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**EXPRESSION ANALYSIS IN SELF-
COMPATIBLE *PETUNIA HYBRIDA* AND
PROMOTER ANALYSIS OF *S*-LOCUS *F*-BOX
GENES/SLFS IN *PETUNIA INFLATA***



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ABSTRACT

Self-incompatibility (SI), a type of intraspecific reproductive barrier occurs widely in flowering plants and helps to avoid inbreeding and protects the genetic diversity of a population. When the pollen lands on a self-pistil, SI will prevent fertilization; when the pollen lands on the pistil of an unrelated plant, fertilization will not be affected. SI consists of two types: Sporophytic SI (SSI) and gametophytic SI (GSI). In GSI it is the haploid genotype of the pollen itself that controls the pollen phenotype. Transgenic studies in *Petunia* have shown that the pistil phenotype is determined by a stylar ribonuclease (*S-RNase*). In the fertilization process, *S-linked F-box genes/SLFs* that are expressed in pollen work together with *S-RNases* to determine the outcome of a pollination. Recently up to 20 *SLF* genes have been reported at the *S*-locus in *Petunia*, but the exact role of individual *SLFs* in controlling the phenotype is still not clear.

Part of this project aims to investigate *SLF* expression between two closely linked varieties of *Petunia hybrida*, which have different phenotypes. A combination of RT-PCR and qRT-PCR using specific primers were used to determine the expression patterns of individual *SLF* genes in these two varieties. The results of RT-PCR and qRT-PCR indicate that all *SLFs* express in these two varieties, and most *SLFs* share significant differences in the expression levels comparison between these two closely linked varieties of *Petunia hybrida*. The partial DNA sequences of *SLFs* in these two varieties are proved that is identical. Therefore, the different expression level of *SLFs* might is the cause of the different SI between these two closely linked varieties of *Petunia hybrida*. The finding of this part of this project might be able to provide some evidence in the evolution theory of SI plants.

On the other hand, there are only two *SLFs* expression analysis papers been published to date from Kao *et al.* ([2004](#)) and Hua *et al.* ([2007](#)), which indicated *SLFs* express in pollen tube, pollen and anther of *Petunia* by RNA gel blot analysis and RT-PCR, but there is no paper to show the expression pattern of *SLFs* using GUS or GFP analysis. This project also aims to explore the promoter analysis of

SLF genes that have recently been identified as part of a genome sequencing effort of *Petunia inflata*. The genomic sequence data were used to characterize the promoter regions to identify shared pollen promoter elements such as those identified for the gene *LAT52* (a tomato pollen gene). Promoter fragments of three selected *SLFs* were cloned and used to prepare *SLFpromoter:GUS* fusion constructs. *Agrobacterium*-based transformation was performed to transfer these constructs into *Petunia hybrida*. The GUS assay was used to test the *SLF* expression pattern in *Petunia*. The result of GUS analysis indicates *SLF3* is expressed in pollen of stamens, which is consistent with the findings from Kao *et al.* ([2004](#)) and Hua *et al.* ([2007](#)). This part also provides a good attempt in other transformation experiments to use the *SLF* promoter of *Petunia inflata*. In addition, the results of both these two parts of this project support each other: the expression of *SLFs* in *Petunia* shows an exponential growth during the maturation of pollen.

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CHAPTER 1 INTRODUCTION

1.1 Overview of self-incompatibility

In most angiosperms, the typical flower is composed of four parts: pistils, stamens, calyx and corolla. The pistil is composed of stigma, style and ovary, and the stamens are comprised of anthers and filaments. Most flowers possess stamens and pistils, and are called perfect or bisexual flowers (Bawa, [1980](#); Renner and Ricklefs, [1995](#)). During the reproductive process of plants, the mature pollen lands on the stigma, in the process called pollination. The process of combination of sperm cells and egg cells is called fertilization. Only after pollination and fertilization can plants generate seeds and produce offspring (Stoddard, [1986](#)). When the pollen lands on the stigma, the pollen tube grows through the stigma to the ovary following the identification of the stigma surface (Taylor and Hepler, [1997](#)). Then, the seeds start to grow after the combination of sperm cells, egg cells and polar nuclei cells (Russell, [1992](#)). There are two types of pollination: self-pollination and cross-pollination. Self-pollination is using self-pollen (pollen comes from the same plant as the pistil) to pollinate onto the stigma; cross-pollination uses non-self-pollen (pollen comes from different plants) to pollinate onto the stigma (Lloyd, [1992](#)).

As one of the responses to self-pollination, self-incompatibility (SI) is a type of intraspecific reproductive barrier in plants. SI usually occurs before fertilization in plants (Takayama and Isogai, [2005](#)). As explained by Kubo and co-workers ([2015](#)), "SI is a genetically controlled reproductive barrier in angiosperms that allows the pistil to reject self-pollen (genetically related) and accept non-self-pollen (genetically unrelated)". SI can help plants to avoid inbreeding and protects the genetic diversity of a population (Li *et al.*, [2017](#)). It is widely known that Darwin first discovered that SI exists widely in flowering plants (Darwin, [1862](#)). According to the statistics from East ([1940](#)), there are over 3000 species of plants that exhibit SI to protect their genetic diversity. In [1949](#), Darlington and Mather indicated that half of angiosperm species present SI phenomenon. More specifically, SI phenomenon is present in 74 angiosperms (Lewis, [1979](#)), and SI exists widely in the *Brassicaceae* and *Solanaceae* (Iwano and Takayama, [2012](#)).

1.2 Classification of self-incompatibility

There are two types of SI in plants: heteromorphic SI and homomorphic SI, which mainly differ according to morphological differences between pistils and stamens (Gibbs, [1986](#)). If SI occurs but there are no morphological differences between pistil and stamens, this type of SI is called homomorphic (Hinata *et al.*, [1993](#)). On the contrary, when pistil and stamens are different in morphology, this kind of SI is called heteromorphic (Stevens and Murray, [1981](#)).

