table 2.1 Incompatibility groups derived from diallel crosses of *Petunia inflata*

Plant ID	Pollen Donor			
	Incompatibility Group I		Incompatibility Group II	
	P2 $PiS_{K1}PiS_d$	P8 $PiS_{K1}PiS_d$	P5 PiS_3PiS_d	P7 PiS_3PiS_d
P2 $PiS_{K1}PiS_d$	-	-	+	+
P8 $PiS_{K1}PiS_d$	-	-	+	+
P5 PiS_3PiS_d	+	+	-	-
P7 PiS_3PiS_d	+	+	-	-

A compatible cross is indicated by (+), and an incompatible cross is indicated by (-). P2, P5, P7 and P8 represent the individual *Petunia inflata* IDs (Dzidzienyo, 2012).

As shown in Table 2.1 the genotype of the parents was known. In this project, seeds produced from these plants (first progeny) were used. In order to obtain all possibilities for heterozygous alleles we sowed nine groups of seeds produced by the parents. The plants parental genotype, plant ID and first progeny ID are shown in Table 2.2.

Table 2.2 *Petunia inflata* crosses showing parental family ID and individual genotype.

Parental Crosses (S genotype) Female X Male	Glasshouse ID	First progeny Glasshouse ID
5 X 2 PiS_3PiS_d X $PiS_{K1}Pi_{Sd}$	P1 (N441)	N441.1
		N441.2
2 X 5 $PiS_{K1}Pi_{Sd}$ X PiS_3PiS_d	P2 (N442)	N442.1
		N442.2
		N442.3
5 X 8 PiS_3PiS_d X $PiS_{K1}Pi_{Sd}$	P3 (N443)	N443.1
		N443.2
8 X 5 $PiS_{K1}Pi_{Sd}$ X PiS_3PiS_d	P4 (N444)	N444.1
		N444.2
		N444.3
2 X 7 $PiS_{K1}Pi_{Sd}$ X PiS_3PiS_d	P5 (N445)	N445.1
		N445.2
		N445.3
		N445.4
7 X 2 PiS_3PiS_d X $PiS_{K1}Pi_{Sd}$	P6 (N446)	N446.1
		N446.2
		N446.3
7 X 8 PiS_3PiS_d X $PiS_{K1}Pi_{Sd}$	P7 (N447)	N447.1
8 X 7 $PiS_{K1}Pi_{Sd}$ X PiS_3PiS_d	P8 (N448)	N448.1
		N448.2
		N448.3
8X2 $PiS_{K1}Pi_{Sd}$ X $PiS_{K1}Pi_{Sd}$	P9 (N449)	N449.1
		N449.2
		N449.3

In addition, four *Petunia hybrida* families (N436, N438, N439 and N440) were raised from seeds obtained from Dr. Tim Robbins. Three plants for each family were grown to carry the PhS_3 and PhS_v alleles (Robbins *et al*, 2000). The parental genotypes crossed, family IDs and first progeny IDs are shown Table 2.3. 10 flowers

from each plant were self-pollinated and all plants showed stable self-incompatibility.

Table 2.3 *Petunia hybrida* parental crosses, family ID and first progeny ID.

Parental Crosses (Bud pollination)	Family ID	First Progeny ID
PhS_3PhS_v X PhS_3PhS_v	N436	N436.4
		N436.5
		N436.6
PhS_3PhS_v X PhS_3PhS_v	N438	N438.4
		N438.5
		N438.6
PhS_3PhS_v X PhS_3PhS_v	N439	N439.4
		N439.5
		N439.6
PhS_3PhS_v X PhS_3PhS_v	N440	N440.4
		N440.5
		N440.6

Moreover, *Petunia inflata* and *Petunia hybrida* stocks were crossed to confirm their compatibility. In addition, self-incompatibility was tested in the first progeny produced from this crosses. Seeds carrying distinct S-alleles were collected from cross pollination and first progeny ID is illustrated in Table 2.4.

Table 2.4 F_1 hybrid parental crosses, family ID and first progeny ID.

Parental Crosses (S genotype)	Family ID	First progeny Glasshouse ID	
N440.5 (PhS_3PhS_v) x N445.4 (PiS_3PiS_{K1})	N450	N450.1	N450.5
		N450.2	N450.6
		N450.3	N450.7
		N450.4	N450.8
N440.6 (PhS_3PhS_v) X N445.1(PiS_3PiS_d)	N451	N451.1	N451.5
		N451.2	N451.6
		N451.3	N451.7
		N451.4	N451.8
N440.4(PhS_3PhS_3) X N445.4 (PiS_3PiS_{K1})	N452	N452.1	N452.6
		N452.2	N452.7
		N452.3	N452.8
		N452.4	N452.9
		N452.5	N452.10
N440.4(PhS_3PhS_3) X N445.1(PiS_3PiS_d)	N453	N453.1	
		N453.2	
		N453.3	
		N453.4	
N439.4(PhS_vPhS_v) X N445.4 (PiS_3PiS_{K1})	N454	N454.1	
		N454.2	
N439.4(PhS_vPhS_v) X N445.1(PiS_3PiS_d)	N455	N455.1	
		N455.2	
		N455.3	
		N455.4	

As the breakdown of SI was noticed with plants holding *PiS$_d$* allele in a F_1 hybrid, cross pollination between F_1 plants was done. The collected seeds were used to produce F_2 hybrids and four families were raised as illustrated in Table 2.5.

Table 2.5 F_2 hybrid parental crosses, family ID and second progeny ID.

Parental crosses S genotype	Family ID	Second Progeny Glasshouse ID					
N453.4(*PhS$_3$PiS$_d$*) x N451.7(*PhS$_3$PiS$_3$*)	N463	N463.1	N463.2	N463.3	N463.4	N463.5	N463.6
		N463.7	N463.8	N463.9	N463.10	N463.11	N463.12
		N463.13	N463.14	N463.15	N463.16	N463.17	N463.18
		N463.19	N463.20	N463.21	N463.22	N463.23	N463.24
N451.6(*PhS$_v$PiS$_d$*) x N450.8(*PhS$_v$PiS$_{k1}$*)	N464	N464.1	N464.2	N464.3	N464.4	N464.5	N464.6
		N464.7	N464.8	N464.9	N464.10	N464.11	N464.12
		N464.13	N464.14	N464.15	N464.16	N464.17	N464.18
		N464.19	N464.20	N464.21	N464.22	N464.23	N464.24
N451.5(*PhS$_v$PiS$_d$*) x N454.1(*PhS$_v$PiS$_{k1}$*)	N465	N465.1	N465.2	N465.3	N465.4	N465.5	N465.6
		N465.7	N465.8	N465.9	N465.10	N465.11	N465.12
		N465.13	N465.14	N465.15	N465.16	N465.17	N465.18
		N465.19	N465.20	N465.21	N465.22	N465.23	N465.24
N451.6 (*PhS$_v$PiS$_d$*) x 454.2(*PhS$_v$PiS$_3$*)	N467	N467.1	N467.2	N467.3	N467.4	N467.5	N467.6
		N467.7	N467.8	N467.9	N467.10	N467.11	N467.12
		N467.13	N467.14	N467.15	N467.16	N467.17	N467.18
		N467.19	N467.20	N467.21	N467.22	N467.23	N467.24

2.3 S-ALLELE PHENOTYPIC ANALYSIS

Regarding *Petunia inflata,* after stigmas showed the presence of exudates as evidence of maturity, cross and self-pollination were carried out. For controlled self-pollination, 10 flowers per plant were pollinated by using the pollen of an individual flower to pollinate the stigma of the same flower. For controlled cross-pollinations, flowers were emasculated three days before anthesis by removing all the stamens to prevent self-pollination but leaving the stigma intact. Pollination was carried out by applying pollen to the stigma using sterilized forceps three days after

emasculation. All pollinations were labelled with a string tag placed around the pedicel recording the date and type of pollination.

The number and size of capsules that were produced per plant were scored to determine the SI/SC phenotype of the plants approximately four weeks after pollination. Plants were characterised as self-compatible (SC), if full seed set occurred in all pollinations determined by the presence of a capsule. On the other hand, if all of the pollinations resulted in no seed set which were indicated by the absence of a capsule; plants were characterized as Self-incompatible (SI). However, if plants produced variable levels of seed set, these were characterized as Pseudo-self-compatible (PSC). The same methods have been used for the phenotypic analysis in *Petunia hybrida*, F_1 hybrids and F_2 hybrids. However, neither *Petunia hybrida* nor F_1 hybrid have shown PSC.

2.4 SELF-INCOMPATIBILITY GENOTYPE DETERMINATION (PCR GENOTYPING)

2.4.1 Genomic DNA Extraction

GeneElute Plant Genomic DNA Miniprep kit (Sigma-Aldrich) was used to harvest DNA from leaves by following the Manufacturer's recommendation, which can be summarized as follows:

Two leaf discs weighing approximately 100 mg of young leaf material were harvested into labelled 1.5 ml microcentrifuge tubes. Liquid nitrogen (N_2) was used to snap freeze the harvested leaves. Sterilized Micro-pestles were used in order to grind the tissue until a fine powder was obtained. The tubes containing the powder were left in the liquid N_2. The tubes were then taken out of the liquid N_2 and 350 µl of lysis A

and 50 µl of lysis B solutions were added to it. By vortexing and inverting, a cloudy lysate precipitate was formed after the addition of lysis B solution. The mixture was incubated at 65°C for 10 minutes with occasional inversion to dissolve the resultant lysate precipitation. 130µl of precipitation solution was added to the mixture and mixed by inversion. Samples were placed on ice for 5 mins. The samples were centrifuged for 5 mins at 13,000 rpm to pellet cellular debris, protein and polysaccharides. The supernatant was carefully pipetted on to a GenElute filtration column (blue insert with a 2 ml collection tube). The samples were centrifuged at 13,000 rpm for 1 min. The blue filtration insert was discarded but the collection tube and the liquid was retained. 700 µl of binding solution was added to the liquid and mixed by gentle inversion. A red GeneElute miniprep binding column was prepared. 500 µl of the Column preparation solution was added to each binding column and centrifuged at 13,000 rpm (1 min). The flow through liquid was discarded.700 µl of the resulting mixture from the addition of Binding Solution step was then carefully pipetted onto the prepared binding column and centrifuged at 13,000 rpm for 1 min. The flow through liquid was discarded and the collection tube retained. The column was returned to the collection tube and the remaining lysate pipetted onto the binding column and centrifugation previous step repeated for another time. The flow-through liquid and the collection tubes were then discarded and the binding column retained. The red insert was placed into a new 2 ml collection tube and 500 µl of the diluted wash solution was applied to the column and centrifuged at maximum speed for 1 min. The flow through liquid was discarded but the red insert was retained. Another 500 µl of Wash Solution was added to the column and centrifuged at maximum speed for 3 min to dry the column (ensuring that the binding column did not touch the flow-through liquid). The binding column was transferred to a new 2 ml collection tube and

50 µl (instead of 100 µl recommended by the manufacturer) of pre-warmed (65°C) Elution Solution added to it and centrifuged at 13,000 for 1 min. The elution step was repeated again and the eluate stored at -20 °C.

2.4.2 DNA quality control and quantification

After DNA extraction, a DNA quantification and purity check was carried out using NanoDrop Spectrophotometer (ND-1000 Spectrophotometer, NanoDrop Technologies, Inc., and Wilmington, USA). In order to further check for quality, 2 µl of the extracted DNA was mixed with 2 µl of 6X loading buffer (See Appendix 2) and electrophoresed on a 1.5% (w/v) agarose gel in 1X TBE buffer (See Appendix 2 and section 2.4.5).

2.4.3 Primer design for allele specific PCR

In order to show allele-specificity and amplify specific S-alleles from plants segregating for known S-RNases, the genotyping primers were designed. Regarding *Petunia inflata* primers, a set of primers were designed for this project by Dr. Daniel Dzidzienyo based on hypervariable regions (See Appendix 4). However, all *Petunia hybrida* primers were designed by Robbins *et al* (2000), except S_3 (PhS3-F2) forward which was designed by Dr. Upendra Devisetty (unpublished). Primers for both stocks are shown in Table 2.6.

Table 2.6 Primers for genotyping populations segregating for *S-RNases* in *Petunia inflata* and *Petunia hybrida*

Primer Name	Direction	Sequence (5'-3')	Tm °C
Sd-F	Forward	AGAAAACTACAAAGACTTAACGG	57.3
Sd-R	Reverse	TACTGATTATCGTAGCGGTTTAC	58.8
Sk-F	Forward	CGGTACCAAGTATAAGATTTTCG	60.3
Sk-R	Reverse	ATTTTTAGATGACCTCGACTAC	55.6
S3-Fn	Forward	TTGCGATGGAGATAAGTTTGTG	63.8
S3-Rn	Reverse	GGTCTTCCTGTAGCTCAGCATT	63.5
PhS3-F2	Forward	ATACGCCTGCAGTACTGCAAGCC	70.1
PhS3-R1	Reverse	CGCATGTATCACTTTGACGACA	65.9
V13-F1	Forward	GGACGAAGCTGATTGAAGG	65.9
V13-R1	Reverse	CGATTTTATATATTGGC	52.8

The Sd-F and Sd-R primers amplify *Petunia* plants carrying the *PiS$_d$-RNase*, Sk-F and Sk-R primers similarly amplify the PiS$_{k1}$-RNase, and, S3-Fn and S3-Rn primers similarly amplify the *PiS$_3$-RNase*. The annealing temperatures used were 52°C, 50°C and 58°C respectively. In addition, PhS3-F2 and PhS3-R1 amplify *Petunia hybrida* plants segregating for Ph$_{S3}$-RNase, also V13-F1 and V13-R primers amplify *PhS$_V$-RNase*. The annealing temperatures used were 64 C$^{\circ}$ and 48 C$^{\circ}$ respectively.

2.4.4 PCR/allele–specific PCR for S-genotyping

Allele-specific primers were used to genotype plants segregating for known S-RNases (see Table 2.6). Genomic PCR reactions were generally prepared in a 25 µl reaction volume consisting of 2.5 µl of 10X PCR buffer, 0.75 µl 50mM $MgCl_2$, 2.5 µl 2 mM dNTPs (Bioline). However, in the middle of the project the company changed the substances and they offered MyTaq reaction buffer as a replacement for (PCR buffer, $MgCl_2$ and dNTPs) and 5µl was used as an ideal amount for ideal results after manipulating the protocol several times (Bioline, London, UK). In addition, 1 µl of 10 µM forward and reverse primers and 0.1µl of Taq DNA polymerase (5 U/µl), 15.15 µl SDW water and 2 µl DNA template were used for preparing master mix. PCR amplification was performed in a PTC-200 Thermal Cycler (MJ Research, Watertown MA, USA) under the following cycling conditions: For *PiS*$_3$, *PiS*$_{k1}$ and *PiS*$_d$ primers an initial 3 min denaturation at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec of annealing at a temperature depending on the Tm (melting temperature) of the primer, 1 min at 72°C and a final extension of 5min at 72°C were used. For the *PhS*$_V$ and *PhS*$_3$ primers, an initial denaturation at 94°C for 2 min, followed by denaturation at 94°C for 1 min, annealing with S_V primer at 48°C or S_3 primer at 64°C for 30 sec, and then for extension at 72°C for 1 min, final extension at 72°C for 5 min and hold at 10°C were used. All PCR products were checked by agarose gel electrophoresis.

2.4.5 Agarose gel electrophoresis

In order to check PCR products, an agarose gel was prepared by mixing 1.5 g agarose with 100 ml of TBE buffer (See Appendix 2). The agarose was dissolved by heating in a microwave for 3 mins. The gel was cooled using tap water and 10 µl

ethidium bromide (EtBr) 500 μg/ml was added. 15 μl of the amplified product (genomic DNA) was mixed with 3 μl (6X) loading buffer (See section 2.4.2) and loaded into the wells of the agarose gel. In addition, 5 μl of HyperLadder II (Bioline, London, UK) was added for size calibration and the gel was run at a constant voltage of 98 V for 1.5 hour. After the electrophoresis, the gel was visualized under ultra violet light and photographed using a digital imaging system (Syngene, Cambridge, UK)

2.5 BINARY VECTORS AND TRANSFORMATION INTO *E.COLI*

We obtained the AhSLF-S2 construct (accession number AJ297974) as used previously by the group from the Chinese Academy of Sciences, Beijing (Qiao *et al*, 2004b). In addition we obtained three constructs containing other *SLF* family members AhSLF-S2C, AhSLF-S4D and AhSLF-S1E with accession number DQ462204, AJ515534 and AJ515535 respectively, which all normally expressed specifically in pollen (Zhou *et al*, 2003). Hypothetical T-DNA maps were created theoretically for each construct in order to design suitable primers as illustrated in Table 2.7 (all maps can be seen in the Appendix 1).

Table 2.7 Primers designed for identifying AhSLF transformed colonies, transgenic plants and the transgene expression.

Primer Name	Direction	Sequence (5'-3')	Tm °C
AhSLF-S1E-F-246	Forward	AGAGGAAGAAGTGTGGGCAA	58.86
AhSLF-S1E-R-1152	Reverse	CACAAGCTGTATTACCTGGCC	58.98
AhSLF-S1E-R-726	Reverse	ACACGGTGGGAGCTTCTTAA	58.95
AhSLF-S2-F-72	Forward	GCTAAGGTTCAGGTGCGTCT	60.00
AhSLF-S2 –F-672	Forward	TGGAACGCAAACTCGACTGA	59.90
AhSLF-S2-R-904	Reverse	ACTCACGCACGCCATATTGA	60.11
AhSLF-S2C-F-247	Forward	TCCCCAGAACTAGAGCAGGT	59.59
AhSLF-S2C-R-1148	Reverse	ACCTGGTTCAAACTGATCAAGC	59.11
AhSLF-S2C-R-828	Reverse	GCATGGACCCAAAAGTGTGAC	60.00
AhSLF-S4D-F-1122	Forward	TTAGGTGGGCGAGGAATTCC	59.46
AhSLF-S4D-R-848	Reverse	TCAGATCGAACGGAGGCAAAA	59.00
AhSLF-S4D-F-674	Forward	TGGAACGCAAACTCGACTGA	59.90
EF1a-R	Reverse	CAGATCGCCTGTCAATCTTGG	59.80
EF1a-F	Forward	CCTGGTCAAATTGGAAACGG	57.30

Some of these reverse primers have been used with M13 reverse (as forward) to amplify *AhSLF-S2*, *AhSLF-S2C*, *AhSLF-S4D* and *AhSLF-S1E* respectively (See appendix 1).

2.5.1 Transformation of *E. coli* DH5α (C) chemically competent cells with Binary vectors.

Tubes of DH5α (C) were obtained from the Biosciences Genomics Facility-The University of Nottingham and 100 ng from each construct were added to them and mixed by stirring gently with pipette tip. The vials were incubated on ice for 30 min, followed by heat shocking for exactly 45 sec in the 42 °C water bath. The vials were

then removed from the water bath and placed on ice. 800 µl of LB medium (See Appendix 2) (at room temperature) was then added to each tube. The vials were then incubated for 1 hour in a 37 °C incubator. Two different volumes (50 µl and 150 µl) of each transformation vial was spread on labelled LB agar plates containing 50 µg/ml of Kanamycin (See Appendix 3). The liquid was then allowed to be absorbed by the LB agar plates and then the plates were inverted and placed in a 37 °C incubator overnight. The colonies were scored on the next day.

2.5.2 Colony Screening by PCR

Colony PCR reactions were generally prepared in a 12.5 µl reaction volume consisting of 1.25 µl of 10X PCR buffer, 0.37 µl 50 mM $MgCl_2$, 1.5 µl 2 mM dNTPs (Bioline, London, UK), 0.5 µl of 10 µM forward and reverse primers and 0.1 µl of Taq DNA polymerase (5U/µl) and 7.75 µl SDW water. Colonies were picked with pipette tips onto a new LB plate containing 50 µg/ml Kanamycin (to be kept as a backup) and the pipette tips placed into PCR reaction mixtures. PCR amplification was performed in a PTC-200 Thermal Cycler (MJ Research, Watertown MA, USA) under the following cycling conditions: an initial 6 min denaturation at 94°C, followed by 35 cycles of 30 sec at 94°C, 30 sec of annealing at a temperature 59°C, 30 sec for extension if the product size was less than 1000bp and 1mins for bigger than 1000bp at 72°C and a final extension of 6 min at 72°C. Gene specific reverse primers with M13 reverse (as forward) were used to identify correctly transformed colonies (See table 2.7).

2.5.3 Glycerol stock preparation of plasmids

In general 750 µl of liquid culture was added to 250 µl of 80% (v/v) sterile glycerol in screw capped tubes. The content of the tubes were then mixed by vigorous shaking and then flash frozen in liquid nitrogen and stored at -80°C. The details of the plasmids were recorded in a laboratory note book for future reference.

2.5.4 Plasmid DNA extraction

The colonies with the expected insert size as revealed by the colony PCR were sampled and grown overnight in 5 ml LB broth containing 50µg/ml of Kanamycin (see appendix 2) using shaking incubator 37°C. Plasmid DNA was isolated using the GeneElute Plasmid DNA Miniprep kit (Sigma-Aldrich) according to the manufacturer's recommendation as summarized:

5 ml of bacterial culture was centrifuged for 25 min at 3000 rpm, the supernatant was removed. 200 µl of Resuspension Solution was added to the pellet and it was completely resuspended by vortexing. After that the mixture was transferred to a microcentrifuge tube. 200 µl of the Lysis Solution was added and mixed by inverting the tube 6-8 times without vortexing. 350 µl of the Neutralization Solution was added and mixed by inverting the tube 4 times without vortexing. The bacterial lysate was centrifuged at 13000 rpm in a micro centrifuge for 10 min. The Miniprep column insert was placed into a 2 ml microcentrifuge tube and 500 µl column preparation solutions was added and centrifuged at 13000 rpm for 1 min. The flow-through liquid was discarded. The cleared lysate was transferred into the Miniprep column insert. The column insert/collection tube was centrifuged at 13000 rpm for 1 min. The Miniprep column insert was removed and the flow-through liquid was discarded from the tube. The column insert was retained and transferred to the

collection tube. 750 µl of the Column Solution containing ethanol was added to the Miniprep column insert. Then, centrifuged at 13000 rpm for 1 min. The Miniprep column insert was removed from the tube and the flow-through liquid was discarded. The Miniprep column insert was placed in the collection tube. In order to remove any remaining ethanol, the Miniprep column insert in the collection tube was centrifuged at 13000 rpm for 2 min and transferred to a new 2 ml collection tube. The plasmid DNA was eluted by adding 30 µl of Sigma water and centrifuged at 13000 rpm for 1 min. The harvested plasmid DNA was stored at -20°C.

2.5.5 Sequencing of plasmid inserts

After identification of the desired transformed colonies their purified plasmid DNA was sent for sequencing. The sequence of the inserted DNA was determined using reverse specific primer for each constructs and M13 reverse (as forward) primers.

2.6 TRANSFORMATION OF *AGROBACTERIUM* (LBA4404) WITH BINARY VECTORS BY ELECTROPORATION

Agrobacterium (LBA4404) was transformed with confirmed constructs by electroporation. These processes can be summerized in the following steps: Tubes of LBA4404 were obtained from the Biosciences Genomics Facility-The University of Nottingham and 100ng from each construct was added to them (and mixed by stirring gently with pipette tip). 20 µl from vials were transferred to Electroporation Cuvettes. Electroporation was carried out by setting the program on the machine to 'Agro' which applied an electric voltage of approximately 2500 volts and 192 Ω for 1 sec. After that, 1 ml from APM media (See Appendix 2) (recommended by Dr.

Ruth Cornock) was added to the vials and mixed by inverting. The vials were transferred to new 25 ml tubes and incubated for 3 hours in a 28 °C shaking incubator. Two different volumes (50µl and 150 µl) and the remaining amount from each transformation vial was spread on labelled LB agar plates (Appendix 2) containing 50 µg/ml of Kanamycin, 50 µg/ml Streptomycin and 25 µg/ml Rifampicin (See Appendix 3). The liquid was then allowed to be absorbed by the LB agar plates and then the plates were inverted and placed in a 28 °C incubator for 72 hour. The colonies were observed after a 3 day period.

2.7 TRANSFORMATION OF *PETUNIA HYBRIDA* LEAF DISCS BY *AGROBACTERIUM* MEDIATED TRANSFER.

2.7.1 Sowing Seed and Growth Condition

As the project needed self-incompatible lines of *Petunia hybrida* with a PhS_3PhS_V genotype, three families were prepared previously from crossing between PhS_3PhS_3 and PhS_vPhS_v SI stocks. Seed were collected from these crosses and sown for this project to provide three stock families (N456, N457, and N458) (Table 2.8).

Table 2.8 Seed lines and their genotypes used in the transformation process.

Parental Crosses and ID	First Progeny ID
PhS_3PhS_3(N439.6) X PhS_vPhS_v (N439.4)	N456 PhS_3PhS_v
PhS_3PhS_3 (N436.6) X PhS_vPhS_v (N439.4)	N457 PhS_3PhS_v
PhS_3PhS_3 (N440.4) X PhS_vPhS_v (N439.4)	N458 PhS_3PhS_v

Regarding sowing seeds and growth condition see section (2.1).

2.7.2 Inoculum Preparation

5-6 colonies from *Agrobacterium tumefaciens* (LBA4404) were put in 50 ml of APM media (See Appendix 2) recommended by Dr. Ruth Cornock (Biosciences Genomics Facility, University of Nottingham) for preparing liquid culture which also contains 50 µg/ml of Kanamycin, 50 µg /ml Streptomycin and 25 µg/ml Rifampicin (See Appendix 3) . The vials was placed at 28°C in a rotary shaker overnight and it was used directly after checking its concentration by Spectrophotometer at 600 nm. As the Optical density (OD) was generally between (0.8-1) according to Dr. Paul Anthonys' recommendations no dilution were required.

2.7.3 Explant Preparation

Leaves were harvested from 4-6 week old *Petunia hybrida* stocks which hold PhS_3PhS_v alleles and placed in a sterilized container. Under the laminar flow hood leaves were surfaced sterilized in 10% (v/v) household bleach solution for approximately 20 min with shaking at varying intervals to ensure that the leaf surface was covered. The leaves were then washed three times using sterile distilled water. After that, the sterilized leaves were then cut under sterile conditions into squares of approximately 2 cm. The mid-rib and edges were avoided as well as any necrotic areas. Approximately 3-4 explants were obtained from each leaf. The surface of each explants were wounded with a scalpel and placed on sterile distilled water to prevent drying out.

Twenty explants were placed directly in four plates of Co-cultivation medium (CCM) (Appendix 2) as a control. The remaining explants were placed in the liquid culture for 15 min. After inoculation the explants were sandwiched between two layers of sterile filter paper to remove the excess inoculum. Approximately 10-15 explants were transferred to each plate containing cultivation medium containing 1μg /ml BAP and 0.01 1μg/ml NAA with the abaxial surface facing downwards. The plates were then sealed with Micropore tape and placed in a growth room at 25 °C with a photoperiod of 16 hour light and 8 hour dark for three days.

After three days the two plates of the control explants were kept in the co-cultivation medium (CCM) to use as a comparison with transgenic plant plants in the future. The other two control plates with other plates were transferred to regeneration and selection medium (RSM) (see appendix 2), containing 1μg/ml BAP 0.01, 1μg/ml NAA, 100 μg /ml and Kanamycin and 200 μg /ml Carbenicillin. The abaxial surfaces were turned down, approximately 8-10 explants were placed per plate and subjected to the same conditions as mentioned previously.

Every two weeks the explants (which created callus) were transferred to fresh RSM (See Appendix 2). The shoots were transferred to rooting media (See Appendix 2) when they reached about 2 cm in length and placed under the same growth conditions until they were large enough for further analysis.

2.8 TRANSFERRING PLANTS TO THE GLASSHOUSE

Kanamycin resistant plants were transferred to the glasshouse (24/22°C, 16/8 hour day/night cycle), when they reached about 10 cm height. Small pots containing

standard potting mix (M3 Levington, John Innes No.3 and Perlite in ratio 3:2:1) were prepared and irrigated until they reached saturation point. The plants were removed from the jars gently and then placed into the pots and covered with a polyethylene bag secured tightly with an elastic band. After one week, one corner of the plastic bag was cut and the other corner after two weeks. All work with transgenic plants was carried out under ACGM code BS139 (T.P. Robbins).

2.9 ANALYSIS OF KANAMYCIN RESISTANT PLANTS

In order to identify transgenic plants transformed with all constructs, all Kanamycin resistant plants were analysed using M13-Reverse (as forward) primer and one of the reverse specific primers for each gene, except the plants transformed with AhSLF-S2,M13 forward (as reverse) and forward specific primers were used (see primer table 2.7). The expected size was obtained from the T-DNA map for each construct (See Appendix 1)

2.10 REVERSE TRANSCRIPTION (RT)-PCR

2.10.1 RNA Extraction

RNA was extracted from pollen of *Petunia hybrida* transgenic plants using a QIAGEN RNeasy® plant Mini kit. About 100 mg anthers were harvested from unopened buds at four different stages separately as illustrated in Figure 2.1. The collected anther samples were put in 1.5 ml microcentrifuge tubes and snap frozen in liquid nitrogen (N_2) and they were ground using a sterile micro-pestle until a fine powder was obtained. 10 µl of β-Mercaptoethanol was mixed with 1 ml of RLT buffer and from this mixture 450 µl was added into the tissue powder and mixed by vortexing and they were incubated for 3 min at 56°C, to help disrupt the tissue. After centrifuging the

samples for 5 min at 13,000 rpm, the supernatant was pipetted carefully into new 1.5 ml microcentrifuge tubes. Then, 225 µl ethanol (96-100%) was applied into the tubes and mixed by pipetting and directly the mixture (usually 650 µl) was transferred directly into an RNeasy spin column (pink) placed in a 2 ml collection tube and they were centrifuged for 15 sec at 13,000 rpm. After discarding the flow through liquid, 350 µl RW1 was added and all tubes were centrifuged at 10,000 rpm for 15 sec, the flow through liquid was discarded again. Inside ice 10 µl of DNase mixed with 70 µl of RDD buffer then from this mixture 75 µl was added into the pink column and the tubes containing the mixture were incubated at 37°C for 1 hour. Another 350 µl of RW1 was applied and the tubes were centrifuged at 10,000 rpm for 15 sec, and the flow through liquid was discarded as well. 500 µl of RPE was added into the pink tubes, and then centrifuged for 15 sec at 10,000 rpm; this step was repeated but they were centrifuged for 2 min. The flow through liquid was discarded and the tubes were centrifuged for 1 min. The pink filters were transferred into new 2 ml collection tubes and then 30 µl of sterilised distilled water was added onto the pink filter and centrifuged for 1 min at 10,000 rpm. The pink filters were discarded and the liquid (RNA) was stored at -80°C.

Figure 2.1 Transgenic buds at four different stages.
1=10-20 mm, 2=20-30 mm, 3=30-40 mm and 4= longer than 40 mm. Bar = 1 Cm

2.10.2 RNA quality control and quantification

A NanoDrop Spectrophotometer (ND-1000 Spectrophotometer, NanoDrop Technologies, Inc., and Wilmington, USA) was used for checking the quantity and purity of RNA using 2 µl of RNA samples. In addition, RNA quality was checked using gel electrophoresis. 3 µl of RNA samples mixed with 2 µl 6X loading buffer, and the mixture was run on 1.5% (w/v) TBE agarose gel (See section 2.4.5) containing ethidium bromide at a concentration of 0.15 µg/ml for more than 1 hour at 90V, and an UV transilluminator was used to visualise the bands.

2.10.3 Synthesis of cDNA for positive RT-PCR (+RT-PCR) and negative RT-PCR (-RT-PCR)

For positive RT-PCR, cDNA was synthesised using Superscript III kit. 1 µg from harvested RNA was prepared using RNA concentration as described in section 2.10.2, 1 µl of oligo (dT) and 1 µl of 10 mM dNTP Mix (dATP, dGTP, dCTP and dTTP at neutral pH) were added into the tubes. RNase free water was added and the

volume was completed to 13 µl. Then the mixture was incubated for 5 min at 65°C and they were put in ice for more than one min. After that, 4 µl of 5X First-Strand Buffer, 1 µl of 0.1M DTT, 1 µl of RNaseOUT (RNase inhibiter) and 1 µl of Super Script III RT were added and mixed by pipetting, the mixture was incubated at 50°C for 60 min. Directly after that, the reaction was inactivated by heating at 70°C for 15 min cDNA was stored at -80°C. Regarding Negative RT-PCR, again 1µg RNA was prepared and then instead using the above kit the volume was completed to 20 µl using RNase free water.

2.10.4 RT-PCR reaction for checking transgene expression

In order to test the expression of the all transgenes, RT-PCR reaction was used to detect AhSLF transgenes in transgenic and control plants of *Petunia hybrida*. Master mix was prepared as described in section 2.5.2 with using 1 µl from cDNA. Initially all cDNA samples were tested using housekeeping gene elongation factor 1 alpha (EF1a) primers. Then, the specific primers for each transgene were used to test the transgene expression. Regarding preparing master mix and PCR condition, the same condition was used as mentioned in section 2.5.2 with using 0.5 µl from cDNA as a template. However, different cycles were tested from 30 to 18.

2.11 QUANTITATIVE REAL TIME PCR (QPCR)

This method has been used as a modern technique to confirm the transgene expression, find the different level of transgene expression in different bud stages and reveal the relation between transgene expression and transgenic plant phenotype. qRT-PCR analyses were performed using the Light Cycler 480 real-time PCR system (Roche Applied Science). Brilliant SYBR Green qPCR master mix (Agilent

Technologies Inc.,) was used. The master mix was prepared in 9 µl reaction volume consisting of 0.5 µl cDNA (see section 2.10.3), 0.2 µl of 10µM forward and reverse primers, 4.5 µl SYBR Green and 3.6 µl sigma water. Initially, primer efficiencies was optimized by diluting one cDNA samples from each transgenic plants three times 1/5, 1/25 and 1/125 in addition to the stock cDNA which was considered to be 1. After testing several annealing temperatures, it was concluded that 60°C and 63°C are the ideal for *AhSLF-S2* and *AhSLF-S1E* respectively, and both *AhSLF-S4D* and *AhSLF-S2C* gave the best results using 62°C.

PCR cycling conditions for amplification were 95°C for 10 min and then 55 cycles of 95°C for 30 sec, 1 min of annealing at a temperature depending on the Tm (melting temperature) of the primers, and 72°C for 1 min). Expression was normalized using the EF1a gene and each sample was repeated three times to obtain accurate results. Light Cycler 480 software was used to analyse the data.

2.12 SELECTING TRANSGENIC SEEDS USING KANAMYCIN

The seeds were collected from crossing transgenic plants *Petunia hybrida* (PhS_3PhS_v) with *Petunia inflata* (PiS_3PiS_{k1}), and other different crosses. In order to surface sterilize seeds, they were put in folded filter paper, labelled and then closed from all corners using paperclips. All folded filter papers were put in 10% (v/v) household bleach for 20 min. After discarding the bleach, the seeds were washed three times using distilled water, the paperclips were opened carefully using sterilized forceps and then the samples were left under a laminar flow hood overnight to dry out. Each group of seed was spread between five plates, on half MS media (See Appendix 2). Two plates without Kanamycin were used as a control to check

the quality of seeds (both non-transgenic and transgenic). Regarding non transgenic seeds, the other three plates (with 50 μg /ml) were used as another control to check the antibiotic quality and to confirm that the non-transgenic seed are sensitive to Kanamycin. With regards to seeds collected from transgenic lines, the other three plates (with 50 μg /ml) were used for selecting transgenic seedlings. The plates were then sealed with Micropore tape and placed in a growth room at 25 °C with a photoperiod of 16 hour light and 8 hour dark. The resistant seedlings were scored four weeks later and the seedlings were transferred to small sterilized pots with full MS media (See Appendix 2). After two weeks, they were transferred to the glasshouse (see section 2.8) but instead of using the pots, trays were used and they were covered by lids for 10-15 day, depending on the growth of the seedlings.

2.13 BIOINFORMATICS

2.13.1 Creating hypothetical T-DNA Maps

The software package "Snapgene" was used to create the T-DNA maps after finding the sequence for the binary vector and gene sequences from the (*National Center for Biotechnology Information)* http://www.ncbi.nlm.nih.gov/

2.13.2 Gene sequencing Confirmation

Sequences obtained from binary vectors were checked using the Biotechnology Information (NCBI) database Global Sequence Alignment Tool.(http://blast.st-va.ncbi.nlm.nih.gov) and The European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/msa/clustalo).

2.13.3 Primer3 software

Primers were designed for the plant transformation part of this project using the Primer3 website (http://primer3.ut.ee/).

2.13.4 Multiple sequences alignment

Multiple sequences alignments was created using The European Bioinformatics Institute website (http://www.ebi.ac.uk/Tools/msa/clustalo).

2.13.5 Statistical analysis

Statistical analysis of segregation ratios was carried out with mean value per analysis and significance of the observed and expected data was determined using Chi-Square Goodness of Fit using IBM SPSS statistics version 22 (Statistical Package For Social Sciences, SPSS) at 5% probability level.

CHAPTER 3 : CHARACTERIZATION OF S-ALLELES IN *PETUNIA INFLATA* AND *PETUNIA HYBRIDA* STOCKS.

3.1 INTRODUCTION

As the main purpose of this project is to test four constructs (AhSLF-S2, AhSLF-S1E, AhSLF-S2C and AhSLF-S4D) with a wide ranges of alleles in *Petunia inflata* and *Petunia hybrida* to control incompatibility, testing the stability of SI in both stocks and F_1 hybrids is a crucial and basic point. Regarding *Petunia inflata*, several plants belonging to nine families were tested for three alleles (*PiS_d-RNase, PiS_3-RNase* and *PiS_{k1}-RNase*). Theses alleles were cloned by Dr. Daniel Dzidzienyo and after comparing with data bases it was revealed that *PiS_d-RNase* is a novel allele (Dzidzienyo, 2012). With regard to *Petunia hybrida*, several plants belonging to four families were raised from seeds obtained from Dr.Tim Robbins. *Petunia hybrida* stocks were screened for *PhS_3-RNase* and *PhS_v-RNase* and both of them were cloned by Robbins *et al*, (2000). Both stocks were genotyped by Genomic PCR using gene specific primers for each individual allele and their phenotypes were tested using self-pollination. Regarding *Petunia inflata* only stable SI lines were selected, but in *Petunia hybrida* stocks, all plants have a stable SI. In addition, the S-RNase allele segregation was found in F_1 hybrids plants using the same techniques. Based on self-pollination results, it was noticed that all plants that inherited *PiS_d* allele became self-compatible. As a result of this observation, the *Petunia inflata* stock that holds *PiS_d* allele has not been tested with transgenes and the behaviour of this allele was further analysed in F_2 hybrids and it will be focused on in the chapter six.

3.2 RESULTS

In order to test the stability of SI in both stocks of *Petunia inflata* and *Petunia hybrida*, several plants were genotyped using gene specific primers to find the S-*RNase* alleles responsible for incompatibility. The plants' phenotypes were tested using self-pollination. In addition, both stocks were crossed to produce F_1 hybrids and the same techniques were used to find different genotypes and phenotypes.

3.2.1 Identification of specific alleles (S-RNases) in *Petunia inflata*.

Nine families (N441, N442, N443, N444, N445, N446, N447, N448 and N449) were raised from *Petunia inflata* seeds obtained from Dr. Daniel Dzidzienyo (See Table 2.2). Initially one plant in each family was screened using gene specific primers and all of them were designed by Dr. Daniel Dzidzienyo (See Table 2.6). These primers were tested by Genomic PCR to identify *PiS_d-RNase*, *PiS_{k1}-RNase* and *PiS_3-RNase* alleles in *Petunia inflata* stocks. Later more plants were genotyped but the PCR data are not shown to avoid repetition.

3.2.1.1 Identification of the PiSd-RNase

In order to identify the *PiS_d-RNase* allele in *Petunia inflata* stock nine plants were screened using *PiS_d-RNase* specific primers, N441.1, N442.1, N443.1, N444.1, N445.1, N446.1, N447.1, N448.1, N449.1. It can be seen that in Figure 3.1 a total of seven out of nine hold *PiS_d-RNase* (N441.1, N443.1, N444.1, N445.1, N446.1, N447.1, and N448.1). This result was repeated twice in order to confirm the plants genotypes are correct.

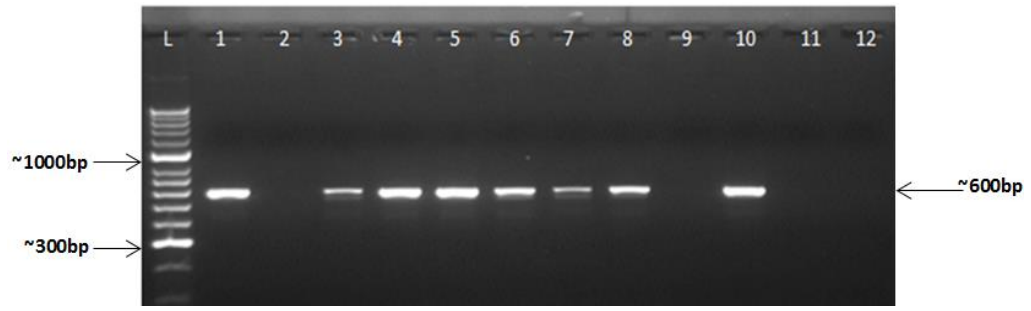


Figure 3.1 Confirmation of the presence of the *PiSd-RNase* in individual *Petunia inflata* plants by Genomic PCR. L represents HyperLadder II (Bioline). Lanes 1-9 represent Plants ID: N441.1, N442.1, N443.1, N444.1, N445.1, N446.1, N447.1, N448.1, and N449.1 respectively. In addition, lanes 10-12 represent: Positive control for *Petunia inflata*, Negative control for *Petunia inflata* (*Petunia hybrida*) and H_2O as a negative control for contamination respectively.

3.2.1.2 Identification of the PiS_{k1}-RNase

PiS_{k1}-RNase specific primers were used to screen nine plants (N441.1, N442.1, N443.1, N444.1, N445.1, N446.1, N447.1, N448.1, and N449.1) by Genomic PCR. As illustrated in Figure 3.2 a total of six plants N441.1, N442.1, N443.1, N446.1, N447.1 and N449.1 out of nine tested hold the *PiS_{k1}-RNase*. In order to ensure reliability of the plant genotype this result was repeated twice.

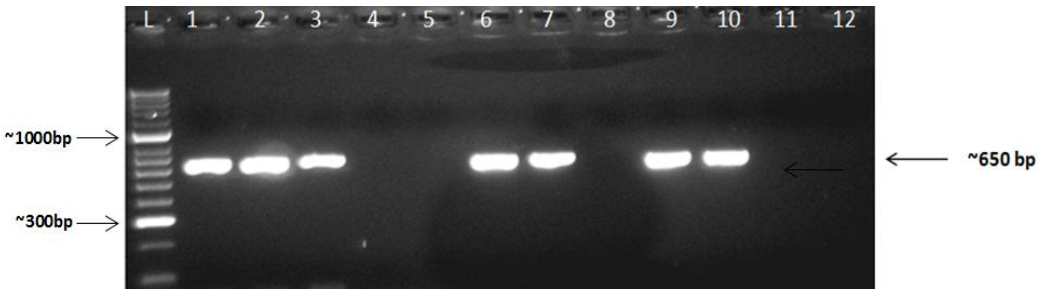


Figure 3.2 Confirmation of the presence of the *PiS_{k1}-RNase* in individual *Petunia inflata* plants by Genomic PCR. L represents HyperLadder II (Bioline). Lanes 1-9 represent Plants ID: N441.1, N442.1, N443.1, N444.1, N445.1, N446.1, N447.1, N448.1, and N449.1 respectively. In addition 10-12 represent: Positive control for *Petunia inflata*, Negative control for *Petunia inflata* (*Petunia hybrida*) and H_2O as a Negative control for contamination respectively.

3.2.1.3 Identification of the PiS_3-RNase

Figure 3.3 shows the presence of *PiS_3-RNase* in four plants (N442.1, N444.1, N445.1 and N448.1) out of nine (N441.1, N442.1, N443.1, N444.1, N445.1, N446.1, N447.1,

N448.1, N449.1) tested using *PiS$_3$-RNase* specific primers by Genomic PCR. This result was repeated twice to ensure the genotype of the plants is correct.

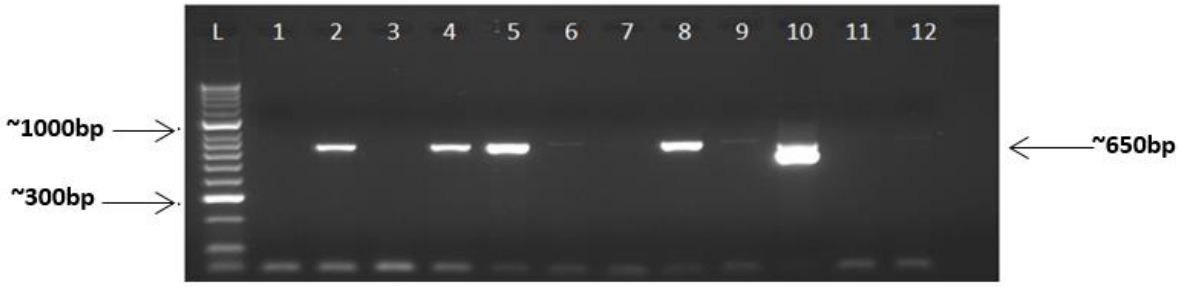


Figure 3.3 Confirmation of the presence of the *PiS$_3$-RNase* in individual *Petunia inflata* plants by Genomic PCR. L represent HyperLadder II (Bioline). Lanes 1-9 represent Plants ID: N441.1, N442.1, N443.1, N444.1, N445.1, N446.1, N447.1, N448.1 and N449.1 respectively. In addition 10-12 represent: Positive control for *Petunia inflata*, Negative control for *Petunia inflata* (*Petunia hybrida*) and H_2O as a negative control for contamination respectively.

3.2.2 S-allele genotype summary for *Petunia inflata* stocks

According to the results shown in the previous sections (3.2.1.1, 3.2.1.2 and 3.2.1.3) the genotype of the plants which were used in this project can be summarized as shown in Table 3.1.

Table 3.1 Summary of *Petunia inflata* genotypes for plants used in this project.

Plant ID	*PiS$_d$*	*PiS$_{k1}$*	*PiS$_3$*	Genotype
N441.1	+	+	-	*PiS$_d$PiS$_{k1}$*
N442.1	-	+	+	*PiS$_{k1}$PiS$_3$*
N443.1	+	+	-	*PiS$_d$PiS$_{k1}$*
N444.1	+	-	+	*PiS$_d$PiS$_3$*
N445.1	+	-	+	*PiS$_d$PiS$_3$*
N446.1	+	+	-	*PiS$_d$PiS$_{k1}$*
N447.1	+	+	-	*PiS$_d$PiS$_{k1}$*
N448.1	+	-	+	*PiS$_d$PiS$_3$*
N449.1	-	+	-	*PiS$_{k1}$PiS$_{k1}$*

It can be seen that the only unexpected result is the genotype of plant N449.1 because it holds just the *PiS$_{k1}$PiS$_{k1}$* allele. The interpretation for this result is that this plant came from crossing between stocks 8 and 2; both are incompatible plants

which have *PiS$_d$PiS$_{k1}$* genotype as shown in Table 2.2. According to self-incompatibility system, crossing between plants which hold the same genotypes should not produce any seed as self-pollen is rejected by the stigma. However, in some cases such crosses can show a breakdown and this phenomenon is called Pseudo self-compatibility. In this case, the compatibility is due to transmission of the PiS$_{k1}$ allele and not *PiS$_d$* allele. PSC plants were discarded from the project directly and only SI plants were kept.

3.2.3 Identification of specific alleles (*PhS-RNases*) in *Petunia hybrida*

Twelve *Petunia hybrida* plants were raised from seeds derived from four families (N436, N438, N439 and N440) obtained from Dr.Tim Robbins which all came from bud pollination (Parents were of *PhS$_3$PhS$_v$*) genotype (See Table 2.3). They were genotyped using gene specific primers for *PhS$_v$-RNase* and *PhS3-RNase* (See Table 2.6) *(*Robbins *et al*, 2000).

3.2.3.1 Identification of the PhS$_v$-RNase

Twelve *Petunia hybrida* plants: N436.4, N436.5, N436.6, N438.4, N438.5 N438.6, N439.4, N439.5, N439.6, N440.4, N440.5 and N440.6 were screened using *PhS$_v$-RNase* specific primers by Genomic PCR. As illustrated in Figure 3.4 all plants hold the *PhS$_v$* allele except N436.6, N439.6 and N440.4. This result was repeated twice to confirm the existence of *PhS$_v$-RNase* allele in these plants.

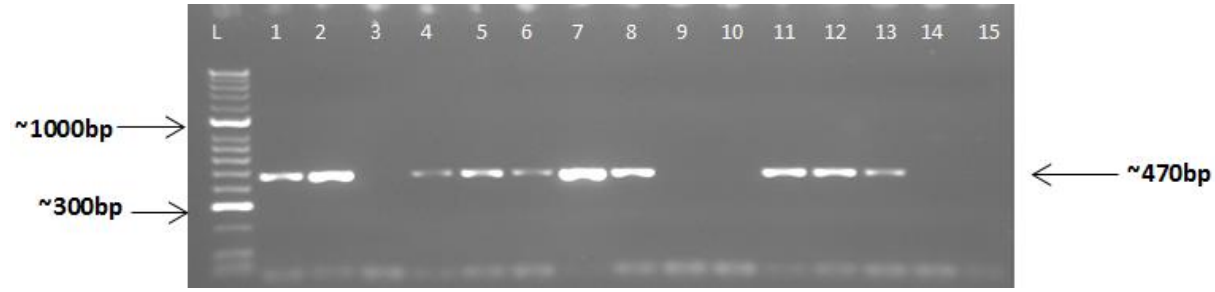


Figure 3.4 Amplification of the *PhS_v-RNase* in a number of individual *Petunia hybrida* plants by Genomic PCR.L represent HyperLadder II (Bioline). Lanes 1-12 represent Plants ID: N436.4, N436.5, N436.6, N438.4, N438.5, N438.6, N439.4, N439.5, N439.6, N440.4, N440.5 and N440.6 respectively. In addition 13-15 represent: Positive control for *PhS_v-RNase* allele, Negative control for *PhS_v-RNase* (*Petunia inflata*) and H_2O as a Negative control for contamination respectively

3.2.3.2 Identification of the PhS3-RNase

The same twelve *Petunia hybrida* plants as mentioned in section 3.2.2.1 were screened using *PhS_3-RNase* gene specific primers by Genomic PCR. As illustrated in Figure 3.5 all plants hold *PhS_3-RNase* allele except N439.4.

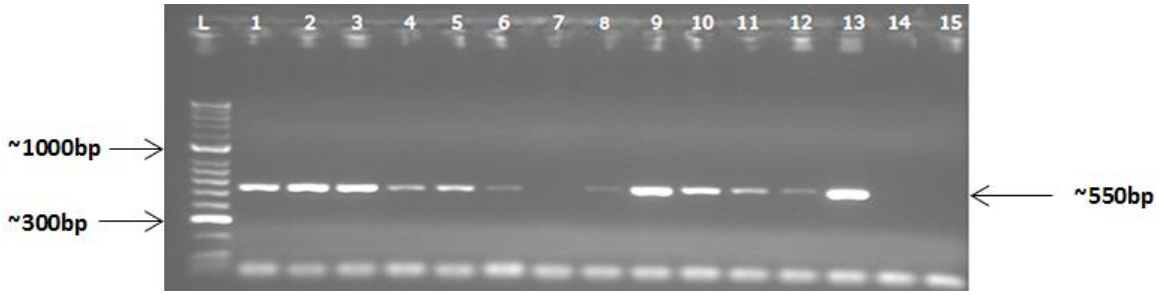


Figure 3.5 Amplification the *PhS_3-RNase* in a number of individual *Petunia hybrida* plants by Genomic PCR. L represent HyperLadder II (Bioline). Lanes 1-12 represent Plants ID: N436.4, N436.5, N436.6, N438.4, N438.5, N438.6, N439.4, N439.5, N439.6, N440.4, N440.5 and N440.6 respectively. In addition 13-15 represent: Positive control for *Ph$_{S3}$-RNase,* Negative control for *Ph$_{S3}$-RNase* (*Petunia inflata)* and H_2O as a negative control for contamination respectively.

3.2.4 S-allele genotypic summary for *Petunia hybrida* stocks

According to the results shown in the previous sections (3.2.3.1 and 3.2.3.2) the genotype of the *Petunia hybrida* stocks which have been used in this project are summarised in Table 3.2. Three different genotypes were obtained *PhS_3PhS_3*, *PhS_vPS$_3$* and *PhS_vPhS_v* because these seeds came from self-bud pollination of

parents which all hold *PhS_vPhS_3* (See Table 2.3). The observed ratio was 3:8:1 for *PhS_3PhS_3*, *PhS_vPS_3* and *PhS_vPhS_v* respectively which is somewhat different from the expected ratio 3:6:3. It can be seen that all plants are heterozygous and hold *PhS_v and PhS_3* alleles except N439.4 which holds only *PhS_v* alleles and N436.6, N439.6 and N440.4 which holds only the *PhS_3* allele which indicates they are homozygous.

Table 3.2 Summary of *Petunia hybrida* genotypes for plants used in this project. The highlighted line in green indicates homozygous plants.

Plant ID	Genotype
N436.4	*PhS_3PhS_v*
N436.5	*PhS_3PhS_v*
N436.6	*PhS_3PhS_3*
N438.4	*PhS_3PhS_v*
N438.5	*PhS_3PhS_v*
N438.6	*PhS_3PhS_v*
N439.4	*PhS_vPhS_v*
N439.5	*PhS_3PhS_v*
N439.6	*PhS_3PhS_3*
N440.4	*PhS_3PhS_3*
N440.5	*PhS_3PhS_v*
N440.6	*PhS_3PhS_v*

3.2.5 Identification of specific alleles (S-RNases) in F_1 hybrids .

In this project several F_1 hybrids were obtained from crosses between *Petunia inflata* and *Petunia hybrida* (Table 2.4). The resulting hybrids have been genotyped using allele specific primers designed and tested for both parental stocks (see sections: 3.2.1, 3.2.3). The segregation of alleles was identified and consequently the F_1 hybrids were genotyped.

3.2.5.1 Identification of the PiS_{k1}-RNase

In order to identify *PiS_{k1}-RNase* in F_1 hybrids twelve plants from three families, N450, N452 and N454 were raised ,which are N450.1, N450.2, N450.3, N450.4, N450.5 , N450.6, N450.7 N450.8, N452.1, N452.2, N454.1 and N454.2 because we

expected they inherited *PiS$_{k1}$* allele from *Petunia inflata* parent (See Table 2.4). They were genotyped using *PiS$_{k1}$-RNase* specific primers (See Table 2.6). Figure 3.6 shows that only two plants N450.8 and N454.1 hold *PiS$_{k1}$*..

Figure 3.6 Identification of F$_1$ hybrid plants carrying *PiS$_{k1}$-RNase*. L represents HyperLadder II (Bioline). Lanes 1-12 represent Plants ID: N450.1, N450.2, N450.3, N450.4, N450.5, N450.6, N450.7 N450.8, N452.1, N452.2, N454.1 and N454.2 respectively. In addition, lanes 13-14 represent Positive control for *Petunia inflata* PiS$_k$1 allele, lanes 15-16 represent Negative control for *PiS$_{k1}$* allele and lane 17 represent H_2O as a Negative control for contamination.

3.2.5.2 Identification of the PiS$_d$-RNase

Twelve plants belonging to two families (N451, N453) were used to identify the PiS$_d$ allele (N451.1, N451.2, N451.3, N451.4, N451.5, N451.6, N451.7 N451.8, N453.1, N453.2, N453.3 and N453.4) which was expected to be inherit from the *Petunia inflata* parent (See Table 2.4). *PiS$_d$-RNase* gene specific primers were used to identify the *PiS$_d$* allele (See Table 2.6). Figure 3.7 illustrates that all plants hold *PiS$_d$-RNase* except N451.1, N451.3, N451.4, N451.7 and N453.1.

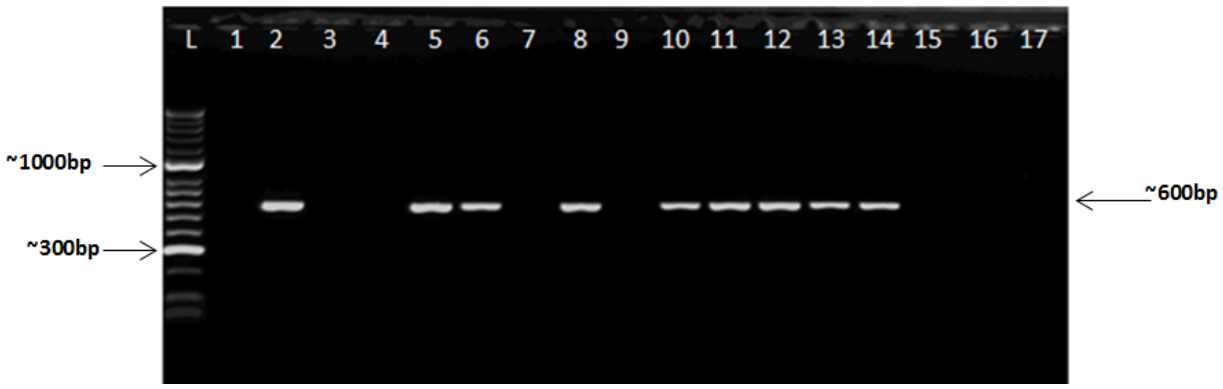


Figure 3.7 Identification of F_1 hybrid plants carrying *PiS_d-RNase*. L represents HyperLadder II (Bioline). Lanes 1-12 represent Plants ID: N451.1, N451.2. N451.3, N451.4, N451.5, N451.6, N451.7 N451.8, N453.1, N453.2, N453.3 and N453.4 respectively. In addition, lanes 13-14 represent Positive control for *Petunia inflata* (PiS_d *allele*), lanes 15-16 represent Negative control for *PiS_d* allele and lane 17 represent H2O as a Negative control for contamination.

3.2.5.3 Identification of the PiS_3-RNase

Twenty four plants derived from four families (N450, N451, N453 and N454) were used to identify *PiS_3-RNase* (N450.1, N450.2, N450.3, N450.4, N450.5, N450.6, N450.7, N450.8, N451.1, N451.2, N451.3, N451.4, N451.5, N451.6, N451.7, N451.8, N452.1, N452.2, N453.1, N453.2, N453.3, N453.4, N454.1 and N454.2) because we expected the plants to inherit this allele from *Petunia inflata* parent in all families (See Table 2.4). Genomic PCR using *PiS_3-RNase* specific primers was used to identify this allele (See Table 2.6). Figure 3.8 shows that all plants hold *PiS_3-RNase* except N450.8, N451.2, N451.5, N451.6, N451.8, N453.2, N453.3, N453.4 and N454.1.

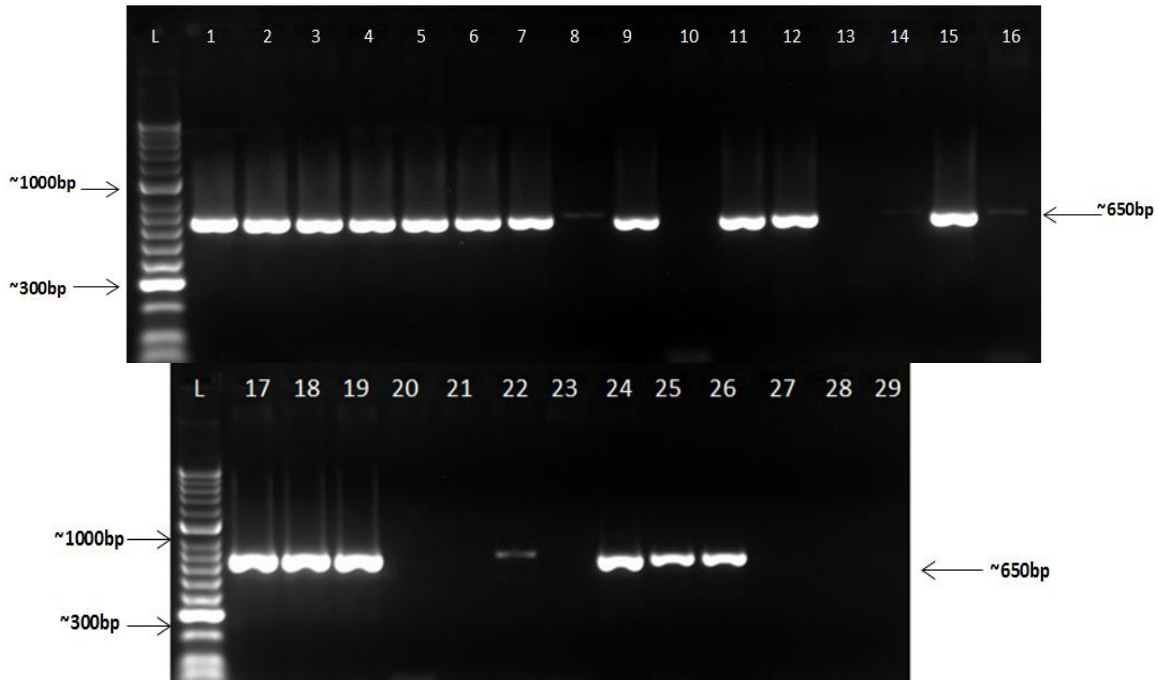


Figure 3.8 Identification of F_1 hybrid plants carrying *PiS_3-RNase.* L represents HyperLadder II (Bioline). Lanes 1-24 represent Plants ID: N450.1, N450.2, N450.3, N450.4, N450.5, N450.6, N450.7, N450.8, N451.1 N451.2, N451.3, N451.4, N451.5, N451.6, N451.7, N451.8, N452.1, N452.2, N453.1, N453.2, N453.3, N453.4, N454.1 and N454.2 respectively. In addition, lanes 25-26 represent Positive control for *Petunia inflata* (PiS_3 allele), lanes 27-28 represent Negative control for PiS_3 *allele* and lane 29 represent H_2O as a Negative control for contamination.

3.2.5.4 Identification of the PhS3-RNase

PhS_3-RNase was identified in 22 plants belonging to four families (N450, N451, N452 and N453) as we expected to be inherited *PhS_3-RNase* from the *Petunia hybrida* parent (see Table 2.4). These plants are N450.1, N450.2, N450.3, N450.4, N450.5, N450.6, N450.7, N450.8, N451.1, N451.2, N451.3, N451.4, N451.5, N451.6, N451.7, N451.8, N452.1, N452.2, N453.1, N453.2, N453.3 and N453.4 .Genomic PCR using *PhS_3-RNase* primers was used to identify this allele (See Table 2.6), as illustrated in Figure 3.9 Only eight plants out of the total did not hold *PhS_3-RNase*; these are N450.3, N450.4 , N450.7, N450.8, N451.4, N451.5, N451.6 and N451.8.

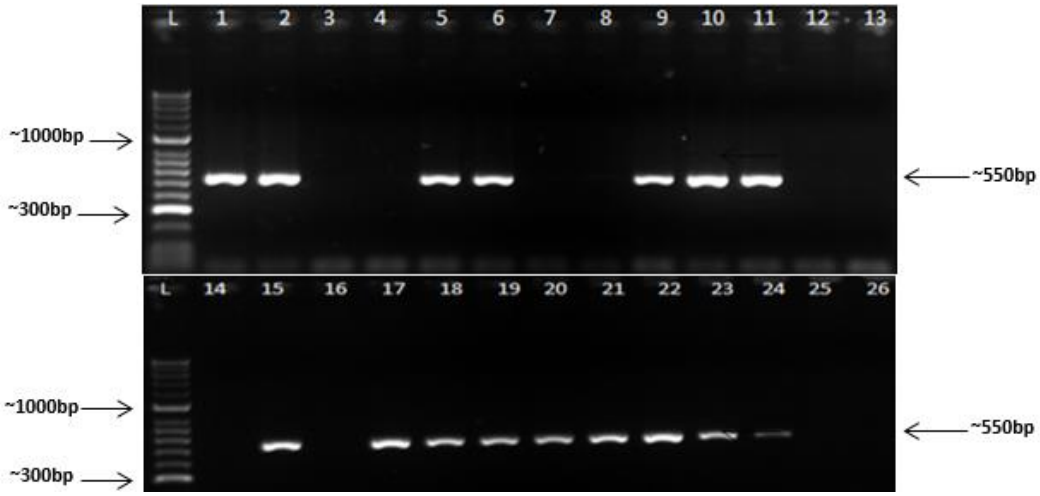


Figure 3.9 Identification of F_1 hybrid plants carrying PhS_3-RNase. L represents HyperLadder II (Bioline). Lanes 1-22 represent Plants ID: N450.1, N450.2, N450.3, N450.4, N450.5, N450.6, N450.7, N450.8, N451.1, N451.2, N451.3, N451.4, N451.5, N451.6, N451.7, N451.8, N452.1, N452.2, N453.1, N453.2, N453.3 and N453.4 respectively. In addition, lanes 23-24 represent Positive control for *Petunia hybrida* (*PhS_3* allele), lanes 25, 26 represent Negative control for *PhS_3* allele and H_2O as a negative control for contamination respectively.

3.2.5.5 Identification of the PhS_V-RNase

Eighteen plants belonging to three families (N450, N451 and N454) were tested to identify *Phs_v-RNase* allele; these are N450.1, N450.2, N450.3, N450.4, N450.5, N450.6, N450.7, N450.8, N451.1, N451.2, N451.3, N451.4, N451.5, N451.6, N451.7, N451.8, N454.1 and N454.2 because it was predicted they inherited this allele from the *Petunia hybrida* parent (See Table 2.4). Genomic PCR using *PhS_v-RNase* was used to identify this allele (See Table 2.6). According to Figure 3.10, it is clear that the following plants hold the *PhS_v-RNase* allele: N450.3, N450.4, N450.7, N450.8, N451.4, N451.5, N451.6, N451.8, N454.1 and N454.2.

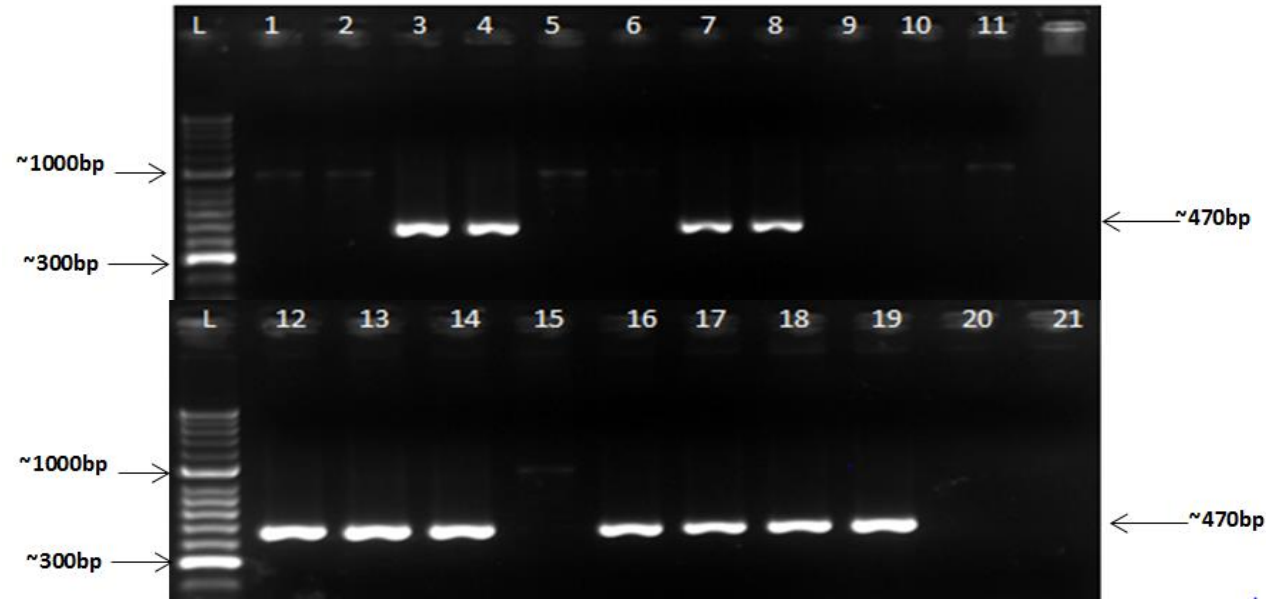


Figure 3.10 Identification of F_1 hybrid plants carrying PhS_v-RNase. L represents HyperLadder II (Bioline). Lanes 1-18 represent Plants ID: N450.1, N450.2, N450.3, N450.4, N450.5, N450.6, N450.7, N450.8, N451.1, N451.2, N451.3, N451.4, N451.5, N451.6, N451.7, N451.8, N454.1 and N454.2 respectively. In addition, lanes 19-21 represent: Positive control for *Petunia hybrida* (*PhS_v* allele), Negative control for *PhS_v allele* and H_2O as a negative control for contamination respectively.

3.2.6 Genotyping of additional plants from F_1 hybrids to identify *PhS_3PiS_{k1}* genotype.

According to the previous results regarding the finding of all possible genotypes in the F_1 hybrids, all genotypes were found except *PhS_3PiS_{k1}*. Consequently the same techniques (See section 3.2.5.1 and 3.2.5.4) were used for genotyping eight plants (N452.3, N452.4, N452.5, N452.6, N452.7, N452.8, N452.9 and N452.10) raised from the N452 family as 50% are expected to give the desired genotype (See Table 2.4). The desired genotype *PhS_3PiS_{k1}* was identified in six plants out of eight tested (N452.3, N452.5, N452.6, N452.7, N452.8 and N452.9), but the PCR data are not shown to avoid repetition.

3.2.7 Summarizing Phenotype and S-allele genotypic summary for F_1 hybrid stocks

Self-pollination was performed for *Petunia inflata* and *Petunia hybrida* stocks using the methods described in section 2.3. Regarding *Petunia inflata* stocks, selected SI plants were used as some plants had undergone a breakdown of SI. In the case of *Petunia hybrida* all plants showed stable SI. Crosses were made between *Petunia hybrida* and *Petunia inflata* (See section 2.3). All six possible genotypes were identified for the F_1 hybrids (*PhS₃PiS₃*, *PhS₃PiS$_d$*, *PhS₃PiSk₁*, *PhS$_v$PiS₃*, *PhS$_v$PiS$_d$* and *PhS$_v$PiS$_{k1}$*) as shown in Table 3.3. Upon testing for SI it was noticed that all progeny have stable SI except for those with the *PiS$_d$* allele. These findings are illustrated in Figure 3.11.

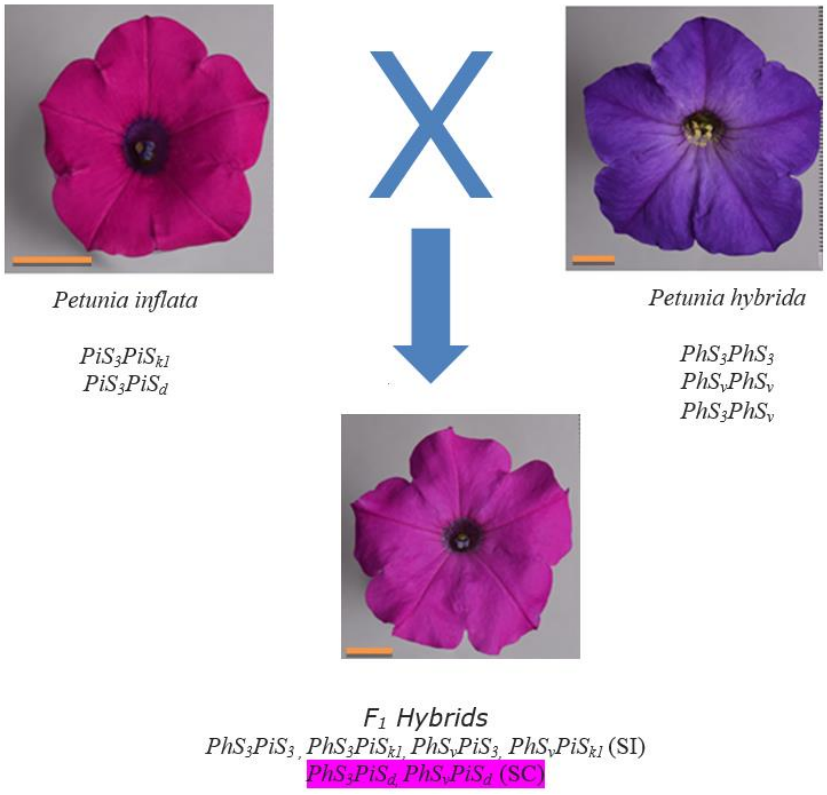


Figure 3.11 Crossing between *Petunia inflata* and *Petunia hybrida* to create F_1 hybrids. The F_1 hybrid shown is an example of the six possible genotypes all of which share a similar size and appearance of the corolla. The self-incompatibility phenotype of the F_1 hybrids is indicated in parentheses (SI or SC). Self compatibile genotypes are highlighted in pink. Bar=1 cm.

According to the results shown in the previous sections (3.2.5.1, 3.2.5.2, 3.2.5.3, 3.2.5.4, 3.2.5.5, 3.2.6) the genotype and phenotype of a total of 32 F_1 hybrid plants which have been generated in this project have been established as summarised in Table 3.3

Table 3.3 Summary of the genotype and phenotype for all F_1 hybrids plants. The highlighted lines in green indicate the plants that inherited the *PiS_d* allele

F1 Hybrids Glasshouse ID	*PiS_{K1}*	*PiS_d*	*PiS_3*	*PhS_3*	*PhS_v*	Genotype	Phenotype SI or SC
N450.1	-	-	+	+	-	*PhS_3PiS_3*	SI
N450.2	-	-	+	+	-	*PhS_3PiS_3*	SI
N450.3	-	-	+	-	+	*PhS_vPiS_3*	SI
N450.4	-	-	+	-	+	*PhS_vPiS_3*	SI
N450.5	-	-	+	+	-	*PhS_3PiS_3*	SI
N450.6	-	-	+	+	-	*PhS_3PiS_3*	SI
N450.7	-	-	+	-	+	*PhS_vPiS_3*	SI
N450.8	+	-	-	-	+	*PhS_vPiS_{K1}*	SI
N451.1	-	-	+	+	-	*PhS_3PiS_3*	SI
N451.2	-	+	-	+	-	*PhS_3PiS_d*	SC
N451.3	-	-	+	+	-	*PhS_3PiS_3*	SI
N451.4	-	-	+	-	+	*PhS_vPiS_3*	SI
N451.5	-	+	-	-	+	*PhS_vPiS_d*	SC
N451.6	-	+	-	-	+	*PhS_vPiS_d*	SC
N451.7	-	-	+	+	-	*PhS_3PiS_3*	SI
N451.8	-	+	-	-	+	*PhS_vPiS_d*	SC
N452.1	-	-	+	+	-	*PhS_3PiS_3*	SI
N452.2	-	-	+	+	-	*PhS_3PiS_3*	SI
N452.3	+	-	-	+	-	*PhS_3PiS_{K1}*	SI
N452.4	-	-	+	+	-	*PhS_3PiS_3*	SI
N452.5	+	-	-	+	-	*PhS_3PiS_{K1}*	SI
N452.6	+	-	-	+	-	*PhS_3PiS_{K1}*	SI
N452.7	+	-	-	+	-	*PhS_3PiS_{K1}*	SI
N452.8	+	-	-	+	-	*PhS_3PiS_{K1}*	SI
N452.9	+	-	-	+	-	*PhS_3PiS_{K1}*	SI
N452.10	-	-	+	+	-	*PhS_3PiS_3*	SI
N453.1	-	-	+	+	-	*PhS_3PiS_3*	SI
N453.2	-	+	-	+	-	*PhS_3PiS_d*	SC
N453.3	-	+	-	+	-	*PhS_3PiS_d*	SC
N453.4	-	+	-	+	-	*PhS_3PiS_d*	SC
N454.1	+	-	-	-	+	*PhS_vPiS_{K1}*	SI
N454.2	-	-	+	-	+	*PhS_vPiS_3*	SI

According to Table 3.3, seven plants (highlighted in green) out of 32 inherited the *PiS_d* allele (N451.2, N451.5, N451.6, N451.8, N453.2, N453.3 and N453.4) and all of them are self-compatible. This indicates a strong relationships between self-

compatibility and this S-allele, particularly since the remainder of the plants that lack this allele are incompatible.

3.3 DISCUSSION

Petunia hybrida was crossed with *Petunia inflata* to test the stability of SI in F_1 hybrid. However, based on the results in Table 3.3 all plants inheriting the PiS_d allele became self-compatible. In nature usually the breakdown of self-incompatibility is observed when the plants do not have a suitable mate or as a result of environmental stress such as high temperature. Self-incompatible plants can change to self-compatible when pollen-part or stylar-part mutations have occurred at the *S*-locus or as a result of the action of modifier genes. Consequently, in nature it is possible to find both self-compatible and self-incompatible species belonging to one genus because self-compatibility is derived from self-incompatibility (Charlesworth and Charlesworth 1979; Charlesworth *et al*. 1990).

Ando *et al* (1998) reported on three natural populations of *Petunia* in Uruguay in terms of self-incompatibility: self-compatible *Petunia parodii*, self-incompatible of *Petunia axillaris* and mixed population contained mostly self-incompatible and some self-compatible plants of *Petunia axillaris*. The identification of both self-incompatible and compatible plants in the same population provides a good opportunity for studying causes of the breakdown of self-incompatibility in nature. Tsukamoto *et al* (1999) collected seeds from one of those mixed populations identified in Uruguay and analyzed thirty three plants. They identified thirty self-

incompatible and three self-compatible plants. Moreover, they revealed that the stylar-part suppression of an *S*-allele (S13-RNase) caused the breakdown of self-incompatibility in the three self-compatible plants. This phenomenon was noticed in other plants belonging to the Solanaceae family. For instance, as a result of lacking RNase activity caused by stylar-part mutation, breakdown of self-incompatibility was observed in the self-compatible line of *Lycopersicon peruvianum* (L.) Mill derived from a mixed population in Peru (Kowyama *et al*. 1994; Royo *et al*. 1994).

Regarding pollen part mutation, the breakdown of self-incompatibility in natural populations caused by the loss of pollen function has been confirmed by Tsukamoto *et al* (2003). They tested 50 plants of *Petunia axillaris* in natural populations, 13 self-incompatible and 37 self-compatible (carrying either an S_{C1}- or an S_{C2}-haplotype), they also found 14 S-haplotypes among these plants. From self-pollination of two of the self-compatible plants, $S_{C1}S_{C1}$ and $S_{C2}S_{C2}$ homozygotes were generated. All lines were tested for compatibility. Interestingly, it was observed that $S_{C1}S_{C1}$ was compatible with all genotypes and $S_{C2}S_{C2}$ accept pollen from all genotypes except $S_{17}S_{17,}$ but $S_{17}S_{17}$ accepted pollen from $S_{C2}S_{C2.}$ After cloning and sequencing cDNA for both genes, it was revealed that their sequences were completely identical and S_{C2}-haplotype was a mutant form of the S_{17}-haplotype and its pollen function was defective.

In this project, the breakdown of SI is related to one allele, (PiS_d), as the other genotypes lacking this allele showed stable SI. This observation can be used to build the hypothesis and identify either female part of this allele (*PiS_d-RNase*) or male part (*SLF*) had lost function. If the female part had lost function, *PiS_d-RNase* would have not degraded the pollen RNA which holds the PiS_d allele. However, if male part

(SLF)s of this gene had lost function, pollen would have escaped from the degradation effects of *PiS$_d$-RNase* and grown successfully. In both cases the pollen tube was able to grow through the style and reach the ovary which leads to successful fertilization and production of seeds. In order to confirm the stability of this observation and test the hypothesis to identify whether the male or female parts of the *PiS$_d$* allele are responsible for this, more plants are needed to be analysed in F_2 hybrids which will be the focus of chapter 6.

CHAPTER 4 : AGROBACTERIUM TRANSFORMATION OF *PETUNIA HYBRIDA* WITH AHSLF CONSTRUCTS.

4.1 INTRODUCTION

Plant transformation can be defined as "the introduction of exogenous genes into plant cells, tissues or organs employing direct or indirect means developed by molecular and cellular biology" (Jenes *et al*., 1993). The principle of transformation for the first time was revealed by F. Griffith in the late 1920s. He observed that pneumococcal cells could convert from a harmless form to a disease-causing type (Griffith, 1928; Avery *et al* 1944).

In spite of the fact that there are several methods for transforming plants such as Chemical procedures, Electroporation, Biolistics: particle bombardment, Vacuum infiltration, Ultrasound-mediated transformation, Shock wave-mediated transformation, Silicon carbide whisker-mediated transformation, Microinjection, Macroinjection, Laser microbeams and Electrophoresis **(**Rivera *et al*.,2012), *Agrobacterium* is by far the most common method for plant genetic engineering. For instance, this method has been attempted by several researchers using various plants such as barley, poplar, *Musa acuminata,* Micro-tom, quaking aspen and *Populus tremula* by Bartlett *et al*, (2008); Leple *et al*., (1992); May *et al*., (1995); Sun *et al*., (2006); Tsai *et al*., (1994); Tzfira *et al*., (1997) respectively.

Agrobacterium tumefaciens is a Gram-negative pathogenic soil bacterium, which has the ability to introduce part of its plasmid DNA (transfer DNA or T-DNA) into the

nuclear genome of infected plant cells. Consequently, it causes crown gall disease in plants by inducing tumours at wound sites of hundreds of different dicotyledonous plants and some monocots and gymnosperms (De Cleene and De Ley, 1976).

The first attempt to produce transgenic plants was about fifty years ago on maize but it was not successful (Coe and Sarkar, 1966). However, at the beginning of 1970s, the first production of recombinant DNA molecules was obtained using restriction enzymes (Meselson and Yuan, 1968; Smith and Welcox, 1970). In addition the invent of tissue culture, regeneration and selection systems had played an important role in this area. Subsequently, several transgenic plants were obtained in the 1980s including maize (Zimmermann and Vienken, 1982; Southgate *et al,* 1998; Armstrong, 1999), tobacco (Bevan *et al*, 1983), petunia (Fraley *et al*, 1983), tomato (Hirsch *et al*, 1985). After 1985, plant transformation was extended to a wide variety of crops including rice (Toriyama *et al*,1988 ; Zhang and Wu,1988 ; Zhang *et al*,1988; Shimamoto *et al*,1989), celery (Catlin *et al*, 1988) , *Brassica napus* (Moloney *et al*,1989) , grape (Perl *et al*, 1996), cassava (Zhang *et al*, 2003) and millets (Ceasar and Ignacimuthu, 2009; and Kothari *et al*,2005) .

Compared to conventional breeding new cultivars can be produced much more quickly using transgenic methods. The main goal for plant transformation are to produce cultivars with novel traits such as drought tolerance, improved nutritional qualities, resistance for pest and diseases and studies of plant metabolism (Rivera *et al*, 2012). However, manipulating reproductive traits like self-incompatibility and male sterility which are important in plant breeding are a desirable objective.

Producing male sterile plants can help plant breeders to avoid emasculation which is time consuming. In addition, male sterile F_1 hybrids can be exploited commercially as the plant cannot produce seeds to be used for next season. Controlling self-incompatibility can help increase the production without spending money and efforts to provide pollinators.

Petunia is an important model for plant transformation and gametophytic self-incompatibility in this plant is very well understood. Consequently, it can be used as a model to manipulate this phenomenon using transgenic methods. For instance, Lee *et al* (1994) introduced antisense S_3-RNase gene into plants of S_2S_3 genotype to inhibit the S_3-RNase; as a result, the transgenic plants lost the ability to reject S_3 pollen. In addition Kao and McCubbin (1996) manipulated the HV region of S_3-RNase and the transgenic plant had lost the ability for rejecting S_3 pollen.

In gametophytic self-incompatibility, one of the most interesting phenomena which can be exploited in breeding programs is competitive interaction. Competitive interaction is a genetic phenomenon, which causes the breakdown of self-incompatibility in pollen and produces self-compatible plants. This phenomenon was revealed in the mid-20th century. It occurred as a result of duplication of the S-locus and was observed in plants from several families such as, Rosaceae (Crane and Lewis 1942), Fabaceae (Brewbaker 1954), Onagraceae (Lewis 1947) and Solanaceae (Livermore and Johnstone 1940; Stout and Chandler 1942; Pandey 1968).

Based on competitive interaction phenomenon, in this project we attempted to control incompatibility in different genetic backgrounds in Petunia using transgenic methods and four SLF genes from *Antirrhinum hispanicum.*

Antirrhinum belongs to the Plantaginaceae family, most species of this genus also possess gametophytic self-incompatibility which is regulated by a single multiallelic S-locus (East, 1940). A novel F-box gene, *AhSLF-S2*, was identified by Lai *et al* (2002) and they confirmed that this gene has expressed specifically in pollen and the tapetum.

Four constructs (*AhSLF-S2*, *AhSLF-S1E*, *AhSLF-S2C* and *AhSLF-S4D*) were obtained from the group of Prof. Yongbiao Xue in the Chinese Academy of Sciences, Beijing. These constructs are all under the control of the pollen-specific promoter, Late Anther Tomato (LAT52).

Previously, *AhSLF-S2* was transformed into self-incompatible *Petunia hybrida* (PhS_3PhS_3) and the plants significantly became self-compatible as a result of the transgene (Qiao *et al*, 2004b). In this project Qiao *et al*, (2004b) work was extended and *Petunia hybrida* (PhS_3PhS_v) was transformed with all these constructs in order to control incompatibility in the different Petunia background.

4.2 RESULTS

4.2.1 Transformation of *E. coli* and *Agrobacterium* with AhSLF Constructs.

All four constructs were transferred to *E. coli* DH5α (DH5813) (see section 2.5.1), the correct transformed colonies were identified by colony PCR, and sequencing the plasmids using the same primers separately (See Appendix 4). Subsequently, the confirmed plasmids were transferred to *Agrobacterium* (LBA4404) (See section 2.6). The correct transformed colonies were again identified using the same primers for each constructs by colony PCR.

4.2.1.1 Transformation of E.coli and Agrobacterium with the AhSLF-S2 construct.

Gene specific primers AhSLF-S2-R-904 reverse with M13 reverse (as forward) were used to identify the correctly transformed colonies (in *E. coli* and *Agrobacterium*) for this construct (see Table 2.7). According to the T-DNA map the size between M13 reverse and AhSLF-S2-R-904 is 1611 bp (See appendix 1). As indicated in Figure 4.1 five colonies gave the expected product size in both *E. coli and Agrobacterium.*

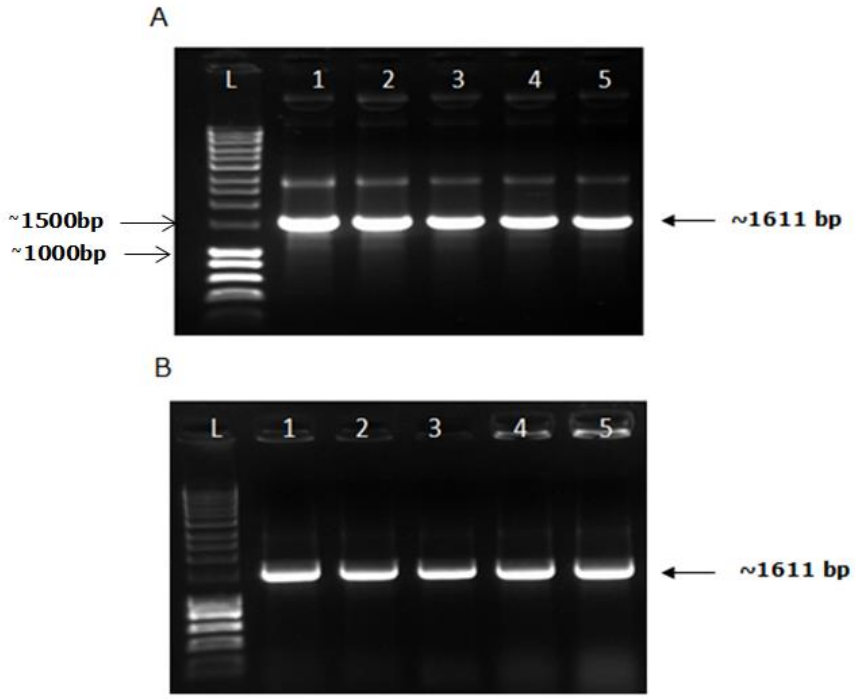


Figure 4.1 Colony PCR confirming the transformed colonies obtained with AhSLF-S2 binary vector. A: AhSLF-S2 transformed into *E. coli*. B: AhSLF-S2 transformed into *Agrobacterium*. L represents HyperLadder I (Bioline). Lanes 1-5 represent five selected colonies.

4.2.1.2 Transformation of E.coli and Agrobacterium with the AhSLF-S2C construct.

The correctly transformed colonies for this construct were identified by colony PCR using Gene specific primers AhSLF-S2C-R-1148 reverse with M13 reverse (as forward) (See Table 2.7). As the distance between M13 reverse and AhSLF-S2C-R-1148 is 1855 bp, this is the expected size of the PCR product (See Appendix 1). Several colonies gave PCR products close to the expected size as shown in Figure 4.2 which indicates that these colonies have been correctly transformed.

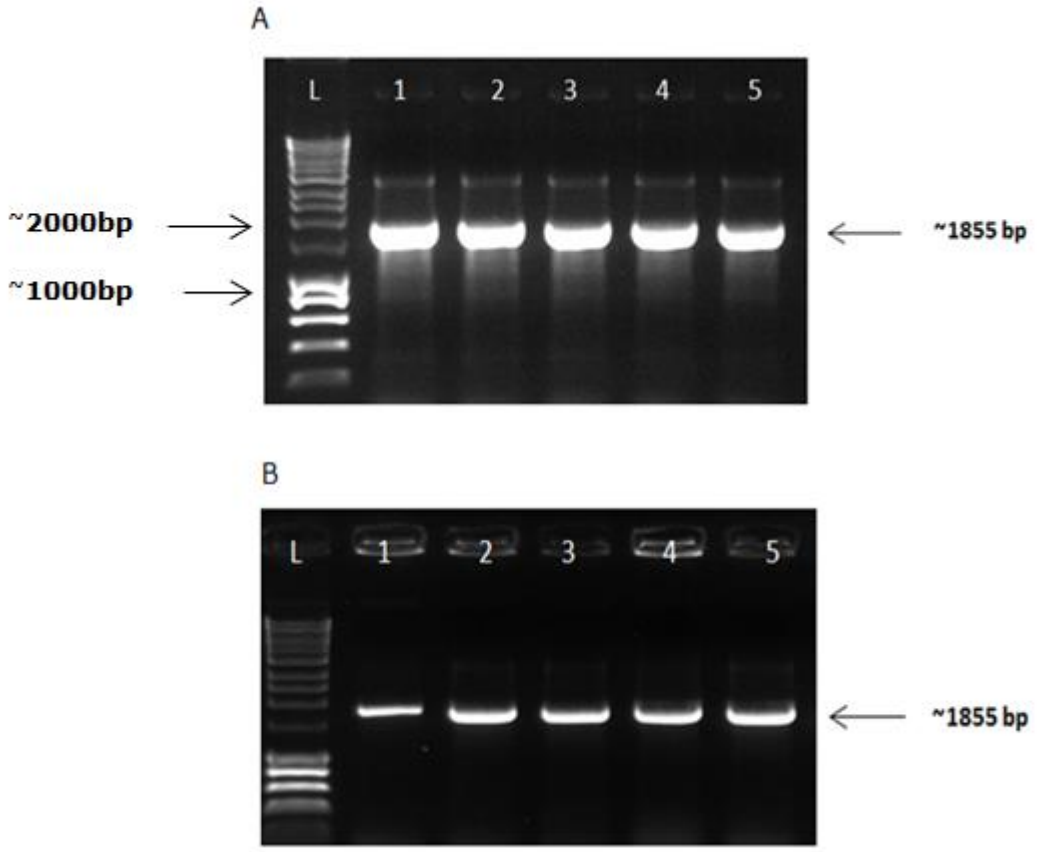


Figure 4.2 Colony PCR confirming the transformed colonies obtained with the AhSLF-S2C binary vector. A: AhSLF-S2C transformed into *E.coli*. B: AhSLF-S2C transformed into *Agrobacterium*. L represents HyperLadder I (Bioline), lanes 1-5 represent five selected colonies.