1.2.1 Heteromorphic self-incompatibility

As heteromorphic SI mechanism uses morphological differences between pistils and stamens to avoid self-pollination, three different factors were discovered to cause SI in heteromorphic plants so far: 1) the different length of pistil and stamens (Stevens and Murray, [1981](#)), 2) the different characteristics of stigma surface (Heslop-Harrison and Shivanna, [1977](#)), and 3) the different size and morphology of pollen (Zavada, [1984](#)). These morphological differences are considered to be a valuable mechanism to promote accurate outcrossing pollination and reduce the interference from self-stamen (Cesaro and Thompson, [2004](#)).

In these three factors causing SI, heteromorphic SI is caused by the different length of pistil and stamens that usually can be divided into two types: distylous and tristylous. Firstly, distylous plants have two phenotype forms of flower: one type, called 'Thrum', has short style and long filaments; the other type called 'Pin' has long style and short filaments (Charlesworth, [1988](#)). These physiological features were observed in *Rubiaceae* (Pailler and Thompson, [1997](#)), *Primulaceae* (Lewis, [1949](#); Li *et al.*, [2007 b](#); Li *et al.*, [2016 a](#)) and *Oxalidaceae* (Weller *et al.*, [2007](#)). Secondly, tristylous plants have different combinations of long, medium and short styles. There are three types of tristylous plants: long style with short or medium stamens; medium style with short or long stamens; and short style with long or medium stamens (Allen and Hiscock, [2008](#)). Because of these different sizes and lengths of style and stamens, the pollen of heteromorphic plants is always hard to pollinate on self-stigma, which also promotes cross-

pollination of heteromorphic plants by insects (Manfield *et al.*, [2005](#); Barrett *et al.*, [2009](#)). Figure 1.1 describes the different types of structures of style and stamens in tristylous and distylous plants (Gardner *et al.*, [2012](#)).

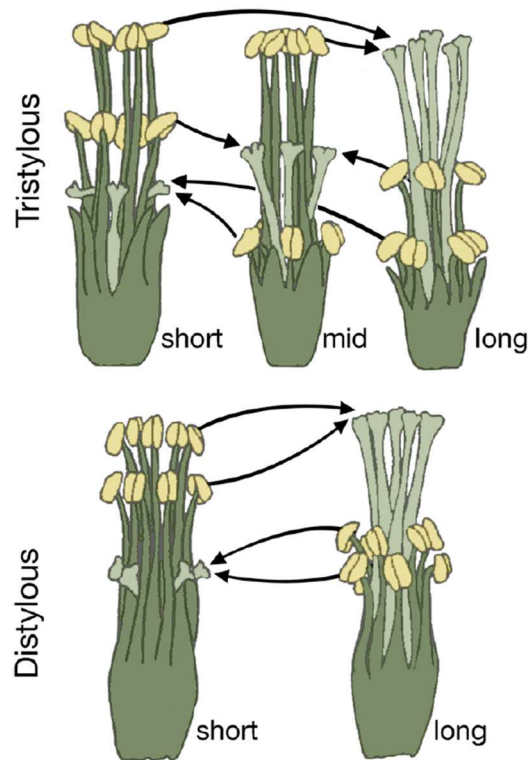


Figure 1.1 **Tristylous and distylous flowers in *Oxalidaceae***. Tristylous flowers have different combinations of long, medium and short styles, and distylous flowers have short and long styles, making self-pollination in these plants difficult. The breakdown of SI will happen only bypollination indicated by arrows (Gardner *et al.*, [2012](#)).

1.2.2 Homomorphic self-incompatibility

The most common type of self-incompatibility is homomorphic SI. Compared with heteromorphic SI, homomorphic SI is not related to any flower's morphological characteristics. The self-incompatibility of most homomorphic SI plants is controlled by one or more S-loci (Hinata *et al.*, [1993](#)). The S-alleles are located in S-loci, and usually contain two types of genes: the pistil S-gene for the determination of pistil-specificity, and the pollen S-gene for the determination of pollen-specificity (Robbins *et al.*, [2000](#)). Different pistil S-genes are closely

associated with a corresponding pollen S-gene, and they constitute different S-haplotypes (Kubo *et al.*, [2010](#)). According to the action of different pollen-specificity determining factors, homomorphic SI can be further divided into gametophytic SI (GSI) and sporophytic SI (SSI) (Gibbs, [1986](#)). The pollen phenotype of sporophytic SI is determined by the S-genotype of the diploid parent (sporophyte). SSI is commonly found in *Brassicaceae* family such as *Brassica napus* L. (Stein *et al.*, [1991](#)) and *Brassica oleracea* (Trick and Flavell, [1989](#)). The pollen phenotype of gametophytic SI is determined by the haploid pollen S-genotype (gametophyte) (Matton *et al.*, [1994](#)). GSI is found widely in many families, including *Solanaceae* (Ioerger *et al.*, [1990](#)), *Rosaceae* (Sassa *et al.*, [2010](#)) and *Papaveraceae* (Steinbachs and Holsinger, [2002](#); Bosch and Franklin-Tong, [2008](#)).

Figure 1.2 shows the outcome of pollinations with different pistil S-genotypes, but using the same S-genotypes pollen in gametophytic SI and sporophytic SI. For gametophytic SI, if the S-genotype of pollen is identified to be the same as the pistil, the growth of the pollen tube will be inhibited. If the genotype of the pollen is different from the pistil, fertilization will occur (Figure 1.2 a). For sporophytic SI, if the genotypes of pollen are totally different from the pistil, the pollen tube will grow normally. If any pollen shares same S-allele with pistil, the growth of pollen tube will be blocked (Figure 1.2 b). The significant difference between sporophytic SI and gametophytic SI is different phenotypes of pollen. For gametophytic SI, the pollen phenotype is determined by its haploid genotype. If the genotype of pollen is different from pistil, the pollen tube will stop growing. For sporophytic SI, the pollen phenotype is determined by the parental genotype. Thus, if the genotype of pollen parent has any S-allele shared with the genotype of the pistil, the growth of pollen tube will be inhibited (Schopfer *et al.*, [1999](#)).