4.2.1.3 Transformation of E.coli and Agrobacterium with the AhSLF-S4D construct.

The confirmation of correctly transformed colonies obtained with the AhSLF-S4D binary vector is illustrated in Figure 4.3. Gene specific primers AhSLF-S4D-R-848 reverse with M13 reverse primer (as forward) were used to identify the correctly transformed colonies (See Table 2.7). According to the T-DNA map the distance between M13 reverse and AhSLF-S4D-R-848 is 1555 bp (See Appendix 1) and approximately the same size products appear in Figure 4.3. Regarding *E.coli* transformation, colonies 1, 2 and 3 are correctly transformed. For *Agrobacterium*

transformation the correctly transformed colonies are 1, 2, 3 and 4 as they gave the expected size (See Appendix 1).

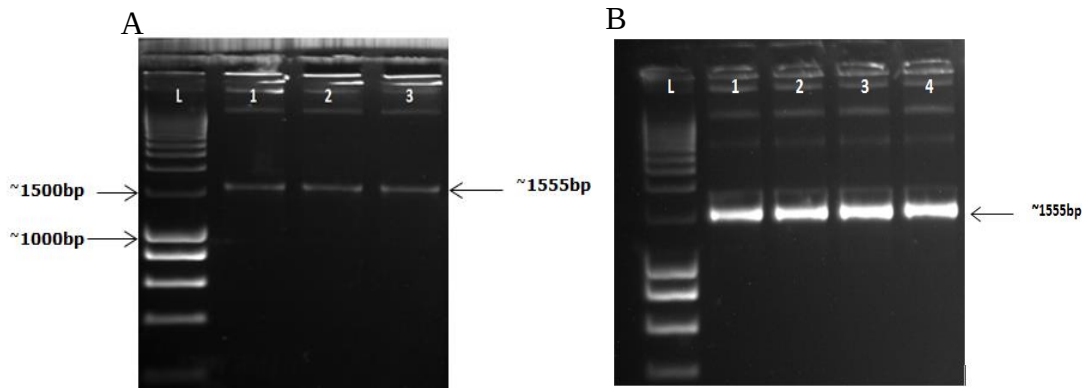


Figure 4.3 Colony PCR confirming the correctly transformed colonies obtained with the binary vector AhSLF-S4D. A and B illustrate transforming *AhSLF-S4D* to E. coli and Agrobacterium respectively. L represents HyperLadder I (Bioline), lanes 1-3, 4 represent selected colonies.

4.2.1.4 Transformation of E. coli and Agrobacterium with the AhSLF-S1E construct.

Gene specific primer AhSLF-S1E-R-1152 reverse with M13 reverse (as forward) were used to identify the colonies correctly transformed with this construct (see Table 2.7). According to the T-DNA map the size between M13 reverse and AhSLF-S1E-R-1152 is 1859 bp (See Appendix 1). This is close to the PCR product size obtained in Figure 4.4. Regarding *E.coli* transformation, only one colony out of five was correctly transformed (Figure 4.4A, Lane 5), consequently the other colonies were discarded. However, for *Agrobacterium* transformation four colonies were selected (Figure 4.4B, Lanes: 1, 2, 4 and 5) as they gave the expected size.

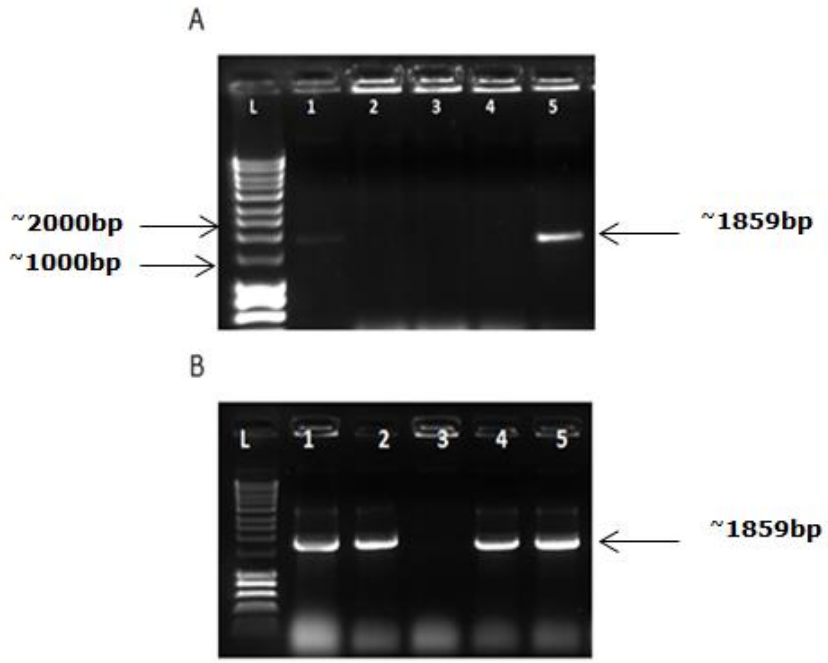


Figure 4.4 Colony PCR confirms the correctly transformed colonies obtained with binary vector AhSLF-S1E. A and B illustrate transforming AhSLF-S1E into *E.coli* and *Agrobacterium* respectively. L represents HyperLadder I (Bioline), lanes 1-5 represent screened colonies.

4.2.2 Agrobacterium transformation of *Petunia hybrida*

Petunia hybrida is considered to be a good model for the analysis of promoter activity and gene expression. Some cultivars of Petunia are easy to transform. In general, generation time is between 3-4 months and it is possible to grow up to 100 plants/m^2 (Gerats *et al*, 1990). A sensitive leaf disc petunia transformation procedure time was first developed by Horsch *et al* (1985). In this project Agrobacterium-mediated transformation of Petunia leaf discs protocol developed by van der Meer (1999) was used to transfer four constructs to *Petunia hybrida* holds (PhS_3PhS_v) (See Section 2.7). Initially more than ten independent transformation experiments were done and in each experiment about 300 explants were infected, but not all of them were successful.

All four constructs (AhSLF-S2, AhSLf-S2C, AhSLF-S4D and AhSF-S1E) were used to transform the *PhS*$_3$*PhS*$_v$ genotype of *Petunia hybrida.* The efficiency was different between experiments and constructs and the results are shown in Table 4.1. Callus and shoot appearing in control (nontransformed) and Kanamycin resistant explants are shown in Figure 4.5.

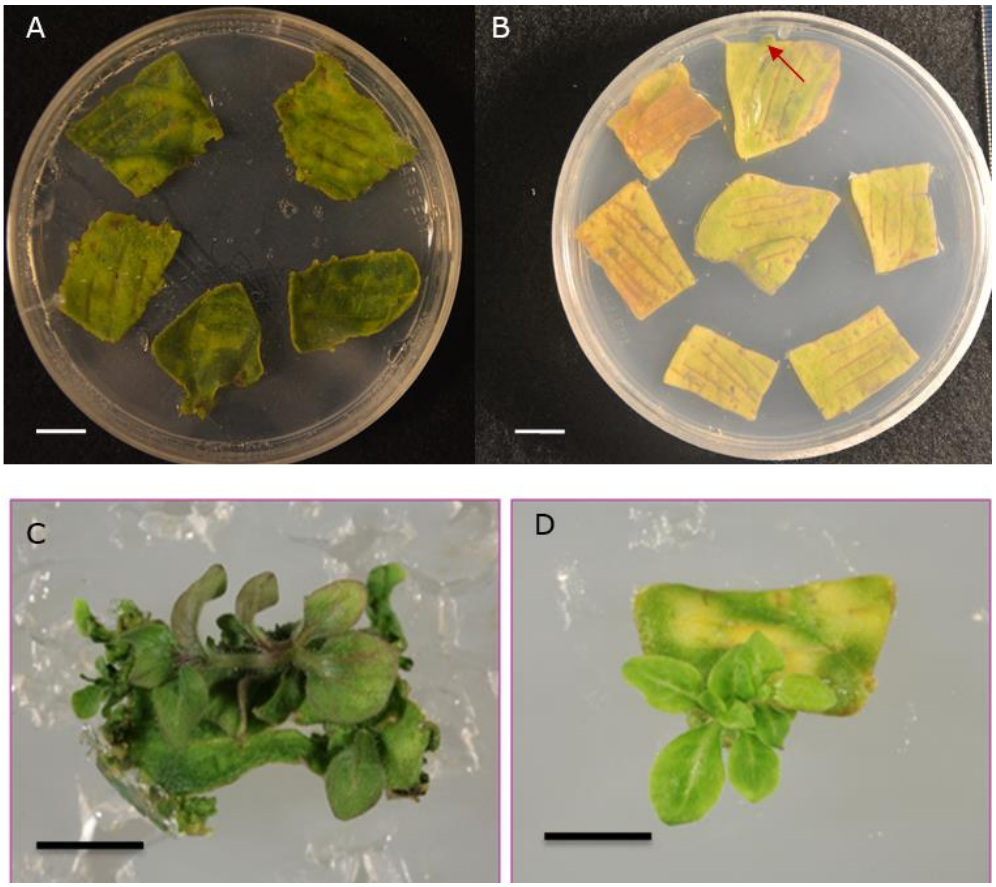
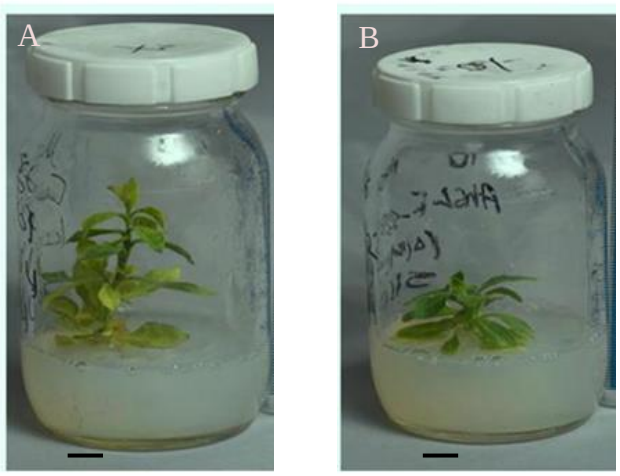


Figure 4.5 Examples of tissue culture results. (A) Control callus (no selection 7 day after transformation) (B) Kanamycin resistant callus (with selection 12 days after transformation) (C) Control shoot (no selection 4 weeks age) (D) Kanamycin resistant shoot (5 weeks age) are shown. Bar =1 cm

When the shoots reached about 1.5 cm they were transferred to the rooting media (See Appendix 2); the root was initiated after about 15 days. Figure 4.6 illustrates a control and a Kanamycin resistant shoot 3 months after transformation.



Control shoot **Kanamycin Resistant shoot**

Figure 4.6 Example of control and Kanamycin resistant plants. (A) Control shoot (no selection) and (B) Kanamycin resistant shoot (with selection) are shown. Bar= 1 cm

Table 4.1 Summary of transformation results for all constructs that have been used in this project

Constructs	The Approximate Number of Explants Used	Number of Explants Callusing	Number and rate of Explants Shooting	The Number of Plants growing in Glasshouse
AhSLF-S2	900	29	20 (69%)	6
AhSLF-S2C	900	5	1 (20%)	1
AhSLF-S1E	900	15	6 (40%)	3
AhSLF-S4D	300	20	3 (15%)	1

It can be seen that at this stage the rate of shoot development from callus was different between the four used constructs and AhSLF-S2C and AhSLF-S4D gave a very low efficiency. Later in this project more transformation experiments were done in order to obtain more transgenic plants and to compare the efficiency between different constructs. Unfortunately, we did not obtain more plants for (AhSLF-S2 and AhSLF-S1E). However, when the inoculation period was changed from 3 days to 6 days the results were changed significantly and 45 calli were obtained for both AhSLF-S4D and AhSLF-S2C. 25 shoots were obtained for the AhSLF-S4D

construct and 13 plants were transferred to the glasshouse. Regarding AhSLF-S2C, 30 shoots were obtained and 20 plants were transferred to the glasshouse. These results are summarized in Table 4.2.

Table 4.2 Summary of transformation results for *AhSLF-S4D* and *AhSL-S2C* constructs.

Constructs	The Approximate Number of Explants Used	Number of Explants Callusing	Number and Rate of Explants Shooting	The Number of Plants Growing in the Glasshouse
AhSLF-S4D	300	45	25 (55%)	13
AhSLF-S2C	300	45	30 (66%)	20

4.2.3 Identification of transgenic plants

Four months after transformation, healthy Kanamycin resistant plants were transferred to the glasshouse (See section 2.8). As it is possible for plants to escape from the antibiotic, using more reliable methods to identify transgenic plants is crucial. Consequently, all Kanamycin resistant plants were screened by Genomic PCR .

4.2.3.1 Identifying transgenic plants transformed with AhSLF-S2

Six Kanamycin resistant plants: AhSLF-S2.16.1, AhSLF-S2.20.1, AhSLF-S2.22.1, AhSLF-S2.24.1, AhSLF-S2.27.1 and AhSLF-S2.28.1 were screened by Genomic PCR using M13 forward (as reverse) and AhSLF-S2 forward specific primer (AhSLF-S2-F-672) (See Table 2.7). According to the T-DNA map for this construct, the expected size is 752 bp (See Appendix 1). As shown in Figure 4.7 a band of nearly the same size was obtained for all plants providing evidence that the T-DNA integrated successfully in all transgenic plants.

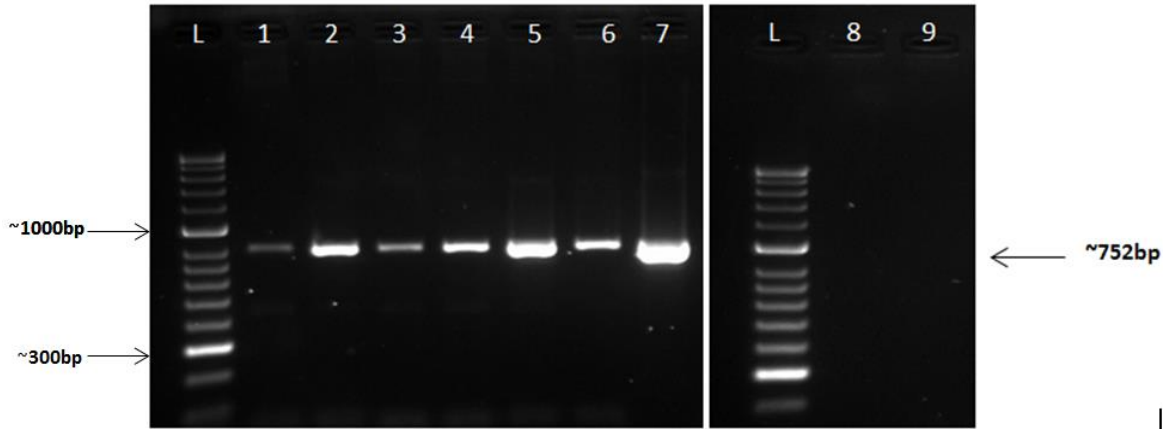


Figure 4.7 Identification of transgenic plants transformed with *AhSLF-S2*. L represent HyperLadder II (Bioline). Lanes 1-6 represent plants ID: AhSLF-S2.16.1, AhSLF-S2.20.1, AhSLF-S2.22.1, AhSLF-S2.24.1, AhSLF-S2.27.1 and AhSLF-S2.28.1. Lanes7-9 represent: Positive control (AhSLF-S2 plasmid DNA), Negative control (Non-transformed *Petunia hybrida* (*PhS*$_3$*PhS*$_v$)) and H_2O as a negative control for contamination respectively.

4.2.3.2 Identifying transgenic plants transformed with AhSLF-S2C

Genomic PCR was used to screen twenty Kanamycin resistant plants: AhSLF-S2C.2.1, AhSLF-S2C.3.1, AhSLF-S2C.4.1, AhSLF-S2C.7.1, AhSLF-S2C.9.1, AhSLF-S2C.10.1, AhSLF-S2C.11.1, AhSLF-S2C.12.1, AhSLF-S2C.14.1, AhSLF-S2C.17.1, AhSLF-S2C.18.1, AhSLF-S2C.19.1, AhSLF-S2C.20.1, AhSLF-S2C.22.1, AhSLF-S2C.23.1, AhSLF-S2C.24.1, AhSLF-S2C.25.1, AhSLF-S2C.26.1, AhSLF-S2C.29.1 and AhSLF-S2C.30.1 putatively transformed with construct AhSLF-S2C. The primers were M13 reverse (as forward) and reverse AhSLF-S2C specific primer (AhSLF-S2C-R- 828) (See Table 2.7). According to the T-DNA map for this construct, the expected size is about 1067 bp (See Appendix 1).

As the expected size was observed for several putative transgenic plants in Figure 4.8, it indicates that these plants are indeed transgenic. Although all plants were

apparently Kanamycin resistant, three out of twenty plants are not transgenic as illustrated in Figure 4.8 (AhSLF-S2C.7.1, AhSLF-S2C.11.1 and AhSLF-S2C.12.1).

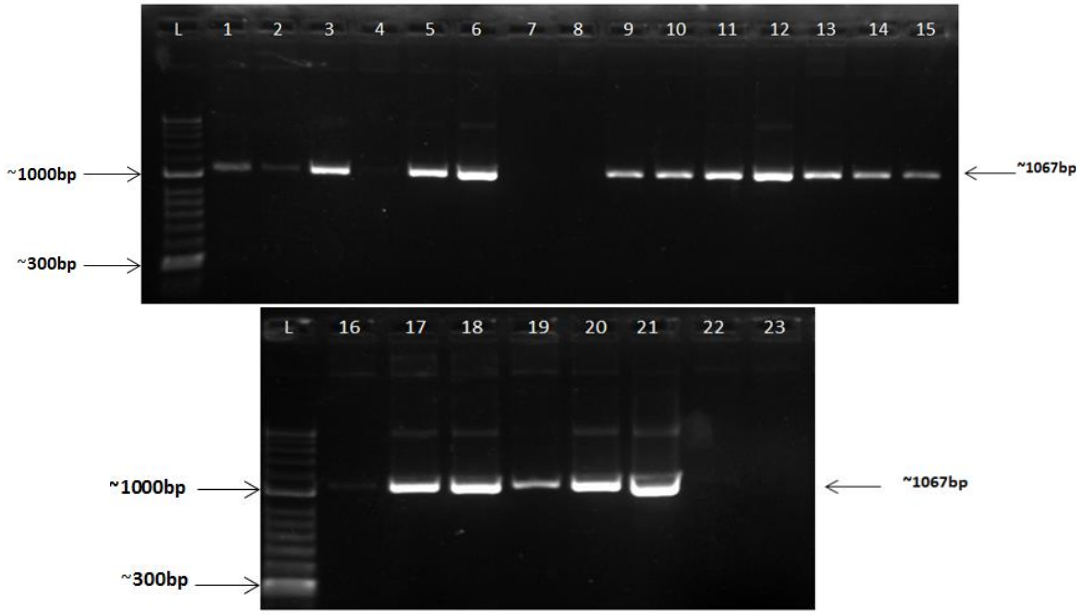


Figure 4.8 Identification of transgenic plants transformed with *AhSLF-S2C*. L represents HyperLadder II (Bioline). Lanes 1-20 represent plants ID: AhSLF-S2C.2.1, AhSLF-S2C.3.1,AhSLF-S2C.4.1,AhSLF-S2C.7.1,AhSLF-S2C.9.1, AhSLF-S2C.10.1, AhSLF-S2C.11, AhSLF-S2C.12.1, AhSLF-S2C.14.1, AhSLF-S2C.17.1, AhSLF-S2C.18.1, AhSLF-S2C.19.1, AhSLF-S2C.20.1, AhSLF-S2C.22.1, AhSLF-S2C.23.1, AhSLF-S2C.24.1, AhSLF-S2C.25.1, AhSLF-S2C.26.1, AhSLF-S2C.29.1, AhSLF-S2C.30.1. Lanes 21 -23 represent: Positive control (AhSLF-S2C plasmid DNA), Negative control (Non-transformed *Petunia hybrida* PhS_3PhS_v) and H2O as a negative control for contamination respectively.

4.2.3.3 Identifying transgenic plants transformed with AhSLF-S4D

M13 reverse (as forward) and AhSLF-S4D reverse specific primers (AhSLF-S4D-R-848) (See Table 2.7) with Genomic PCR were used to screen thirteen Kanamycin resistance plants: AhSLF-S4D.1.1, AhSLF-S4D.2.1, AhSLF-S4D.3.1, AhSLF-S4D.4.1, AhSLF-S4D.5.1, AhSLF-S4D.6.1, AhSLF-S4D.7.1, AhSLF-S4D.8.1, AhSLF-S4D.10.1, AhSLF-S4D.11.1, AhSLF-S4D.12.1, AhSLF-S4D.13.1 and AhSLF-S4D.15.1. The expected size, about 1555bp according to T-DNA map (See Appendix 1) was obtained, providing good evidence to identify transgenic plants.

According to Figure 4.9 all plants are transgenic except AhSLF-S4D.4.1 and AhSLF-S4D.10.1.

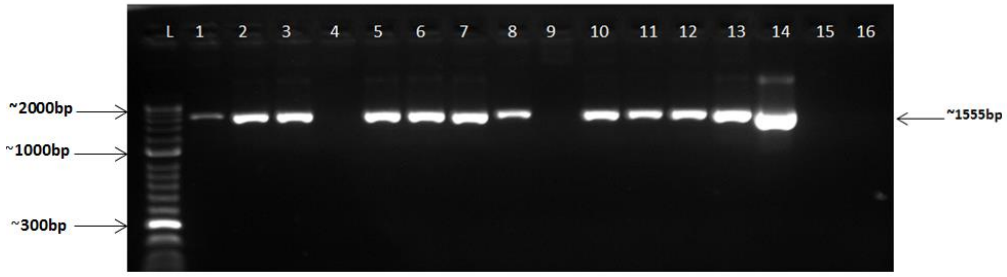


Figure 4.9 Identification of transgenic plants transformed with *AhSLF-S4D*. L represents HyperLadder II (Bioline). Lanes 1-13 represent plants ID: AhSLF-S4D.1.1, AhSLF-S4D.2.1, AhSLF-S4D.3.1, AhSLF-S4D.4.1, AhSLF-S4D.5.1, AhSLF-S4D.6.1, AhSLF-S4D.7.1, AhSLF-S4D.8.1, AhSLF-S4D.10.1, AhSLF-S4D.11.1, AhSLF-S4D.12.1, AhSLF-S4D.13.1, AhSLF-S4D.15.1. Lanes 14, 15 and 16 represent: Positive control (AhSLF-S4D plasmid DNA), Negative control (Nontransformed *Petunia hybrida* (*PhS*$_3$*PhS*$_v$) and H_2O as a negative control for contamination respectively.

4.2.3.4 Identifying transgenic plants transformed with AhSLF-S1E

Three Kanamycin resistant plants AhSLF-S1E.15.1, AhSLF-S1E.16.1 and AhSLF-S1E.31.1 transformed with AhSLF-S1E were identified using M13 reverse (as forward) and AhSLF-S1E specific (AhSLF-S1E-R-726) primers (See Table 2.7) by Genomic PCR. According to the T-DNA map (See appendix 1) for this construct the expected size is 1142 bp. As nearly the same size was obtained for all plants, it means all of them are transgenic as shown in Figure 4.10.

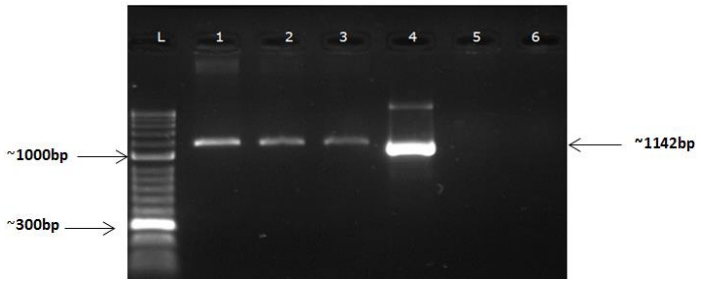


Figure 4.10 Identification of transgenic plants transformed with *AhSLF-S1E*. L represents HyperLadder II (Bioline) Lanes 1-3 represent plants ID: AhSLF-S1E.15.1, AhSLF-S1E.16.1 and AhSLF-S1E.31.1. Lanes 4 - 6 represent: Positive control (AhSLF-S1E plasmid DNA), Negative control (Non-transformed *Petunia hybrida* (*PhS*$_3$*PhS*$_v$)) and H2O as a negative control for contamination respectively.

4.2.4 AhSLF expression analysis using RT-PCR.

Reverse transcription (RT)-PCR has been used to identify the transgene expression which is driven by LAT52 promoter in pollen (See section 2.10), RNA was harvested from anthers in four different flower bud stages, stage 1 (10-20mm), stage 2 (20-30mm) , stage 3 (30-40mm) and stage four (more than 40mm but the bud was still unopened (see section 2.10.1) according to methods taken from Czyzyk (2010).

Gene expression was tested (see section 2.10.4) in three plants transformed with *AhSLF-S2* and *AhSLF-S1E*. Regarding *AhSLF-S2C* and *AhSLF-S4D*, gene expression was tested in three plants in four different bud stages and for the remainder of the plants in the stage four only. Initially, all cDNA samples were screened using the housekeeping gene, elongation factor 1 alpha *(*EF1a) to check the quality of cDNA Czyzyk (2010). Figure 4.11 shows EF1a expression in four bud stages and leaf of AhSLF-S2.24.1 in addition to stage four of control (Non-transgenic) plant which indicates similar expression in all tissue. In addition, no expression in –RT confirms that there is no contamination with genomic DNA. However, the rest of the Figures regarding other samples were omitted to avoid repetition.

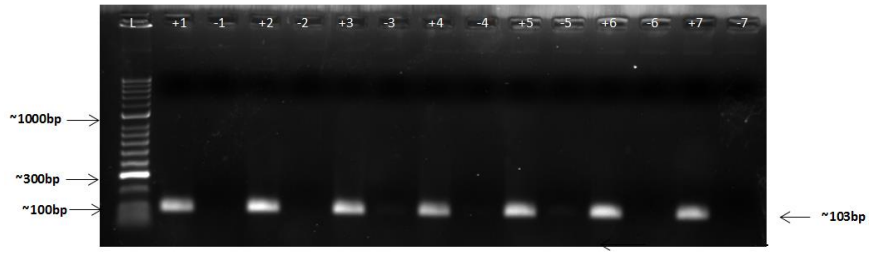


Figure 4.11 Ef1a expression in the pollen and leaf of transgenic plants AhSLF-S2.24.1 from four different bud stages and control plant (nontransgenic). L represent Hyperladder II (Bioline). 1-4 indicates four bud stages and 5 represent leaf for AhSLF-S2.24.1. 6 and 7 represent pollen and leaf for Control plant (nontransformed). + and - indicates +RT-PCR and –RT-PCR respectively.

Moreover, gene specific primers for each transgene were then used for checking the gene expression using standard RT-PCR 30 cycles.

4.2.4.1 Testing gene expression in transgenic plants transformed with AhSLF-S2

Gene expression was tested in three plants transformed with this gene (AhSLF-S2.16.1, AhSLF-S2.20.1 and AhSLF-S2.24.1) (see section 4.2.3.1). Gene specific primers (AhSLF-S2-F-72 and AhSLF-S2-R-904) with RT-PCR were used. According to the T-DNA map for this gene the expected size is 833bp. As illustrated in Figure 4.12 (A, B, C and D), nearly the same size was obtained in all three transgenic plants, which is evidence that the transgene expressed correctly in all four stages. There is no expression obtained in the leaf which is expected as the AhSLF-S2 is driven by LAT52 which is a pollen specific promoter. Moreover, other transgenic plants transformed with this transgene (AhSLF-S2.22.1, AhSLF-S2.27.1 and AhSLF-S2.28.1 have been tested for expression in the same four stages and they all gave the positive band but the PCR data are omitted to avoid repetitions.

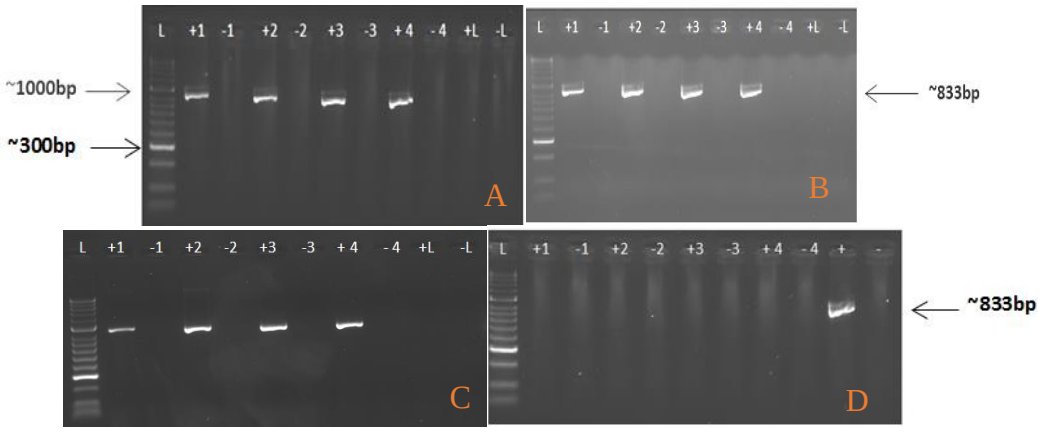


Figure 4.12 Gene expression in the pollen of transgenic plants transformed with *AhSLF-S2* from four different bud stages. A,B,C and D represent plants ID: AhSLF-S2.16.1, AhSLF-S2.20.1, AhSLF-S2.24.1 and control plants (nontransformed) respectively. L represent HyperLadder II (Bioline). 1-4 indicates four bud stages, L indicates expression in the leaf, - and + illustrates -RT-PCR and + RT-PCR respectively. + represent positive control (Plasmid DNA) and – represent H_2O as a negative control for contamination.

4.2.4.2 Testing gene expression in transgenic plants transformed with AhSLF-S2C

Gene specific primers for this gene (AhSLF-S2C-F-247 and AhSLF-S2C-R-828) were used to test the gene expression in 11 plants (AhSLF-S2C.2.1, AhSLF-S2C.10.1, AhSLF-S2C.20.1, AhSLF-S2C.17.1, AhSLF-S2C.18.1, AhSLF-S2C.19.1, AhSLF-S2C.22.1, AhSLF-S2C.23.1, AhSLF-S2C.25.1, AhSLF-S2C.28.1, AhSLF-S2C.29 transformed with this transgene only in the last stage of flower bud (stage four). According to the T-DNA map for this gene the expected size (114bp) was obtained (see Figure 4.13) and the transgene is expressed in all transgenic plants except AhSLF-S2C.20.1.

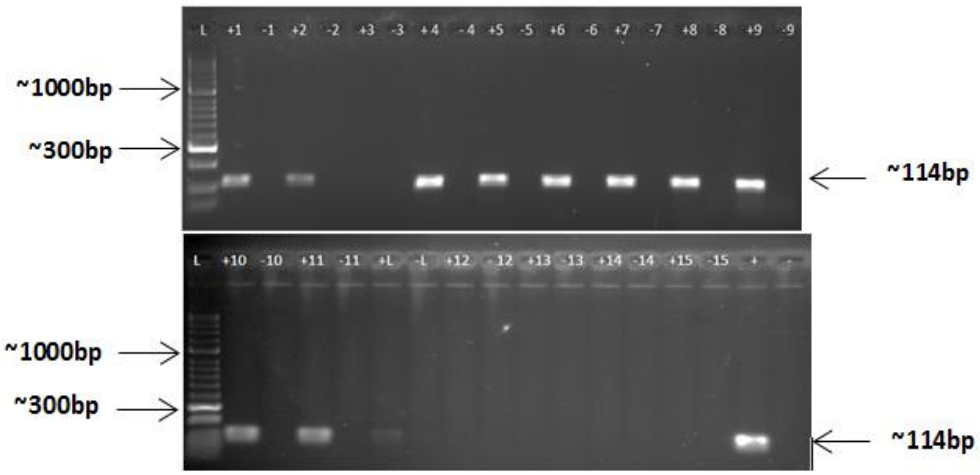


Figure 4.13 Gene expression in the pollen of transgenic plants transformed with *AhSLF-S2C* from last flower bud stages. L represent Hyppr Ladder II (Bioline). Lanes 1-11 indicates plants ID: AhSLF-S2C.2.1, AhSLF-S2C.10.1, AhSLF-S2C.20.1, AhSLF-S2C.17.1, AhSLF-S2C.18.1, AhSLF-S2C.19.1, AhSLF-S2C.22.1, AhSLF-S2C.23.1, AhSLF-S2C.25.1, AhSLF-S2C.28.1 and AhSLF-S2C.29. L indicates expression in the leaf. 12-15 represent: unopened flower buds in four different bud stages in control plant (nontransformed plant). + and - indicates +RT-PCR and –RT-PCR respectively. + indicates positive control (plasmid DNA) and - represent H_2O as a negative control for contamination.

In addition, gene expression was analysed in three selected transgenic plants (AhSLF-S2C.2.1, AhSLF-S2C.17.1 and AhSLF-S2C.23.1) in all bud stages (first, second, third and four). It can be observed that in Figure 4.14 this transgene is expressed correctly in the pollen of all three transgenic plants in all stages. There is no expression in leaf as this gene is driven by pollen specific promoter LAT52.

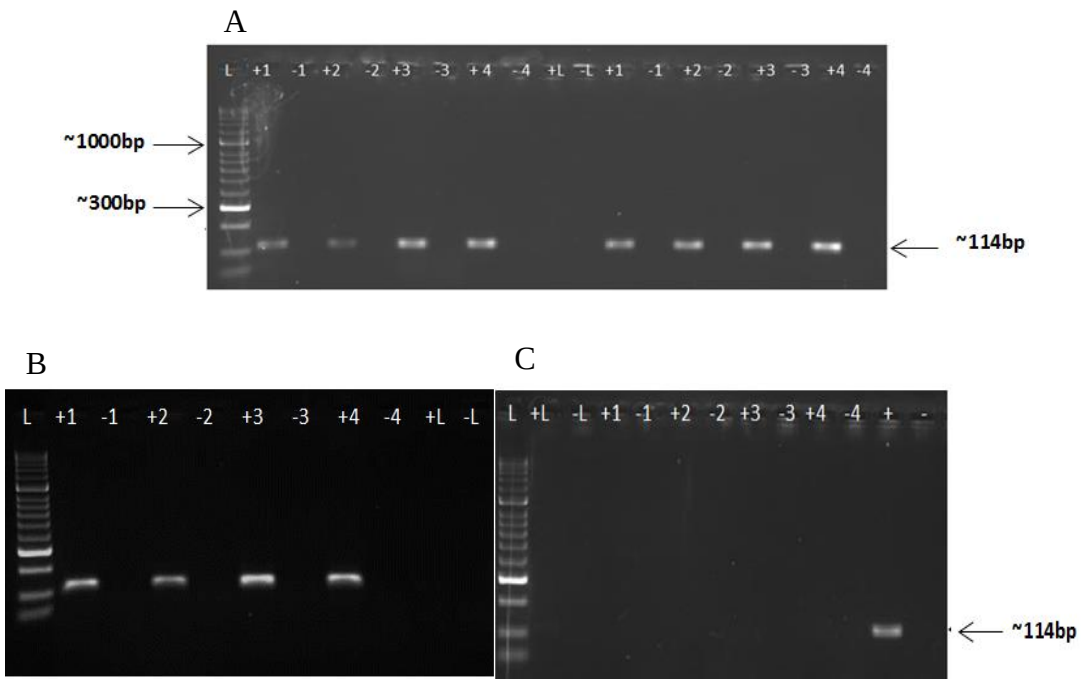


Figure 4.14 Gene expression in the pollen of transgenic plants transformed with *AhSLF-S2C* from four different bud stages. L represent Hyper ladder II (Bioline). A represent plants ID: AhSLF-S2C.2.1 and AhSLF-S2C.23.1, B indicates AhSLF-S2C.17.1 and C represent control plants (nontransformed). Lanes 1-4 indicates four flower bud stages. L indicates the transgene expression in the leaf. + and - illustrates +RT-PCR and RT-PCR respectively. + represent positive control (Plasmid DNA) and – represent H_2O as a negative control for contamination.

4.2.4.3 Testing gene expression in transgenic plants transformed with AhSLF-S4D

Gene expression was tested in seven plants (AhSLF-S4D.1.1, AhSLF-S4D.3.1, AhSLF-S4D.8.1, AhSLF-S4D.12.1, AhSLF-S4D.13.1, AhSLF-S4D.14.1, AhSLF-S4D.28.1*)* transformed with this transgene only in the last bud stage (stage four) using gene specific primers (AhSLF-S4D-R-848 and AhSLF-S4D-F-1122). According to the T-DNA map for this gene the expected size is 845 bp and a product of approximately this size was obtained in Figure 4.15 and the transgene was expressed correctly in all transgenic plants except AhSLF-S4D.28.1.

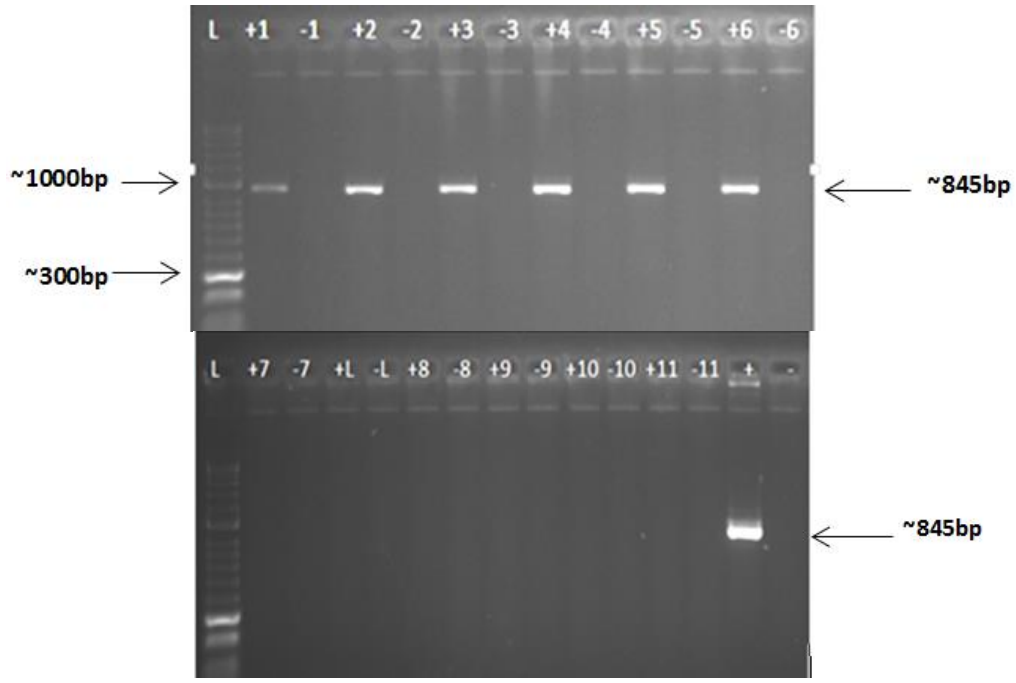


Figure 4.15 Gene expression in the pollen of transgenic plants transformed with *AhSLF-S4D* from later flower bud stage (stage four). L represent HyperLadder II (Bioline). + and - indicate +RT-PCR and –RT-PCR respectively. Lane 1-7 represent plants ID: AhSLF-S4D.1.1, AhSLF-S4D.3.1, AhSLF-S4D.8.1, AhSLF-S4D.12.1, AhSLF-S4D.13.1, AhSLF-S4D.14.1 and AhSLF-S4D.28.1 respectively. L indicates the transgene expression in the leaf. Lanes 8-11 indicates buds in four different stages in control plant (nontransformed plant). + indicates positive control (plasmid DNA) and - represent H_2O as a negative control for contamination.

Moreover, gene expression was tested in all four bud stages for three selected trangenic plants (AhSLF-S4D.1.1, AhSLF-S4D.3.1 and AhSLF-S4D.13.1) using the same primers and the same expected size 845 bp was obtained as illustrated in Figure 4.16. There is no expression in the leaf tissue because this gene is driven by the LAT52 promoter.

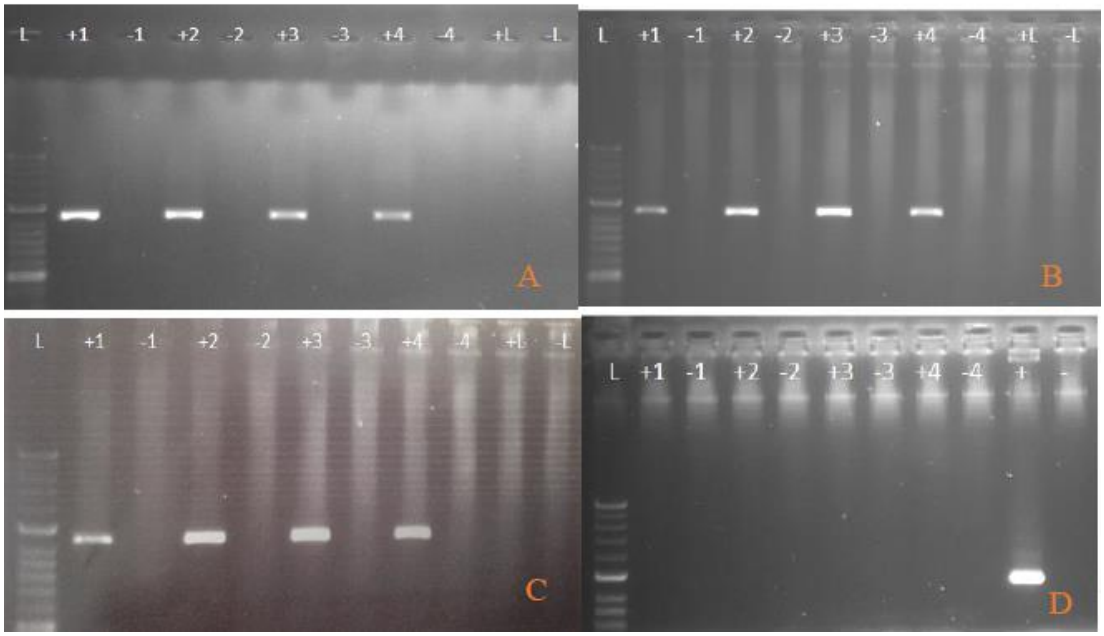


Figure 4.16 Gene expression in the pollen of transgenic plants transformed with *AhSLF-S4D* from four different bud stages. L represent HyperLadder II (Bioline). A, B and C represent plants ID: AhSLF-S4D.1.1, AhSLF-S4D.3.1 and AhSLF-S4D.13.1 respectivly. D represents control plants (nontransformed). Lanes 1-4 indicate four bud stages, L indicates expression in the leaf. + and - illustrates + RT-PCR and -RT-PCR and respectively. + represents positive control (Plasmid DNA) and – represents H_2O as a negative control for contamination.

4.2.4.4 Testing gene expression in transgenic plants transformed with AhSLF-S1E

Figure 4.17 shows the gene expression in all three plants obtained for this transgene (AhSLF-S1E.15.1, AhSLF-S1E.16.1 and AhSLF-S1E.31.1) in four bud stages using gene specific primers for this transgene (AhSLF-S1E-F-246 and AhSLF-S1E.R-726). According to the T-DNA map for this gene the expected size is 189 bp and a product of approximately this size was obtained in all three transgenic plants which indicates that the transgene was expressed correctly in all stages and no expression was obtained in leaf because this gene is driven by the pollen specific promoter LAT52.

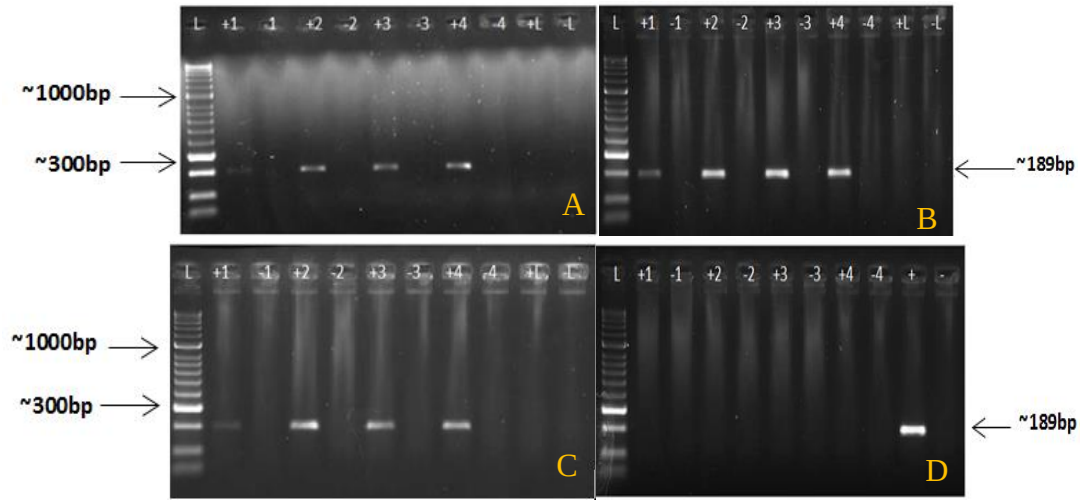


Figure 4.17 Gene expression in the pollen of transgenic plants transformed with *AhSLF-S1E* from four different bud stages. L represent HyperLadder II (Bioline). A, B and C represent plants ID: AhSLF-S1E.15.1, AhSLF-S1E.16.1 and AhSLF-S1E.31.1. D represent control plants (nontransformed). Lanes 1-4 indicate four bud stages and L indicates expression in the leaf . + and - illustrate + RT-PCR and -RT-PCR respectively. + indicates positive control (Plasmid DNA) and – represents H_2O as a negative control for contamination.

From all expression results (Figure 4.12, 4.14 and 4.16) it can be concluded that there are not signficant differences appear in the level of expression in the four different stages of flower bud development. However, regarding Figure 4.17 which illustrates AhSLF-S1E expression in three transgenic plants (AhSLF-S1E.15.1, AhSLF-S1E.16.1 and AhSLF-S1E.31.1), the level of expression in the first stages were weaker than the older stages for all plants. In all transgenic plants, the transgenes are not expressed in the leaf because they are all driven by LAT 52 which is a pollen specific promoter.

4.2.5 Using semi-quantitative RT-PCR to test the transgene expression level in different bud stages.

In order to obtain more sensitive results regarding transgene expression in the four different bud stages, semi-quantitative RT-PCR was performed. Cycle numbers of 28, 25, 20, and 18 were tested. It was noticed that there was no significant difference between standard RT-PCR 30 cycles and 28, 25 cycles. However, all stages gave

very weak bands using 20 cycles and bands at all stages were not seenusing 18 cycles. Consequently, we have analysed the level of expression in four bud stages using qPCR and this will be discussed in the next section (4.2.6).

4.2.6 Testing the transgene expression level in different bud stages using Quantitative RT-PCR (qPCR).

In order to identify the different level of the transgenes expression in different flower bud stages, one transgenic plant for each transgene (AhSLF-S2, AhSLF-S2C, AhSLF-S1E and AhSLF-S4D) was selected and the transgene expression level was tested in four different stages (see section 2.10.1). This method was taken from Czyzyk (2010). qRT-PCR analyses were performed using the Light Cycler 480 real-time PCR system. Expression was normalized using the EF1a gene and each sample was repeated three times to obtain the accurate results. Light Cycler 480 software was used to analyse the data (See section 2.11).

Specific primers were designed for each transgene to give small size PCR products and their efficiency was optimized by temperature manipulation several times (section 2.11). According to Figure 4.18 A, B, C and D in all transgenic plants the level of the transgene expression is increased significantly from stage one to stage four and there is no expression in leaf as all transgenes are driven by the pollen specific promoter LAT 52.

In addition the lower starting point of expression is observed in the first stage for both (A: AhSLF-S2.20.1 and B: AhSLF-S2C.23.1) but the level of expression is increased above fold in AhSLF-S1E.16.1 and AhSLF-S4D.3.1.

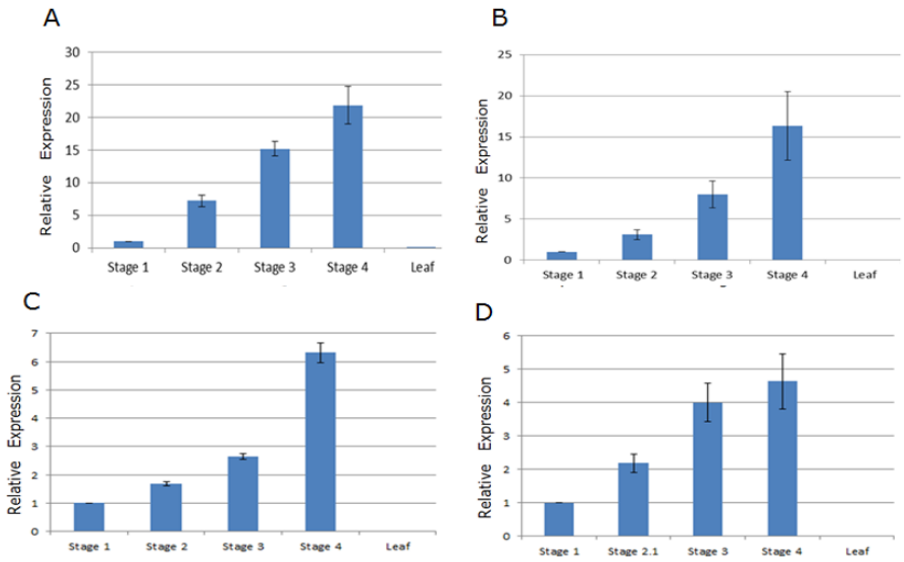


Figure 4.18 Transgene expression analysis in four unopened flower bud stages using qPCR. A: AhSLF-S2.20.1, B: AhSLF-S2C.23.1, C: AhSLF-S1E.16.1 and D: AhSLF-S1E.3.1. Relative quantity chart obtained from LightCycler480 software analysis of qRT-PCR. Stage 1 in all plants used as calibrator and it has assign value of 1.0. House keeping gene *EF1a* was used as normalisation assay. Bars indicate the error calculated based on average and standard deviation.

4.3 DISCUSSION

Different number of transgenic plants were obtained from different constructs. According to Van der Meer (1999) about 20 transgenic lines can be obtained from 100 explants. However, our results are significantly different which may be due to the different cultivar selected for transformation. In the previous study a low frequency was reported for a S_vS_v stock compared to a standard cultivar V26 (Harbord *et al*, 2000).

In spite of the fact that the inoculation period recommended by van der Meer (1999) is three days, based on our results increasing this period to six days increased efficiency significantly.

This improvement in the transformation efficiency can be interpreted as: First, increasing inoculation period from three to six days gave more chance for Agrobacterium to infect the explants in the absence of both antibiotic (Kanamycin and Carbenicillin). Second, the explants had more time to get benefit from both plant hormones 6-Benzylaminopurine (BAP), and 1-Naphthaleneacetic acid (NAA). Third, the absence of antibiotic (Kanamycin) during the inoculation period reduced plants stress. These factors all together can help the appearance of more calli.

Although all plants were apparently Kanamycin resistant, some of them did not give positive results using genomic PCR. In order to make this clear all results are summarized in Table 4.3.

Table 4.3 summarizing the results for apparently and confirmed transgenic plants

Constructs	Apparently Kanamycin Resistant Plants	Confirmed Transgenic Plants
AhSLF-S2	6	6
AhSLF-S2C	20	17
AhSLF-S1E	3	3
AhSLF-S4D	13	11

According to Table 4.3 not all apparently Kanamycin resistant plants are transgenic which means they escaped from Kanamycin or it is possible only the Kanamycin resistant part (NPTII) of the construct was transferred to the plants and not the *AhSLF* genes.

Regarding the transgene expression, all transgenic plants obtained for *AhSLF-S2* and *AhSLF-S1E* gave the positive result as show in Figures 4.12 and 4.17 respectively. However, for the other constructs: AhSLF-S2C.20.1 and AhSF-S4D.28.1 did not have expression as is shown in Figure 4.13 and 4.15 respectively. It is possible that all transgenic insertions behave independently from each other. After transformation, it is possible that the foreign DNA is inserted at random locations in the host chromosome(s) and, as a result, independent transgenes can show variability in expression despite carrying the same cassette of foreign gene sequences depending on position effect (Prols and Meyer, 1992). The level of transgene expression can also be different between the transgenic plants because of copy number in addition to any position effect. Moreover, gene silencing can arise due to position effect or complex T-DNA insertion, if the transgene position is in the heterochromatic region it will be suppressed. These complications can affect the transgenic plant phenotype. Consequently, it is possible for homozygous plant to give different phenotype compare to hemizygous plants contained the same transgene. This hypothesis can be tested by analysing T_0 transgenic plants using self-pollination. In addition, the transgene behaviour can be analysed in F_1 hybrids and some of in T_1 and T_2 generation which will be discussed in the next chapter.

CHAPTER 5 : TESTING FOR SELF-COMPATIBILITY DUE TO COMPETITIVE INTERACTION IN TRANSGENIC PLANTS

5.1 INTRODUCTION

The main aim of this project was to control self-incompatibility in *Petunia* using four heterologous constructs (AhSLF-S2, AhSLF-S2C, AhSLF-S4D and AhSLF-S1E). Several transgenic plants were obtained for each construct as described in Chapter Four. The idea is to introduce a heteroallelic SLF into the pollen which should cause the plant phenotype to be converted from self-incompatible to self-compatible based on the phenomenon of 'competitive interaction'.

Competitive interaction is a well-established genetic phenomenon which leads to the breakdown of gametophytic self-incompatibility in pollen and produces self-compatible plants. It usually occurs as a result of duplication of the entire S-locus. The pollen production for both homoallelic and heteroallelic genotypes from a tetraploid individual is expected to be 50:50. For example if a plant has the sporophytic genotype $S_1S_1S_2S_2$ the expected genotype for male gametes will be S_1S_1, S_2S_2 and S_1S_2 in a ratio of 1:1:2 (Stone, 2002).

In self-pollination between tetraploid plants and homoallelic pollen grains, pollen grain growth is arrested in styles exactly as it is observed in normal plants (Stone, 2002). On the other hand, heteroallelic pollen (pollen grains with two different S-alleles) is not arrested and results in successful fertilization (See Figure 5.1A).

Pollination of a diploid style with the same heteroallelic pollen leads to the same result (production of seeds). This confirmed that self-compatibility behaviour that was observed and did not result from tetraploidy in the styles. This result confirmed that the duplication of the S-locus only influenced the pollen component of SI and not the style component (Stone, 2002).

Stout and Chandler (1942) analysed progeny arrays from three types of crosses and found the effect of duplication on the function of the S-locus in *Petunia.* Chawla *et al*. (1997) used modern molecular techniques in *Lycopersicon peruvianum* (*Solanum peruvianum*) illustrating that S-protein expression in the style was unaffected by tetraploidy, and pollen tubes holding two different S-alleles were able to penetrate styles of any genotype. Moreover, artificially generated polyploidy showed that bearing two different S-alleles in the pollen confers self-compatibility (Entani *et al*, 1999). In this study the chromosome numbers of SI diploid *Petunia hybrida* of both heterozygous and homozygous *S-haplotypes* were doubled and tetraploid plants bearing only one S-allele (For instance, $S_1S_1S_1S_1$) remained self-incompatible, whereas the heteroallelic tetraploids were self-compatible.

Golz *et al* (2001) studied *Nicotiana alata* plants with an additional S allele generated by irradiation of pollen and showed that pollen-part mutations in several different genetic backgrounds were caused by duplications of S-alleles. They observed the breakdown of self-incompatibility as a result of the presence of two non-matching *S*-alleles in the same pollen grain. Consequently, inheritance of the duplication was consistent with a competitive interaction model.

Sijacic *et al* (2004) used a transgenic approach to induce competitive interaction. They introduced the S_2-allele of *Petunia inflata PiSLF* (*PiSLF2*) into plants of genoptye S_1S_1, S_1S_2 and S_2S_3. The presence of *PiSLF2* in S_1 and S_3 pollen grain and not S_2 pollen grains caused the breakdown of SI. These results were predicted by competitive interaction and confirmed via progeny analysis. For instance, self-pollination of a $PiSLF_2/S_2S_3$ transgenic plant produced S_2S_3 and S_3S_3 progeny plants and not S_2S_2 progeny plants. All progeny plants carried the PiSLF2 transgene. This phenomenon is explained schematically in Figure 5.1.C

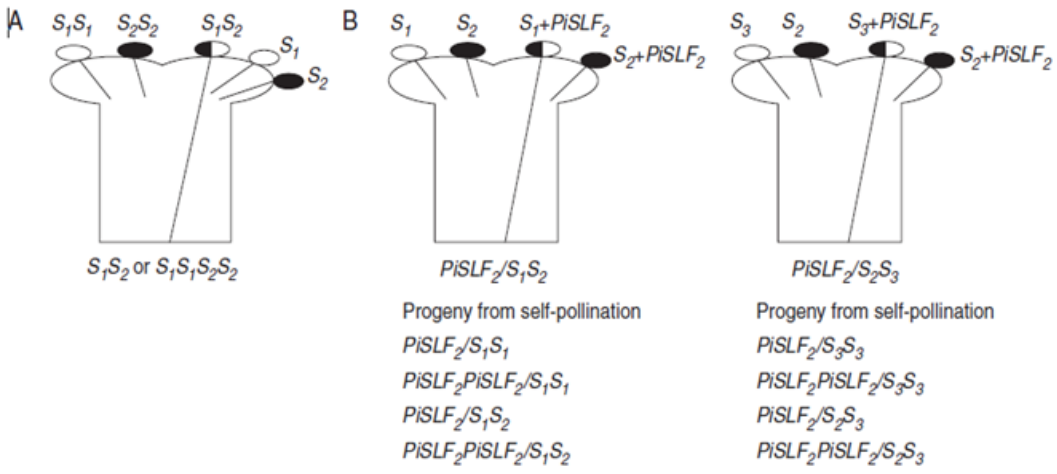


Figure 5.1 Competitive interaction and its use in establishing the function of PiSLF. (A) Self-pollination of S_1S_2 diploid results in SI, but in an $S_1S_1S_2S_2$ tetraploid only the homoallelic pollen (S_1S_1 and S_2S_2) are rejected. According to the phenomenon of competitive interaction the heteroallelic S_1S_2 pollen is accepted resulting in SC. (B) Both left and right pictures illustrate the self-pollination for transgenic plants $PiSLF_2/S_1S_2$ and $PiSLF_2/S_2S_3$ respectively. It can be seen that in both plants only heteroallelic genotypes are accepted: S_1+*PiSLF*$_2$ *and* S_3+*PiSLF*$_2$ .This is consistent with the prediction made by competitive interaction that pollen carrying two different *S*-alleles results in a breakdown of incompatibility (Modified from Meng *et al*, 2011).

In spite of the fact that there are many models for the interaction between male and female determinants in GSI, not all of them are able to explain the molecular mechanism behind competitive interaction. One of the models for interpreting this

phenomenon was identified by Hua *et al*, (2008) who described a "modified protein-degradation model".

The biochemical mechanism of S-RNase-based SI with the interaction occurring in the cytoplasm of the pollen tube is illustrated in Figure 5.2.

In the case of an incompatible pollination, as illustrated in Figure 5.2A, in an S_1 pollen tube, the weak interaction between the SBRD (S-RNase- Binding-Regulating Domain) of PiSLF1 and the S-allele-specific domain of S_1-RNase results in an unfavourable interaction between the SBD (S-RNase-binding domain) of PiSLF1 and S_1-RNase. Consequently, the PiSLF1-S_1-RNase complex would not be stable and produces the free form of S_1-RNase molecules which leads to degradation of pollen RNA and results in the growth inhibition of the S_1 pollen tube.

Regarding a compatible pollination, in an S_1 pollen tube, PiSLF1 can interact strongly with a non-self S-RNase, S_2-RNase, as illustrated in Figure 5.2 B. The SBD of PiSLF1 and a domain common to all S-RNases interacts strongly which is not be affected by the SBRD of PiSLF1 and the S-allele-specificity domain of S_2-RNase. Consequently, S1 pollen tube growth occurs as a result of this strong interaction which leads to the formation of stable PiSLF1-S_2-RNase complex and allows the assembly of the PiSLF-containing E3-like complex, which would ubiquitinate S_2-RNase and target it for degradation.

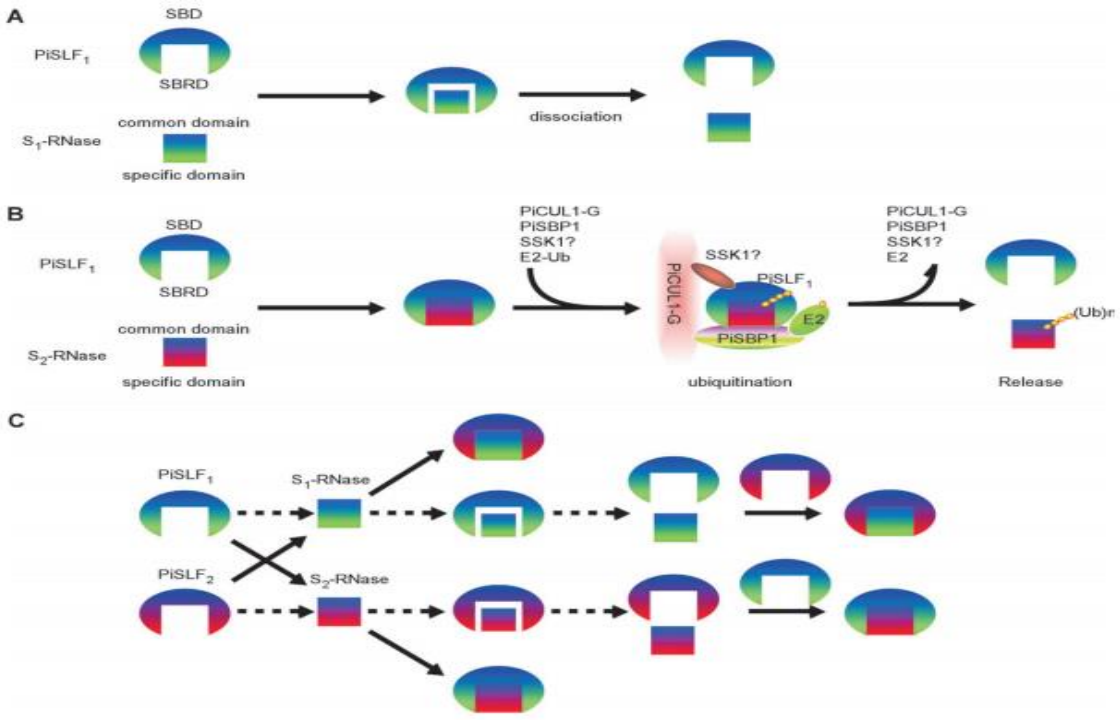


Figure 5.2 A Biochemical Model for S-RNase-based SI. (A) The predicted fate of S_1-RNase molecules in the cytoplasm of an incompatible S_1 pollen tube. According to this model in the interaction between SBRD (S-RNase-Binding-Regulating Domain) of PiSLF1 and S-allele specific domain is unfavourable, the interaction between SBD (S-RNase-binding domain) of PiSLF1 is weak too. This leads to an unstable complex between PiSLF-S_1-RNase resulting in a free form of the S_1-RNase. Consequently, the pollen is inhibited as a result of degradation of its RNA. (B) The predicted fate of S_2-RNase molecules in the cytoplasm of a compatible S_1 pollen tube. The strong interaction between PiSLF1 and S_2-RNase is not affected by the interaction SBRD of PiSLF1 with S-allele specifity domain of S2-RNase. (C) The predicted fates of S_1-RNase and S_2-RNase molecules in the cytoplasm of a heteroallelic *S_1S_2* pollen tube. In the cytoplasm two stable complexes can form as a result of preferential interaction of PiSLF1 and PiSLF2 with S_2-RNase and S_1-RNase respectively (indicated with solid arrows). As illustrated in figure (B) each complex would subsequently assemble into a putative PiSLF-containing E3-like complex (not shown) to mediate ubiquitination and degradation of both S_1-RNase and S_2-RNase. An unstable complex can be produced as a result of the interaction between PiSLF1 and S_1-RNase and between PiSLF2 and S_2-RNase (indicated with broken arrows). Regarding the dissociated S_1-RNase and S_2-RNase, these can preferentially interact with their respective non-self PiSLF, and would suffer the same fate of degradation (Hua *et al*, 2008).

With regard to competitive interaction, the pollination of an *S_1S_2* pistil by *S_1S_2* heteroallelic pollen tubes carrying both PiSLF1 and PiSLF2 is illustrated in Figure 5.2 C and their respective self-S-RNases, S_1-RN*ase* and S_2-RNase, are present in the cytoplasm of the same pollen tube. Two stable complexes PiSLF1-S_2-RNase and

PiSLF2-S_1-RNase can form after the interaction between PiSLF1 and PiSLF2 and their respective non-self S-RNases S_1-RNase and S_2-RNase through their SBDs. The same situation can occur for all disassociated S_1-RNase and S_2-RNase. Thus, S_1-RNase and S_2-RNase would be ubiquitinated and degraded as explained for the compatible pollination (Figure 5.2B). On the other hand, the interaction might occur between SLF1 or PiSLF2 and respective self S-RNases, but the stable complex would not be produced as was mentioned in incompatible pollination (Figure 5.2 A).

The phenomenon of competitive interaction has been used in several transgenic experiments to study SLF function in *Petunia* (Sijacic *et al*., 2004; Kubo *et al*., 2010; Qiao *et al*, 2004b). Qiao *et al*, (2004b) transformed an *Antirrhinum* transformation competent artificial chromosome (TAC) clone containing both AhS_2-RNase and AhSLF-S_2 into a self-incompatible line of *Petunia hybrida*. In another experiment, they transformed the *AhSLF-S_2* cDNA driven by a pollen-specific promoter (LAT52) into the same line. In both circumstances, *Petunia* plants significantly became self-compatible as transgenes were expressed correctly in the reproductive tissues (pollen). This was proposed to result from competitive interaction between the AhSLF transgene and the endogenous PhSLF genes of the transformation host.

Although this phenomenon was observed in the Rosaceae family by Crane and Lewis (1942) and this family has an S-RNase system, several researchers observed different responses regarding the breakdown of SI and competitive interaction in the plants belonging to this family. For instance Hauck *et al*., (2006) demonstrated that heteroallelic pollen in sour cherry pollen self-incompatible. However, Huang *et al*.,

(2008) illustrated that the competitive interaction between two of the functional alleles confer self-compatibility on Chinese cherry cultivar "NC". Moreover, some reports show that two different S-haplotypes do not show competitive interaction (Bosˇkovic´ *et al*. 2006).

However, Mase and Sawamura (2014) confirmed that SC of *Pyrus pyrifolia* was not caused by a mutation of a pollen S factor in either S- haplotype but it appears as a result of a duplication of the S-haplotype which made it possible to produce S-heteroallelic pollen that results in competitive interaction.

In this PhD project the work of Qiao *et al*, (2004b) has been extended to include three more Antirrhinum SLF genes: *AhSLF-S2C*, *AhSLF-S4D* and *AhSLF-S1E* as described by Zhou *et al* (2003) to be transformed into a *Petunia hybrida* background. In this chapter the phenotype of primary transformants is described and progeny studied for inheritance of the transgenes and any correlation with self-compatibility.

5.2 RESULTS

5.2.1 Testing self-incompatibility in T_0 transgenic plants

From the data of Qiao *et al*, (2004b) it was expected that the phenotype of the transgenic plants would be changed from incompatible to compatible as a result of the competitive interaction phenomenon. To test this ten flowers from each transgenic plant were self-pollinated (see Appendix 5, Table 1). However, the phenotypes were not changed and all of them remained self-incompatible.

As tissue specific and normal developmental expression of the transgene is observed, it was not clear why they did not give the expected phenotype (Self-compatible). In order to answer this question more analysis was done, which will be focused on in the next sections.

5.2.2 Identification of F_1 hybrids plants inheriting transgenes (*AhSLF-S2*, *AhSLF-S2C*, *AhSLF-S4D* and *AhSLF-S1E*).

To analyse the interaction between the transgenes and a wide range of alleles (PhS_v, PhS_3, PiS_3, PiS_{K1}) in F_1 hybrids, transgenic plants (PhS_3PhS_V) were selected and used as a female (♀) and crossed with *Petunia inflata* (PiS_3PiS_{K1}) family number (N445.3) as a male (♂). Harvested seeds were plated on half MS media (See Appendix 2) containing 50 μg /ml Kanamycin (Figure 5.3.C). In these experiments two types of controls were used, firstly plating without Kanamycin (Figure 5.3. A, B) to test both control and the transgenic seed for germination respectively, and secondly plating with Kanamycin to test the effectiveness of the antibiotic and the sensitivity of non-transformed seeds (Figure 5.3.D). Kanamycin sensitive and resistant seedlings were scored after four weeks.

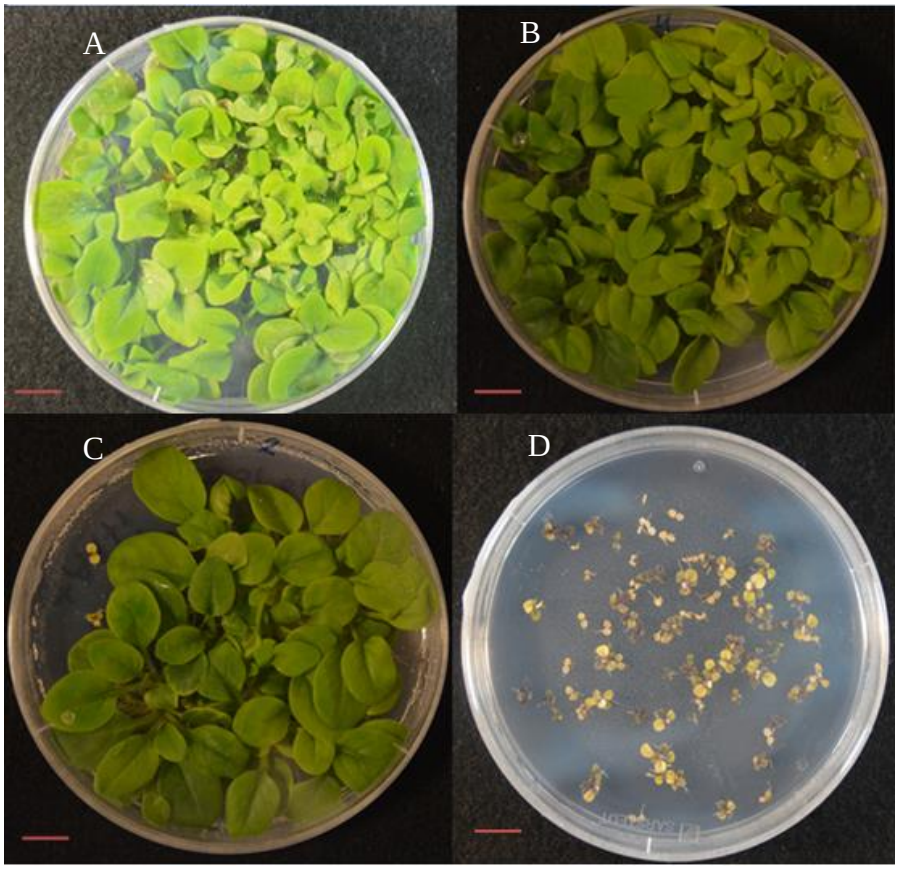


Figure 5.3 Germination of Petunia (F_1 hybrids) seeds on control and selective media. A: Control seedlings (nontransformed) without Kanamycin. B: Control transformed seedlings (without Kanamycin). C: Transformed seedlings using 50 µg/ml Kanamycin. D: Control seedlings (sensitive) (nontransformed) using 50 µg /ml Kanamycin. Bar= 1 cm

After scoring seedlings, the resistant seedlings were transferred to sterilized plastic pots containing Co-Cultivation Medium (See Appendix 2) without antibiotic to give them an opportunity to become established before they were transferred to the glasshouse (see section 2.8). Transgenic plants were confirmed using Genomic PCR. In addition, in order to test the interaction between the transgene (SLF) and all endogenous (SLF), twenty four F_1 hybrids transgenic plants were genotyped using *S-RNase* specific primers (*PhS_v, PhS_3, PiS_3, PiS_{K1}*). However, to avoid repetition the PCR data are not shown and plant genotypes are summarized in sections 5.2.2.2, 5.2.2.4, 5.2.2.6 and 5.2.2.8.

5.2.2.1 Identification of F_1 hybrids inheriting AhSLF-S2 transgene

In order to identify F_1 hybrids which inherited this transgene, two families were raised. The N468 family was raised from crossing between AhSLF-S2.28.1 (PhS_3PhS_v) (Female to harvest more seeds as the flower is bigger than *Petunia inflata*) with N445.3 (PiS_3PiS_{k1}) (Male). Similarly, the N474 family was raised from crossing between AhSLF-S2.22.14 (PhS_3PhS_v) with N445.3 (PiS_3PiS_{k1}). F_1 hybrid transgenic seedlings were selected using 50 µg/ml Kanamycin (see section 2.12). Kanamycin resistant and sensitive seedlings were scored after four weeks and the results are illustrated in Table 5.1.

Table 5.1 Kanamycin resistance assay for F_1 hybrid progeny inheriting *AhSLF-S2*.

Plant Line	Replicate Number	Kan ^{R}	Kan ^{S}
Control Nontransformed seed	1	0	100%
	2	0	100%
	3	0	100%
N468	1	54(72%)	21(28%)
	2	50(71%)	20(29%)
	3	14(45%)	17(55%)
	Total	104	41
	X^2 Value for 1:1 Ratio	27.372	
	X^2 Value for 3:1 Ratio	0.830	
N474	1	43(57%)	32(43%)
	2	24(51%)	23(49%)
	3	25(56%)	20(44%)
	Total	92	75
	X^2 Value for 1:1 Ratio	1.731	
	X^2 Value for 3:1 Ratio	80.641	

Kan ^{R} = Kanamycin Resistant Kan ^{S} = Kanamycin Sensitive

X^2 of 3.84 is significant at the 0.05 probability level and degree of freedom 1 only in the ratio 3:1 and 1:1 for N468 and N474 respectively. In addition the X^2 value for 1:1 segregation is 27.372 in N468 family is above 3.48 which is sufficient for rejecting Null hypothesis.

According to Table 5.1 in N468 family the total number of Kanamycin resistant and sensitive seedlings ratio are considered only in the first and second replicates as the seeds scored in the third replicate gave an odd segregation ratio as a limited number of seed was used. The X^2 value obtained for 1:1 ratio is 27.372 which is greater than X^2 of 3.84 at the 0.05 probability level. Consequently, there is a significant difference between the observed and expected ratio. The observed ratio for Kanamycin resistant seedling and Kanamycin sensitive seedlings are closer to 3:1 ratio and X^2 value for 3:1 ratio, 0.830, confirms that as it is less than X^2 of 3.84 at the 0.05 probability level. Regarding N474, the X^2 value obtained for a 1:1 ratio is 1.731 which is smaller than X^2 of 3.84 at the 0.05 probability level, it indicates that there is no significant differences between the observed and expected ratio of seedlings. In addition the X^2 value for 3:1 ratio of 80.641 is significantly larger than X^2 of 3.84 at the 0.05 probability level. These results indicate that AhSLF-S2.28.1 is a two locus transformant but AhSLF-S2.22.14 is a single locus transformant.

In order to confirm that the Kanamycin resistant seedlings are transgenic, twenty six plants (N468.1- N468.14, N474.1- N474.12) were screened by Genomic PCR using M13 Forward (as a reverse) and AhSLF-S2 forward specific primers (AhSLF-S2-F-672). The expected size of 752 bp was obtained (Figure 5.4 and 5.5) indicating that all F_1 progeny plants inherited the transgene successfully.

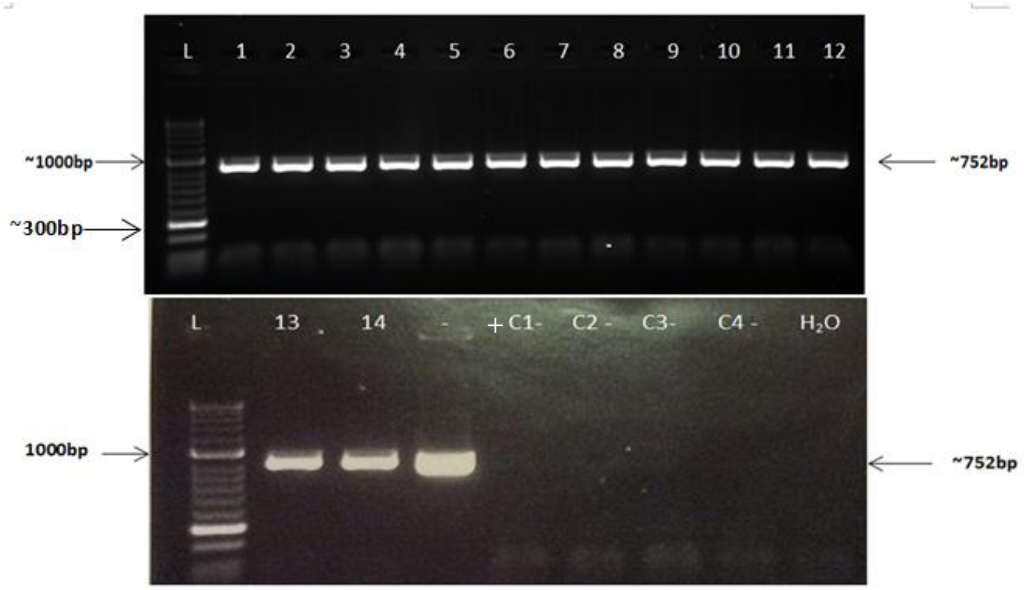


Figure 5.4 Identification of transgenic plants in N468 family inheriting *AhSLF-S2*. L represent HyperLadder II (Bioline) and lanes 1-14 represent plants ID: N468.1, N468.2, N468.3, N468.4, N468.5, N468.6, N468.7, N468.8, N468.9, N468.10, N468.11, N468.12, N468.13 and N468.14. + represents plasmid DNA (AhSLF-S2). C1- to C4- represent four controls (nontransformed F_1 hybrid plants), N450.8 (PhS_vPiS_{k1}), N450.3 (PhS_vPiS_3), N452.5 (PhS_3PiS_{K1}) and N452.1 (PhS_3PiS_3) respectively, and H_2O indicates negative control for contamination.

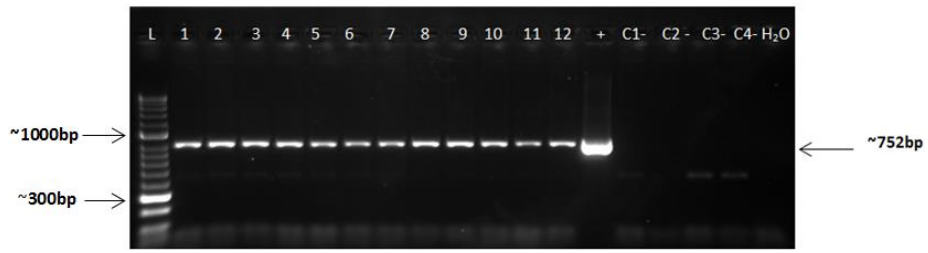


Figure 5.5 Identification of transgenic plants in N474 family inheriting AhSLF-S2. L represents HyperLadder II (Bioline) and lanes 1-12 represent plants ID: N474.1, N474.2, N474.3, N474.4, N474.5, N474.6, N474.7, N474.8, N474.9, N474.10, N474.11 and N474.12. + represents plasmid DNA. C1- to C4- represent four controls (nontransformed F_1 hybrid plants), N450.8 (PhS_vPiS_{k1}), N450.3 (PhS_vPiS_3), N452.5 (PhS_3PiS_{K1}) and N452.1 (PhS_3PiS_3) respectively. H_2O indicates negative control for contamination.

5.2.2.2 Identification of S alleles (PiS_3, PiS_{k1}, PhS_3, PhS_v) in F_1 hybrids inheriting AhSLF-S2.

S-RNase specific primers were used to identify PiS_3, PiS_{k1}, PhS_3 and PhS_v alleles in 23 plants for both N468 and N474 families which inherited the AhSLF-S2 transgene. All possible genotypes were found to test the interaction between the transgene and S

alleles. In order to avoid repetition the PCR data are not shown and all plant genotypes are summarised in Table 5.2.

Table 5.2 Summary of F_1 hybrid genotype inheriting *AhSLF-S2*

Plant ID	Genotype	Plant ID	Genotype
N468.1	PhS_3PiS_{K1}	N468.14	PhS_vPiS_{k1}
N468.2	PhS_3PiS_{K1}	N474.1	PhS_vPiS_{k1}
N468.3	PhS_3PiS_{K1}	N474.2	PhS_vPiS_3
N468.4	PhS_vPiS_{k1}	N474.3	PhS_3PiS_3
N468.5	PhS_vPiS_{k1}	N474.4	PhS_vPiS_3
N468.6	PhS_3PiS_{K1}	N474.5	PhS_vPiS_3
N468.7	PhS_vPiS_{k1}	N474.6	PhS_vPiS_{k1}
N468.8	PhS_3PiS_{K1}	N474.7	PhS_vPiS_{k1}
N468.9	PhS_3PiS_{K1}	N474.8	PhS_3PiS_{K1}
N468.10	PhS_vPiS_{k1}	N474.10	PhS_3PiS_3
N468.11	PhS_3PiS_{K1}	X^2 value for 1:1:1:1 Ratio	5.348
N468.12	PhS_vPiS_{k1}		
N468.13	PhS_3PiS_3		

X^2 of 7.815 is significant at the 0.05 probability level under three degrees of freedom

As both N468 and N474 families came from crossing transgenic *Petunia hybrida* PhS_3PhS_v and *Petunia inflata* PiS_3PiS_{K1}, in theory the segregation ratio for each allele should be 1:1:1:1 However, based on the Table 5.2 the results are different. Regarding N468 family, the ratio for segregation of PhS_3 and *PhSv* alleles is 8:6 which is close to the expected ratio but most plants inherited PiS_{K1} and only one plant N468.13 holds the PiS_3 allele. Meanwhile, the segregation for PhS_V allele is twice compared to PhS_3 allele in the N474 family, but nearly expected ratio was obtained for PiS_3 and PiS_{K1} which is 5:4. The expected ratio is 1:1:1:1 for all possible genotype but based on our results in Table 5.2 it is 8:9:3:3 for PhS_3PiS_{k1}, PhS_vPiS_{k1}, PhS_3PiS_3 and PhS_vPiS_3 respectively. The X^2 value for 1:1:1:1 ratio is 5.348 which is smaller than the X^2 of 7.815 for the 0.05 probability level which means there is no significant differences between the observed and expected plant genotypes.

5.2.2.3 Identification of F_1 hybrids inheriting AhSLF-S2C transgene

In order to test the ability of this transgene to be inherited in F_1 hybrid progeny, N481 family were raised from seeds obtained from crossing AhSLF-S2C.2.1 (PhS_3PhS_v) (♀) with N445.3 (PiS_3PiS_{k1}) (♂). Transgenic seedlings were selected using 50 µg /ml Kanamycin (see section 2.12). After four weeks, Kanamycin resistant and sensitive seedlings were scored as shown in Table 5.3.

Table 5.3 Kanamycin resistant assay for F_1 hybrid progeny inheriting *AhSLF-S2C* .

Plant Line	Replicate Number	Kan ^{R}	Kan ^{S}
Control (Nontransformed)	1	0	100
	2	0	100
	3	0	100
N481	1	31 (46%)	36(54%)
	2	22(42%)	30(58%)
	3	25(42%)	35(58%)
	Total	78	101
	X^2 Value for 1:1 Ratio	2.955	
	X^2 Value for 3:1 Ratio	94.274	

Kan ^{R} = Kanamycin Resistant Kan ^{S} = Kanamycin Sensitive

X^2 of 3.84 is significant at the 0.05 probability level and one degree of freedom only for segregation ratio 1:1.

According to Table 5.3 the X^2 value obtained for a 1:1 ratio is 2.955 which is less than X^2 value of 3.84 at the 0.05 probability level and indicates that there is no significant difference between the expected and observed Kanamycin resistant and sensitive seedlings. In addition, the X^2 value for 3:1 segregation is 94.274 which is significantly greater than X^2 of 3.84 at the 0.05 probability level which confirms the segreagation ratio of Kanamycin resistant and senstive seedling is closer to 1:1

instead of 3:1. Consequently, the transgenic plant AhSLF-S2C.2.1 (*PhS₃PhSᵥ*) contained only one Kanamycin resistance locus.

Genomic PCR using M13 reverse (as forward) and AhSLF-S2C (AhSLF-S2C-R-828) reverse primers were used to further confirm that the Kanamycin resistant seedlings are transgenic. Twenty three plants were screened (N481.2- N481.24). The expected product size is 1067 bp and approximately this size was obtained, as shown in Figure 5.6

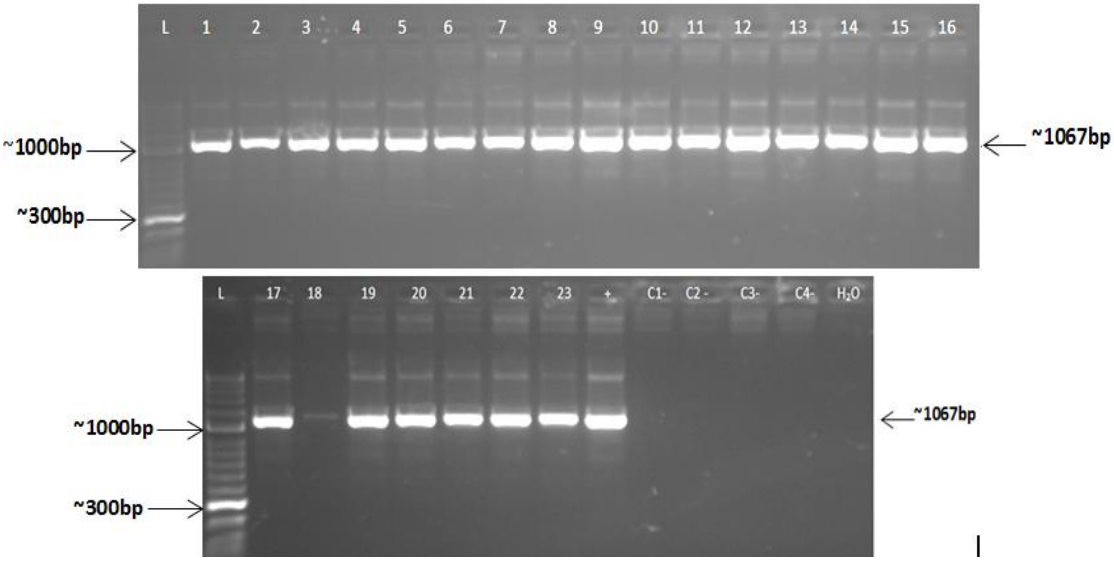


Figure 5.6 Identification of transgenic plants in N481 family inheriting AhSLF-S2C. L represents HyperLadder II (Bioline). Lanes 1-23 represent plants ID: N481.2, N481.3, N481.4, N481.5, N481.6, N481.7, N481.8, N481.9, N481.10, N481.11, N481.12, N481.13, N481.14, N481.15, N481.16, N481.17, N481.18, N481.19, N481.20, N481.21, N481.22 ,N481.23 and N481.24. + represent plasmid DNA (AhSLF-S2C). C1- to C4- represent four controls (nontransformed F_1 hybrid plants), N450.8 (*PhSᵥPiSₖ₁*), N450.3 (*PhSᵥPiS₃*), N452.5 (*PhS₃PiSₖ₁*) and N452.1 (*PhS₃PiS₃*) respectively. H_2O indicates negative control for contamination.

5.2.2.4 Identification of S-alleles (PiS_3, PiS_{k1}, PhS_3, PhS_v) in F_1 hybrids inheriting AhSLF-S2C.

S-RNase specific primers were used for genotyping 23 plants inheriting AhSLF-S2C transgene. All possible genotypes were found to test the interaction between *AhSLF-S2C* with S-alleles. The results are summarized in Table 5.4

Table 5.4 Summary of F_1 hybrid genotypes inheriting *AhSLF-S2C*

Plant ID	Genotype	Plant ID	Genotype
N481.2	PhS_vPiS_3	N481.14	PhS_3PiS_{k1}
N481.3	PhS_3PiS_{k1}	N481.15	PhS_vPiS_{k1}
N481.4	PhS_vPiS_{k1}	N481.16	PhS_vPiS_{k1}
N481.5	$Ph_{Sv}Pi_{Sk1}$	N481.17	PhS_3PiS_{k1}
N481.6	PhS_3PiS_{k1}	N481.18	PhS_vPiS_3
N481.7	PhS_vPiS_3	N481.19	PhS_3PiS_{k1}
N481.8	PhS_vPiS_3	N481.20	PhS_vPiS_{k1}
N481.9	PhS_vPiS_3	N481.21	PhS_vPiS_{k1}
N481.10	PhS_vPiS_{k1}	N481.22	PhS_vPiS_3
N481.11	PhS_3PiS_3	N481.23	PhS_3PiS_{k1}
N481.12	PhS_vPiS_3	N481.24	PhS_vPiS_3
N481.13	PhS_vPiS_{k1}	X^2 Value for 1:1:1:1 Ratio	5.696

X^2 of 7.815 is significant at the 0.05 probability level under three degrees of freedom

According to Table 5.4 the segregation for PhS_v allele is double when compared to the PhS_3 allele and the segregation ratio for PiS_{k1} and PiS_3 is 14:9. Moreover, the expected genotype ratio for PhS_vPiS_3, PhS_vPiS_{k1}, PhS_3PiS_{k1} and PhS_3PiS_3 is 1:1:1:1, but the results show 8:8:6:1 respectively. As the X^2 value for a 1:1:1:1 ratio is 5.696 which is less than X^2 of 7.815 at the 0.05 probability level, there is no significant difference between the expected and observed genotype ratio.