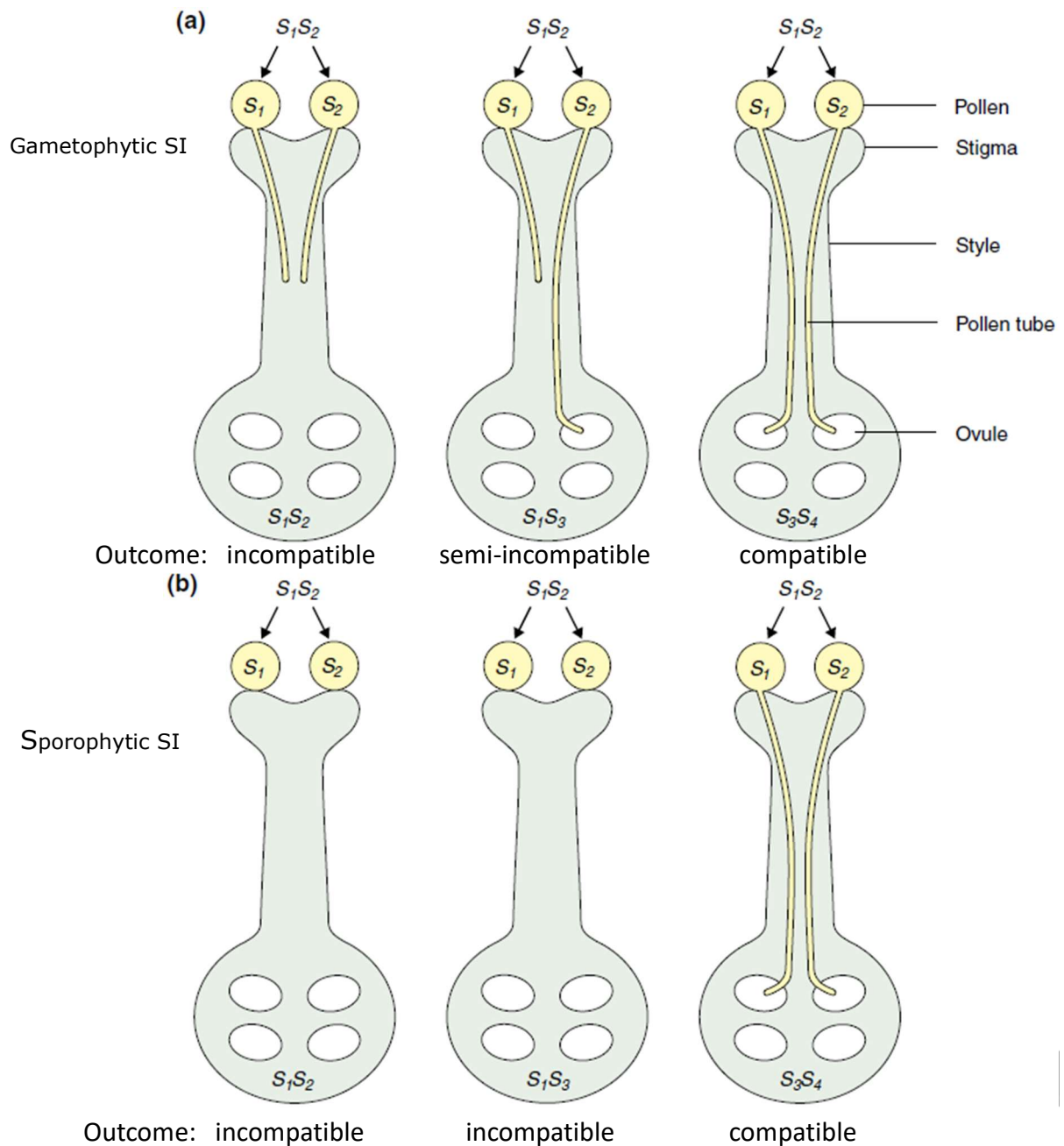


Figure 1.2 **The genetic control of gametophytic system and sporophytic system of SI.** (a): In gametophytic SI, when pollen of S_1 and S_2 lands on stigma of S_1S_2 , pollen tubes does not grow; when pollen of S_1 and S_2 lands on stigma of S_1S_3 , the S_2 pollen grows as a normal pollen tube, but S_1 pollen does not grow as far as the ovary; when pollen of S_1 and S_2 lands on stigma of S_3S_4 , both pollen tubes grow normally. (b): In sporophytic SI, when the pollen from S_1S_2 lands on stigma of S_1S_2 , pollen tubes do not grow, when it lands on S_1S_3 stigma the growth of pollen tube is inhibited; Only when pollen from S_1S_2 and pistil genotype is S_3S_4 , will normal pollen tube growth and fertilization occur (Hiscock, [2002](#)).

1.3 Sporophytic self-incompatibility

Sporophytic SI (SSI) is one of the types of homomorphic SI. Most SSI plants are controlled by a single locus, such as the *Brassicaceae* (Uyenoyama, [1995](#)) or *Compositae* family (Reinartz and Les, [1994](#)). As a special case, Sesame (*Sesamum indicum*) has SSI but it is controlled by multiple loci (Ram *et al.*, [2006](#)). There are two types of relationships between SSI heterozygous S-alleles: independent and dominant/recessive relationship. Independent relationship means that different heterozygous alleles in male determinant are independent and there is no interaction (Thompson, [1957](#)). Dominant and recessive relationship is indicated if one of two different alleles is activated, another allele will completely or partly lose activity (Thompson, [1957](#)). Thompson ([1972](#)) also indicated that the interaction between two dominant alleles (one is dominant allele in style and another is dominant in pollen) might lead to the decrease of incompatibility, and even convert incompatibility to compatibility.

SSI exists in *Brassicaceae*, *Asteraceae*, *Auriculata*, *Betulaceae* and *Araucariaceae* (Charlesworth, [1988](#)). The compatibility of *Brassicaceae* is better explained at the molecular level than others (Baldwin and Schoen, [2017](#)). *Brassica* is the common model in *Brassicaceae* for studying SSI (Stein *et al.*, [1991](#); Ma *et al.*, [2016](#)). There are over 60 alleles of the S-locus were identified in SSI plants (Ockendon, [1974](#)). Most of pistil S-alleles and pollen S-allele were successfully identified from *Brassica*, such as SLG-2, SLG-22 and SLG-29 (Dwyer *et al.*, [1991](#)) was identified from *Brassica oleracea*, S-receptor kinase (SRK) was identified from *Brassica napus* (Stein *et al.*, [1993](#)), and S-locus protein 11 (SP11; or called S-locus cysteine-rich (SCR)) was identified from *Brassica campestris* (Suzuki *et al.*, [1999](#); Schopfer *et al.*, [1999](#)).

1.4 Gametophytic self-incompatibility

Gametophytic SI (GSI) is widely found in the majority of SI plants (Allen and Hiscock, [2008](#)). Some GSI plants are controlled by a single S-locus, like the

Solanaceae (Ioerger *et al.*, [1991](#)). Some GSI plants are controlled by two or more *S*-locus. For example, the SI of *Poaceae* relates to two loci: the *S*-locus and the *Z*-locus (Baumann *et al.*, [2000](#)). Only if the *S*-allele in pollen and *Z*-allele in stigma are different, will fertilization continue normally (Hayman and Richter, [1992](#)). Furthermore, the plants from *Chenopodiaceae* and *Ranunculaceae* are controlled by multiple *S*-loci (ØSTERBYE, [1978](#)). There are several types of SI signal transduction systems in GSI, such as *S*-RNase-based SI and Ca^{2+} signalling SI systems (Franklin-Tong and Franklin, [2003](#)). The Ca^{2+} signalling SI system (representative family: *Papaver*) involves the SI signalling and programmed cell death (PCD) of actin cytoskeleton (Wheeler *et al.*, [2001](#); Wheeler *et al.*, [2009](#); Li *et al.*, [2007 a](#)). Because this project does not involve this specific system, its further details are not described here. The representative families of *S*-RNase-based SI system are *Solanaceae* and *Rosaceae* (Franklin-Tong and Franklin, [2003](#)). In *S*-RNase-based SI system, SI is considered to be controlled by single *S*-locus, and two determinants locate in there: female determinant and male determinant (*S*-RNases and SLFs) (Sims and Robbins, [2009](#)).

1.4.1 Gametophytic SI in *S*-RNase-based plants: *S*-RNases (the female determinant)

1.4.1.1 The discovery and molecular structure of *S*-RNases

SI in *S*-RNase-based SI plants is controlled by two determinants, and the female determinant (*S*-RNase) was discovered earlier than the male determinant. The *S*-RNase was identified in the pistil of tobacco in 1986 as the female determinant of SI (Anderson *et al.*, [1986](#)), and has been studied extensively (Moore *et al.*, [1990](#); Kuroda *et al.*, [1995](#); Ishimizu *et al.*, [1996](#)). Several proteins were detected by electrophoresis gels from the pistil of *Petunia hybrida*, and it was found that these proteins are glycoproteins with low molecular weight that are recognizable and unique (Bredemeijer and Blaas, [1981](#)). In a subsequent study, these glycoproteins were confirmed to have close relationships with *S*_{1L}-allele, *S*_{2L}-allele and *S*_{3L}-allele (Broothaerts *et al.*, [1990](#)). Through the expression studies of these three

glycoproteins, it was confirmed that they are only expressed in pistil rather than in the stamens or the entire flower. Therefore, the gene encoding these three glycoprotein were initially named as *S*-pistil (Broothaerts *et al.*, [1990](#)). From functional studies it was found that *S*-pistil needs glycosylation to play its role in tobacco during SI (Woodward *et al.*, [1989](#)), and this also was confirmed in *Petunia* (Broothaerts *et al.*, [1991](#)). In addition, the expression of *S*-*pistil* showed an increasing trend in flower progression from the immature to mature state and it reaches its peak when the flower is fully matured (Broothaerts *et al.*, [1991](#)). After structural comparison and functional test experiments, *S*-pistil was confirmed as a ribonuclease that specifically inhibits the growth of the pollen tube (Broothaerts *et al.*, [1991](#)). In addition, because *S*-pistil and fungal T2-type ribonucleases are highly similar in function, 'S-RNases' is a most commonly used term to refer to this female determinant in S-RNase based SI (Sims and Robbins, [2009](#)).

As mentioned above, the function of S-RNases is considered to be a rejection of the growth of pollen tube (McClure *et al.*, [1989](#)), which is necessary in SI reaction of *Petunia inflata* (Huang *et al.*, [1994](#)). The knockdown of *S*-RNase caused the pistil to lose the ability to inhibit fertilization of same *S*-type of pollen, indicating that the enzymatic activity of S-RNase plays a vital role during SI (Lee *et al.*, [1994](#)). In additions, the S-RNases was shown to non-specifically degrade RNA in pollen tube, which means the recognition from different pollen is dependent on other decisive factors (McClure *et al.*, [1990](#)).