5.2.2.5 Identification of F_1 hybrids inheriting AhSLF-S4D transgene.

Transgenic plants AhSLF-S4D.3.1 (PhS_3PhS_v) (♀) were crossed with N445.3 (PiS_3PiS_{k1}) (♂) in order to test the ability of this transgene to be inherited in F_1

hybrid progeny (N489 family) were produced. Seedlings were selected using 50 µg /ml Kanamycin (see section 2.12). Kanamycin resistant and sensitive seedlings were scored after four weeks and the results are summarized in Table 5.5.

Table 5.5 Kanamycin resistant assay for F_1 hybrid progeny inheriting *AhSLF-S4D*.

Plant Line	Replicate Number	Kan ^{R}	Kan ^{S}
Control (Nontransformed)	1	0	100
	2	0	100
	3	0	100
N489	1	25(67%)	12(33%)
	2	74(76%)	24(24%)
	3	46(81%)	11 (19%)
	Total	145 (75%)	47 (24%)
	X^2 Value for 1:1 Ratio	50.021	
	X^2 Value for 3:1 Ratio	0.028	

Kan ^{R} = Kanamycin Resistant Kan^S = Kanamycin Sensitive

X^2 of 3.84 is significant at the 0.05 probability level and one degree of freedom for segregation ratio 3:1.

According to the results in Table 5.5 the total number of Kanamycin resistant and sensitive seedlings is 145 and 47 respectively. As the X^2 value for 1:1 ratio is 50.021 and significantly greater than X^2 of 3.84 at the 0.05 probability level there is a significant difference between the expected and observed ratio for 1:1 segregation. However, the observed ratio is closer to 3:1 segregation as the obtained X^2 value is 0.028 which is smaller than X^2 of 3.84 at the 0.05 probability level. These results indicate that the transgenic plant AhSLF-S4D.3.1 contained two kanamycin resistant loci.

In order to confirm that the resistant seedlings are transgenic 24 plants (N489.1-N489.24) were screened by genomic PCR using M13 reverse (as forward) and gene specific primer (AhSLF-S4D-R-848). The expected size is 1554 bp and approximately this size was obtained, as shown in Figure 5.7.

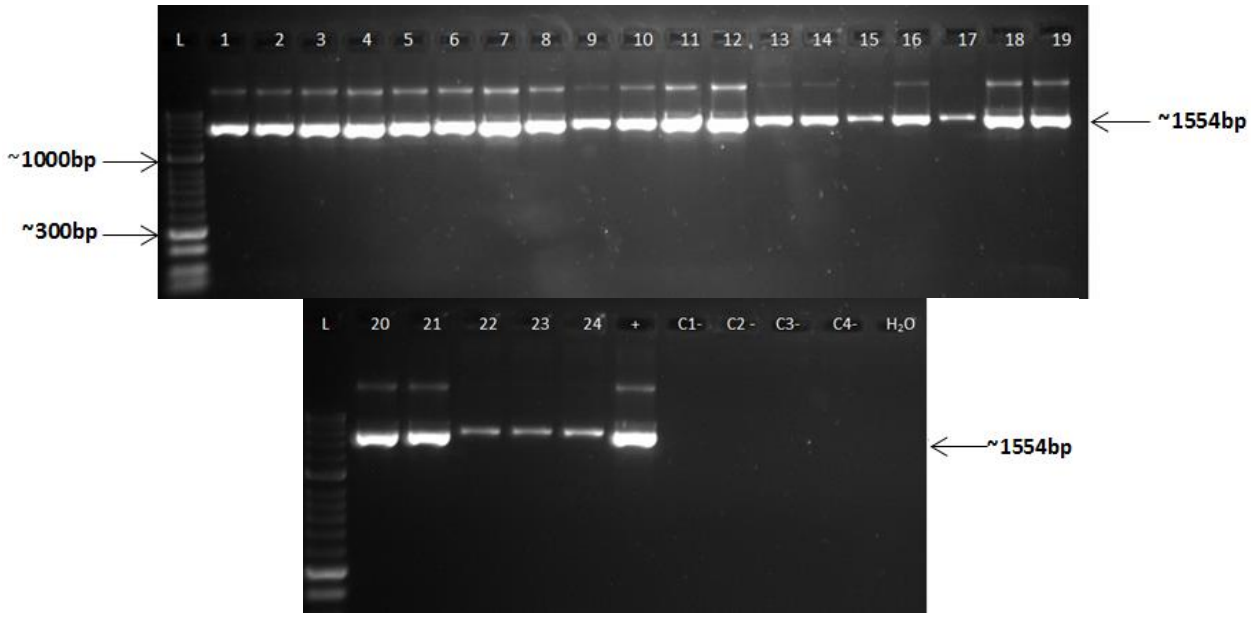


Figure 5.7 Identification of transgenic plants in N489 family inheriting *AhSLF-S4D*. L represent HyperLadder II (Bioline) and lanes 1-24 represent plants ID :N489.1, N489.2, N489.3, N489.4, N489.5, N489.6, N489.7, N489.8, N489.9, N489.10, N489.11, N489.12, N489.13, N489.14, N489.15, N489.16, N489.17, N489.18, N489.19, N489.20, N489.21, N489.22, N489.23 and N489.24 respectively. + represents plasmid DNA (AhSLF-S4D). C1- to C4- represent four controls (nontransformed F_1 hybrid plants) N450.8 (PhS_vPiS_{k1}), N450.3 (PhS_vPiS_3), N452.5 (PhS_3PiS_{K1}) and N452.1 (PhS_3PiS_3) respectively. H_2O indicates negative control for contamination.

5.2.2.6 Identification of S alleles (PiS_3, PiS_{k1}, PhS_3, PhS_v) in F_1 hybrids inheriting AhSLF-S4D.

S-RNase specific primers were used for genotyping all twenty three plants inheriting AhSLF-S4D and all possible genotypes were found to test the interaction between AhSLF-S4D with all S-alleles. The results are summarised in Table 5.6.

Table 5.6 Summary of F_1 hybrid genotype inherited *AhSLF-S4D*

Plant ID	Genotype	Plant ID	Genotype
N489.1	PhS_vPiS_3	N489.14	PhS_3PiS_{k1}
N489.2	PhS_vPiS_{k1}	N489.15	PhS_3PiS_3
N489.3	PhS_3PiS_3	N489.16	PhS_3PiS_{k1}
N489.4	PhS_vPiS_{k1}	N489.17	PhS_3PiS_{k1}
N489.5	PhS_vPiS_{k1}	N489.18	PhS_3PiS_{k1}
N489.7	PhS_vPiS_3	N489.19	PhS_3PiS_{k1}
N489.8	PhS_3PiS_3	N489.20	PhS_3PiS_3
N489.9	PhS_3PiS_{k1}	N489.21	PhS_3PiS_{k1}
N489.10	PhS_3PiS_{k1}	N489.22	PhS_3PiS_{k1}
N489.11	PhS_vPiS_{k1}	N489.23	PhS_vPiS_{k1}
N489.12	PhS_3PiS_{k1}	N489.24	PhS_3PiS_{k1}
N489.13	PhS_3PiS_{k1}	X^2 Value for 1:1:1:1 Ratio	9.870

X^2 of 7.815 is not significant at the 0.05 probability level under three degrees of freedom

Based on the results shown in Table 5.6 we can conclude that the segregation ratio for PhS_V and PiS_{K1} is more than double compared to PhS_3 and PiS_3 respectively which is different from the expected ratio of 1:1 for each pair of alleles. In addition, the observed ratio for all possible genotypes (PhS_3PiS_3, PhS_3PiS_{k1}, PhS_vPiS_3, PhS_vPiS_{k1}) is 4:12:2:5 respectively which is significantly different from the expected ratio 1:1:1:1 because the X^2 value for 1:1:1:1 segregation is 9.870 which is slightly above the X^2 of 7.815 at the 0.05 probability level.

5.2.2.7 Identification of F_1 hybrids inheriting AhSLF-S1E transgene.

In order to test the ability of this transgene to be inherited by the F_1 hybrid, one plant from T_0 raised from this transgene (AhSLF-S1E.16.1) (♀) was crossed with N445.3 (PiS_3PiS_{k1}) (♂) to create the N482 family. Kanamycin resistant and sensitive seedlings were selected using 50 µg /ml Kanamycin and they were scored after four weeks and the results are summarized in Table 5.7.

Table 5.7 Kanamycin resistant assay for F_1 hybrid progeny inheriting *AhSLF-S1E*

Plant Line	Replicate Number	Kan ^{R}	Kan ^{S}
Control (Nontransformed)	1	0	100
	2	0	100
	3	0	100
N482	1	54(58%)	39(42%)
	2	42(52%)	38(48%)
	3	25(56%)	20(44%)
	Total	121	97
	X^2 Value for 1:1 Ratio	2.642	
	X^2 Value for 3:1 Ratio	44.190	

Kan ^{R} = Kanamycin Resistant Kan ^{S} = Kanamycin Sensitive

X^2 of 3.84 is significant at the 0.05 probability level and one degree of freedom for segregation ratio 1:1.

According to Table 5.7 the total ratio for Kanamycin resistant and sensitive seedlings is 121:97. This ratio is closer to 1:1 segregation ratio as the X^2 value for this ratio is 2.642 which is smaller than X^2 of 3.84 at the 0.05 probability level and the X^2 value for 3:1 segregation of 44.190 is significantly greater than X^2 of 3.84 at the 0.05 probability level. These results indicate thatthe transgenic plant (AhSLF-S1E.16.1) contained one kanamycin resistant locus.

From Kanamycin resistant seedlings 24 plants were selected and they were screened by genomic PCR using M13 reverse (as forward) with gene specific primers (AhSLF-S1E-R-726). The expected size is 1142 bp; a band of approximately this size was obtained as illustrated in Figure 5.8.

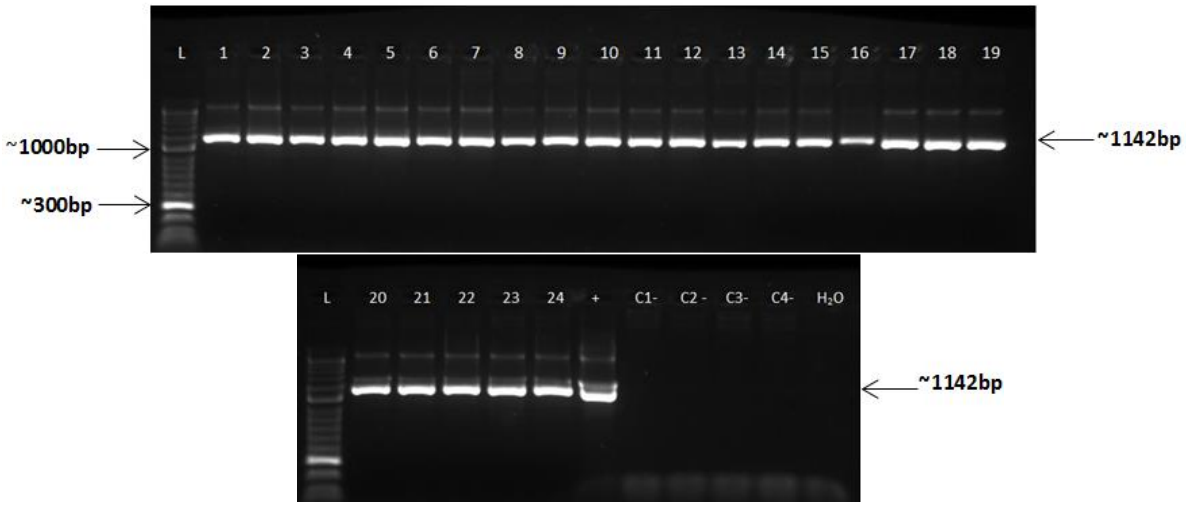


Figure 5.8 Identification of transgenic plants in N482 family inheriting *AhSLF-S1E*. L represents HyperLadder II (Bioline) and lanes 1-24 represent plants ID : N482.1, N482.2, N482.3, N482.4, N482.5, N482.6, N482.7, N482.8, N482.9, N482.10, N482.11, N482.12, N482.13, N482.14, N482.15, N482.16, N482.17, N482.18, N482.19, N482.20, N482.21, N482.22, N482.23 and N482.24). + represents plasmid DNA. C1- to C4- represent four controls (nontransformed F_1 hybrid plants), N450.5 (PhS_3PiS_3), N450.8 (PhS_vPiS_{k1}), N450.7 (PhS_vPiS_3) and N452.3 (PhS_3PiS_{K1}) respectively. H_2O indicates negative control for contamination.

5.2.2.8 Identification of Salleles (PiS_3, PiS_{k1}, PhS_3, PhS_v) in F_1 hybrids plants inheriting AhSLF-S1E.

S-RNase specific primers were used for genotyping this family in order to test the interaction between AhSLF-S1E and PiS_3, PiS_{k1}, PhS_3, PhS_v alleles. The results are summarized in Table 5.8.

Table 5.8 Summary of F_1 hybrid genotypes inheriting *AhSLF-S1E*

Plant ID	Genotype	Plant ID	Genotype
N482.1	PhS_3PiS_{k1}	N482.14	PhS_3PiS_{k1}
N482.2	PhS_3PiS_3	N482.15	PhS_vPiS_{k1}
N482.3	PhS_vPiS_{k1}	N482.16	PhS_3PiS_{k1}
N482.4	PhS_vPiS_{k1}	N482.17	PhS_3PiS_{k1}
N482.5	PhS_vPiS_{k1}	N482.18	PhS_vPiS_3
N482.6	PhS_3PiS_{k1}	N482.19	PhS_3PiS_{k1}
N482.7	PhS_vPiS_{k1}	N482.20	PhS_3PiS_{k1}
N482.8	PhS_3PiS_{k1}	N482.21	PhS_3PiS_{k1}
N482.9	PhS_vPiS_3	N482.22	PhS_vPiS_{k1}
N482.10	PhS_vPiS_3	N482.23	PhS_vPiS_{k1}
N482.11	PhS_vPiS_{k1}	N482.24	PhS_vPiS_{k1}
N482.12	PhS_vPiS_{k1}	X^2 Value for 1:1:1:1 Ratio	11.33
N482.13	PhS_vPiS_{k1}		

X^2 of 7.815 is not significant at the 0.05 probability level under three degrees of freedom

According to the results shown in Table 5.8. *PiS$_{k1}$* allele is inherited nearly five times more than *PiS$_3$* and both *PhS$_v$* and *PhS$_3$* are inherited in 14 and 10 plants respectively. Consequently, the observed genotype ratio is 11:9:3:1 for *PhS$_v$PiS$_{k1}$*, *PhS$_3$PiS$_{k1}$*, *PhS$_v$PiS$_3$* and *PhS$_3$PiS$_3$* respectively. This ratio is significantly different from the expected ratio of 1:1:1:1 as the X^2 value for this ratio is 11.33 which is greater than X^2 of 7.815 at the 0.05 probability level.

5.2.3 Testing compatibility in F_1 hybrid plants inheriting transgenes *AhSLF-S2*, *AhSLF-S2C*, *AhSLF-S4D* and *AhSLF-S1E*.

In order to test the interaction between the four transgenes (*AhSLF-S2, AhSLF-S2C, AhSLF-S4D* and *AhSLF-S1E*) with wide ranges of *S*-alleles based on the competitive interaction phenomenon, ten flowers for each plants were self-pollinated. However, no competitive interaction for each family was observed (See Appendix 5, Table 2)

5.2.4 Identification of T_1 transgenic plants derived from *AhSLF-S2* transformant.

As the competitive interaction has not observed in T_0 Transgenic plants and all plants remained self-incompatible, bud pollination was done in order to harvest some self seeds to analyse the transgene behaviour in T_1 plants. Four transgenic plants from the T_0 line transformed with the *AhSLF-S2* gene (AhSLF-S2-28.2, AhSLF-S2-27.1, AhSLF-S2-22.1 and AhSLF-S2-24.1) were selected and some seeds were obtained following bud pollination. Consequently, four families N469, N470, N472 and N473 were raised. A different number of plants were obtained for each family, as they came from bud pollination which usually produces small amounts of seed. The parental plants, family ID and number of plants raised for each transformant are illustrated in Table 5.9

Table 5.9 Summary of T_1 plants raised from T_0 (*AhSLF-S2*) seeds

T_0 Transformant	T_1 Family Number	Number of Plants
AhSLF-S2-28.2	N469	28
AhSLF-S2-27.1	N470	10
AhSLF-S2-22.1	N472	6
AhSLF-S2-24.1	N473	31

As the amount of seeds obtained from bud pollination is limited, they were not selected by Kanamycin. All seeds were sown and they were screened by genomic PCR using M13 Forward (as reverse) and Forward AhSLF-S2 specific primers (AhSLF-F-S2-672). Figure 5.9 indicates that the transgene is inherited by some T_1 progeny successfully. As shown in Figure 5.9, all plants inherited the transgene (AhSLF-S2) except N469.3, N469.4, N469.9, N469.13, N469.16, N469.22, N469.26, N470.7, N470.10, N472.2 and N472.3).

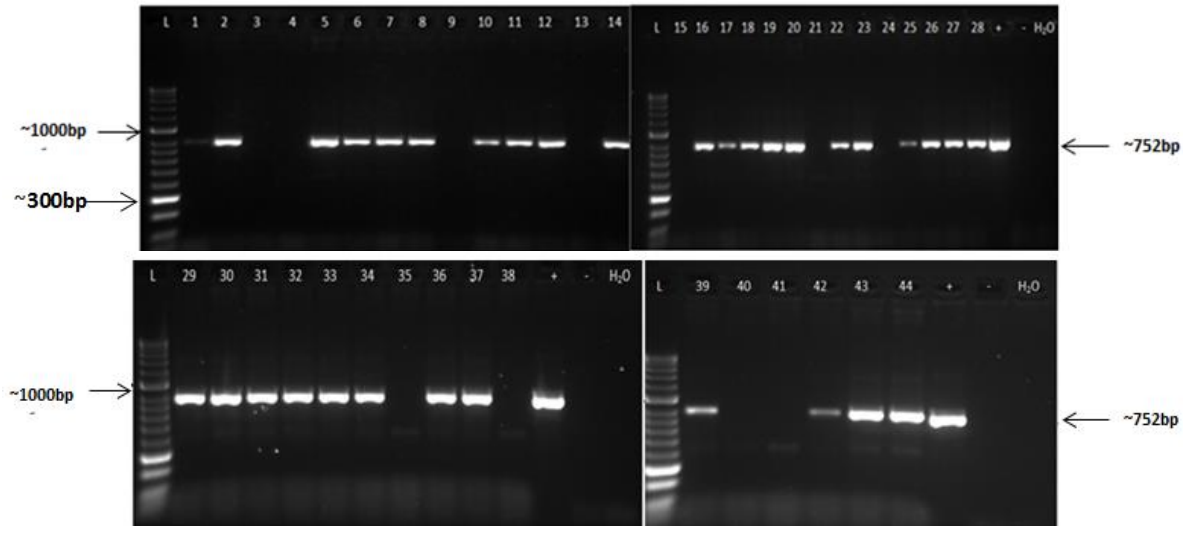


Figure 5.9 Identification of transgenic plants in N469, N470 and N472 families derived from AhSLF-S2 Transformants. L represent HyperLadder II and lanes 1-44 represent plants ID:N469.1, N469.2, N469.3, N469.4, N469.5, N469.6, N469.7, N469.8, N469.9, N469.10, N469.11, N469.12, N469.13, N469.15, N469.16, N469.17, N469.18, N469.19, N469.20, N469.21, N469.22, N469.23, N469.25, N469.26, N469.27, N469.28, N469.29, N469.30, N470.1,N470.2, N470.3, N470.4, N470.5, N470.6, N470.7, N470.8, N470.9, N470.10, N472.1, N472.2, N472.3, N472.4, N472.5 and N472.6 respectively . + represent plasmid DNA (Positive Control). Represent negative control (non-transformed plant, N440.6 (*PhS_3PhS_v*)). H_2O indicates negative control for contamination.

More plants were raised from seed obtained from bud pollination of the AhSLF-S2-24.1 family and N473 family. 31 plants were screened for the transgene as described in the previous section. Figure 5.10 shows that all plants inherited the transgene AhSLF-S2 except N473.2, N473.4, N473.5, N473.6, N473.13, N473.14, N473.16, N473.17, N473.24, N473.26 and N473.30.

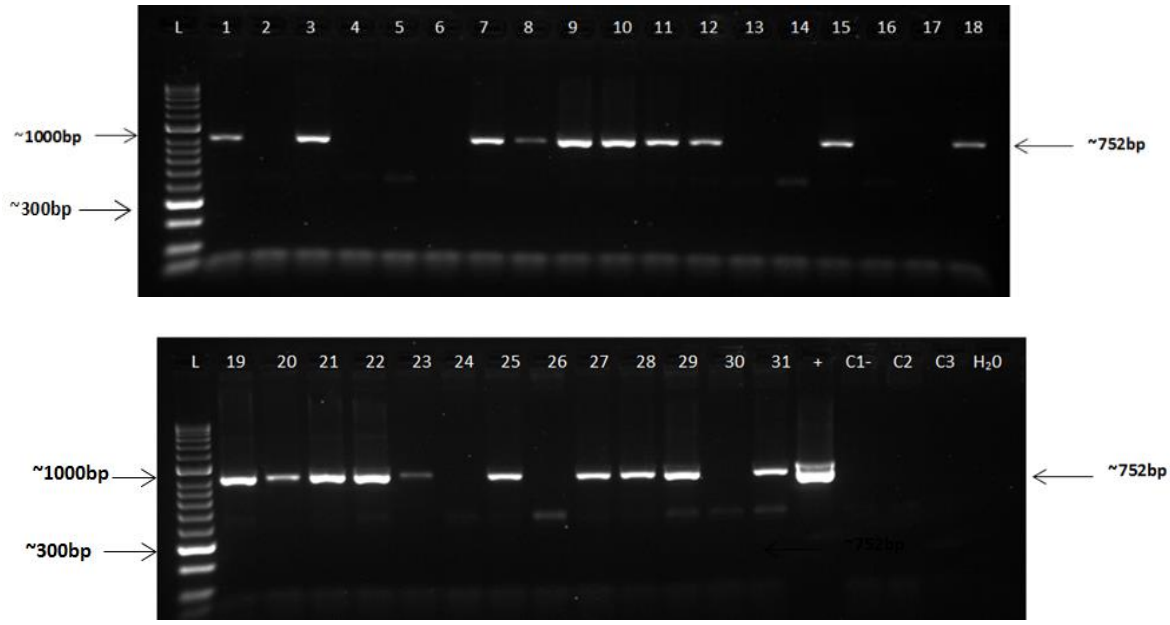


Figure 5.10 Identification of transgenic plants in N473 family derived from AhSLF-S2 transformant. L represent HyperLadder II (Bioline) and lanes 1-31 represent plants ID: N473.1, N473.2, N473.3, N473.4, N473.5, N473.6, N473.7 , N473.8, N473.9, N473.10, N473.11, N473.12, N473.13, N473.14, N473.15, N473.16, N473.17, N473.18, N473.19, N473.20, N473.21, N473.22, N473.23, N473.24, N473.25, N473.26, N473.27, N473.28, N473.29, N473.30 and N473.31 . + represent plasmid DNA (Positive Control). C1– to C3- represent negative control (nontransformed plant) N440.6 (*PhS*$_3$*PhS*$_v$), N440.4 (*PhS*$_3$*PhS*$_3$) and N439.4 (*PhS*$_v$*PhS*$_v$). H_2O indicates Negative control for contamination.

5.2.5 Identification of *PhS*$_3$ and *PhS*$_v$ in T_1 plants derived from AhSLF-S2 transformants

Qiao *et al*, (2004b) transformed *Petunia hybrida* plants of genotype *PhS*$_3$*PhS*$_3$ using the same AhSLF-S2 construct used in this thesis and the transgenic plants became self-compatible in contrast to the results reported in this thesis. However, one difference between the two studies is that a different genotype (*PhS*$_3$*PhS*$_V$) was used

in this project (See Section 5.2.1). In order to test whether either plant genotype (homozygous and heterozygous) has an effect on the transgene behaviour, all plants in the four T_1 families described above (N469, N470, N472 and N473) were screened using gene specific primers for *PhS₃* and *PhSᵥ* alleles by genomic PCR. The PCR data were omitted to avoid repetition. The results are summarized in Table 5.

Table 5.10 Summary of T_1 plants S-genotype derived from AhSLF-S2 transformant. The highlighted lines in blue indicate non-transgenic plants.

Plant ID	Genotype	Plant ID	Genotype	Plant ID	Genotype
N469. 1	*PhS₃PhSᵥ*	N469.28	*PhS₃PhS₃*	N473.7	*PhSᵥPhSᵥ*
N469.2	*PhS₃PhSᵥ*	N469.29	*PhS₃PhSᵥ*	N473.8	*PhS₃PhSᵥ*
N469.3	*PhSᵥPhSᵥ*	N469.30	*PhS₃PhSᵥ*	N473.9	*PhS₃PhS₃*
N469.4	*PhS₃PhSᵥ*	N470.1	*PhSᵥPhSᵥ*	N473.10	*PhSᵥPhSᵥ*
N469.5	*PhS₃PhSᵥ*	N470.2	*PhS₃PhSᵥ*	N473.11	*PhSᵥPhSᵥ*
N469.6	*PhSᵥPhSᵥ*	N470.3	*PhS₃PhSᵥ*	N473.12	*PhS₃PhSᵥ*
N469.7	*PhSᵥPhSᵥ*	N470.4	*PhS₃PhSᵥ*	N473.13	*PhS₃PhSᵥ*
N469.8	*PhSᵥPhSᵥ*	N470.5	*PhS₃PhSᵥ*	N473.14	*PhS₃PhSᵥ*
N469.9	*PhS₃PhSᵥ*	N470.6	*PhSᵥPhSᵥ*	N473.15	*PhS₃PhSᵥ*
N469.10	*PhS₃PhSᵥ*	N470.7	*PhS₃PhSᵥ*	N473.16	*PhSᵥPhSᵥ*
N469.11	*PhS₃PhSᵥ*	N470.8	*PhS₃PhSᵥ*	N473.17	*PhS₃PhSᵥ*
N469.12	*PhS₃PhSᵥ*	N470.9	*PhS₃PhSᵥ*	N473.18	*PhS₃PhSᵥ*
N469.13	*PhS₃PhSᵥ*	N470.10	*PhS₃PhSᵥ*	N473.19	*PhS₃PhSᵥ*
N469.15	*PhS₃PhSᵥ*	N472.1	*PhS₃PhSᵥ*	N473.20	*PhS₃PhSᵥ*
N469.16	*PhSᵥPhSᵥ*	N472.2	*PhSᵥPhSᵥ*	N473.21	*PhS₃PhSᵥ*
N469.17	*PhSᵥPhSᵥ*	N472.3	*PhS₃PhSᵥ*	N473.22	*PhS₃PhSᵥ*
N469.18	*PhS₃PhSᵥ*	N472.4	*PhS₃PhSᵥ*	N473.23	*PhS₃PhSᵥ*
N469.19	*PhS₃PhS₃*	N472.5	*PhS₃PhSᵥ*	N473.24	*PhS₃PhSᵥ*
N469.20	*PhS₃PhS₃*	N472.6	*PhS₃PhSᵥ*	N473.25	*PhS₃PhSᵥ*
N469.21	*PhS₃PhSᵥ*	N473.1	*PhS₃PhS₃*	N473.26	*PhS₃PhSᵥ*
N469.22	*PhS₃PhSᵥ*	N473.2	*PhS₃PhSᵥ*	N473.27	*PhS₃PhSᵥ*
N469.23	*PhS₃PhSᵥ*	N473.3	*PhS₃PhSᵥ*	N473.28	*PhS₃PhSᵥ*
N469.25	*PhS₃PhSᵥ*	N473.4	*PhSᵥPhSᵥ*	N473.29	*PhSᵥPhSᵥ*
N469.26	*PhS₃PhSᵥ*	N473.5	*PhSᵥPhSᵥ*	N473.30	*PhSᵥPhSᵥ*
N469.27	*PhS₃PhSᵥ*	N473.6	*PhS₃PhS₃*	N473.31	*PhSᵥPhSᵥ*

The highlighted plants are not transgenic as they have not inherited the transgene according to the genomic PCR results illustrated in Figures 5.9 and 5.10. According to Table 5, it seems that *PhS₃* allele is rejected more than *PhSV* allele. Significantly, five plants of the plants of *PhS₃PhS₃* genotype also inherited the *AhSLF-S2* transgene. These are indistinguishable in S-genotype from the T_0 generation described by Qiao *et al*, (2004b).

5.2.6 Testing compatibility in T_1 plants derived from *AhSLF-S2* transformant.

Ten flowers were self-pollinated in all plants belonging to the N469, N470, N472 and N473 families, however, all plants remained self-incompatible (See Appendix 5, Table 3). This indicated that no competitive interaction was observed between the AhSLF-S2 transgene and the endogenous (PhSLF genes) in four independent transformants. The reason for a lack of competitive interaction in the experiments described here is unclear although it is possible that Qiao *et al* (2004b) selected for specific transformants with a high expression level.

5.2.7 Identification of T_1 plants derived from *AhSLF-S1E* Transformant.

As all three transgenic plants transformed with the *AhSLF-S1E* gene (AhSLF-S1E.15.1, AhSLF-S1E.16.1 and AhSLF-S1E.31.1) remained self-incompatible in the T_0 generation, bud pollinations were performed in order to analyse the transgene behaviour in T1 progeny. The N471 family was raised from bud pollination of AhSLF-S1E.16.1 and 11 plants were obtained and they were screened by genomic PCR using M13 reverse (as forward) with gene specific primers (AhSLF-S1E-R-726). The expected size is 1142 bp and approximately this size was obtained as illustrated in Figure 5.11 for nine plants out of eleven (N471.1, N471.2, N471.3, N471.5, N471.6, N471.7, N471.8, N471.10 and N471.11) indicating that these T_1 progeny are transgenic.

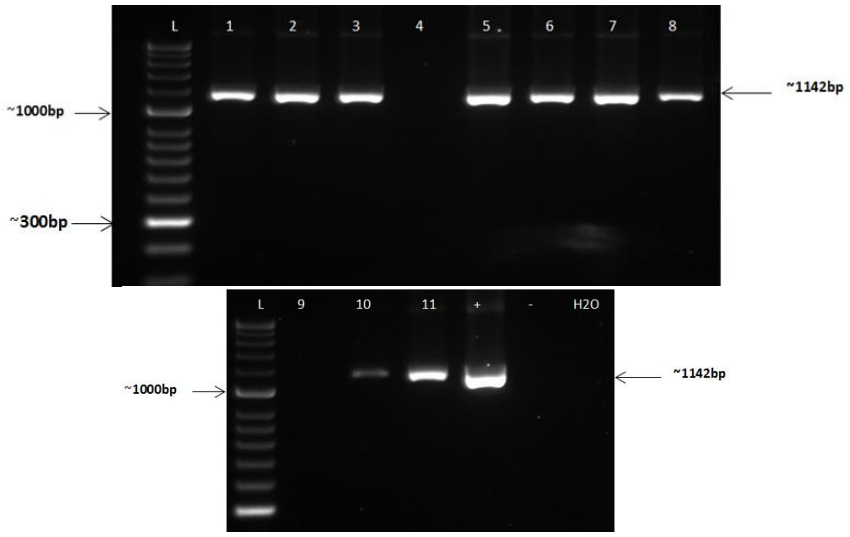


Figure 5.11 Identification of T_1 transgenic plants in N471 family derived from AhSLF-S1E.16.1 transformant. L represents HyperLadder II (Bioline) and lanes 1-11 represent plants ID: N471.1, N471.2, N471.3, N471.4, N471.5, N471.6, N471.7, N471.8, N471.9, N471.10 and N471.11 respectively. + represents plasmid DNA (Positive Control) - represents negative control (nontransformed plant) N440.6 (*PhS_3PhS_v*). H_2O indicates negative control for contamination.

5.2.8 Identification of *PhS_3* and *PhS_v* alleles in T_1 plants derived from AhSLF-S1E transformant.

In order to identify whether a plant S-genotype (homozygous or heterozygous) has an effect on transgene behaviour, the N471 family was screened using gene specific primers for *PhS_3* and *PhS_v* alleles. The PCR gel images were omitted for brevity but the results are summarized in Table 5.11.

Table 5.11 Summary of T_1 plants derived from AhSLF-S1E.16.1 transformant. The highlighted line in blue indicates non-transgenic plants.

Plant ID	Genotype
N471.1	*PhS_3PhS_v*
N471.2	*PhS_3PhS_v*
N471.3	*PhS_3PhS_v*
N471.4	*PhS_vPhS_v*
N471.5	*PhS_3PhS_v*
N471.6	*PhS_vPhS_v*
N471.7	*PhS_vPhS_v*
N471.8	*PhS_3PhS_v*
N471.9	*PhS_3PhS_v*
N471.10	*PhS_3PhS_v*
N471.11	*PhS_3PhS_v*

5.2.9 Testing compatibility in T_1 plants derived from the *AhSLF-S1E* transformant.

Ten flowers were self-pollinated in all plants belong to N471 family. Interestingly, it was observed that N471.1 was self-compatible (see Figure 5.12) and the remainder of the plants of the N471 family remained incompatible. This observation could be consistent with competitive interaction if it was interpreted that this particular progeny plant was homozygous for the transgene. In this hypothesis the transgene homozygosity would result from self-pollination and other T_1 progeny would need to have contained only one copy of the transgene (equivalent to the T_0 transformant). However, this hypothesis needs to be tested using additional methods and this is the focus of sections 5.2.9.1, 5.2.9.2 and 5.2.9.3.

Figure 5.12 Seed capsule produced from self-pollination of the N471.1 plant. Bar=5 mm

5.2.9.1 Using reciprocal crosses to test competitive interaction in N471.1 plant.

Competitive interaction is a genetic phenomenon which leads to the breakdown of gametophytic self-incompatibility in pollen and produces self-compatible plants. This phenomenon occurs as a result of heteroallelic pollen only (see Figure 5.1) and does not require a duplication of S-alleles in the style. Reciprocal crosses were used to test competitive interaction in the N471.1 plant. Five flowers for three non-transformed plants holding the same genotype (PhS_3PhS_v) were cross pollinated using the N471.1 transgenic line as pollen donor and all crosses resulted in large capsules (See Figure 5.13), which means they are fully cross-compatible. However, crossing N471.1 using these three lines as pollen donors did not produce any capsules which indicates that compatibility only resulted from heteroallelic pollen of N471.1 plant and not pistil as it was expected, which confirms competitive interaction.

Figure 5.13 Seed capsule produced using cross pollination between (PhS_3PhS_V) (non transformed plants) as a female and N471.1 plant as male. Bar= 5 mm

5.2.9.2 Testing compatibility in T_2 plants derived from AhSLF-S1E transgene.

Further analysis of the competitive interaction in N471.1 was performed. The N487 family was raised from self-seed collected from N471.1 and they were 100% Kanamycin resistant which confirms that this plant is homozygous for the transgene (See Figure 5.14)

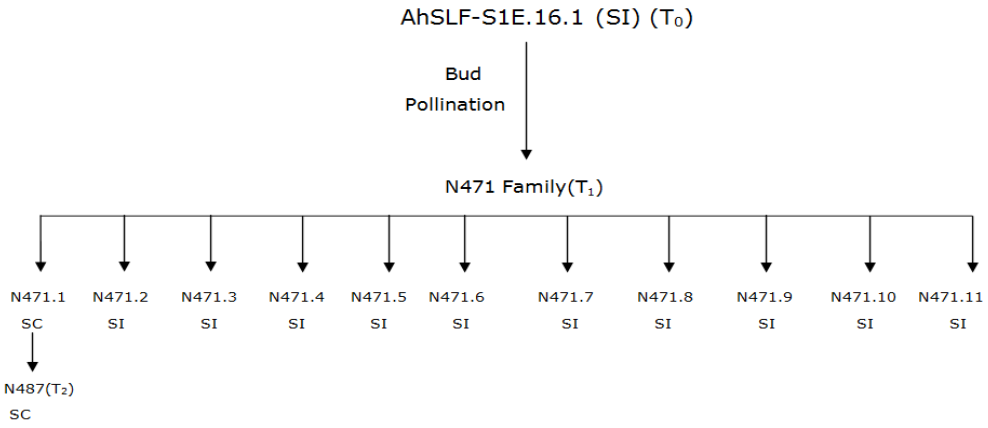


Figure 5.14 Diagram illustrating the N487 family pedigree

In addition, 14 plants from the T_2 generation were selected and five flowers for each of them were self-pollinated. Interestingly, it was noticed that all of them are self-compatible as they produced a large capsule (See Figure 5.15)

Figure 5.15 Capsule produced from self-pollination in the plant belongs to N487 family. Bar=5 mm

5.2.9.3 Testing homozygosity in the N471 family by crossing with Petunia inflata

As mentioned in section 5.2.12, N471.1 was the only self-compatible plant obtained out of 11 T_1 progeny belonging to the same family (N471). It was proposed that the homozygosity for the transgene (*AhSLF-S1E*) in N471.1 might cause self-compatibility.

This hypothesis was applied to other siblings of the N471 family. Several plants were crossed with *Petunia inflata* in order to provide more seeds as crossing between different compatible species produces large capsules. Collected seeds were selected on Kanamycin media to determine the ratio of seedlings inheriting the transgene.

If the seedlings show 100% resistance to Kanamycin it means the parent plant was homozygous for the transgene. However, if the 50% of the seedlings are resistant to Kanamycin it means the parents are hemizygous for the transgene. The results obtained for the N471 family are summarized in Table 5.12.

Table 5.12 Summary of testing for homozygosity in T_1 transgenic plants inheriting the AhSLF-S1E transgene by crossing with *Petunia inflata*. The highlighted lines in green, yellow and red illustrate homozygous, hemizygous and non-transgenic plants respectively.

Plant Line	Number of Replication	Kan ^{R}	Kan ^{S}	Genotype
N471.1	1	56 (100%)	0	Homozygous
	2	72 (100%)	0	
	3	45 (100%)	0	
N471.2	1	75 (100%)	0	Homozygous
	2	100(100%)	0	
	3	88 (100%)	0	
N471.3	1	31 (46%)	36 (54%)	Hemizygous
	2	36 (56%)	28 (44%)	
	3	30 (57%)	22 (43%)	
	X^2 Value for 1:1 Ratio	0.661		
	X^2 Value for 3:1 Ratio	47.215		
N471.4	1	0	100	Non-transgenic
	2	0	100	
	3	0	100	
N471.7	1	80 (100%)	0	Homozygous
	2	74 (100%)	0	
	3	70 (100%)	0	
N471.8	1	95 (100%)	0	Homozygous
	2	106(100%)	0	
	3	110(100%)	0	
N471.9	1	0	100	Non-transgenic
	2	0	100	
	3	0	100	
N471.10	1	51 (73%)	19 (27%)	Hemizygous
	2	78 (77%)	23 (23%)	
	3	27 (44%)	34 (56%)	
	X^2 Value for 1:1 Ratio	27.586		
	X^2 Value for 3:1 Ratio	7.448		
N471.11	1	100	0	Homozygous
	2	100	0	
	3	100	0	

Kan ^{R} = Kanamycin Resistant Kan ^{S} = Kanamycin Sensitive

X^2 of 3.84 is significant at the 0.05 probability level and degree of freedom 1 only for segregation ratio 1:1 in N471.3.

According to the results shown in Table 5.12, five plants (highlighted in green) out of nine are homozygous for the transgene (*AhSLF-S1E*). Both N471.3 and N471.10 (highlighted in yellow) are hemizygous. Regarding N471.3, the X^2 value for 1:1 and 3:1 segregation is 0.661 and 47.215 respectively. This result indicates that N471.3 contained one copy of the transgene as 0.661 is smaller than X^2 of 3.84 at the 0.05

probability level but X^2 value for 3:1 segregation is 47.215 greater than 3.84 which confirms that the T_0 parent for this plant, AhSLF-S1E 16.1, contained one loci.

The X^2 for 1:1 and 3:1 segregation in N471.10 is 27.586 and 7.448 respectively which both of them are greater than X^2 of 3.84 at the 0.05 probability level. This means this segregation ratio is not consist with neither 1:1 nor 3:1. This odd result can be interpreted as Kanamycin resistant seed germinated faster , so some sensitives were unscored which had an effect on segregation ratio.

All seedlings produced from crossing between N471.1 plant and *Petunia inflata* were Kanamycin resistant. This observation confirms that N471.1 is homozygous for the transgene (*AhSLF-S1E*), which causes compatibility according to our hypothesis. Moreover, twenty four plants from this seedling were grown and ten flowers for each plants were self-pollinated. It was observed that they are all self-incompatible, which confirms the relation between homozygosity and self-compatibility in N471.1 plant as all plants in F_1 hybrids became self-incompatible as a result of the transgene segregation resulting in hemizygosity.

However, screening the seedlings produced from crossing other siblings (N471.2, N471.7, N471.8 and N471.11) belonging to the same family with *Petunia inflata* gave 100% Kanamycin resistance, which challenges the relationship between transgene homozygosity and self-compatibility observed in the N471.1 plant. As an alternative approach, the level of expression was compared between N471.1 and the other sibling plants in an attempt to reveal the possible reason behind self-compatibility in N471.1.

Seedlings produced from crosses between N471.4 and N471.9 with *Petunia inflata* gave 100% Kanamycin sensitive which is expected as both of them did not gave the band using genomic PCR (See Figure 5.11).

5.2.9.4 Comparing AhSLF-S1E expression in homozygous plants derived from the N471 family using qRT-PCR.

qRT-PCR analyses were performed using the Light Cycler 480 real-time PCR system. Expression was normalized using the EF1a gene and each sample was repeated three times to obtain accurate results. Light Cycler 480 software was used to analyse the data (See section 2.11). Transgene expression was compared between N471.1 and N471.2, N471.7, N471.8 and N471.11 using qPCR. The N471.1 plant was used as a positive control (with expression normalized to one).

According to Figure 5.16 the level of expression in the N471.1 plant is much higher than the other sibling plants which could be the underlying reason for self-compatibilty in this particular plant. The low level of expression in N471.2, N471.7, N471.7, N471.8 and N471.11 is consistent with the observation that these plants remain self-incompatibile.

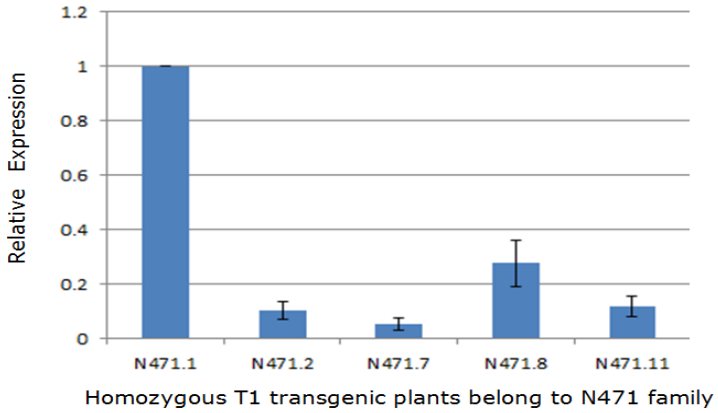


Figure 5.16 AhSLF-S1E transgene expression analysis using qRT-PCR in homozygous plants belonging to the N471 family. Relative quantity chart obtained from LightCycler480 software analysis of qRT-PCR. N471.1 was used as a calibrator and it has been assigned a value of 1.0. House keeping gene EF1a was used as normalisation assay. Bars indicate the error calculated based on average and standard deviation.

From these results it can be concluded that there is a strong relation between homozygosity and compatibilty in N471.1 plant. However, homozygosity *per se* cannot be sufficient for self-compatibilty. It is possible that epigenetic factors have a role in determining compatibilty but the detailed mechanism is not clear.

5.3 DISCUSSION

Competitive interaction is an interesting phenomenon, which can be exploited to control self-incompatibility. This phenomenon was revealed in the mid -20th century. It occurred as a result of duplication of the S-locus and it was observed in plants from several families such as Fabaceae (Brewbaker, 1954), Onagraceae (Lewis, 1947) and Solanaceae (Livermore and Johnstone 1940; Stout and Chandler 1942; Pandey 1968).

Based on this phenomenon we have attempted to control self-incompatibility in *Petunia hybrida* using a heterologous transgenic approach. Initially *Petunia hybrida*

was transformed using four constructs (*AhSLF-S2*, *AhSLF-S2C*, *AhSLF-S4D* and *AhSLF-S1E*) which are all driven by the pollen specific promoter (LAT52). Different numbers of transgenic plants were obtained for each construct.

In spite of the fact that the *AhSLF-S2* transgene was expressed correctly in the reproductive tissues (See section 5.2.1.1) the host phenotype in T_0 generation remained self-incompatible. This result is not consist with the findings of Qiao *et al*, (2004b) as they transformed *Petunia hybrida* PhS_3PhS_3 plants using the AhSLF-S2 construct and plants significantly became self-compatible. One possible explanation is that we have used a different host genotype in the primary transformation, PhS_3PhS_v instead of *Petunia hybrida* PhS_3PhS_3. In order to test this possibility the hypothesis for the relation between the homozygosity in *Petunia hybrida* (PhS_3PhS_3) and the breakdown of self-incompatibility using AhSLF-S2 was tested. T_1 transgenic plants were raised from bud pollination of (AhSLF-S2-28.2, AhSLF-S2-27.1, AhSLF-S2-22.1 and AhSLF-S2-24.1) plants see section (5.2.7) and 44 plants were raised. Transgenic plants were screened using Genomic PCR with M13 Forward (as reverse) and Forward specific primers (AhSLF-F-S2-672), and their genotypes were identified using gene specific primers for PhS_3 and PhS_v alleles (See Table 5.10). However, all plants regardless of their genotype remain self-incompatible. This result is not consistent with the findings of Qiao *et al*, (2004b) as they obtained self-compatible plants after transformation of *Petunia hybrida* PhS_3PhS_3 with AhSLF-S_2 construct.