1.4.1.2 The molecular structure of S-RNases

S-RNase proteins have a similar protein structure to fungal T2-type ribonucleases (Ida *et al.*, [2001](#)), which can be divided into two functional regions: five conservative and two hypervariable regions. The primary structure is shown in Figure 1.3. The main structural areas of S-RNases are 5 conserved (C1-C5) and 2 hypervariable regions (HV_a and HV_b). The conserved regions of S-RNases were considered to maintain the basic function of S-RNases (Ai *et al.*, [1990](#); Huang *et al.*, [1994](#)). Further conserved region study indicated that these regions determine

the activity of S-RNase (Huang *et al.*, [1994](#)). The codon of 'HIS-93' in the conserved region has been considered to be involved in ribonuclease activity (Ai *et al.*, [1990](#)). Replacing the codon of 'HIS-93' in S₃-RNase of *Petunia inflata* with the codon of asparagine, found that the S₃-RNase of transgenic plant lost the ribonuclease activity and failed to reject S₃ pollen (Huang *et al.*, [1994](#)). This is because 'HIS-93' is essential for the enzyme activity of S₃-RNase.

The hypervariable regions of S-RNases HVa and HVb are located in between two conserved regions C2 and C3. These two hypervariable regions (HVa and HVb) were considered as the main factors to refuse self-pollen rather than the carbohydrate portion of S-RNases (Karunanandaa *et al.*, [1994](#); Kao and McCubbin, [1996](#)). These two regions also be considered that is the main region for distinguishing the specificity of different S-RNases (Ioerger *et al.*, [1991](#); Xue *et al.*, [1996](#)). Matton *et al.* ([1997](#)) used *Solanum chacoense*, a wild potato species (a member of *Solanaceae*), as a system to replace different amino acids among different hypervariable regions. It was indicated that the specificities of S₁₁-RNase and S₁₃-RNase could be inter-converted, which proved the hypervariable region plays an essential role in the specificity of the S-RNase. Moreover, there is a signal peptide near the N-terminus of S-RNase that was found to be responsible for guiding the protein into the pollen tube (Kao and McCubbin, [1996](#)).

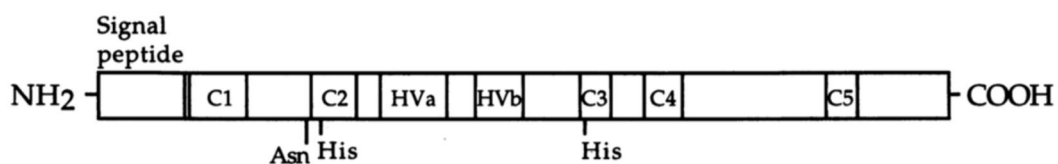


Figure 1.3 **The structure of S-RNase protein in GSI plants.** HVa and HVb indicate hypervariable regions, and C1-C5 refer to conserved regions (Kao and McCubbin, [1996](#)).

Ida and his co-workers ([2001](#)) showed the tertiary molecular structure of S-RNase via X-ray crystallography (Figure 1.4): 8 α -helical structures and 7 β -sheets folded together. Two of the eight α -helices were confirmed as part of the two hypervariable regions HVa and HVb. These two hypervariable regions are in the outer part of the S-RNase molecule, which allows S-RNase molecules to come to contact with solvents easily. The amino acid sequence comparison of two S-

RNases (S_{11} -RNase and S_{13} -RNase from *Solanum chacoense*) indicated that they only have 10 amino acids that are different, and all these differences come from hypervariable regions. Moreover, when three of these ten amino acids were cloned from S_{11} -RNase into S_{13} -RNase, the mutated S_{13} '-RNase obtained the ability from both S-RNases to refuse pollens contained S_{11} -allele or S_{13} -allele (Matton *et al.*, 1999). It should be noted that HVa region is positively charged and HVb is negatively charged, while other regions are neutrally uncharged (Ida *et al.*, 2001; Sims and Robbins, 2009).

The conserved region also plays an essential role in the function of S-RNases. Some amino acid residues were found to contribute to the catalytic function of S-RNases, for example, the C2 and C3 regions from the conserved region were found to contain two histidine residues (His32 and His91). His32 and His91 are together with another residue Lys90 to form the catalytic site of the ribonuclease (Ida *et al.*, 2001). In addition, five amino acid residues (Tyr4, Gln6, Trp35, Asp37 and Arg42) and another eight amino acid residues (Thr10, Phe15, Gln69, Leu70, Lys71, Phe72, Pro82 and Ser83) from conserved region consisting of two ends of a rift, and this rift can bind to other nucleotide substrates (Ida *et al.*, 2001).

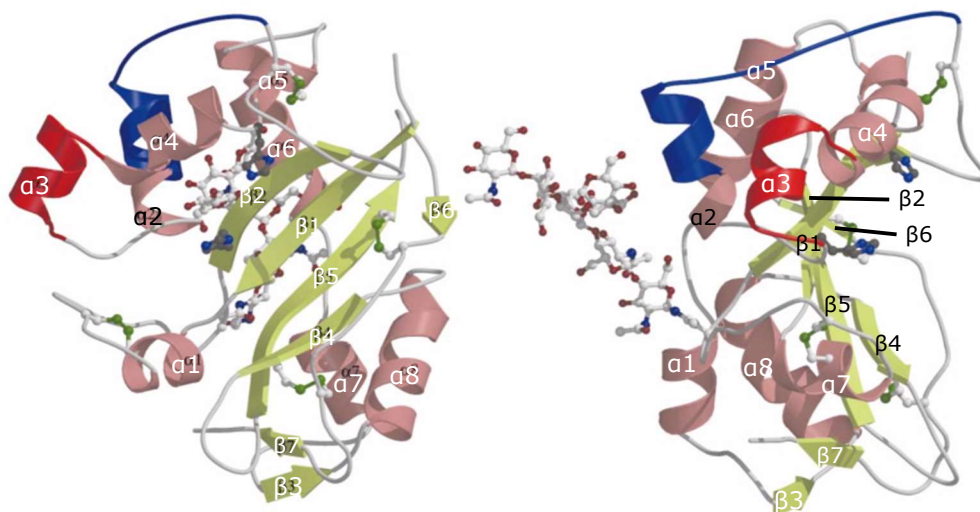


Figure 1.4 **The front (left) and back (right) of S-RNase structure of tobacco.** The $\alpha 1$ - $\alpha 8$ refers to the 8 α -helixes, $\beta 1$ - $\beta 7$ refers to the 7 β -sheets. HVa region ($\alpha 2$) is marked as blue and HVb region ($\alpha 3$) is marked as red. The structure of red ball, white stick and blue ball are the catalytic residues, disulphide bonds and carbohydrates, respectively (Ida *et al.*, 2001).

1.4.1.3 The diversity of *S*-alleles and *S*-RNases

The identification of pollen in most GSI plants during SI requires *S*-RNases and SLFs to jointly determine the outcome (Hua *et al.*, [2007](#)). This can be likened to locks and keys, and when the “key” is matched with the “lock”, the growth of pollen tube will be inhibited. In order to have enough types of “keys” and “locks” for their species, most GSI plants will increase the *S*-allele number to ensure the diversity of SI (De Nettancourt, [1997](#)). For the *Petunia* genus, near 40 different *S*-alleles have been found in all wild species (Tsukamoto *et al.*, 2003). In the case of *Petunia hybrida*, several common varieties were found to contain about ten different *S*-alleles (Robbins *et al.*, [2000](#)). In fact, the diversity of *S*-alleles is much lower than the diversity of its wild species (Sims and Robbins, [2009](#)), this indicates that GSI plants usually have some way to ensure the wild species have enough *S*-allele diversity.

The naming of *S*-RNases is usually assisted with the names of different *S*-alleles, because of the uniqueness of *S*-RNases. It was shown that each *S*-haplotype only contains one kind of *S*-RNase (Kubo *et al.*, [2010](#)). Therefore, using the same symbol of *S*-allele to name *S*-RNase is a direct way to distinguish *S*-RNase. Meanwhile, this is also related to previous studies in which researchers thought that *S*-RNase was the only gene that is located in *S*-allele, and is also controlling SI (Clark and Sims, [1994](#)). The naming of *S*-alleles and *S*-RNases share a same symbol. For example, the *S*-RNase that is isolated from *S*_o-allele is called *S*_o-RNase.

As described above, the diversity of *S*-RNase is controlled through some important amino acids in hypervariable regions (Ida *et al.*, [2001](#)). The discovery of different *S*-RNases was mainly around the 1990s, after which *S*-RNase was considered as the regulator of the SI system (McClure *et al.*, [1989](#)). Broothaerts *et al.* ([1990](#)) identified three glycoproteins from three *Petunia hybrida* varieties which come from Linsken’s group, and named them as *S*_{1L}-RNase, *S*_{2L}-RNase and *S*_{3L}-RNase. Clark and his co-workers ([1990](#)) extracted the RNA from the pistil of three *Petunia hybrida* varieties which contain *S*₁-allele, *S*₂-allele and *S*₃-allele (these three varieties come from Flaschenriem and Ascher, [1979](#)), and obtained the cDNA of

*S*₁-RNase, *S*₂-RNase and *S*₃-RNase by cloning. *S*_x-RNase (Ai *et al.*, [1992](#)) and *S*_v-RNase (Robbins *et al.*, [2000](#)) were subsequently identified and shown to be distinct by means of sequencing. In fact, the *S*-RNases identified by Broothaerts *et al.* (1989) and Clark *et al.* ([1990](#)) were all named as *S*₁-RNase, *S*₂-RNase, and *S*₃-RNase, but have different sequences. To avoid confusion, Robbins and co-workers ([2000](#)) suggested distinguishing these *S*-RNase by adding an "L" (Linsken's initial) as an extra postfix for the *S*-RNases identified by Broothaerts *et al.* ([1990](#)). Similar methods were also used to identify many *S*-RNases from *Petunia inflata*: *S*_{1l}-RNase, *S*_{2l}-RNase and *S*_{3l}-RNase (Ai *et al.*, [1990](#)). Additionally, when comparing these *S*-RNases found from *Petunia inflata* and *Petunia hybrida*, the N-terminal sequences of these *S*-RNases were found to be similar but still unique to that species (Sims and Robbins, [2009](#)).

*S*_b-RNase involved in this project was first identified from the *S*-allele of a self-incompatible *Petunia* variety by Broothaerts and his co-workers ([1991](#)). Among the 20 N-terminal amino acid sequences of the *S*_b-RNase, there are seven identical amino acids in *S*_b-RNase when comparing with the sequence of other *S*-RNases, which suggested that *S*_b-RNase and other *S*-RNases have a similarity of amino acid sequence (Broothaerts *et al.*, [1991](#)). *S*_o-RNase was first separated as a 31kDa protein from a self-compatible *Petunia* variety, and it was confirmed that this is a new pistil *S*-protein after its sequencing, and was named as *S*_o-RNase (Ai *et al.*, [1991](#)). Kubo and his co-workers ([2015](#)) also found an *S*-RNase which has a 99% similar genomic sequence with *S*_o-RNase from a different cultivated line of *Petunia*, and this *S*-RNase was named as *S*_{om}-RNase. In fact, *S*_{om}-RNase and *S*_o-RNase just have different names named by different groups, because their sequences are identical no matter genomic sequences or amino acid sequences (Czyzyk, [2010](#)).

1.4.2 Gametophytic self-incompatibility in *S-RNase* system: SLFs (the male determinant)

1.4.2.1 The discovery of SLFs

Before the male determinants were observed from pollen, SI was considered to be controlled by the *S-RNase* alone (Clark and Sims, [1994](#)). Evidence for a separated male determinant of the *S-RNase* system was first observed from *Pyrus serotina* (Sassa *et al.*, [1997](#); Tao and Iezzoni, [2010](#)). Sassa and co-workers ([1997](#)) found a mutation of *S-RNase* in *Pyrus serotina*, which lost SI ability in stigma but the SI ability in pollen was still maintained. This indicated that there is a different *S*-gene that exists in pollen to co-control SI with *S-RNases* and this phenomenon was also observed in *Petunia* (Sassa *et al.*, [1997](#); Dowd *et al.*, [2000](#)).