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From these results we can conclude that, it is possible for AhSLF-S_2 transgenic plants to show compatibility but perhaps only in particular epigenetic conditions. In this

project only six independent transformants were obtained for this transgene. If we obtained more transgenic plants, we would have more chance to observe a compatible plant. The number of transformants screened by Qiao *et al*, (2004b) in order to observe competitive interaction was not reported.

In further progeny studies, one plant for each construct was selected and crossed with a *Petunia inflata* line to test the interaction between the transgene and a wide range of S-alleles and test the ability of the transgene to be inherited in a different species (*Petunia inflata*). In spite of the fact that this transgene was inherited successfully by *Petunia inflata* (See sections 5.2.5.1) all F_1 hybrid plants remained self-incompatible.

Regarding the AhSLF-S1E, AhSLF-S2C and AhSLF-S4D constructs, all plants in T_0 generation remained self-incompatible. The T_1 generation was only analysed for *AhSLF-S1E* because the transgenic plants for the other two constructs were obtained in the later stages of the project and harvesting seeds from bud pollination is not straightforward. Bud pollination was done for AhSLF-S1E.16.1 and eleven T_1 progeny were analysed. The transgenic plants were analysed using transgene specific primers and PhS_3 and PhS_V allele specific primers as summarized in Table 5.13.

Table 5.13 Summary of S-alleles and T-DNA genotype and testing for compatibility for N471 derived from AhSLF-S1E transgene. The plants highlighted in blue are not transgenic.

Plant ID	Genotype	Testing for compatibility
N471.1	PhS_3PhS_v	SC
N471.2	PhS_3PhS_v	SI
N471.3	PhS_3PhS_v	SI
N471.4	PhS_vPhS_v	SI
N471.5	PhS_3PhS_v	SI
N471.6	PhS_vPhS_v	SI
N471.7	PhS_vPhS_v	SI
N471.8	PhS_3PhS_v	SI
N471.9	PhS_3PhS_v	SI
N471.10	PhS_3PhS_v	SI
N471.11	PhS_3PhS_v	SI

Interestingly, it was noticed that N471.1 plant became self-compatible while the remainder of the siblings remained self-incompatible. One possibility is that self-compatibility in N471.1 could be due to the transgene copy number (homozygosity). In order to test this hypothesis, (the relationship between compatibility in N471.1 plant and homozygosity) several techniques have been used. Firstly, this plant was crossed with *Petunia inflata* and collected seeds were screened by Kanamycin and it was noticed that the F_1 hybrid seeds were all Kanamycin resistant which confirms that N471.1 is homozygous for the transgene (*AhSLF-S1E*). 24 Kanamycin resistance seedlings were grown and after self-pollination it was noticed that all of them were self-incompatible which means the transgene lost its effect after segregation for one copy.

This observation supports the relationship between self-compatibility and homozygosity in the N471.1 plant. Secondly, T_2 harvested seeds from this plant (N471.1) were screened using Kanamycin and it was noticed that all seedlings were Kanamycin resistant. Compatibility was tested in 14 plants using self-pollination, it was noticed that all of them are self-compatible see (section 5.2.8.2), this observation

again supports the relationship between compatibility and homozygosity in the N471.1 plant.

In order to reveal if self-compatibility in N471.1 is due to competitive interaction, reciprocal crosses were done (see section 5.2.8.1) and it was noticed that only transgenic pollen (heteroallelic pollen) in N471.1 caused compatibility and the pistil in the same plant did not have any role in compatibility.

The same results were obtained previously by Sijacic *et al* (2004), and it was revealed that the presence of PiSLF2 in S_1 and S_3 pollen grains caused the breakdown of SI. This observation could be interpreted based on the modified protein-degradation model described by Hua *et al*, (2008) as follows. In the cytoplasm of heteroallelic pollen (AhSLF-S1E+PhSLF(S_3) or PhSLF(S_V)) both *PhS_3-RNase* and *PhS_v-RNase* are present. Two stable complexes AhSLF-S1E-PhS_3-RNase and *AhSLF-S1E-PhSv-RNase* can form after the interaction between AhSLF-S1E with PhS_3-RNases and PhS_v-RNases through their SBD (S-RNase-binding domain) which would ubiquitinate PhS_3-RNase and PhS_v-RNase to target them for degradation. Consequently the pollen tube can grow which leads to compatibility.

Moreover, several sibling plants belonging to the same family (N471) were crossed with *Petunia inflata* line (See Table 5.12) and collected seeds from each plant were selected by Kanamycin. Surprisingly, it was noticed that, although N471.2, N471.7, N471.8 and N471.11 were self-incompatible, F_1 hybrid seeds were all Kanamycin resistant, exactly the same as N471.1 which was self-compatible. This observation indicates that although homozygosity is essential for the presence of compatibility,

additional epigenetic factors can have a role. Consequently, we have tested the level of expression of AhSLF-S1E in N471.1 compared to N471.2, N471.7, N471.8 and N471.11. Interestingly, it was noticed that the expression level in N471.1 is much higher compared to N471.2, N471.7, N471.8 and N471.11 (See Figure 5.16).

From all these results we can conclude that, all transgenes are able to inherit to another species (*Petunia inflata*). However, regarding *AhSLF-S2*, in the primary transformation we have obtained different results from previous research and only one plant from the T_1 generation derived from *AhSLF-S1E* was self-compatible. We can interpret both results as the transgene behaves in different way in different epigenetic backgrounds.

CHAPTER 6 : BREAKDOWN OF SELF-INCOMPATIBILITY ASSOCIATED WITH *PiS$_d$* ALLELE.

6.1 INTRODUCTION

Petunia inflata and *Petunia hybrida* were crossed to produce F_1 hybrids and it was noticed that all plants inheriting the *PiS$_d$* allele became self-compatible as described in section 3.2.7. In order to confirm that the incompatibility was lost because of a change of function of the *PiS$_d$* allele, crosses were done between semi-compatible plants to allow segregation of the *PiS$_d$* allele. Moreover, in order to understand whether the *PiS$_d$* had lost function in female or male parts, reciprocal crosses were done between self-compatible and self-incompatible plants holding the same F_2 hybrid genotypes.

There are various factors behind the breakdown of self-incompatibility which could be natural or artificial. In spite of the fact that the duplication of S-locus can be done artificially using radiation, chemical substances and transgenic methods, it is relatively very rare in nature. However, in natural populations, mutations that cause loss of S-RNase activity and mutations that do not cause loss of S-RNase activity are well documented (Stone, 2002). As the presence of the S-locus alone is not enough for a plant to show SI it needs the cooperation with other genes which are called modifier genes; mutation of these genes cause the breakdown of SI as well.

Both true self compatibility and pseudo self-compatibility (PSC) can be defined as the breakdown of self-incompatibility when incompatible plants revert to set seeds after self or cross pollination with other individuals bearing the same S-allele. However, PSC plants set more seeds after crossing with plants carrying distinct S-alleles (Ascher, 1984). PSC is an intermediate state between SI and SC and it can arise in the presence of non-functional maternal or paternal control. In addition, environmental factors can cause this phenomenon, especially in species with a weak incompatibility. Moreover, the random segregation of a modifier gene, the variation between S allele ability to allow the pollen tube to grow in incompatible pistil, mutation, polyploidy and interspecific hybridization, can be considered as the most evident factors behind this phenomenon (Denward, 1963).

Takahashi (1973) reported that pseudo-compatibility in *Petunia hybrida* is probably caused by a polygenic system rather than by S alleles. According to Robacker and Ascher (1982), studies in *Nemesia* revealed that the PSC was not attributed to female and male function. Moreover, Hiscock (2000) obtained the same result with *Senecio*.

PSC has been studied in several species including *Petunia hybrida* (Dana and Ascher 1985). In spite of the fact that PSC is widespread in *Petunia hybrida*, it does not appear as a result of a hybrid origin of this species. This phenomenon is widely expressed in parents of the hybrid, for instance, *Petunia integrifolia* (Dana and Ascher 1985). Furthermore, PSC can be associated with the particular S-allele S_0 (Ai *et al* 1991

6.2 RESULTS

In order to further confirm the relationship between the breakdown of self-incompatibility and the *PiS$_d$* allele, cross pollinations have been done between different semi-compatible genotypes in the F_1 hybrid generation to produce F_2 progeny. Four families N463, N464, N465 and N467 were generated from crossing between semi-compatible plants in F_1 hybrid (See Table 2.5) in order to allow the *PiS$_d$* allele to be tested in a segregating family. The semi-compatible nature of the cross ensures that only a single allele is inherited through the pollen to simplify analysis. In each family it is possible to test for cosegregation between the SC phenotype and the *PiS$_d$* allele.

6.2.1 Identification of *PiS$_3$*, *PiS$_d$* and *PhS$_3$* alleles and compatibility test in N463 family.

This family of 24 plants came from crossing between N453.4 (*PhS$_3$PiS$_d$*) as a female and N451.7 (*PhS$_3$PiS$_3$*) as a male (see Table 2.5). As illustrated in Figure 6.1 all plants inherited *PiS$_3$* as expected because it is the only allele that should come from the male parent as all pollen grains holding *PhS$_3$* are not able to grow because their RNA was degraded by *PhS$_3$-RNase*.

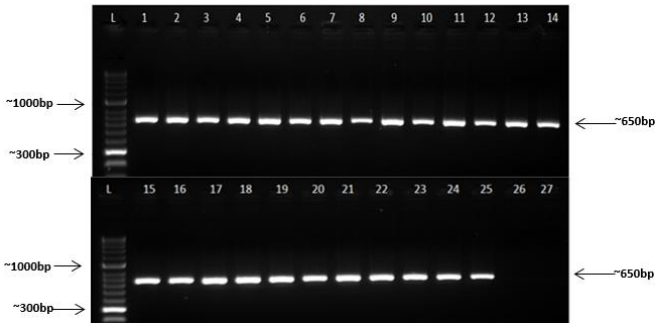


Figure 6.1 Identification of F_2 hybrid plants (N463) carrying *PiS$_3$-RNase*. L represents HyperLadder II (Bioline) and lanes 1-24 represent plants ID: N463.1 to N463.24. Lanes 25 - 27 represent: Positive control, Negative control for the *PiS3* allele and H_2O as a negative control for contamination, respectively.

Regarding the segregation for *PhS₃* and *PiS_d* in theory they should segregate 50:50 because they came through female. However, as shown in Figure 6.2, 16 plants out of 24 inherited *PiS_d:* N463.2, N463.4, N463.5, N463.6, N463.7, N463.8, N463.9, N463.10, N463.13, N463.15, N463.16, N463.18, N463.21, N463.22, N463.23 and N463.24. Consequently, the ratio for segregation is different from expected ratio and twice as many segregants carry the *PiS_d* compared to the *PhS₃* allele.

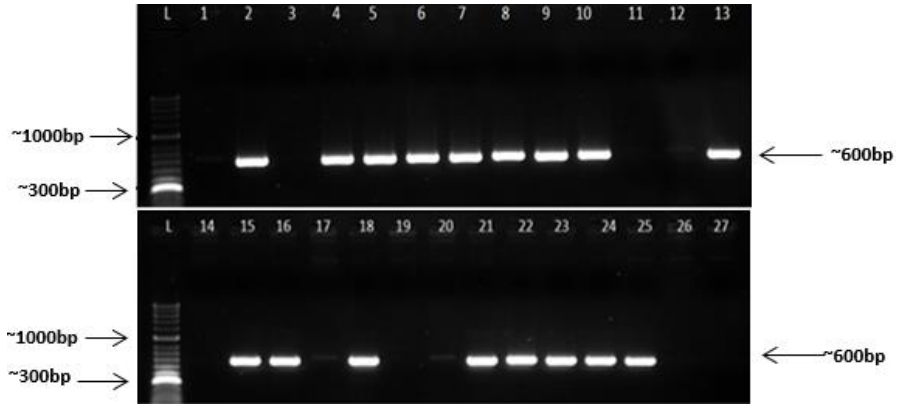


Figure 6.2 Identification of F₂ hybrid plants (N463) carrying *PiS_d-RNase*. L represent HyperLadder II (Bioline) and lanes 1-24 represent plants ID: N463.1 to N463.24, lanes 25 - 27 represent Positive control, Negative control for the *Pis_d* allele and H_2O as a negative control for contamination.

The remainder of the plants: N463.1, N463.3, N463.11, N463.12, N463.14, N463.17, N463.19 and N463.20 hold *PhS₃* as confirmed in Figure 6.3

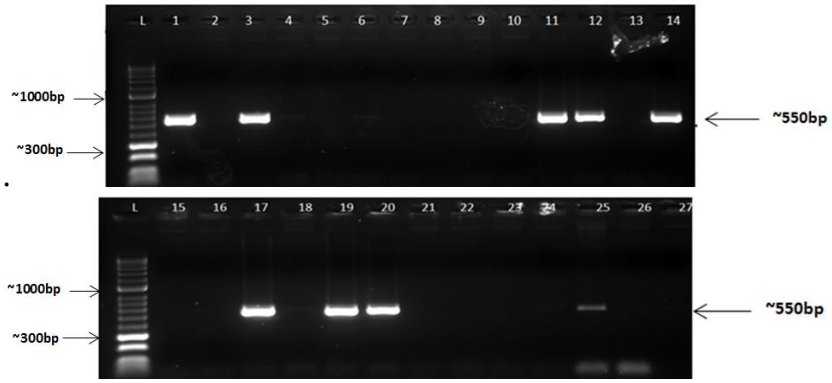


Figure 6.3 Identification of F₂ hybrid plants (N463) carrying *PhS₃-RNase*. L represents HyperLadder II (Bioline) , lanes 1-24 represent plants ID: N463.1 to N463.24, lanes 25 -27 represent Positive control , Negative control of *PhS₃* allele and H_2O as a Negative control for contamination respectively.

Self-pollination has been done to test the self-compatibility of plants (see section 2.3). A summary of the N463 plant genotypes and phenotypes is shown in Table 6.1

Table 6.1 Summary of F_2 hybrid genotypes and phenotypes for N463 family. All highlighted lines indicate self-compatible and self-incompatible plants hold *PiS_3PiS_d* highlighted in green and blue respectively**.**

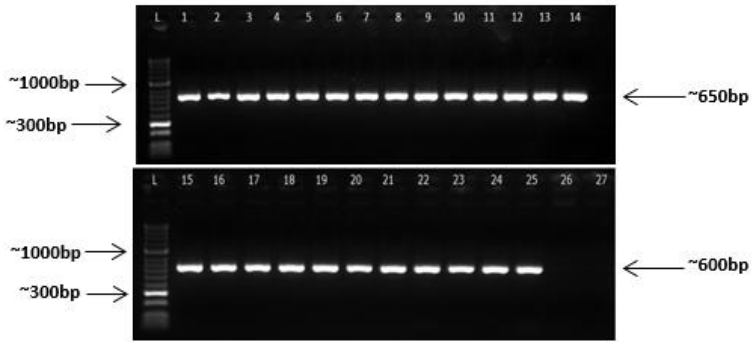
F2 Progeny Glasshouse ID	*PhS_3*	*PiS_3*	*PiS_d*	Genotype	Phenotype
N463.1	+	+	-	*PiS_3PhS_3*	SI
N463.2	-	+	+	*PiS_3PiS_d*	SC
N463.3	+	+	-	*PiS_3PhS_3*	SI
N463.4	-	+	+	*PiS_3PiS_d*	SC
N463.5	-	+	+	*PiS_3PiS_d*	SI
N463.6	-	+	+	*PiS_3PiS_d*	SC
N463.7	-	+	+	*PiS_3PiS_d*	SC
N463.8	-	+	+	*PiS_3PiS_d*	SC
N463.9	-	+	+	*PiS_3PiS_d*	SC
N463.10	-	+	+	*PiS_3PiS_d*	SC
N463.11	+	+	-	*PiS_3PhS_3*	SI
N463.12	+	+	-	*PiS_3PhS_3*	SI
N463.13	-	+	+	*PiS_3PiS_d*	SC
N463.14	+	+	-	*PiS_3PhS_3*	SI
N463.15	-	+	+	*PiS_3PiS_d*	SC
N463.16	-	+	+	*PiS_3PiS_d*	SC
N463.17	+	+	-	*PiS_3PhS_3*	SI
N463.18	-	+	+	*PiS_3PiS_d*	SC
N463.19	+	+	-	*PiS_3PhS_3*	SI
N463.20	+	+	-	*PiS_3PhS_3*	SI
N463.21	-	+	+	*PiS_3PiS_d*	SC
N463.22	-	+	+	*PiS_3PiS_d*	SI
N463.23	-	+	+	*PiS_3PiS_d*	SC
N463.24	-	+	+	*PiS_3PiS_d*	SI
X^2 Value for 1:1 Ratio	2.667				

X^2 of 3.84 is significant at the 0.05 probability level and one degree of freedom for segregation ratio 1:1

According to Table 6.1 it can be seen that all plants inherited the PiS_3 allele as it is the only allele coming from male parent as the PhS_3 was rejected. Regarding the PiS_d and PhS_3 alleles the segregation ratio is 16:8 this ratio is not significantly different from the expected ratio 1:1 as the X^2 Value for expected ratio 1:1 is 2.667 which is smaller than X^2 of 3.84 at the 0.05 probability level. The majority of plants that inherited the PiS_d became self-compatible (highlighted in green) which confirms the relationships between this allele and compatibility. However, three plants out of 16 from all plants inheriting PiS_d (highlighted in blue) were self-incompatible.

6.2.2 Identification of PiS_{k1}, PiS_d and PhS_v alleles and testing compatibility in N464 and N465 families.

The N464 family came from a cross between N451.6 (PhS_vPiS_d) and N450.8 (PhS_vPiS_{k1}), whereas the N465 family came from a cross between N451.5 (PhS_vPiS_d) and N454.1 (PhS_vPiS_{k1}) (see Table 2.5). In both families the parents which hold PiS_d were used as a female. Both crosses are expected to be semi-compatible because female and male parents share the PhS_v allele. As both families have the same background not all PCR data regarding the N465 family are shown to avoid repetition. It can be seen that in Figure 6.4 all plants inherited $PiSk_1$ as predicted.



Figuer 6.4 Identification of F_2 hybrid plants N464 carrying *PiS_{k1}-RNase.* L represents HyperLadder II (Bioline) and lanes 1-24 represent plants ID: N464.1 to N464.24. Lanes 25 -27 represent Positive control, Negative control for the PiS_{k1} allele and 27 represents H_2O as a negative control for contamination respectively.

As illustrated in Figure 6.5, in the N464 family the following plants inherited the *PiS$_d$* allele: N464.2, N464.3, N464.6, N464.8, N464.11, N464.12, N464.13, N464.19, N464.20, and N464.23.

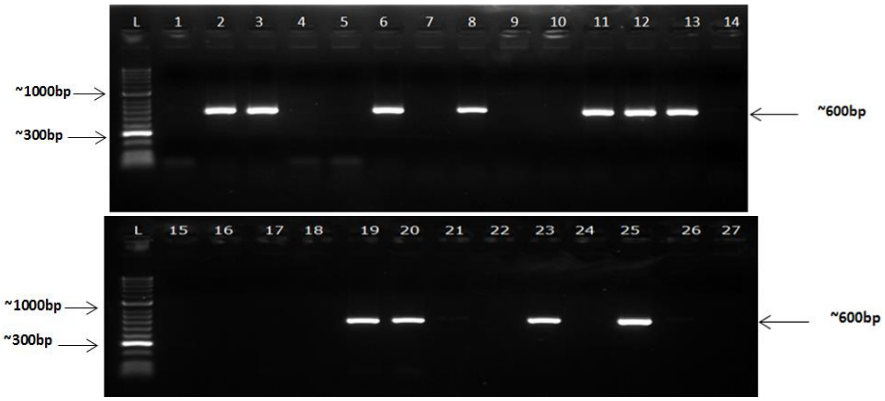


Figure 6.5 Identification of F$_2$ hybrid plants in N464 carrying *PiS$_d$-RNase*. L represents HyperLadder II (Bioline) and lanes 1-24 represent plants ID: N464.1 to N464.24 .Lanes 25 -27 represent Positive control, Negative control for the Pi$_{Sd}$ allele respectively and 27 represent H_2O as a Negative control for contamination respectively.

The reminder of the plants hold *PhS$_v$* allele as shown in Figure 6.6

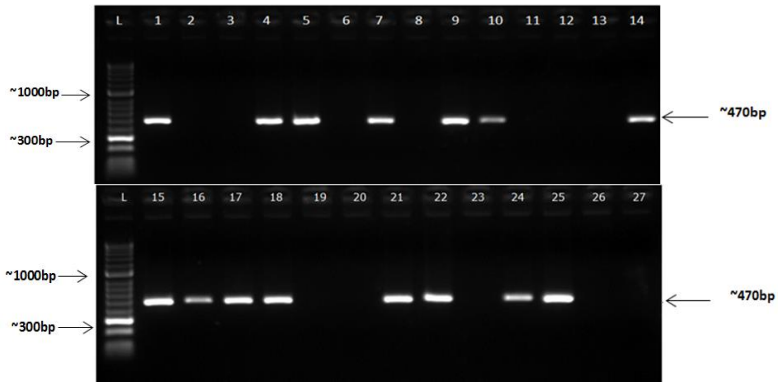


Figure 6.6 Identification of F$_2$ hybrid plants (N464) carrying *PhS$_v$-RNase*. L represent HyperLadder II (Bioline) and lanes 1-24 represent plants ID: N464.1 to N464.24. Lanes 25 -27 represent Positive control Negative control for the *Ph$_{Sv}$* allele and H_2O as a Negative control for contamination, respectively.

Self-pollination has been done to test the phenotypes for both families (see section 2.3). A summary of the plant genotypes and phenotypes is given in Tables 6.2 and 6.3.

Table 6.2 Summary of F_2 hybrid genotypes and phenotypes for the N464 family. All highlighted lines indicate self-compatible and self-incompatible plants hold $PiS_{k1}PiS_d$ highlighted in green and blue respectively.

F_2 Progeny Glasshouse ID	PhS_v	PiS_{k1}	PiS_d	Genotype	Phenotype
N464.1	+	+	-	$PiS_{k1}PhS_v$	SI
N464.2	-	+	+	$PiS_{k1}PiS_d$	SC
N464.3	-	+	+	$PiS_{k1}PiS_d$	SC
N464.4	+	+	-	$PiS_{k1}PhS_v$	SI
N464.5	+	+	-	$PiS_{k1}PhS_v$	SI
N464.6	-	+	+	$PiS_{k1}PiS_d$	SC
N464.7	+	+	-	$PiS_{k1}PhS_v$	SI
N464.8	-	+	+	$PiS_{k1}PiS_d$	SC
N464.9	+	+	-	$PiS_{k1}PhS_v$	SI
N464.10	+	+	-	$PiS_{k1}PhS_v$	SI
N464.11	-	+	+	$PiS_{k1}PiS_d$	SI
N464.12	-	+	+	$PiS_{k1}PiS_d$	SC
N464.13	-	+	+	$PiS_{k1}PiS_d$	SC
N464.14	+	+	-	$PiS_{k1}PhS_v$	SI
N464.15	+	+	-	$PiS_{k1}PhS_v$	SI
N464.16	+	+	-	$PiS_{k1}PhS_v$	SI
N464.17	+	+	-	$PiS_{k1}PhS_v$	SI
N464.18	+	+	-	$PiS_{k1}PhS_v$	SI
N464.19	-	+	+	$PiS_{k1}PiS_d$	SC
N464.20	+	+	-	$PiS_{k1}PiS_d$	SC
N464.21	+	+	-	$PiS_{k1}PhS_v$	SI
N464.22	+	+	-	$PiS_{k1}PhS_v$	SI
N464.23		+	+	$PiS_{k1}PiS_d$	SC
N464.24	+	+		$PiS_{k1}PhS_v$	SI
X^2 Value for 1:1 Ratio	0.667				

X^2 of 3.84 is significant at the 0.05 probability level and one degree of freedom for segregation ratio 1:1

Table 6.3 Summary of F_2 hybrid genotypes and phenotypes for the N465 family. All highlighted plants hold $PiS_{k1}PiS_d$ self-compatible and self-incompatible plants highlighted in green and blue respectively.

F_2 Progeny Glasshouse ID	PhS_v	PiS_{k1}	PiS_d	Genotype	Phenotype
N465.1	+	+	-	$PiS_{k1}PhS_v$	SI
N465.2	-	+	+	$PiS_{k1}PiS_d$	SC
N465.3	+	+	-	$PiS_{k1}PhS_v$	SI
N465.4	+	+	-	$PiS_{k1}PhS_v$	SI
N465.5	-	+	+	$PiS_{k1}PiS_d$	SC
N465.6	-	+	+	$PiS_{k1}PiS_d$	SC
N465.7	-	+	+	$PiS_{k1}PiS_d$	SC
N465.8	+	+	-	$PiS_{k1}PhS_v$	SI
N465.9	+	+	-	$PiS_{k1}PhS_v$	SI
N465.10	+	+	-	$PiS_{k1}PhS_v$	SI
N465.11	-	+	+	$PiS_{k1}PiS_d$	SC
N465.12	+	+	-	$PiS_{k1}PhS_v$	SI
N465.13	+	+	-	$PiS_{k1}PhS_v$	SI
N465.14	-	+	+	$PiS_{k1}PiS_d$	SC
N465.15	+	+	-	$PiS_{k1}PhS_v$	SI
N465.16	+	+	-	$PiS_{k1}PhS_v$	SI
N465.17	-	+	+	$PiS_{k1}PiS_d$	SC
N465.18	+	+	-	$PiS_{k1}PhS_v$	SI
N465.19	+	+	-	$PiS_{k1}PhS_v$	SI
N465.20	-	+	+	$PiS_{k1}PiS_d$	SI
N465.21	-	+	+	$PiS_{k1}PiS_d$	SC
N465.22	-	+	+	$PiS_{k1}PiS_d$	SC
N465.23	+	+	-	$PiS_{k1}PhS_v$	SI
N465.24	-	+	+	$PiS_{k1}PiS_d$	SI
X^2 Value for 1:1 Ratio	0.167				

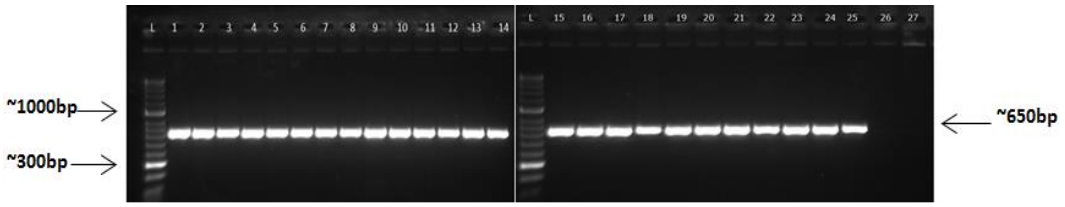
X^2 of 3.84 is significant at the 0.05 probability level and one degree of freedom for segregation ratio 1:1

According to Tables 6.2 and 6.3 all F_2 progeny inherited PiS_{k1} as expected. Regarding the observed segregation ratio for the PhS_v and PiS_d alleles in N464 family is 14:10. This ratio is not significantly different from the expected ratio 1:1 as the X^2 value for expected ratio is 0.667 which is smaller than X^2 of 3.84 at the 0.05 probability level. In case of N465 family, 12 and 13 plants hold PiS_d and PhS_v alleles respectively. This ratio is not significantly different from the predicted ratio 1:1 as the as the X^2 value 0.167 is smaller than X^2 of 3.84 at the 0.05 probability level.

In both families a total of 18 progeny inheriting PiS_d were self-compatibile (highlighted in green). However, a total of three plants were self-incompatible (highlighted in blue) despite inheriting this allele.

6.2.3 Identification of PiS_3, PiS_d and PhS_v alleles and compatibility test in the N467 family.

To further extend the co-segregation analysis of the PiS_d allele and self-compatibility an additional family arising from a semi-compatible pollination was examined. This family came from crossing between N451.6 (PhS_vPiS_d) and N454.2 (PhS_vPiS_3) (see Table 2.5). As N454.2 was used as a male, all plants in this family inherited PiS_3 (see Figure 6.7). All pollen holding Ph_{Sv} were rejected by the female parent N451.6.



Figuer 6.7 Identification of F_2 hybrid plants N467 carrying *PiS_3-RNase*. L represents Hyperladder II (Bioline) and lanes 1-24 represent plants ID: N467.1 to N467.24 and 25-27 represent positive control, negative control of the PiS_3 allele and H_2O as a Negative control for contamination, respectively.

In theory both PhS_v and PiS_d (coming from the female parent) should segregate 1:1. However, as illustrated in Figure 6.8, only 10 plants hold PiS_d which are N467.3,

N467.8, N467.10, N467.11, N467.14, N467.15, N467.17, N467.19, N467.20 and N467.23. Consequently the rest of the progeny should hold *PhSv* as shown in Figure 6.9.

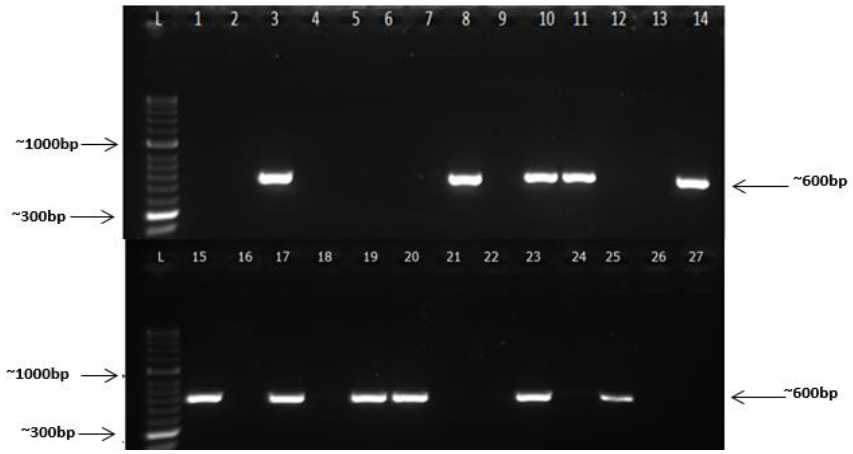


Figure 6.8 Identification of F_2 hybrid plants N467 carrying *PiS_d-RNase*. L represents Hyperladder II (Bioline) and lanes 1-24 represent plants ID: N467.1-N467.24 and 25-27 represent positive control, negative control for the *PiS_d* allele and H_2O as a negative control for contamination, respectively

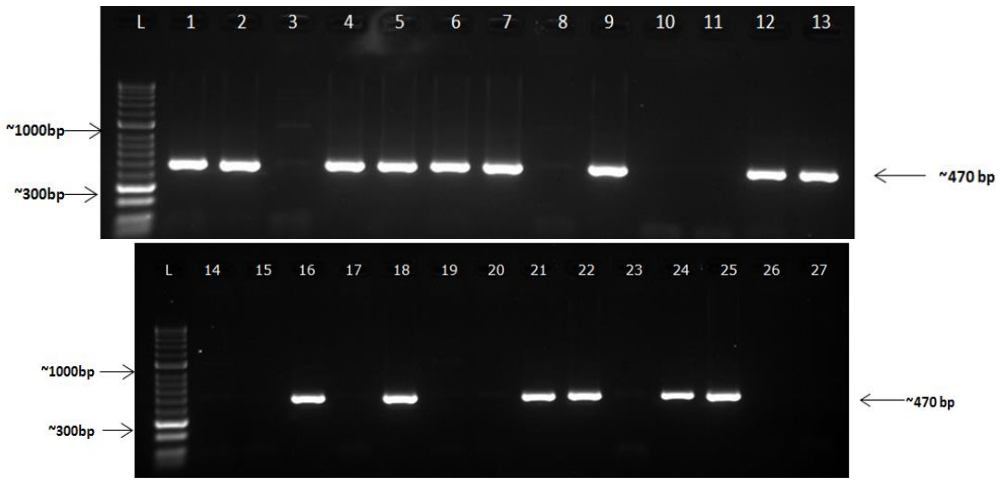


Figure 6.9 Identification of F_2 hybrid plants N467 carrying *PhS_v-RNase*. L represents Hyperladder II and lanes 1-24 represent plants ID N467.1 to N467.24 and 25, 26 represent positive and negative control of *PhS_v* allele respectively and 27 represent H2O as a negative control for contamination.

Self-pollination was performed to test all plants in the N467 family (see section 2.3).A summary of the plant genotypes and phenotypes is shown in Table 6.4

Table 6.4 Summary of F_2 hybrid genotypes and phenotypes for N467 families. All highlighted plants hold *PiS_3PiS_d* self-compatible and self-incompatible plants highlighted in green and blue respectively**.**

F_2 Progeny Glasshouse ID	*PhS_v*	*PiS_3*	*PiS_d*	Genotype	Phenotype
N467.1	+	+	-	*PhS_vPiS_3*	SI
N467.2	+	+	-	*PhS_vPiS_3*	SI
N467.3		+	+	*PiS_3PiS_d*	SC
N467.4	+	+	-	*PhS_vPiS_3*	SI
N467.5	+	+	-	*PhS_vPiS_3*	SI
N467.6	+	+	-	*PhS_vPiS_3*	SI
N467.7	+	+	-	*PhS_vPiS_3*	SI
N467.8		+	+	*PiS_3PiS_d*	SC
N467.9	+	+	-	*PhS_vPiS_3*	SI
N467.10	-	+	+	*PiS_3PiS_d*	SC
N467.11	-	+	+	*PiS_3PiS_d*	SC
N467.12	+	+	-	*PhS_vPiS_3*	SI
N467.13	+	+	-	*PhS_vPiS_3*	SI
N467.14	-	+	+	*PiS_3PiS_d*	SI
N467.15	-	+	+	*PiS_3PiS_d*	SC
N467.16	+	+	-	*PhS_vPiS_3*	SI
N467.17		+	+	*PiS_3PiS_d*	SC
N467.18	+	+	-	*PhS_vPiS_3*	SI
N467.19		+	+	*PiS_3PiS_d*	SC
N467.20		+	+	*PiS_3PiS_d*	SC
N467.21	+	+	-	*PhS_vPiS_3*	SI
N467.22	-	+	-	*PhS_vPiS_3*	SI
N467.23		+	+	*PiS_3PiS_d*	SC
N467.24	+	+	-	*PhS_vPiS_3*	SI
X^2 Value for 1:1 Ratio	0.667				

X^2 of 3.84 is significant at the 0.05 probability level and degree of freedom 1 for segregation ratio 1:1

Based on Table 6.4 all plants inherited *PiS*$_3$ as expected because it is the only allele inherited from the male parent due to semi-compatibility. The segregation ratio for the *PhS*$_v$ and *PiS*$_d$ alleles is 14:10 which is not significantly different from the predicted ratio 1:1 as the X^2 value 0.667 is smaller than X^2 of 3.84 at the 0.05 probability level. Most plants inherited *PiS*$_d$ were self-compatible (Highlighted in green) and only one plant (Highlighted in blue) was incompatible.

6.2.4 Reciprocal crossing to reveal the *PiS*$_d$ allele parent associated with self-compatibility.

As illustrated in Tables 6.1, 6.2, 6.3 and 6.4, the majority of F_2 plants inheriting the *PiS*$_d$ allele were self-compatible and only a few were self-incompatible. This observation was exploited to reveal whether the male or female part of this particular haplotype had lost function using reciprocal crosses (see section 2.3) and the results are summarised in Table 6.5.

Table 6.5 Reciprocal crosses results between SC and SI plants belonging to N463, N465 and N467 families.

Plant ID			Pollen Donor					
			N463.5 PiS_3PiS_d (SI)	N463.4 PiS_3PiS_d (SC)	N463.6 PiS_3PiS_d (SC)	N463.7 PiS_3PiS_d (SC)	N463.8 PiS_3PiS_d (SC)	N463.9 PiS_3PiS_d (SC)
N463.5	PiS_3PiS_d	(SI)	-	+	+	+	+	+
N463.4	PiS_3PiS_d	(SC)	-	+	+	+	+	+
N463.6	PiS_3PiS_d	(SC)	-	+	+	+	+	+
N463.7	PiS_3PiS_d	(SC)	-	+	+	+	+	+
N463.8	PiS_3PiS_d	(SC)	-	+	+	+	+	+
N463.9	PiS_3PiS_d	(SC)	-	+	+	+	+	+
Plant ID			Pollen Donor					
			N465.20 $PiS_{k1}PiS_d$ (SI)	N465.2 $PiS_{k1}PiS_d$ (SC)	N465.5 $PiS_{k1}PiS_d$ (SC)	N465.6 $PiS_{k1}PiS_d$ (SC)	N465.7 $PiS_{k1}PiS_d$ (SC)	N465.14 $PiS_{k1}PiS_d$ (SC)
N465.20	$PiS_{k1}PiS_d$	(SI)	-	+	+	+	+	+
N465.2	$PiS_{k1}PiS_d$	(SC)	-	+	+	+	+	+
N465.5	$PiS_{k1}PiS_d$	(SC)	-	+	+	+	+	+
N465.6	$PiS_{k1}PiS_d$	(SC)	-	+	+	+	+	+
N465.7	$PiS_{k1}PiS_d$	(SC)	-	+	+	+	+	+
N465.14	$PiS_{k1}PiS_d$	(SC)	-	+	+	+	+	+
Plant ID			Pollen Donor					
			N467.14 PiS_3PiS_d (SI)	N467.3 PiS_3PiS_d (SC)	N467.8 PiS_3PiS_d (SC)	N467.11 PiS_3PiS_d (SC)	N467.15 PiS_3PiS_d (SC)	N467.19 PiS_3PiS_d (SC)
N467.14	PiS_3PiS_d	(SI)	-	+	+	+	+	+
N467.3	PiS_3PiS_d	(SC)	-	+	+	+	+	+
N467.8	PiS_3PiS_d	(SC)	-	+	+	+	+	+
N467.11	PiS_3PiS_d	(SC)	-	+	+	+	+	+
N467.15	PiS_3PiS_d	(SC)	-	+	+	+	+	+
N467.19	PiS_3PiS_d	(SC)	-	+	+	+	+	+

Compatible cross is indicated by (+), and an incompatible cross is indicated by (-).

Based on the results shown in Table 6.5, in all three F_2 families (N463, N465 and N467) crossing five self-compatible plants using a self-incompatible sibling as the pollen donor resulted in no capsule. However, when the self-compatible plants were used as pollen donors, the self-incompatible line produced capsules. This observation indicates that in all three families the self-compatibility is due to a loss of pollen function.

6.3 DISCUSSION

From these results we can conclude that self-compatibility appeared in these F_2 families because the PiS_d allele alone had lost function and the remainder of the

segregating alleles (*PiS*$_3$, *PiS*$_{k1}$, *PhS*$_v$, *PhS*$_3$) are all associated with stable self-incompatibility and retained their function in F_1 and F_2 hybrids. This observation supports the hypothesis that self-compatibility appears because of *PiS*$_d$ allele in F_1 and F_2 hybrids.

It can be seen that in all four families the majority of plants inheriting the *PiS*$_d$ allele became self-compatible with the exception of seven segregants (N463.5, N463.22, N463.24, N464.11, N465.20, N465.24 and N467.14). These seven exceptions arose from a total of 57 segregants inheriting the *PiS*$_d$ allele and represent just 12.3% of progeny that share this genotype. However, appearing self-compatible and self-incompatible plants holding the same genotype was exploited to identify either male or female part in *PiS*$_d$ allele had lost function using reciprocal crosses. Based on the results shown in Table 6.5 the male part of *PiS*$_d$ allele had lost function.

A breakdown of self-incompatibility during controlled crosses has been reported by many researchers, they often claimed that it was caused by a change in S-allele. Some have proposed that mutation in an S-allele in either the female, male or both parts leads to Pseudo Self-compatibility (Lewis 1949; Lewis 1951; Pandey 1956; Brewbaker and Natarajan 1960; Denward 1963; Pandey 1970). In addition, Atwood (1942) claimed that several additive genes can determine Pseudo Self-compatibility, so it is a quantitative character which means there is the relationship between the S–allele and the amount of Pseudo Self-compatibility. Subsequently, Takahashi (1973) claimed that there is a substance in the style of a self-incompatible plant which

prevents the growth of pollen tubes after self-pollination and Pseudo Self-compatibility can appear once the activity of this substance is decreased.

Classical breeding experiments revealed other factors which cooperate with the S-locus to determine SI and they are called modifier loci because they affect SI or they modify the response of SI and yet they are unlinked to the S-locus (Mather, 1943; Martin, 1961., 1968; Townsend, 1969; Takahashi, 1973; Kaothien-Nakayama *et al*, 2010). Such modifier genes or loci can influence the function of either the pistil or pollen components of the S-locus. In this thesis the confirmation that the breakdown of self-incompatibility resulted from the loss of pollen part function in the PiS_d allele indicates it could be related with a pollen modifier gene which has not been identified yet.

McClure *et al* (2000) classified these modifier genes into three groups. Group 1 factors directly influence the expression of the S-locus. Group 2 and 3 factors are required for pollen rejection. Mutation in Group 1 or 2 factors could lead to change from SI to SC behaviour (Cruz-Garcia *et al*, 2003). The HT-B protein and the 120 kDa glycoprotein are examples of two pistil modifier genes. These are two style-expressed proteins that have been shown to be required for self–incompatibility using RNAi (McClure *et al*, 1999 and Hancock *et al*, 2005). Recently Tovar-Méndez (2016) revealed that HT proteins are involved in both S-RNase-dependent and S-RNase-independent pollen rejection.

Regarding the Rosaceae family, recently the mechanism that causes self-compatibility was characterised by Li *et al* (2016b) in apple cultivar, 'CAU-1' (S_1S_9) using self-incompatible line, 'Fuji' (S_1S_9) as a control. It was revealed that the expression level of the pollen-expressed hexose transporter, MdHT1 which interact with S-RNases in 'CAU-1' is significantly lower than in 'Fuji' pollen tubes. This finding supports the view that S-locus external factors are crucial for SI in Rosaceae family. It is possible that during SI response MdHT1 participate in producing S-RNase, consequently reducing its expression might lead to preventing the releasing of S-RNase into the cytoplasm of pollen tubes and protecting pollen from the cytotoxicity of S-RNase which cause compatibility.

In this project, the appearance of self-compatibility in F_1 hybrids could result from epigenetic changes in expression of the SLF genes belonging to the PiS_d allele or haplotype or alternatively modifier genes. In plant evolution, interspecific hybridization has played an important role as a major agent of rapid speciation. After crossing between self-incompatible plants, this prezygotic barrier must be overcome to allow self-pollination and useful information regarding this phenomenon was reported by Nasrallah *et al* (2007). They crossed self-incompatible and compatible species in each of two crucifer genera and analysed loss of SI in synthetic hybrids, *Arabidopsis thaliana–lyrata* and *Capsella rubella–grandiflora*. They revealed that, in both cases, loss of SI upon interspecific hybridization was due to reversible changes in expression of the S-locus recognition genes, *SRK* or *SCR* (Nasrallah *et al*, 2007).

CHAPTER 7 : GENERAL CONCLUSION AND DISCUSSION

7.1 GENETIC BREAKDOWN OF SELF INCOMPATIBILITY

As the main aim of this project was to control self-incompatibility in various genetic backgrounds of Petunia, initially three stable self-incompatible lines for *Petunia hybrida* (*PhS$_3$PhS$_v$*) and *Petunia inflata* (*PiS$_3$PiS$_{K1}$*) and (*PiS$_3$PiS$_d$*) were generated. In order to test incompatibility in (*P.hybrida* x *P.inflata* F_1 hybrid) they were crossed and it was noticed that all plants inheriting the *PiS$_d$* allele` became self-compatible. This observation was further confirmed using cross pollination between semi-compatible plants to obtain a 1:1 segregation for *PiS$_d$* allele in F_2 hybrid and four families were raised N463, N464, N465 and N467.

Interestingly, it was observed that all plants lacking the *PiS$_d$* allele were self-incompatible, which confirms the association of *PiS$_d$* with compatibility. Moreover, most plants inheriting the *PiSd* allele were self-compatible with just a few exceptions (N463.5, N463.22, N463.24, N464.11, N465.20, N465.24 and N467.14). In order to reveal whether male or female parts of the *PiS$_d$* haplotype had lost function, reciprocal crosses were done between self-compatible and self-incompatible plants holding the same genotypes in F_2 hybrid. It was observed that using only self-compatible plants as a pollen donor causes compatibility which means *PiS$_d$-RNase* remains functional and the pollen function of the *PiS$_d$* haplotype is lost.

Researchers have attempted to understand the breakdown of self-incompatibility in Petunia in previous studies. For instance, Ai *et al* (1991) crossed self-compatible *Petunia hybrida* (S_xS_0) and self-incompatible *Petunia inflata* (S_2S_2). They noticed that in F_1 hybrids all plants of the S_2S_0 genotype were self-compatible which indicat that the S_0 allele was non-functional. However, an equal number of compatible and incompatible plants holding S_2S_x were obtained. This observation strongly indicates that the presence of a functional allele is not sufficient for self-incompatibility (Ai *et al*, 1991).

As defined by Ai *et al* (1991) unlinked modifiers can affect incompatibility and this can be seen in Solanaceae and Brassica systems. Initially, the existence of modifier loci was revealed from classical breeding experiments. These factors are called modifiers because they affect SI or they modify the response of SI and they are unlinked to the S-locus and can influence pistil or pollen components. In GSI system these modifiers were reported by Mather (1943); Martin (1961., 1968); Townsend (1969); Takahashi (1973); Kaothien-Nakayama (2010) and in SSI, THL1 , THL2 and ARCI modifiers were revealed by Bower *et al* (1996) and Goring (2000) respectively.