Lai and co-workers ([2002](#)) identified an F-box gene cluster at the *Antirrhinum* *S*-locus and named it as *AhSLF-S₂* (*Antirrhinum hispanicum* *SLF-S₂*). *SLFs* are considered as the best candidates of SI male determinant, for several reasons (Qiao *et al.*, [2004](#)). Firstly, the proteins of *SLFs* only exist in pollen (Sijacic *et al.*, [2004](#)), which is in line with the expectations of the expression position of SI male determinant. Secondly, *SLF* genes are located at the *S*-locus, which are physically close to the *S-RNase* compared with other candidates (Kubo *et al.*, [2010](#)). Thirdly, *SLFs* are directly involved in the recognition progress of *S-RNases* in pollen tube and the controlling of pollen tube growth (Wang and Xue, [2005](#); Takayama and Isogai, [2005](#); Kubo *et al.*, [2010](#)). Fourthly, *SLFs* have less allelic sequence polymorphism than *S-RNases*. However, *SLFs* have the ability to recognize different kinds of non-self *S-RNases* (Wang *et al.*, [2004](#); Kubo *et al.*, [2010](#)). Kubo and co-workers ([2015](#)) identified 16 *SLFs* in *Petunia hybrida*, which indicated that multiple *SLFs* are able to recognize all non-self *S-RNases* by some combination method. All the evidence stated above supports the assertion that *SLFs* are the male determinants of SI in the *S-RNase* system.

1.4.2.2 *S-linked F-box genes (SLFs)*

There are nearly 700 and 800 F-box genes identified in *Arabidopsis* and rice respectively (Kuroda *et al.*, [2002](#); Xu *et al.*, [2009](#)), such a high number suggests the F-box genes family can be considered as one of the supergene families in plants. However, these F-box genes have diverse functions. F-box proteins can regulate many physiological processes in plants, such as hormone regulation (Potuschak *et al.*, [2003](#); Dharmasiri *et al.*, [2005](#)), light signal transduction (Stolpe *et al.*, [2005](#)) and floral organ development (Chae *et al.*, [2008](#); González-Carranza *et al.*, [2007](#)). The N-terminal region of F-box proteins is composed of 40-50 amino acids (Cui *et al.*, [2015](#)), the C-terminal region usually contains some secondary structures that closely react with other proteins, and these structures aid the identification of substrates (Jin *et al.*, [2005](#)).

Some F-box proteins are considered to be involved in the ubiquitination protease process (King *et al.*, [1996](#); Skowyra *et al.*, [1997](#)). Ubiquitin is a highly conserved protein, and the main function of ubiquitin is to mark the proteins that need to be degraded by the 26S proteasome (Skowyra *et al.*, [1997](#)). Sequence analysis shows that about 6% of the proteins encoded in *Arabidopsis* play a role in the ubiquitin-proteasome degradation pathway (Dreher and Callis, [2007](#)). During this degradation pathway, the targeting of protein (ubiquitination) usually requires the participation of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), ubiquitin ligase (E3) (Hershko *et al.*, [1983](#)). E3 is substrate-specific and can recognize the specific proteins, and then helps to target these proteins (Hershko *et al.*, [1983](#)). During the ubiquitination, SCF complex is formed to combine with the substrate and consists of four subunits, which contain Cullin (CUL1), Suppressor of kinetochore protein 1 (SKP1), Ring-box1 protein (RBX1) and F-box protein (Jin *et al.*, [2005](#); Entani *et al.*, [2014](#)). The three subunits: CUL1, SKP1, and RBX1 form a general skeleton, and recognize different substrates by binding to different F-box proteins (Jin *et al.*, [2005](#); Zhao *et al.*, [2010](#)).

As described above, the main function of *S-linked F-box genes (SLFs)* in S-RNase based SI is as an important part of SCF complex to identify different S-RNases, and the discovery of SCF complexes in the extracts of styles supports this (Entani *et al.*, [2014](#)). During the fertilization process, the S-RNase is cytotoxic to the self-pollen, through the degradation of RNA in the pollen tube, which stops the pollen tube growth and fertilization (Goldraij *et al.*, [2006](#)). S-RNases are indiscriminate to degrade RNA in pollen tubes and are unable to distinguish self-pollen and non-self-pollen. SLFs play the role to distinguish different S-RNases from 'self' and 'non-self' (Sims and Robbins, [2009](#)). SLFs and other three subunits together form an SCF complex (also known as SCF^{SLF} complex) to discriminate different S-RNases (Li *et al.*, [2014](#); Entani *et al.*, [2014](#)). In more detail, different S-RNases are distinguished by the SLF protein on the SCF complex (Wang *et al.*, [2004](#)). SLFs are also considered as ubiquitin ligases of E3 during ubiquitination that happens in the pollen tube (Hua and Vierstra, [2011](#)). Entani and his co-workers ([2014](#)) isolated SCF^{SLF} complexes from pollen, and these SCF^{SLF} complexes were observed to work together with the 26S protease to degrade different S-RNases *in vitro*. This phenomenon was also confirmed by using peach as plant material (Chen *et al.*, [2018](#)).