With regard to pistil modifier SI genes, McClure *et al* (1999) and Hancock *et al* (2005) have identified both HT-B and the 120 kDa glycoprotein. These are two style-expressed proteins required for self-incompatibility. Self-incompatible plants become self-compatible and lose their ability to reject pollen following down regulation of these proteins using the antisense technique (McClure *et al*, 1999). Similar observations were recorded with down regulation of the 120 KDa protein in

Nicotiana using RNAi (Hancock *et al*., 2005). The 120kDa glycoprotein was found in the extracellular matrix (ECM) of the style where it is taken up by the growing pollen tubes (Lind *et al*, 1994, 1996). Cruz-Garcia *et al* (2005) revealed a direct interaction between the120 kDa glycoprotein and S-RNases by using an S-RNase affinity column. In compatible pollen tubes, both proteins accumulate with S-RNases in a vacuole-like compartment. According to Goldraij *et al* (2006), in incompatible pollen tubes the 120 kDa protein can also be detected during early stages of the SI response.

Further approaches illustrated the importance of HT-B by analysing various SC and SI species of tomato and its cultivars. Kondo and co-workers (2002a, 2002b) reported that SC tomato species had different defects in expression of both S-RNase and HT-B that prevented expression of both normal proteins.

Busot *et al* (2008) revealed the proteinase inhibitor, (*NaStEP*) *Nicotiana alata* Stigma-Expressed Protein as a strong modifier gene candidate using immunohistochemical and protein blot analyses, which is highly expressed in the stigma of self-incompatible *Nicotiana* species but not in self-compatible *spp*. In addition, the activity of NaStEP was illustrated by Jiménez-Durán *et al* (2013). *NaStEP* is considered as a novel pistil-modifier gene, both compatible and incompatible pollen tubes are able to take it up, but its suppression in *Nicotiana spp.* transgenic plants disrupts S-specific pollen rejection. Furthermore, pollination of the NaStEP-suppressed pistils with either compatible or incompatible pollen led to reduced HT-B levels within the pollen tubes. However, in wild-type self-incompatible *N. alata* HT-B degradation occurs preferentially in compatible

pollinations. Consequently, the stability of HT-B inside pollen tubes during the rejection response needs the presence of NaStEP, but the underlying mechanism is currently unknown (Jiménez-Durán *et al*, 2013).

A candidate for pollen SI modifier genes, *SSK1* (SLF-interacting SKP1-like1) was identified. *SSK1* was revealed to be a homolog of *SKP1* by a yeast two-hybrid system screening of *Antirrhinum hispanicum* pollen cDNA library using AhSLF-S2 as bait (Huang *et al*, 2006). Subsequently Zhao *et al* (2010) identified *PhSSK1* (*Petunia hybrida* SLF-interacting Skp1-like1) which is expressed specifically in pollen, equivalent of AhSSK1 in *Antirrhinum hispanicum* and acts as an adaptor in an SCF (Skp1-Cullin1-F-box) SLF complex, but they were not able to confirm the role of *AhSSK1* in self-incompatibility as PhSSK1-RNAi transgenic plants remained self-incompatible which indicates that *SSK1* is not considered as a pollen modifier. Till now the pollen modifier gene has not been identified.

In spite of the fact that different molecules are proposed as the candidate Cullin1 as another component of SCF^{SLF}, the exact results have not confirmed. Recently, Kubo *et al* (2016) identified five Cullin1s from Petunia pollen and only PhCUL1-P was co-immunoprecipitated with S_7-SLF2. According to an *in vitro* protein-binding assay PhSSK1 specifically, forms a complex with PhCUL1-P in an SLF-dependent manner. In cross-compatible pollination, it was noticed that the fertility of transgenic pollen was suppressed by Knockdown of PhCUL1-P. This result confirmed that SCF^{SLF} selectively uses PhCUL1-P.

Co-immunoprecipitation Co-IP) and mass spectrometry (MS) were used to identify PiCUL1-P (a pollen-specific Cullin1), PiSSK1 (a pollen-specific Skp1-like protein) and PiRBX1 (an Rbx1) in the SCF complex in *Petunia inflata* which mediates ubiquitination of protein substrates for degradation by the 26S proteasome. It was revealed that all complex components except Rbx1 have evolved in SI (Li *et al*, 2014). The same techniques with some modification were recently used by Li *et al* (2016c) and reported that all 17 types of *SLF*s (*SLF1* to *SLF17*) and an SLF-like protein, SLFLike1 are co-immunoprecipitated with PiSSK1:FLAG:GFP in *Petunia inflata.*

We have confirmed that the breakdown of self-incompatibility associated with PiS_d allele in F_1 hybrid resulted from the pollen part. This is based on the use of self-incompatible plants as a pollen donor for pollination of self-compatible plants resulting in no capsule. It is evident that pistil modifier genes are functional in both self-compatible and incompatible plants and there is no relation between compatibility and pistil modifier genes. However, it is possible a modifier gene in pollen has lost function and causes compatibility, although till now it has not been identified. One argument against this is that the PiS_d segregation modifier is S-linked.

In addition, the appearance of self-compatibility in F_1 hybrids may result from epigenetic factors which can lead to change in expression of the SLF genes belonging to the PiS_d allele that determines specificity in the SI response. Wright (2004) compared self-compatible (S_0S_0) and self- incompatible (S_bS_b) lines of *Petunia hybrida* in order to understand the molecular basis of self-compatibility as

both S_b and S_0 alleles are identical. He concluded that the compatibility was not caused by to duplication or deletion of S-RNase but was related to the methylation differences between two alleles.

Regarding the reasons behind the breakdown of self-incompatibility in other species, Bernatzky et *al* (1995) crossed self-compatible *Lycopersicon esculent* with self-incompatible Lycopersicon *hirsutum*; they noticed that all F_1 hybrids plants were self-incompatible despite containing one allele from *Lycopersicon hirsutum*. However, in progeny raised from backcrossing of F_1 hybrids and *Lycopersicon hirsutum* a strong correlation between the presence of the S allele from *L. hirsutum* and self-incompatibility was observed. Surprisingly, they noticed that this relationship was uncoupled in some plants that were homozygous for the functional allele, from *Lycopersicon hirsutum* but were self-compatible. This observation means functional *S*-allele haplotypes *per se* are not sufficient for incompatibility and there may be a mutation in the background or modifier not that leads to compatibility and it was not very clear that time.

In spite of the fact that the Rosaceae family is phylogenetically quite distinct from the Solanaceae family, they share the same molecule class of protein as the GSI pistil determinant (Chase *et al*., 1993; Igic and Kohn, 2001). There are several important fruit crop plants that belong to Rosacea family such as apple, pears, quinces, apricots, plums, cherries, peaches, raspberries, loquats, strawberries and almonds and some of them are self-incompatible. As incompatibility causes problem in commercial orchards, researchers have attempted to understand the reason behind the breakdown of self-incompatibility in order to exploit it in breeding programme.

Ushijima *et al* (2004) and Sonneveld *et al* (2005) observed that pollen of Prunus self-compatible mutants can lose its function as a result of mutations, insertions, deletions or rearrangement in *S*-haplotype-specific F-box (SFB) genes.

Although S-locus products (S-RNase and F-box proteins) are essential for the gametophytic self-incompatibility in the Rosaceae family, S-locus unlinked factors are also required for self-incompatibility as in the Solanaceae family. A breakdown of self-incompatibility is associated with a pollen-part mutation unlinked to the S-locus in apricot (*Prunus armeniaca* L.) cv. 'Canino' (Zuriaga *et al*, 2012), which means identification of the modifier genes in this family can play an important role in overcoming self-incompatibility. Interestingly, a marker linked to self-compatibility, EMPaS02, in *Prunus avium* (sweet cherry) was identified by Cachi and Wünsch (2011) and the map for mutated modifier gene M-locus in *P. armeniaca* (apricot) has been reported by Zuriaga *et al*. (2012).

7.2 PETUNIA TRANSFORMATION EFFICIENCY

In order to control self-incompatibility in Petunia, *Petunia hybrida* holding the PhS_3 and PhS_v alleles was transformed with four constructs (AhSLF-S2, AhSLF-S1E, AhSLF-S2C and AhSLF-S4D) driven by the LAT52 promoter and obtained from Prof. Y. Xue (Chinese Academy of Sciences, Beijing). *E. coli* strain DH5 α (DH5813) and Agrobacterium strain (LBA4404) which were both obtained from the Biosciences Genomics Facility-The University of Nottingham were used in this study. After transformation of *E. coli* with binary vectors, the correct transformed colonies were selected using genomic PCR and their stability was confirmed by

sequencing the extracted plasmid. Subsequently, they were transformed into *Agrobacterium* strain (LBA4404) and the correct transformed colonies were identified using genomic PCR. The protocol developed by van der Meer (1999) was used for Agrobacterium -Mediated Transformation of *Petunia* Leaf Discs.

Different numbers of transgenic plants were obtained from each construct. Initially six and three transgenic plants for *AhSLF-S2* and *AhSLF-S1E* respectively and only only transgenic plant for each of *AhSLF-S2C* and *AhSLF-S4D* were obtained following several experiments from a total of more than 900 explants were used for each constructs. These results are significantly different from the van der Meer protocol (1999) as she claimed 20 transgenic lines can be obtained from 100 explants. The reason for this in fact is due to the use of *Petunia hybrida* c.v. W115 (Mitchell) by van der Meer protocol (1999) which is relatively easy to transform. Much lower efficiencies were reported for an S_VS_V stock (SI) compared to a standard cultivar V26 (Harbord *et al*, 2000).

Interestingly, it was noticed in this study that by increasing the inoculation period from 3 days to 6 days the efficiency was significantly increased. 13 and 20 Kanamycin resistant plants were obtained for AhSLF-S4D and AhSLF-S2C respectively. One possible reason for this may be that the absence of both antibiotics (Kanamycin and Carbenicillin) reduced the stress on the plants during the inoculation period. The presence of both plant growth regulators, Benzylaminopurine (BAP) (a plant growth hormone that initiates cell division), and 1-Naphthaleneacetic acid (NAA) (essential hormone to the plants survival) can lead to the appearance and

development of more calli. Moreover, Agrobacterium can have more chance to grow and infect explants in the absence of Carbenicillin.

7.3 COMPARISON OF PETUNIA AHSLF TRANSGENICS WITH RELATED STUDIES

Based on the competitive interaction phenomenon which leads to breakdown of self-incompatibility as a result of duplication of *S*-allele, *Petunia hybrida* (*PhS*$_3$*PhS*$_v$) was transformed with four constructs (*AhSLF-S2*, *AhSLF-S1E*, *AhSLF-S2C* and *AhSLF-S4D*) and different numbers of transgenic plants were obtained. They were identified by genomic PCR using an M13 primer with a transgene specific primer to ensure the integrity of the transgenes. The transgene expression was tested using gene specific primers (RT-PCR) and qPCR, the transgenes were expressed successfully in most of them.

However, after testing the transgenic plant phenotypes using self-pollination they all remained self-incompatible. Previously, *Petunia hybrida* (*PhS*$_3$*PhS*$_3$) was transformed with *AhSLF-S2* by Qiao *et al* (2004b) and they observed the breakdown of self-incompatibility in five transgenic plants. Consequently, we can conclude that different genotypes of *Petunia hybrida* have different responses to the heterologous transgenes. In order to understand the reason behind this observation, more analysis has been done and some plants transgenic for *AhSLF-S2* and *AhSLF-S1E* were bud pollinated to analyse T_1 generation. However, no relation was observed between Petunia genotype and the transgenes as all plants that inherited *AhSLF-S2* remained incompatible.

Interestingly, it was noticed that one plant (N471.1) which inherited AhSLF-S1E became self-compatible and reciprocal crosses confirmed competitive interaction which means compatibility resulted from heteroallelic pollen. Based on this observation a hypothesis is proposed for the relationship between the copy number and phenotype. According to this hypothesis transgenic plants remained self-incompatible because they contained only one copy of the transgene or in other words they are hemizygous for the transgene. In the case of N471.1 we proposed that the compatibility is a result of homozygosity for the transgene.

This hypothesis was tested using different methods. Firstly, seeds collected from N471.1 were screened with Kanamycin and they were all 100% resistant which confirms the homozygosity in N471.1 plant. Meanwhile, after self-pollination of 14 plants (T_2), it was observed that they are all self-compatible and reciprocal crosses confirmed that the compatibility resulted from heteroallelic pollen. Secondly, N471.1 was crossed with *Petunia inflata* and seeds collected were all Kanamycin resistant which another confirmation of homozygosity in N471.1 is. Furthermore, 24 F_1 hybrids plants were self-pollinated and all of them were self-incompatible which indicates compatibility only appears in the presence of two copies of the transgene.

The hypothesis was further tested by crossing other plants belonging to N471 with *Petunia inflata*. Surprisingly, according to our results obtained from screening F_1 hybrid seeds using Kanamycin some of the generated families gave 100% resistant results. Although this point caused doubts about the accuracy of our hypothesis, the evidence for the relation between the homozygosity of N471.1 plant can still be considered as we obtained self-compatible plants in the T_2 generation and not in F_1

hybrids, which means once the transgene segregated to one copy in F_1 hybrids does not affect incompatibility. In addition, we have compared the level of the transgene expression in all transgenic plants belonging to the N471 family that gave 100% Kanamycin resistant progenies using qPCR. Interestingly, N471.1 plant gave the highest level of expression. Consequently, we can confirm that there is a relationship between homozygosity, high transgene expression and self-compatibility in the N471.1 plant. Regarding the other plants belonging to N471.1 which gave 100% Kanamycin resistant progenies and they were all self-incompatible, they gave various levels of expression. These results can be interpreted as the transgene expression was inhibited because of epigenetic factors which can stably alter the expression of the transgenes and result in incompatibility. DNA methylation and histone modifications are two molecular mechanisms that mediate epigenetic phenomena. Riggs (1975) and Holliday *et al* (1996) proposed that the stable maintenance of a particular gene expression pattern through mitotic cell division might result from DNA methylation. Subsequently, this concept has been supported by ample evidences and DNA methylation is considered to be the main contributor to the stability of gene expression states. DNA methylation establishes a silent chromatin state by collaborating with proteins that modify nucleosomes (Wolffe and Matzke, 1999; Urnov *et al*, 2001). Previously the effect of methylation states on plant response to the transgene has been observed. For instance, Meyer *et al* (1987) transformed *Petunia hybrida* mutant RL01 white with maize A1 cDNA which is driven by the CaMV 35S RNA promoter and leads to the production of pelargonidin derivatives, resulting in a brick red flower phenotype. Differential expression patterns, namely red, variegated, and the 'white' were observed among the transgenic petunia plants. The transgene expression was investigated by analysing the pigmentation pattern of

the petals. It was noticed that there is an inverse relationship between number of integrated A1 copies and the uniformity of floral pelargonidin pigmentation. In addition, a relationship between the methylation status of the 35S RNA promoter and the instability of the floral pelargonidin coloration was found. It was concluded that the status of promoter methylation controlling the expression of the A1 gene seems to be influenced by the copy number and the chromosomal position of the transferred gene (Linn *et al*, 1990).

Regarding controlling self-incompatibility in *Petunia inflata* competitive interaction was observed by Sijacic *et al* (2004). They transformed three different genotypes S_1S_1, S_1S_2 and S_2S_3 with PiSLF2. They noticed that the presence of PiSLF2 in S_1 and S_3 pollen grains and not S_2 pollen grains caused the breakdown of SI. The results were confirmed via progeny analysis and it was observed that self-pollination of a PiSLF2/ S_2S_3 transgenic plant produced S_2S_3 and S_3S_3 and not S_2S_2 progeny plants. All progeny plants carried the PiSLF2 transgene. In addition, Kubo *et al* (2010) transformed three Petunia genotypes S_5S_7, S_7S_{11}, and S_5S_{19} plants with S7-SLF1 transgene and all plants remained self-incompatible. However, when they transformed the different genotype S_7S_9 with the same transgene (S7-SLF1) all plants exhibited breakdown of SI and reciprocal crosses with wild-type S_7S_9 plants showed that S7-SLF1 caused breakdown of pollen, but not of the style. In addition from progeny analysis they observed only S_7S_9 and S_9S_9 plants and not S_7S_7 which indicates that only the S_9 allele carried S7-SLF1. They also noticed that the S7-SLF1 causes competitive interaction in the S17 haplotype. Competitive interaction in *Petunia inflata* was further tested by introducing S2-SLF1 and S3-SLF1 and they observed only S2-SLF1 causes compatibility in the S3 haplotype (See Figure 1.10).

Comparing these observations with our results, we can conclude that hybrid nature may make epigenetic differences which can lead to different response for the transgene.

In conclusion, self-incompatibility can be exploited in many breeding programmes. Understanding the ideal methods for overcoming this phenomenon can be obtained through more research at the molecular level. Petunia can be used as a very good model for studying self-incompatibility and the methods of overcoming this phenomenon. The obtained information can be transferred to the other plants belonging to the Solanaceae and the Rosaceae family. Controlling self-incompatibility is very important in fruit crops belonging to the Rosaceae family. Attempts have been made to manipulate the SFB gene. Ushijima *et al* (2004) and Sonneveld *et al* (2005) identified that mutations, insertions or deletions in S-haplotype-specific F-box (SFB) genes can lead to a loss of pollen-S function in Prunus self-compatible mutants. Moreover, it was noticed that the breakdown of self-incompatibility in apricot (*Prunus armeniaca*), Carrot (SCSC) and Canino (S2SC) is associated with insertion of 358-bp in the SFBC gene (Vilanova *et al*, 2006).

CHAPTER 8 : FUTURE WORK

According to all results obtained from this project to date, there are several points worthy of further investigation:

1-According to my studies there is a strong relationship between the *PiS$_d$* allele and a breakdown of self-incompatibility in F_1 hybrids (*Petunia hybrida* X *Petunia inflata*). It has been confirmed that this breakdown resulted from the pollen part. More analysis could be done in order to understand whether this breakdown is because of specific SLF genes or pollen modifier genes. In spite of the fact that currently the Petunia genome is sequenced by Bombarely *et al* (2016), cloning the pollen modifier gene is still challenging as it has not been identified in other plants. However, SLF genes were identified for *PiS$_3$* haplotype (accession number M67991) by Williams *et al* (2014b), according to Blast search and alignment we observed that it is identical to our *PiS$_3$* haplotype (accession number AB094599.1). Consequently, it is possible to follow the methods described by Williams *et al* (2014b) for cloning all SLF genes in *PiS$_d$* haplotype but it would take considerable effort. Moreover, all SLFs in self-compatible and incompatible lines need to be compared to identify any change in SLF gene expression. This will require the development of a suite of SLF gene-specific RT-PCR primers. Understanding the reason behind the breakdown of self-incompatibility in *PiS$_d$* allele might provide useful information that can be used when producing F_1 hybrid seeds in ornamental plants using strategies similar to those that are well established in Brassica.

2-Previously the breakdown of self-incompatibility was observed after transforming *Petunia hybrida* genotype *PhS$_3$PhS$_3$* with AhSLF-S2 which confirms a competitive

interaction phenomenon as described by Qiao *et al* (2004b). However, in this project *Petunia hybrida* genotype PhS_3PhS_v was transformed with four constructs and no competitive interaction was observed in the T_0 generation. In the beginning of the project *SLF* genes for these two haplotypes were not identified. Interestingly, Kubo *et al* (2015) identified 16–20 SLF genes belonging to eight different S-haplotypes of Petunia. According to Blast search and alignment we noticed that PhS_5*-RNase* (accession number AB016522), PhS_9*-RNase* (accession number AB016523) are identical to PhS_v*-RNase* (accession number AJ271062.1) and PhS_3*-RNase* (accession number AJ271065.1) respectively, which means currently sequences for all *SLF* genes are available. Consequently, it is possible to make alignments for all *SLF* and design primers based on untranslated regions to compare the level of expression for all *SLF* using qPCR and test for any change in the expression.

3-In this thesis it is proposed that there is a relationship between homozygosity and self-compatibility in the N471.1 plant. This concept could be applied to the remainder of the AhSLF constructs. F_1 hybrid transgenic plants holding different genotype and hemizygous for the transgene were crossed and plants were grown. Consequently, homozygous plants could be analysed for competitive interaction. Any breakdown of self-incompatibility in homozygous lines could be tested for a correlation with AhSLF expression level as has been described for AhSLFS1E

4- There are several important fruit crops which belong to the Rosaceae family such as apple, pears, quinces, apricots, plums, cherries, peaches, raspberries, loquats, strawberries and almonds and some cultivars are self-incompatible. As controlling self-incompatibility in Rosaceae family is considered to be important in horticulture,

using S-heteroallelic pollen which causes competitive interaction can be exploited for this purpose as both Solanaceae and Rosaceae family share GSI. Previously, Huang *et al*., (2008) observed the competitive interaction between two of the functional alleles in Chinese cherry cultivar "NC" which led to compatibility. However, the transgenic plant phenotypes observed in this project might give a clear idea about the complication and challenges that may be faced in the future research regarding manipulation of this important trait in the Rosaceae family.

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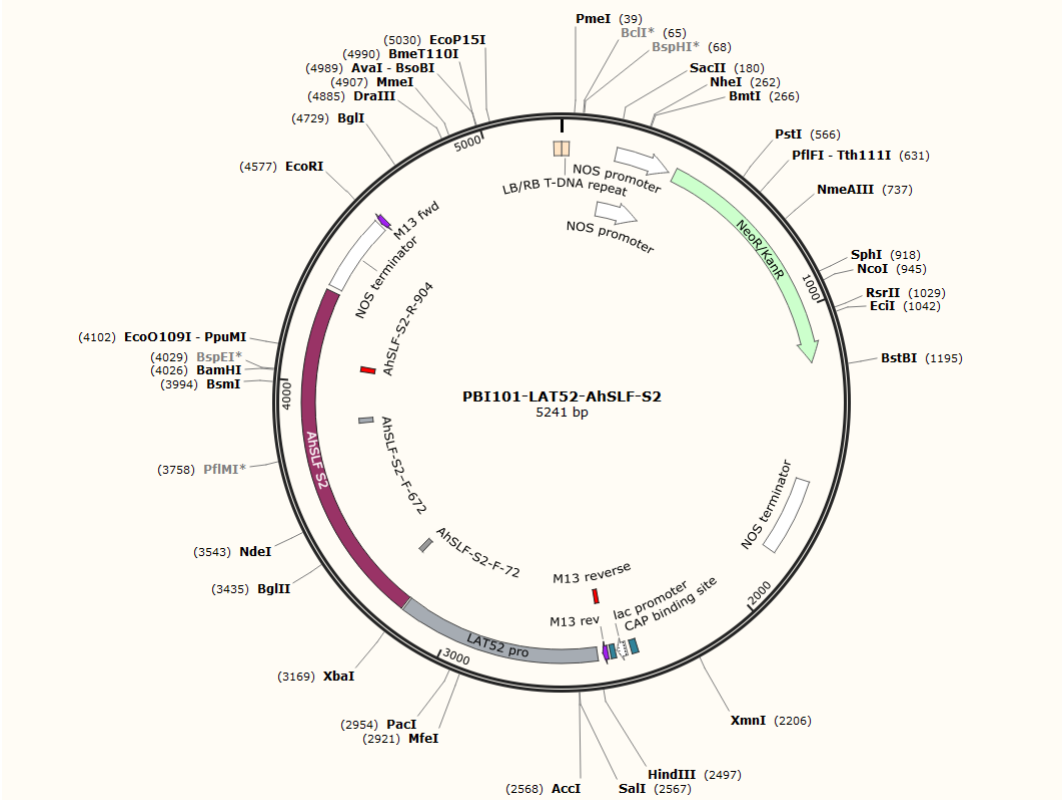
APPENDICES

APPENDIX 1 T-DNA MAPS

PBI101-LAT52-AhSLF-S2



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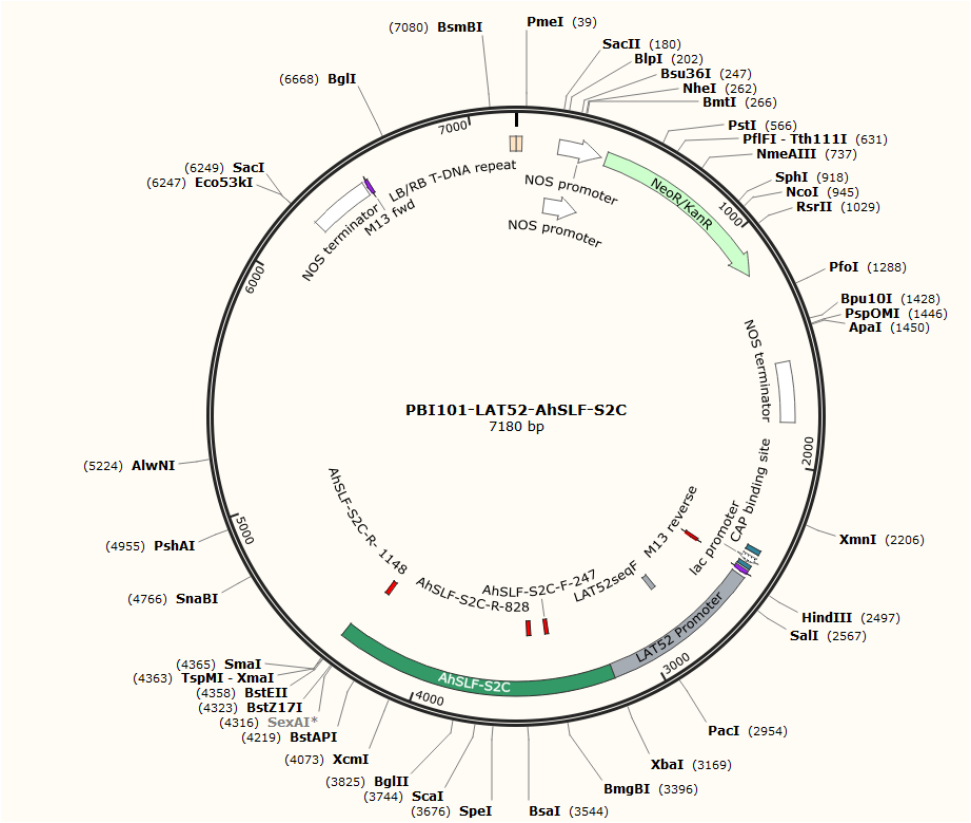

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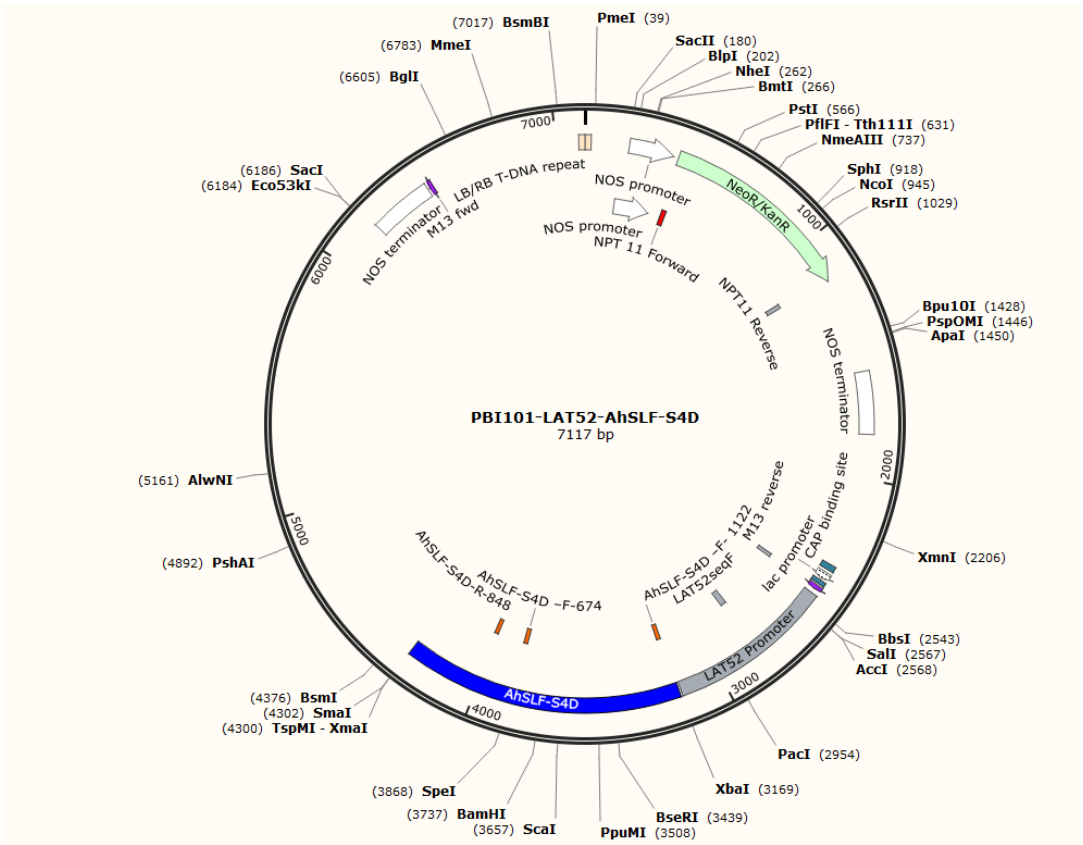
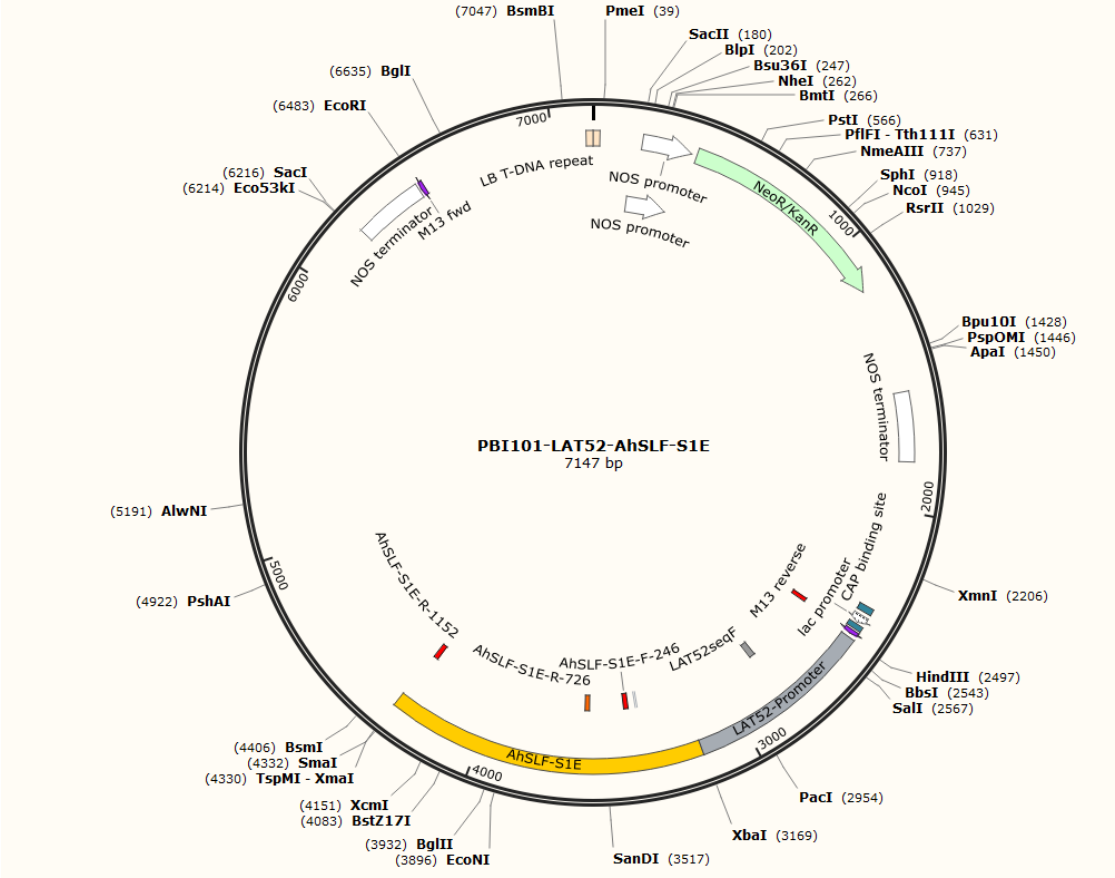


PBI101-LAT52-AhSLF-S1E



APPENDIX 2 MEDIA RECIPES

lOX TBE buffer

108 g Tris base

55 g Boric acid

9.12 g EDTA

PH 8

Make up to 1 litre with distilled water; dissolve well and store at room temperature.

LB broth

10 g Peptone

5 g Yeast extract

10 g NaCl

Make up to 1 litre with distilled water. Autoclave and store at room temperature

LB Agar

10 g Peptone

5 g Yeast Extract

10 g NaCl

15 g Agar

Make up to 1 litre with distilled water. Autoclave and store at room temperature

APM media

5 g Yeast extract

0.5 g Casein

8 g Mannnitol

2 g Ammonium Sulphate

5 g NaCl

Make up to 1 litre with distilled water. Autoclave and store in the fridge.

Co-Cultivation Medium (CCM)

4.4 g MS salts and vitamins

30 g sucrose

Adjust pH to 5.8 with KOH and HCl

8 g plant tissue culture agar.

Make up to 1 litre with distilled water. Autoclave and store in the fridge.

Regeneration and Selection Medium (RSM)

4.4 g Murashinge and Skoog Basal Medium

30 g sucrose

Make up to 1 litre with distilled water

Adjust pH to 5.8 with KOH and HCl

8 g plant tissue culture agar. Autoclave and store in the fridge.

Half MS Media for Selecting Transgenic Seedlings

2.46 g Murashinge and Skoog Basal Medium (MS)

5 g Sucrose

Make up to 1 litre with distilled water

Adjust pH to 5.8 with KOH and HCl

7 g plant tissue culture agar. Autoclave and store in the fridge.

Rooting Media

4.4 g Murashinge and Skoog Basal Medium

30 g sucrose

Make up to 1 litre with distilled water

Adjust pH to 5.8 with KOH and HCl

8 g plant tissue culture agar. Autoclave and store in the fridge.

APPENDIX 3 ANTIBIOTIC RECIPES

Kanamycin (50 mg/ ml stock con)

0.5 g Kanamycin

10 ml sterile water

Filter sterilise

Store at -20 °C

Streptomycin (50mg/ ml stock con)

0.5 g Streptomycin

10 ml sterile water

Filter sterilise

Store at -20 °C

Rifampicin (25mg/ ml stock concentration)

0.25 g Rifampicin

10 ml of DMSO

Store at -20 °C

Carbenicillin (200mg/ml stock concentration)

1 g Carbenicillin

5 ml 50% Ethanol

Filter sterilise

Store at -20 °C

APPENDIX 4 SEQUENCES

Petunia inflata multiple sequences alignment for *PiS$_3$-RNase*, *PiS$_{k1}$-RNase* and *PiS$_d$-RNase*

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PiSd-RNase      ------------------------------------------------------------
PiS3-RNase      ---ATTGGGCGAACTAGAAATGTTTAAATCACAGCTCATATCAGCTCTTTTCATTTCACT
PiSk1-RNase     GATTTGATCGAGCTTGAAAATGTTTAAATGGCGGATCGTGTCAGCTCTTGTCATTTTAAA

PiSd-RNase      ------------------------------------------------------------
PiS3-RNase      TTTTGCCTTTTCTCCCGTTTGTGCGAATTTTGACTACATCCAACTCGTTTTAACTTGGCC
PiSk1-RNase     TTTTGCTCTTTTTCAAGTTTACGGGGATTTTGATTACTTGCAACTTGTTTTAACTTGGCC

PiSd-RNase      ------------------------------------------------------------
PiS3-RNase      AGCATCTTTTTGCTATAGGCCTAAAAATATTTGCAGGAGAATACCCAACAACTTTACAAT
PiSk1-RNase     AGCATCCTTTTGCTACCAGCCAAAAGATATTTGCAAGAGAACAGTGAACAACTTTACTAT

PiSd-RNase      ---------------TGATAACGTCAGTACACCGCTGAATTTTTGTGGTGCCAAAGAAGA
PiS3-RNase      TCATGGGCTTTGGCCCGAAAAGGAGCACTTTCGTCTGGAGTTTTGCGATGGAGATAAGTT
PiSk1-RNase     TCACGGGCTTTGGCCGGAAAAAAAGGGGTTTCGTCTGGAGTTCTGTAGTGGCGGTACCAA
                             ** **          *  *** * ** **   **

PiSd-RNase      AAACTACAAAGACTTAACGGATGATAACAAAAAGAATAATTTGTATAAACGTTGGCCTGA
PiS3-RNase      TGTGTCGTT---CAGCTTAAAAGATAGAATTGTCAATGATTTGGAGCGCCACTGGGTTCA
PiSk1-RNase     GT---ATAA---GATTTTCGAGGACAATATGGTCAATGATCTGGAACGCCACTGGTTACA
                             * ** *  *     *** ** ** *    *  ***    *

PiSd-RNase      CTTGACCACCGATGAAGCTGTATGTTTGGAAAAGCAAGATTTCTGGAGACATGAGTATAA
PiS3-RNase      AATGAAGTTCGATGAAAAATTCGCCAAGATTAAACAACCACTCTGGACCCATGAATACAA
PiSk1-RNase     AATGAAGTTCGATGAAAATTATGCTAAGAAACATCAACCTCTCTGGAGCTATCAATACAG
                  ***    *******          *    * ***    ******   ** * ** *

PiSd-RNase      TAAGCATGGAACGTGTTGTTTAGGTAGCTACAATGAAGATCAATACTTTCATTTAGCCAT
PiS3-RNase      TAAACATGGAATATGCTCTTCAAATCTCTACGATCAGAGGGCATATTTTTTGTTAGCCAT
PiSk1-RNase     AAAGCATGGAATGTGCTGTTATAAACTCTACAATCAGAATGCATATTTCTTATTAGCAAT
                 ** *******  ** * **       **** ** *      *** **    ***** **

PiSd-RNase      GGCCCTAAAAGACAAGTATGATCTTCTAACATCTTTGAGAAAGCATGGAATTAGTCCTGG
PiS3-RNase      GCGCCTGAAAGATAAATTTGATCTTTTGACTACTCTCAGAACCCATGGAATTACTCCGGG
PiSk1-RNase     GCGCTTAAAAGATAAACTTGATCTTTTGACGACTCTAAGAACTCATGGAATTACTCCGGG
                *  * * ***** **   ******* * **  ** * ****  ********** *** **
```

***The Highlighted squences are *PiS-Rnase* sepecific primers**

```
PiSd-RNase      CTGGCAATATACCGTTCAGAAAATCAATAGCACCATCAAGACAATAACTCGAGGG---TA
PiS3-RNase      AACAAAACATACATTTGGTGAAATCCAAAAAGCCATAAAGACGGTCACTAATAACAAAGA
PiSk1-RNase     AACAAAGCATACATTTAGTGAAATCCAAAAAGCCATAAAGACGGTCACCAATAACAAAGA
                     *  ****  **    ***** * *   **** *****  * **           *

PiSd-RNase      TCCTAACCTCTCGTGCACTAA-----------GAAAATGGAACTATGGGAGATAGGAAT
PiS3-RNase      TCCTGACCTCAAGTGCGTTGAACATATCAAAGGAGTAAAGGAACTAAAAGAAGTAGGCAT
PiSk1-RNase     TCCTGACCTCAAGTGCGTCGAACATATCAAAGGAGTAAAGGAACTAAATGAAATAGGAAT
                **** *****  ****    *               ** *******   **  **** **

PiSd-RNase      ATGTTTCGACTCGACGGTACAAAAGGTGATCGATTGTCCTCATCCTAAGACATGCGCGCC
PiS3-RNase      ATGTTTTACCCCAGCGGCAGATAGTTTTCATGATTGTCGTCACAGCAACACATGCGATGA
PiSk1-RNase     ATGTTTTAACCCCGCGGCAGATAGTTTTCATGATTGTCGTCACAGCTATACATGCGATGA
                ******   * *  *** * * *   *    ******* ***     * *******

PiSd-RNase      GATGAA---------AATTATGTTTCCATAAAT-------AAAATTTCATTTTCTCTCTT
PiS3-RNase      AACGGACAGCACGAAGATTCTGTTTCGGTGATGAATGATTCTCATTTCTTTTTTTTCTGT
PiSk1-RNase     AACAGACAGCACGAAGACCCTGTTTCGGCTATGAATGGTTATCATTTCTTTTTCTTTCTT
                 *   *          *   ******    *            ***** **** *    *

PiSd-RNase      ATGTAAACCGCTACGATAATCA----GTACAATCAAATAAAATTTTATTTGAAAATT---
PiS3-RNase      TGGTTCTTTCTAA-------------GAAAAAGAAAA---------------TGCTGAGC
PiSk1-RNase     TTTTTCCTTCTGTTGGCTCTTACTAAGAAAAAGTGTAGTCGAGGTCATCTAAAAATAAAA
                   *                      * * **    *                  *

PiSd-RNase      TGATATCAAATTTATAAAAACTACAATTGAATTTAAACATTCTATTCTGTAAAAAAAAAA
PiS3-RNase      TACAGGAAGACCTCTTTATGGAACAAGTGAAATAAATTTATCATCTATTTGCGAAAAAAA
PiSk1-RNase     TTGTACCGGACCTCTTTGTGGAACAAGTGAAATAAATTTATTATCGATTTGCGAAAAAAA
                *        *  * *       **** **** * **    *      * *   *******

PiSd-RNase      AAAAAAAAAAAAAAAAAA
PiS3-RNase      AAAAAAAAAAAAAAA---
PiSk1-RNase     AAAAAAAAAA--------
                **********
```

***The Highlighted squences are *PiS-Rnase* sepecific primers**

Plasmid DNA Sequencing Results

AhSLF-S2 with M13 Reverse

```
M13-Reverse     ------------------------------------------------------------
AhSLF-S2        CAGGAAACAGCTATGACCATGATTACGCCAAGCTTCCTATACCCCTTGGATAAGGGTAGC

M13-Reverse     ------------------------------------------------------------AGAA
AhSLF-S2        TCTATCTATATAGTCAATTATTGTCTTCTGTCTGTTGGTGTCGACATACTCGACTCAGAA
                                                                        ****

M13-Reverse     GGTATTGAGGAATGATCGATTCTGGGTCATTTGTGTGGTTAATCACCCTCCAAATCAACT
AhSLF-S2        GGTATTGAGGAATGATCGATTCTGGGTCATTTGTGTGGTTAATCACCCTCCAAATCAACT
                ************************************************************

M13-Reverse     AAGTCATCCTGAAGGACAATATCCTATTTTTTCTCTCGTAGGTTTATCATTTAAATTACT
AhSLF-S2        AAGTCATCCTGAAGGACAATATCCTATTTTTTCTCTCGTAGGTTTATCATTTAAATTACT
                ************************************************************

M13-Reverse     ATCGCGTGATAATTTTGTAACGTAGAAAAATAATACCATTAATCCAAACGTTATATTCAT
AhSLF-S2        ATCGCGTGATAATTTTGTAACGTAGAAAAATAATACCATTAATCCAAACGTTATATTCAT
                ************************************************************

M13-Reverse     TAAAATAATTATGATACATTTAAAAATATTTCGTGACCTCTCAATTATTGCAAATTCTAA
AhSLF-S2        TAAAATAATTATGATACATTTAAAAATATTTCGTGACCTCTCAATTATTGCAAATTCTAA
                ************************************************************

M13-Reverse     GCCATCCCAAGTTTTGAGGCTAATTTTTTTTTACTATACTATTTTTACAACCACAAAAACA
AhSLF-S2        GCCATCCCAAGTTTTGAGGCTAATTTTTTTTTACTATACTATTTTTACAACCACAAAAACA
                ************************************************************

M13-Reverse     TAAAAAATAAAAAATAAAAAAAAATAAACCGAGTCAATTGCTACAATCACTTCATTATTAA
AhSLF-S2        TAAAAAATAAAAAATAAAAAAAAATAAACCGAGTCAATTGCTACAATCACTTCATTATTAA
                ************************************************************

M13-Reverse     TTTTAATTAATATTATGTGGTTATATATGAAACTGTTAGAGAAATAATAGCTCCACCATA
AhSLF-S2        TTTTAATTAATATTATGTGGTTATATATGAAACTGTTAGAGAAATAATAGCTCCACCATA
                ************************************************************

M13-Reverse     TTTTTTTCTTAATTTATTTTCACTATAAAAAGGCTATTTCATTATAATCAAAACAAGACA
AhSLF-S2        TTTTTTTCTCAATTTATTTTCACTATAAAAAGGCTATTTCATTATAATCAAAACAAGACA
                ********* ****************************************************


M13-Reverse      CACACAAAGAGAAGGAGCAATAAAATAAAAGTAAACAACAATTTGTGTGTTTAAAAAAAA
AhSLF-S2         CACACAAAGAGAAGGAGCAATAAAATAAAAGTAAACAACAATTTGTGTGTTTAAAAAAAA
                 ************************************************************

M13-Reverse      AAAAAAAGTACACACACCAAAAAAAAAAATTCCAATTTAAACTCGAGGTACCTCTAGAAT
AhSLF-S2         AAAAAAAGTACACACACCAAAAAAAAAAATTCCAATT-----------TAAATCTAGAAT
                 ************************************          **  ********


M13-Reverse      GATGGATCGACGATTTCCGCGACAGGATGTAATTAGTGAAATTTTGTTATTCTCGTCAGT
AhSLF-S2         GATGGATCGACGATTTCCGCGACAGGATGTAATTAGTGAAATTTTGTTATTCTCGTCAGT
                 ************************************************************
```

```
M13-Reverse      GAAATCCCTGCTAAGGTTCAGGTGCGTCTCAAAGTCTTGGTGTTCTCTAATCAAAAGTAA
AhSLF-S2         GAAATCCCTGCTAAGGTTCAGGTGCGTCTCAAAGTCTTGGTGTTCTCTAATCAAAAGTAA
                 ************************************************************

M13-Reverse      TGACTTCATAGATAATCATCTCCTAAGACGACAAACAAATGGTAATGTAATGGTTGTAAA
AhSLF-S2         TGACTTCATAGATAATCATCTCCTAAGACGACAAACAAATGGTAATGTAATGGTTGTAAA
                 ************************************************************

M13-Reverse      GCGGTATGTACGGACACCAGAAAGGGATATGTTTTCCTTCTACAATATCAATTTCTCCAG
AhSLF-S2         GCGGTATGTACGGACACCAGAAAGGGATATGTTTTCCTT----------------------
                 ************************************

M13-Reverse      AACTAGACGAATTGTTGCCAGATCTACCCAATCCATATTTCAAGAATATAAAATTCGACT
AhSLF-S2         ------------------------------------------------------------

M13-Reverse      ACGATTATTTTTTACTTACCTCAAC
AhSLF-S2         -------------------------
```

AhSLF-S2 with AhSLF-R-904

```
S2-R-904         ------------------------------------------------------------
AhSLF-S2         ACTCACGCACGCCATATTGATTCATAACCCAAATGTCAAATAATTCCGGATCCTCCATCC

S2-R-904         ----------------------AGGTATTCATTCAAGGACATGAGAGATAAAAAACTGT
AhSLF-S2         ATTCTTTATATCGAACCATAGCAAGGCATTCATTCAAGGACATGAGAGATAAAAAACTGT
                                     *** **********************************

S2-R-904         TAGAGAATTGCGCTAAACAATGAGGGTACGCCATCTCCTTAAAAACCTCCGTGATTATAT
AhSLF-S2         TAGAGAATTGCGCTAAACAATGAGGGTACGCCATCTCCTTAAAAACCTCCGTGATTATAT
                 ************************************************************

S2-R-904         CAAAAGTAAGGATAAAATCTGCATAAAAAATATCAGTCGAGTTTGCGTTCCAGTGGAAAG
AhSLF-S2         CAAAAGTAAGGATAAAATCTGCATAAAAAATATCAGTCGAGTTTGCGTTCCAGTGGAAAG
                 ************************************************************

S2-R-904         CACCTTTGAAGAAGAGCTCGTTACAAGGAAAGTGACAAATGTACTTAATAGGTGTAGAAT
AhSLF-S2         CACCTTTGAAGAAGAGCTCGTTACAAGGAAAGTGACAAATGTACTTAATAGGTGTAGAAT
                 ************************************************************

S2-R-904         CATCCTCGATATGTTTCCAGGAGTTGGTGTCTGAATAGTACACGTATATGTTGATATGAT
AhSLF-S2         CATCCTCGATATGTTTCCAGGAGTTGGTGTCTGAATAGTACACGTATATGTTGATATGAT
                 ************************************************************

S2-R-904         GATCCTCAGGCCCGACACTCTCTATCAAGACAACTTTGTAACAATCGTTACAAGTATTAC
AhSLF-S2         GATCCTCAGGCCCGACACTCTCTATCAAGACAACTTTGTAACAATCGTTACAAGTATTAC
                 ************************************************************
```

```
S2-R-904        CAAATCCGTATCCTATAATGTCGGTGCAGTGACCCTCTGGGTTAGCAAAAGGCGTAGGTG
AhSLF-S2        CAAATCCGTATCCTATAATGTCGGTGCAGTGACCCTCTGGGTTAGCAAAAGGCGTAGGTG
                ************************************************************

S2-R-904        GAAGCCGCTTGATTTCTCGCAGTGCAGGATTGGACAAAAGAACGCAATCACCATATGCTA
AhSLF-S2        GAAGCCGCTTGATTTCTCGCAGTGCAGGATTGGACAAAAGAACGCAATCACCATATGCTA
                ************************************************************

S2-R-904        AGCAGATGAGCCCGTTGCATGGGCCCATTAGGTTGACACGTTGAGGTAAGTAAAAATAAT
AhSLF-S2        AGCAGATGAGCCCGTTGCATGGGCCCATTAGGTTGACACGTTGAGGTAAGTAAAAATAAT
                ************************************************************


S2-R-904        CGTAGTCGAATTTTATATTCTTGAAATATGGATTGGGTAGATCTGGCAACAATTCGTCTA
AhSLF-S2        CGTAGTCGAATTTTATATTCTTGAAATATGGATTGGGTAGATCTGGCAACAATTCGTCTA
                ************************************************************

S2-R-904        GTTCTGGAGAATTGATATTGTAGAAGGAAAACATATCCCTTTCTGGTGTCCGTACATACC
AhSLF-S2        GTTCTGGAGAATTGATATTGTAGAAGGAAAACATATCCCTTTCTGGTGTCCGTACATACC
                ************************************************************

S2-R-904        GCTTTTACAACCATTACATTACCATTTGTTTGTCGTCTTAGGAGATGATTATCTATGAAG
AhSLF-S2        GCTT-TACAACCATTACATTACCATTTGTTTGTCGTCTTAGGAGATGATTATCTATGAAG
                **** ********************************************************

S2-R-904        TCATTACTTTTGATTAGAGAACACCAAGACTTTGAGACGCACCTGAACCTTAGCAGGGAT
AhSLF-S2        TCATTACTTTTGATTAGAGAACACCAAGACTTTGAGACGCACCTGAACCTTAGC------
                **************************************************

S2-R-904        TTCACTGACGAGAATAACAAAATTTCACTAATTACATCCTGTCGCGGAAATCGTCGATCC
AhSLF-S2        ------------------------------------------------------------

S2-R-904        ATCATTCTAGAGGTACCTCGA
AhSLF-S2        --------------------
```

AhSLF-S1E with M13 Reverse

```
M13-Reverese    ------------------------------------------------------------
AhSLF-S1E       CAGGAAACAGCTATGACCATGATTACGCCAAGCTTCCTATACCCCTTGGATAAGGGTAGC

M13-Reverese    ------------------------------------------------------------
AhSLF-S1E       TCTATCTATATAGTCAATTATTGTCTTCTGTCTGTTGGTGTCGACATACTCGACTCAGAA

M13-Reverese    ---ATTGAGGAATGATCGATTCTGGGTCATTTGTGTGGTTAATCACCCTCCAAATCAACT
AhSLF-S1E       GGTATTGAGGAATGATCGATTCTGGGTCATTTGTGTGGTTAATCACCCTCCAAATCAACT
                   ************************************************************

M13-Reverese    AAGTCATCCTGAAGGACAATATCCTATTTTTTCTCTCGTAGGTTTATCATTTAAATTACT
AhSLF-S1E       AAGTCATCCTGAAGGACAATATCCTATTTTTTCTCTCGTAGGTTTATCATTTAAATTACT
                ************************************************************

M13-Reverese    ATCGCGTGATAATTTTGTAACGTAGAAAAATAATACCATTAATCCAAACGTTATATTCAT
AhSLF-S1E       ATCGCGTGATAATTTTGTAACGTAGAAAAATAATACCATTAATCCAAACGTTATATTCAT
                ************************************************************

M13-Reverese    TAAAATAATTATGATACATTTAAAAATATTTCGTGACCTCTCAATTATTGCAAATTCTAA
AhSLF-S1E       TAAAATAATTATGATACATTTAAAAATATTTCGTGACCTCTCAATTATTGCAAATTCTAA
                ************************************************************

M13-Reverese    GCCATCCCAAGTTTTGAGGCTAATTTTTTTTACTATACTATTTTTACAACCACAAAAACA
AhSLF-S1E       GCCATCCCAAGTTTTGAGGCTAATTTTTTTTACTATACTATTTTTACAACCACAAAAACA
                ************************************************************

M13-Reverese    TAAAAAATAAAAAATAAAAAAAATAAACCGAGTCAATTGCTACAATCACTTCATTATTAA
AhSLF-S1E       TAAAAAATAAAAAATAAAAAAAATAAACCGAGTCAATTGCTACAATCACTTCATTATTAA
                ************************************************************

M13-Reverese    TTTTAATTAATATTATGTGGTTATATATGAAACTGTTAGAGAAATAATAGCTCCACCATA
AhSLF-S1E       TTTTAATTAATATTATGTGGTTATATATGAAACTGTTAGAGAAATAATAGCTCCACCATA
                ************************************************************

M13-Reverese    TTTTTTTCTTAATTTATTTTCACTATAAAAAGGCTATTTCATTATAATCAAAACAAGACA
AhSLF-S1E       TTTTTTTCTCAATTTATTTTCACTATAAAAAGGCTATTTCATTATAATCAAAACAAGACA
                ********* ********************************************************

M13-Reverese    CACACAAAGAGAAGGAGCAATAAAATAAAAGTAAACAACAATTTGTGTGTTTAAAAAAAA
AhSLF-S1E       CACACAAAGAGAAGGAGCAATAAAATAAAAGTAAACAACAATTTGTGTGTTTAAAAAAAA
                ************************************************************

M13-Reverese    AAAAAAGT-ACACACACCAAAAAAAAAAATTCCAATTTAAACTCGAGGTACCTCTAGAAT
AhSLF-S1E       AAAAAAAGTACACACACCAAAAAAAAAAATT-----------CCAATTTAAATCTAGAAT
                ******   ********************** * *  **  ********

M13-Reverese    GATAGATTCGCTGCTTCCACTGGACATGGTAATTGAGATTATGGTTCAATTGCCAGTTAA
AhSLF-S1E       GATAGATTCGCTGCTTCCACTGGACATGGTAATTGAGATTATGGTTCAATTGCCAGTTAA
                ************************************************************

M13-Reverese    ATCACTTGTTCGATTTAGATGCGTATCAAAAATCTTTCTGTGTTATAATCAAAAGCTCAA
AhSLF-S1E       ATCACTTGTTCGATTTAGATGCGTATCAA-AATCTTTCTGTGTTATAATCAAAAGCTCAA
                ***************************** ******************************

M13-Reverese    ACTTTATCAACAACCACTTTCTCAGACGGCAAACGAGAGACACCTTACTCCTAATTAGAC
AhSLF-S1E       ACTTTATCAACAACCACTTTCTCAGACGGCAAACGAGAGACACCTTACTCCTAATTAGAC
                ************************************************************

M13-Reverese    GATATTTTCCTTCACCACAGGACGATGATGCATTGTCTTTCCACAAACCAGATTCTCCCG
AhSLF-S1E       GATATTTTCCTTCA----------------------------------------------
                **************
```

```
M13-Reverese      GGACTAGAGGAAGAAGTGTGGCCAAAGTTGTCCATTCCATTCTTAAGTGATTTGAGGTTA
AhSLF-S1E         ------------------------------------------------------------

M13-Reverese      AGAT
AhSLF-S1E         ----
```

AhSLF-S1E with AhSLF-S1E-R-1152

```
S1E-R-1152        ------------------------------------------------------------
AhSLF-S1E         CACAAGCTGTATTACCTGGCCTAAATTGATTAAGCTCTCGTTGTAAATAAGAACTCGCAA

S1E-R-1152        -----------------------------------------------GAAGGGCGCAAGA
AhSLF-S1E         GGACTCTTCAGTTCCATCAACTTGAAATCGCTTAATTTCATCGGTGTGAAGGGCGCAAGA
                                                                  ************

S1E-R-1152        CGCCAATTGGCCATTGCTGTTATCTACCAACAACCAATCATCATTCTTCCAGAATATGAA
AhSLF-S1E         CGCCAATTGGCCATTGCTGTTATCTACCAACAACCAATCATCATTCTTCCAGAATATGAA
                  ************************************************************

S1E-R-1152        TGGACGTATTACTAGATGGGGTCCTAATACGAAGTCTTTGGTCCAAGAATCACTGTTGCC
AhSLF-S1E         TGGACGTATTACTAGATGGGGTCCTAATACGAAGTCTTTGGTCCAAGAATCACTGTTGCC
                  ************************************************************

S1E-R-1152        GTATACTTTCATCACCCAAATCTCAATGAATTGAGGATCTTTCGTCGAATCATTGTACCG
AhSLF-S1E         GTATACTTTCATCACCCAAATCTCAATGAATTGAGGATCTTTCGTCGAATCATTGTACCG
                  ************************************************************

S1E-R-1152        AATAACGGAAAGGCACTCACTAAGTGCAGTGAGACACAAACAACCTCCATATAGTTCCCT
AhSLF-S1E         AATAACGGAAAGGCACTCACTAAGTGCAGTGAGACACAAACAACCTCCATATAGTTCCCT
                  ************************************************************

S1E-R-1152        AAAACCATCAGGATATTCAAACTCACTAAAGATCTCGGTACTTATGTCAAATGTAAGGAT
AhSLF-S1E         AAAACCATCAGGATATTCAAACTCACTAAAGATCTCGGTACTTATGTCAAATGTAAGGAT
                  ************************************************************

S1E-R-1152        ACTCCCTGGATAAGGAATACCAAAAACACCCGCGTTCCAGTGGCAAGCGCCATTAAAGAA
AhSLF-S1E         ACTCCCTGGATAAGGAATACCAAAAACACCCGCGTTCCAGTGGCAAGCGCCATTAAAGAA
                  ************************************************************

S1E-R-1152        AACATCTGTACAAGGAGAGGAAAATATATATTGAACTAAAACTGCTTCTCCTTCTATAAG
AhSLF-S1E         AACATCTGTACAAGGAGAGGAAAATATATATTGAACTAAAACTGCTTCTCCTTCTATAAG
                  ************************************************************
```

```
S1E-R-1152      CCTCCAGGATTGGGTGTTTGAATTGTACAAATGGACCATGACGTATGGTTTATCGCGATT
AhSLF-S1E       CCTCCAGGATTGGGTGTTTGAATTGTACAAATGGACCATGATGTAAGGTTTATCGCGATT
                **************************************** *** **************

S1E-R-1152      GTAATCGCTCACGGACTTAATAGTACGTACTATGACAATCTTGAAAAAATTGCTATCACA
AhSLF-S1E       GTAATCGCTCACGGACTTAATAGTACGTACTAGGACAATCTTGAAAAAATTGCTATCACA
                ******************************** **************************

S1E-R-1152      ATTCCCAAATCCCTGACCTATAATGTTGGAGTAAAAACGTTTCGGGCAGCAGAAAGGACA
AhSLF-S1E       ATTCCCAAATCCCTGACCTATAATG-----------------------------------
                ************************

S1E-R-1152      CGGTGGGAGCTTCTTAAATTCGCGCAATGCTGGATTACATAAAATAATGAAGTCATCATA
AhSLF-S1E       ------------------------------------------------------------

S1E-R-1152      GAATATGCATAT
AhSLF-S1E       ------------
```

AhSLF-S2C with M13-Reverse

```
M13-Reverse     ------------------------------------------------------------
AhSLF-S2C       CAGGAAACAGCTATGACCATGATTACGCCAAGCTTCCTATACCCCTTGGATAAGGGTAGC

M13-Reverse     ----------------------------------------TGTCGACTACTCGACTCAGAA
AhSLF-S2C       TCTATCTATATAGTCAATTATTGTCTTCTGTCTGTTGGTGTCGACATACTCGACTCAGAA
                                                        **************

M13-Reverse     GGTATTGAGGAATGATCGATTCTGGGTCATTTGTGTGGTTAATCACCCTCCAAATCAACT
AhSLF-S2C       GGTATTGAGGAATGATCGATTCTGGGTCATTTGTGTGGTTAATCACCCTCCAAATCAACT
                ************************************************************

M13-Reverse     AAGTCATCCTGAAGGACAATATCCTATTTTTTCTCTCGTAGGTTTATCATTTAAATTACT
AhSLF-S2C       AAGTCATCCTGAAGGACAATATCCTATTTTTTCTCTCGTAGGTTTATCATTTAAATTACT
                ************************************************************

M13-Reverse     ATCGCGTGATAATTTTGTAACGTAGAAAAATAATACCATTAATCCAAACGTTATATTCAT
AhSLF-S2C       ATCGCGTGATAATTTTGTAACGTAGAAAAATAATACCATTAATCCAAACGTTATATTCAT
                ************************************************************

M13-Reverse     TAAAATAATTATGATACATTTAAAAATATTTCGTGACCTCTCAATTATTGCAAATTCTAA
AhSLF-S2C       TAAAATAATTATGATACATTTAAAAATATTTCGTGACCTCTCAATTATTGCAAATTCTAA
                ************************************************************

M13-Reverse     GCCATCCCAAGTTTTGAGGCTAATTTTTTTTACTATACTATTTTTACAACCACAAAAACA
AhSLF-S2C       GCCATCCCAAGTTTTGAGGCTAATTTTTTTTACTATACTATTTTTACAACCACAAAAACA
                ************************************************************

M13-Reverse     TAAAAAATAAAAAATAAAAAAAATAAACCGAGTCAATTGCTACAATCACTTCATTATTAA
AhSLF-S2C       TAAAAAATAAAAAATAAAAAAAATAAACCGAGTCAATTGCTACAATCACTTCATTATTAA
                ************************************************************

M13-Reverse     TTTTAATTAATATTATGTGGTTATATATGAAACTGTTAGAGAAATAATAGCTCCACCATA
AhSLF-S2C       TTTTAATTAATATTATGTGGTTATATATGAAACTGTTAGAGAAATAATAGCTCCACCATA
                ************************************************************
```

```
M13-Reverse      TTTTTTTCTTAATTTATTTTCACTATAAAAAGGCTATTTCATTATAATCAAAACAAGACA
AhSLF-S2C        TTTTTTTCTCAATTTATTTTCACTATAAAAAGGCTATTTCATTATAATCAAAACAAGACA
                 ********* **************************************************

M13-Reverse      CACACAAAGAGAAGGAGCAATAAAAAA-AAGTAAACAACAATTTGTGTGTTTAAAAAAAA
AhSLF-S2C        CACACAAAGAGAAGGAGCAATAAAATAAAAGTAAACAACAATTTGTGTGTTTAAAAAAAA
                 ************************* * ********************************

M13-Reverse      AAAAAAAGTACACACACCAAAAAAAAAAATTCCAATTTAAACTCGAGGTACCTCTAGAAT
AhSLF-S2C        AAAAAAAGTACACACACCAAAAAAAAAAATTCCAATTTAAATCTAGAAT----------G
                 ****************************************       *

M13-Reverse      GGAGAAATTGCAGATGGCTATCCATTA-GGGGATGAAAACTCATTGAGATTCTCTTACAT
AhSLF-S2C        GAGAAAATTGCAGATGGCCATCCATTAGGGGATGATAAACTCATTGAGATTCTCTTACAT
                 *   ************** ******** ***     ************************

M13-Reverse      CTTCCAGTAAGGTCACTGGTGAGATTTAAGTGTGTCTCCATATCATGGTATAATTTGATT
AhSLF-S2C        CTTCCAGTAAGGTCACTGGTGAGATTTAAGTGTGTCTCCATATCATGGTATAATTTGATT
                 ************************************************************

M13-Reverse      AGAAGTCCTACATTTTGCAACAAACACTTTCTTACATCTCGGAGAAATGATAGTGTGTTG
AhSLF-S2C        AGAAGTCCTACATTTTGCAACAAACACTTTCTTACATCTCGGAGAAATGATAGTGTGTTG
                 ************************************************************

M13-Reverse      CTGGTAAGGCGCTTTTTGCGTCCACCAGAAGACGAAACGTGCTTTCCTTCCACGATGTGA
AhSLF-S2C        CTGGTAAGGCGCTTTT----------------------------------------------
                 ****************

M13-Reverse      ATTCCCCAGAACTAGAGCAGGTGGCTCCAAATTTGTCCATTCCTTTCTTAAAGGATATAA
AhSLF-S2C        ------------------------------------------------------------

M13-Reverse      GGC
AhSLF-S2C        ---
```

AhSLF-S2C with AhSLF-S2C-R-1148

```
S2C-R-1148       ----------------------------------------GCTCTCT--GGTTTTTTCACT
AhSLF-S2C        ACCTGGTTCAAACTGATCAAGCTCTCCTTGTAAATCATAGCTCTCATGGTTTTTTCAACT
                                                        ******:  * ***** .***

S2C-R-1148       CCATAGATTGGTAATCGCCTAAACTCATTTGTATGAAGTGCACAAGTTGCCAACTGGCCG
AhSLF-S2C        CCATAGATTGGTAATCGCCTAAACTCATTTGTATGAAGTGCACAAGTTGCCAACTGGCCG
                 ************************************************************

S2C-R-1148       TCGCTGCTTTCAACGAACAACCATTCATCATTCTTCCACGGCAATATACAACTTACGACT
AhSLF-S2C        TCGCTGCTTTCAACGAACAACCATTCATCATTCTTCCACGGCAATATACAACTTACGACT
                 ************************************************************

S2C-R-1148       TGGTAAGGCCCAACTGTAAACTGTTTTGTCCATGACTCACCAAAGCCATATTTCTTCATC
AhSLF-S2C        TGGTAAGGCCCAACTGTAAACTGTTTTGTCCATGACTCACCAAAGCCATATTTCTTCATC
                 ************************************************************

S2C-R-1148       ACCCAAATGTCAAGTGGTTGTGGCTCTTCCTTCGAGTCCTTGTATAGAATAATTGCAAAG
AhSLF-S2C        ACCCAAATGTCAAGTGGTTGTGGCTCTTCCTTCGAGTCCTTGTATAGAATAATTGCAAAG
                 ************************************************************
```

```
S2C-R-1148      TATTCTAACTGTCCAAAAGTCTCAGTGTTTAGGTTGAAAGTAAGAATGGCTTTTCGAGAA
AhSLF-S2C       TATTCTAACTGTCCAAAAGTCTCAGTGTTTAGGTTGAAAGTAAGAATGGCTTTTCGAGAA
                ************************************************************

S2C-R-1148      GAGTAATCATATGATGCTGCATTCCAATGAAGATTACCGTGGAAGAAACGTTGAGAACAA
AhSLF-S2C       GAGTAATCATATGATGCTGCATTCCAATGAAGATTACCGTGGAAGAAACGTTGAGAACAA
                ************************************************************

S2C-R-1148      GGATAGTCCCAAAGATCTTTAAAAGATAATGTACCAACATCATTAGTAATCCCCCATGAG
AhSLF-S2C       GGATAGTCCCAAAGATCTTTAAAAGATAATGTACCAACATCATTAGTAATCCTCCATGAG
                ************************************************** *******

S2C-R-1148      TTTGTGTTTGAATTGTACAAATGAACAAGTGTGTAAGTACTATAATCATATACATCATCA
AhSLF-S2C       TTTGTGTTTGAATTGTACAAATGAACAAGTGTGTAAGTACTATAATCATATACATCATCA
                ************************************************************

S2C-R-1148      AACCAAATACTTCGGATCAACACGACCTTGAAATCTGTCATACTAGTACAACCGAATCCA
AhSLF-S2C       AACCAAATACTTCGGATCAACACGACCTTGAAATCTGTCATACTAGTACAACCGAATCCA
                ************************************************************

S2C-R-1148      GACCCTATAATCCTATCGAAGAAACCCTTTGGGGACACATATGGACAAGGTTGAAGCCGC
AhSLF-S2C       GACCCTATAATCCTATCGAAGAAACCCTTTGGGGACACATATGGACAAGGTTGAAGCCGC
                ************************************************************

S2C-R-1148      TTGAATTCCCTTAATGTTGGACAACAAAAAATGAGGAATTCAGCATGAGTTATGCATAGG
AhSLF-S2C       TTGAATTCCCTTAATGTTGGACAACAAAAAATGAGGAATTCAGCATGAGTTATGCATAGG
                ************************************************************

S2C-R-1148      AGACCATTGCATGGACCCAAAAGTGTGACACCTTCAGGGAAGTATGGCCTGTTGTATCTA
AhSLF-S2C       AGACCATTGCATGGACCCAAAAGTGTGACACCTTCAGGGAAGTATGGCCTGTTGTATCTA
                ************************************************************

S2C-R-1148      AGCCTTATATCCTTTAAGAAAGGAATGGACAAATTTGGAGCCACCTGCTCTAGTTCTGGG
AhSLF-S2C       AGCCTTATATCCTTTAAGAAAGGAATGGACAAATTTGGAGCCACCTGCTCTAGTTCTGGG
                ************************************************************

S2C-R-1148      GAATTCACATCGTGGAAGGAAAGCACGTCTTCGTCTTCTGGTGGACGCAAAAAGCGCCTT
AhSLF-S2C       GAATTCACATCGTGGAAGGAAAGCACGTCTTCGTCTTCTGGTGGACGC------------
                ********************************************

S2C-R-1148      ACCAGCAACACACTATCATTTCTCCGAGAT
AhSLF-S2C       ------------------------------
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AhSLF-S4D with M13 Reverse

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M13-Reverse     ------------------------------------------------------------
AhSLF-S4D       CAGGAAACAGCTATGACCATGATTACGCCAAGCTTCCTATACCCCTTGGATAAGGGTAGC

M13-Reverse     ------------------------------------------------------------
AhSLF-S4D       TCTATCTATATAGTCAATTATTGTCTTCTGTCTGTTGGTGTCGACATACTCGACTCAGAA

M13-Reverse     GGTATTGAGGAATGATCGATTCTGGGTCATTTGTGTGGTTAATCACCCTCCAAATCAACT
AhSLF-S4D       GGTATTGAGGAATGATCGATTCTGGGTCATTTGTGTGGTTAATCACCCTCCAAATCAACT
                ************************************************************
M13-Reverse     AAGTCATCCTGAAGGACAATATCCTATTTTTTCTCTCGTAGGTTTATCATTTAAATTACT
AhSLF-S4D       AAGTCATCCTGAAGGACAATATCCTATTTTTTCTCTCGTAGGTTTATCATTTAAATTACT
                ************************************************************

M13-Reverse     ATCGCGTGATAATTTTGTAACGTAGAAAAATAATACCATTAATCCAAACGTTATATTCAT
AhSLF-S4D       ATCGCGTGATAATTTTGTAACGTAGAAAAATAATACCATTAATCCAAACGTTATATTCAT
                ************************************************************

M13-Reverse     TAAAATAATTATGATACATTTAAAAATATTTCGTGACCTCTCAATTATTGCAAATTCTAA
AhSLF-S4D       TAAAATAATTATGATACATTTAAAAATATTTCGTGACCTCTCAATTATTGCAAATTCTAA
                ************************************************************

M13-Reverse     GCCATCCCAAGTTTTGAGGCTAATTTTTTTTTACTATACTATTTTTACAACCACAAAAACA
AhSLF-S4D       GCCATCCCAAGTTTTGAGGCTAATTTTTTTTTACTATACTATTTTTACAACCACAAAAACA
                ************************************************************

M13-Reverse     TAAAAAATAAAAAATAAAAAAAAATAAACCGAGTCAATTGCTACAATCACTTCATTATTAA
AhSLF-S4D       TAAAAAATAAAAAATAAAAAAAAATAAACCGAGTCAATTGCTACAATCACTTCATTATTAA
                ************************************************************

M13-Reverse     TTTTAATTAATATTATGTGGTTATATATGAAACTGTTAGAGAAATAATAGCTCCACCATA
AhSLF-S4D       TTTTAATTAATATTATGTGGTTATATATGAAACTGTTAGAGAAATAATAGCTCCACCATA
                ************************************************************

M13-Reverse     TTTTTTTCTTAATTTATTTTCACTATAAAAAGGCTATTTCATTATAATCAAAACAAGACA
AhSLF-S4D       TTTTTTTCTCAATTTATTTTCACTATAAAAAGGCTATTTCATTATAATCAAAACAAGACA
                ********* ********************************************************

M13-Reverse     CACACAAAGAGAAGGAGCAATAAAATAAAAGTAAACAACAATTTGTGTGTTTAAAAAAAA
AhSLF-S4D       CACACAAAGAGAAGGAGCAATAAAATAAAAGTAAACAACAATTTGTGTGTTTAAAAAAAA
                ************************************************************

M13-Reverse     AAAAAAAGTACACACACCAAAAAAAAAAAATTCCAATTTAAACTCGAGGTACCTCTAGAAT
AhSLF-S4D       AAAAAAAGTACACACACCAAAAAAAAAAAATTCCAATTTAAATC------------TAGAA
                ****************************************                 *

M13-Reverse     GTTTAGGTGGGCGAGGAATTCTTGAGGATATCTTGAAAGAAATTCTTGTGTGGCTTCCAG
AhSLF-S4D       TGTTAGGTGGGCGAGGAATTCCTGAGGATATCTTGAAAGAAATTCTTGTGTGGCTTCCAG
                  ****************** ************************************

M13-Reverse     TAAAGTCACTAATTCGATTGAAGTGTGCCTCAAAGCATTTAGATATGTCGATCAAAAGCC
AhSLF-S4D       TAAAGTCACTAATTCGATTGAAGTGTGCCTCAAAGCATTTAGATATGTTGATCAAAAGCC
                ********************************************** ***********

M13-Reverse     AGGCTTTTATCACCAGCCACATGATCAAACAGCGGAGAAACGATGGTATGTTATTGGTAA
AhSLF-S4D       AGGCTTTTATCACCAGCCACATGATCAAACAGCGGAGAAACGATGGTATGTTATTGGTAA
                ************************************************************
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M13-Reverse     GACGCATTTTGCCTCCATCAACATACAATGATGTATTTTCTTTCCACGATGTGAATTCTC
AhSLF-S4D       GACGCATTTTGCCTCCATCAACATACAATGATGTA-------------------------
                **********************************

M13-Reverse     CAGAATTAGAGGAGGGTTTGCCAAAACTTCCTTATTACATTATTGTCTAATCCTGATGAA
AhSLF-S4D       ------------------------------------------------------------

M13-Reverse     GCATCTTTCAACCCAAATATGTTG
AhSLF-S4D       -------------------------
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AhSLF-S4D with AhSLF-S4D-R-848

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S4D-R-848       ------------------------------------------------GAAGTCCAGTG
AhSLF-S4D       TCAGATCGAACGGAGGCAAAACATTCATTTAGGATCATAAAATTATGCTGAAGTCCAGTG
                                                               ***********

S4D-R-848       CATAACTTGAAACCACTAGGGATGCTCAAATTGTCCAAAAACCTCAGTGCTTACATCAAA
AhSLF-S4D       CATAACTTGAAACC-ACTAGGATGCTCAAATTGTCCAAAAACCTCAGTGCTTACATCAAA
                **************     ****************************************

S4D-R-848       AGTAAGGATAACATCTGGAGTAGTCTCCCCACTAGTTCGCCCATTCCAGTGACAAGCACC
AhSLF-S4D       AGTAAGGATAACATCTGGAGTAGTCTCCCCACTAGTTCGCCCATTCCAGTGACAAGCACC
                ************************************************************

S4D-R-848       TTTAAAGAAGAGTTCAGAGCATTGGTAAGAGAAAACGACAGGCATGACTATTGCAAAGTC
AhSLF-S4D       TTTAAAGAAGAGTTCAGAGCATTGGTAAGAGAAAACGACAGGCATGACTATTGCAAAGTC
                ************************************************************

S4D-R-848       ATTTATTTCCCTCCAGGAATCGTTGTTTGAATTGTACAGATGGATCCTATGTTGGGCGTC
AhSLF-S4D       ATTTATTTCCCTCCAGGAATCGTTGTTTGAATTGTACAGATGGATCCTATGTTGGGCGTC
                ************************************************************

S4D-R-848       ACGACCATCAACGCGGGCAGTGTACAATGTATTCATTAAAATAACCTTAAAATTGTTGGT
AhSLF-S4D       ACGACCATCAACGCGGGCAGTGTACAATGTATTCATTAAAATAACCTTAAAATTGTTGGT
                ************************************************************

S4D-R-848       ACAAGTACTACCAAATCCCCCTCCAGTACGTATGCTATAACAAGGTGGTCGGCATGAAAT
AhSLF-S4D       ACAAGTACTACCAAATCCCCCTCCAGTACGTATGCTATAACAAGGTGGTCGGCATGAAAT
                ************************************************************

S4D-R-848       TGGGGCAGAAGGAAGCTTCCTAAATTCCCGCAATGCGGGGTTGCATAAAATAATATCCTC
AhSLF-S4D       TGGGGCAGAAGGAAGCTTCCTAAATTCCCGCAATGCGGGGTTGCATAAAATAATATCCTC
                ************************************************************

S4D-R-848       TTGCCCAGTTATGCAAACAATCCCATTGCAAGGTCCCAAAACATCAACAATATTTGGGTT
AhSLF-S4D       TTGCCCAGTTATGCAAACAATCCCATTGCAAGGTCCCAAAACATCAACAATATTTGGGTT
                ************************************************************

S4D-R-848       GAAAGATGCTTCATCAGGATTAGACAATAATGTAATAGGAAGTTTTGGCAAAACCTCCTC
AhSLF-S4D       GAAAGATGCTTCATCAGGATTAGACAATAATGTAATAGGAAGTTTTGGCAAAACCTCCTC
                ************************************************************
```

```
S4D-R-848       TAATTCTGGAGAATTCACATCGTGGAAAGAAAATACATCATTGTATGTTGATGGAGGCAA
AhSLF-S4D       TAATTCTGGAGAATTCACATCGTGGAAAGAAAATACATCATTGTATGTTGATGGAGGCAA
                ************************************************************

S4D-R-848       AATGCGTCTTACCAATAACATACCATCGTTTCTCCGCTGTTTGATCATGTGGCTGGTGAT
AhSLF-S4D       AATGCGTCTTACCAATAACATACCATCGTTTCTCCGCTGTTTGATCATGTGGCTGGTGAT
                ************************************************************

S4D-R-848       AAAAGCCTGGCTTTTGATCGACATATCTAAATGCTTTGAGGCACACTTCAATCGAATTAG
AhSLF-S4D       AAAAGCCTGGCTTTTGATCAACATATCTAAATGCTTTGAGGCACACTTCAATCGAATTAG
                ****************** ****************************************

S4D-R-848       TGACTTTACTGGAAGCCACACAAGAATTTCTTTCAAGATATCCTCAAGAATTCCTCGCCC
AhSLF-S4D       TGACTTTACTGGAAGCCACACAAGAATTTCTTTCAAGATATCCTCAGGAATTCCTCGCCC
                ******************************************** ************

S4D-R-848       ACCTAAACATTCTAGAGGTACCTCGAGTTTAAATTGGAATTTTTTTTTTTTGGTGTGTGT
AhSLF-S4D       ACCTAACAT-----------------------------------------------------
                ******

S4D-R-848       ACTTTTTTTTTTTTTTTTTAACACACAATT
AhSLF-S4D       ------------------------------
```

*All primers used for sequencing highlighted with Red.

*In all sequencing results (CTCGAGGTACC) is obtained and it is absent in original sequences (obtained from database). It is possible that this particular sequences were used as a primer for linking between promoter and each specific gene but the gap is different in the different constructs because of the software adjustment.

Appendix 5

Table 1 Summery of T_0 transgenic plants self-pollination results

Plant ID	Genotype	Number of Pollinated Flowers	Results	Plant ID	Genotype	Number of Pollinated Flowers	Results
AhSLF-S2.16.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S2C.24.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2.20.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S2C.25.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2.22.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S2C.26.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2.24.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S2C.29.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2.27.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S2C.30.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2.28.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S4D.1.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2C.2.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S4D.2.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2C.3.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S4D.3.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2C.4.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S4D.5.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2C.9.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S4D.6.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2C.10.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S4D.7.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2C.14.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S4D.11.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2C.17.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S4D.12.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2C.18.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S4D.13.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2C.19.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S4D.15.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2C.20.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S1E.15.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2C.22.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S1E.16.1	*PhS₃PhS$_V$*	10	SI
AhSLF-S2C.23.1	*PhS₃PhS$_V$*	10	SI	AhSLF-S1E.31.1	*PhS₃PhS$_V$*	10	SI

Table 2 Summery of F_1 transgenic plants self-pollination results.

Plant ID	Genotype	Number of Pollinated flowers	Results	Plant ID	Genotype	Number of Pollinated flowers	Results
N468.1	*PhS$_3$PiS$_{K1}$*	10	SI	N489.2	*PhS$_v$PiS$_{K1}$*	10	SI
N468.2	*PhS$_3$PiS$_{K1}$*	10	SI	N489.3	*PhS$_3$PiS$_3$*	10	SI
N468.3	*PhS$_3$PiS$_{K1}$*	10	SI	N489.4	*PhS$_v$PiS$_{K1}$*	10	SI
N468.4	*PhS$_v$PiS$_{K1}$*	10	SI	N489.5	*PhS$_v$PiS$_{K1}$*	10	SI
N468.5	*PhS$_v$PiS$_{K1}$*	10	SI	N489.7	*PhS$_v$PiS$_3$*	10	SI
N468.6	*PhS$_3$PiS$_{K1}$*	10	SI	N489.8	*PhS$_3$PiS$_3$*	10	SI
N468.7	*PhS$_v$PiS$_{K1}$*	10	SI	N489.9	*PhS$_3$PiS$_{k1}$*	10	SI
N468.8	*PhS$_3$PiS$_{K1}$*	10	SI	N489.10	*PhS3PiS$_{k1}$*	10	SI
N468.9	*PhS$_3$PiS$_{K1}$*	10	SI	N489.11	*PhS$_v$PiS$_{K1}$*	10	SI
N468.10	*PhS$_v$PiS$_{K1}$*	10	SI	N489.12	*PhS$_3$PiS$_{k1}$*	10	SI
N468.11	*PhS$_3$PiS$_{K1}$*	10	SI	N489.13	*PhS$_3$PiS$_{k1}$*	10	SI
N468.12	*PhS$_v$PiS$_{K1}$*	10	SI	N489.14	*PhS$_3$PiS$_{k1}$*	10	SI
N468.13	*PhS$_3$PiS$_3$*	10	SI	N489.15	*PhS$_3$PiS$_3$*	10	SI
N468.14	*PhS$_v$PiS$_{K1}$*	10	SI	N489.16	*PhS$_3$PiS$_{k1}$*	10	SI
N474.1	*PhS$_v$PiS$_{K1}$*	10	SI	N489.17	*PhS$_3$PiS$_{k1}$*	10	SI
N474.2	*PhS$_v$PiS$_3$*	10	SI	N489.18	*PhS$_3$PiS$_{k1}$*	10	SI
N474.3	*PhS$_3$PiS$_3$*	10	SI	N489.19	*PhS$_3$PiS$_{k1}$*	10	SI
N474.4	*PhS$_v$PiS$_3$*	10	SI	N489.20	*PhS$_3$PiS$_3$*	10	SI
N474.5	*PhS$_v$PiS$_3$*	10	SI	N489.21	*PhS$_3$PiS$_{k1}$*	10	SI
N474.6	*PhS$_v$PiS$_{K1}$*	10	SI	N489.22	*PhS$_3$PiS$_{k1}$*	10	SI
N474.7	*PhS$_v$PiS$_{K1}$*	10	SI	N489.23	*PhS$_v$PiS$_{k1}$*	10	SI
N474.8	*PhS$_3$PiS$_{K1}$*	10	SI	N489.24	*PhS$_3$PiS$_{k1}$*	10	SI
N474.10	*PhS$_3$PiS$_3$*	10	SI	N482.1	*PhS$_3$PiS$_{k1}$*	10	SI
N481.2	*PhS$_v$PiS$_3$*	10	SI	N482.2	*PhS$_3$PiS$_3$*	10	SI
N481.3	*PhS$_3$PiS$_{K1}$*	10	SI	N482.3	*PhS$_v$PiS$_{K1}$*	10	SI
N481.4	*PhS$_v$PiS$_{K1}$*	10	SI	N482.4	*PhS$_v$PiS$_{K1}$*	10	SI
N481.5	*PhS$_v$PiS$_{K1}$*	10	SI	N482.5	*PhS$_v$PiS$_{K1}$*	10	SI
N481.6	*PhS$_3$PiS$_{K1}$*	10	SI	N482.6	*PhS$_3$PiS$_{k1}$*	10	SI
N481.7	*PhS$_v$PiS$_3$*	10	SI	N482.7	*PhS$_v$PiS$_{K1}$*	10	SI
N481.8	*PhS$_v$PiS$_3$*	10	SI	N482.8	*PhS$_3$PiS$_{k1}$*	10	SI
N481.9	*PhS$_v$PiS$_3$*	10	SI	N482.9	*PhS$_v$PiS$_3$*	10	SI
N481.10	*PhS$_v$PiS$_{K1}$*	10	SI	N482.10	*PhS$_v$PiS$_3$*	10	SI
N481.11	*PhS$_3$PiS$_3$*	10	SI	N482.11	*PhS$_v$PiS$_{K1}$*	10	SI
N481.12	*PhS$_v$PiS$_3$*	10	SI	N482.12	*PhS$_v$PiS$_{K1}$*	10	SI
N481.13	*PhS$_v$PiS$_{K1}$*	10	SI	N482.13	*PhS$_v$PiS$_{K1}$*	10	SI
N481.14	*PhS$_3$PiS$_{K1}$*	10	SI	N482.14	*Ph$_{S3}$Pi$_{Sk1}$*	10	SI
N481.15	*PhS$_v$PiS$_{K1}$*	10	SI	N482.15	*PhS$_v$PiS$_{K1}$*	10	SI
N481.16	*PhS$_v$PiS$_{K1}$*	10	SI	N482.16	*Ph$_{S3}$Pi$_{Sk1}$*	10	SI
N481.17	*PhS$_3$PiS$_{K1}$*	10	SI	N482.17	*Ph$_{S3}$Pi$_{Sk1}$*	10	SI
N481.18	*PhS$_v$PiS$_3$*	10	SI	N482.18	*PhS$_v$PiS$_3$*	10	SI
N481.19	*PhS$_3$PiS$_{K1}$*	10	SI	N482.19	*Ph$_{S3}$Pi$_{Sk1}$*	10	SI
N481.20	*PhS$_v$PiS$_{K1}$*	10	SI	N482.20	*Ph$_{S3}$Pi$_{Sk1}$*	10	SI
N481.21	*PhS$_v$PiS$_{K1}$*	10	SI	N482.21	*Ph$_{S3}$Pi$_{Sk1}$*	10	SI
N481.22	*PhS$_v$PiS$_3$*	10	SI	N482.22	*PhS$_v$PiS$_{K1}$*	10	SI
N481.23	*PhS$_3$PiS$_{K1}$*	10	SI	N482.23	*PhS$_v$PiS$_{K1}$*	10	SI
N481.24	*PhS$_v$PiS$_3$*	10	SI	N482.24	*PhS$_v$PiS$_{K1}$*	10	SI
N489.1	*PhS$_v$PiS$_3$*	10	SI				

Table 3 Testing compatibility in T_1 plants derived from *AhSLF-S2* transformant

Plant ID	Genotype	Number of Pollinated Flowers	Results	Plant ID	Genotype	Number of Pollinated Flowers	Results
N469. 1	PhS_3PhS_v	10	SI	N470.9	PhS_3PhS_v	10	SI
N469.2	PhS_3PhS_v	10	SI	N472.1	PhS_3PhS_v	10	SI
N469.5	PhS_3PhS_v	10	SI	N472.4	PhS_3PhS_v	10	SI
N469.6	PhS_vPhS_v	10	SI	N472.5	PhS_3PhS_v	10	SI
N469.7	PhS_vPhS_v	10	SI	N472.6	PhS_3PhS_v	10	SI
N469.8	PhS_vPhS_v	10	SI	N473.1	PhS_3PhS_3	10	SI
N469.10	PhS_3PhS_v	10	SI	N473.3	PhS_3PhS_v	10	SI
N469.11	PhS_3PhS_v	10	SI	N473.7	PhS_vPhS_v	10	SI
N469.12	PhS_3PhS_v	10	SI	N473.8	PhS_3PhS_v	10	SI
N469.15	PhS_3PhS_v	10	SI	N473.9	PhS_3PhS_3	10	SI
N469.17	PhS_vPhS_v	10	SI	N473.10	PhS_vPhS_v	10	SI
N469.18	PhS_3PhS_v	10	SI	N473.11	PhS_vPhS_v	10	SI
N469.19	PhS_3PhS_3	10	SI	N473.12	PhS_3PhS_v	10	SI
N469.20	PhS_3PhS_3	10	SI	N473.14	PhS_3PhS_v	10	SI
N469.21	PhS_3PhS_v	10	SI	N473.15	PhS_3PhS_v	10	SI
N469.23	PhS_3PhS_v	10	SI	N473.16	PhS_vPhS_v	10	SI
N469.25	PhS_3PhS_v	10	SI	N473.18	PhS_3PhS_v	10	SI
N469.27	PhS_3PhS_v	10	SI	N473.19	PhS_3PhS_v	10	SI
N469.28	PhS_3PhS_3	10	SI	N473.20	PhS_3PhS_v	10	SI
N469.29	PhS_3PhS_v	10	SI	N473.21	PhS_3PhS_v	10	SI
N469.30	PhS_3PhS_v	10	SI	N473.22	PhS_3PhS_v	10	SI
N470.1	PhS_vPhS_v	10	SI	N473.23	PhS_3PhS_v	10	SI
N470.2	PhS_3PhS_v	10	SI	N473.25	PhS_3PhS_v	10	SI
N470.3	PhS_3PhS_v	10	SI	N473.27	PhS_3PhS_v	10	SI
N470.4	PhS_3PhS_v	10	SI	N473.28	PhS_3PhS_v	10	SI
N470.5	PhS_3PhS_v	10	SI	N473.29	PhS_vPhS_v	10	SI
N470.6	PhS_vPhS_v	10	SI	N473.31	PhS_vPhS_v	10	SI
N470.8	PhS_3PhS_v	10	SI				