1.4.2.3 The protein structure of SLFs

The protein structure of SLFs consists of two parts: several α -helices in the N-terminal and several β -sheets in the C-terminal of the *SLF* protein (Li *et al.*, [2017](#)). A conservative F-box domain is located in α -helices, and the function of this F-box domain is to ensure that the SLF have basic functions as F-box gene. The β -sheets contain an FBA domain (F-box associated domain), which is the "hypervariable region" of SLF, mainly encoding the specific residues to allow each SLF to differentiate different SLFs. In fact, this domain also has been shown to be the main region that identifies and interacts with the two hypervariable region of S-RNase HVa and HVb (Li *et al.*, [2017](#)). The Figure 1.5 shows the schematic diagram of SLF structure (Figure 1.5 b) and the three-dimensional structure diagram to demonstrate how SLF interact with S-RNase (Figure 1.5 a).

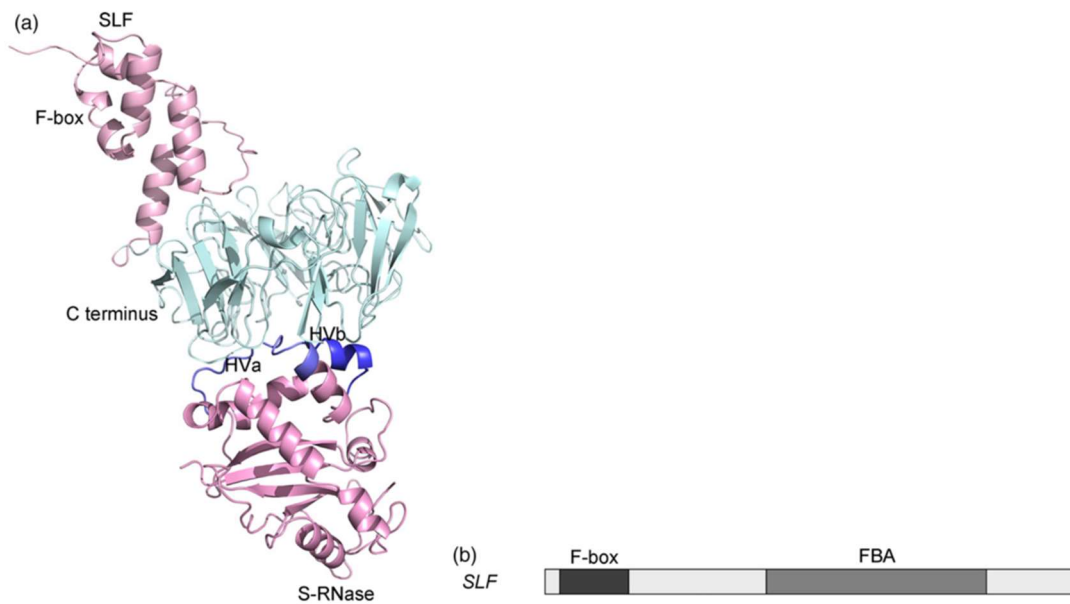


Figure 1.5 The three-dimensional structure diagram describing the interaction of SLF S-RNase (a) and the schematic diagram of SLF structure (b). In the 3D protein structure model (a), the pink helices of SLF represent the α -helices of SLF and the cyan-blue structure represents the C-terminus which contain FBA domain. The dark-blue helix represents the hypervariable region of S-RNase HVa and HVb, and the pink structure is the conserved region of S-RNase. In the schematic diagram of SLF, F-box domain and FBA domain are included (Li *et al.*, [2017](#)).

1.4.2.4 The diversity of *SLFs*

Compared with *S-RNases*, there is no certain number of *SLFs* for a given haplotype to date. This is because one *S*-allele or *S*-haplotype contains many *SLFs*, and there are over ten *SLFs* identified in *Petunia* (Sim and Robbins, [2009](#)). For example, 17 *SLFs* were isolated from *S*₂-haplotype and *S*₃-haplotype of *Petunia inflata* (William *et al.*, 2014), but surprisingly the *S*₆-haplotype of *Petunia inflata* was found to contain 29 *SLFs* (Bombarely *et al.*, [2016](#)). In *Petunia hybrida*, Kubo and his co-workers ([2015](#)) identified 16-19 *SLFs* in five self-incompatible haplotypes (e.g. *S*₅-haplotype, *S*₇-haplotype) and two self-compatible haplotypes (*S*_m-haplotype and *S*_{om}-haplotype). In addition, 18-20 *SLFs* were found in *S*₁₇-haplotype, *S*₁₉-haplotype and some haplotypes of *Petunia axillaris* (Kubo *et al.*, [2015](#)).

To sum up, different *Petunia* haplotypes were found to have different numbers of *SLF* genes, the sequences of these *SLFs* are also different, thus, although they have the same name such as *SLF1*, *SLF2* etc., they cannot be considered as the same *SLFs* if they come from different haplotypes. For example, the sequence of *SLF1* from *S*₇-haplotype of *Petunia hybrida* is different from the *SLF1* from *S*₀-haplotype of *Petunia hybrida*, but they have a close relationship on the phylogenetic tree (Kubo *et al.*, [2015](#)). Also, it has been determined that all *SLFs* are expressed simultaneously in the pollen and pollen tube (Li *et al.*, [2016 b](#); this study). Using immunoprecipitation, it was shown that 17 *SLF* genes identified from *S*₂-haplotype and *S*₃-haplotypes of *Petunia inflata* were all synthesized into proteins during the SI process only in the pollens (Li *et al.*, [2016 b](#)).

1.5 Gametophytic self-incompatibility models in *S-RNase* system

1.5.1 Recent gametophytic self-incompatibility models in *S-RNase* system

The function of male and female determinants in the *S-RNase* system is considered to be removing the cytotoxicity of non-self *S-RNase* and degradation of growth-related RNA (McClure *et al.*, [1989](#); Entani *et al.*, [2014](#)). From the perspective of cytotoxicity of *S-RNases*, the cytotoxicity of non-self *S-RNases* is decreased by ubiquitination involving multiple SCF^{SLF} complexes, and non-self *S-RNases* are subsequently degraded by the 26S proteasome. Self *S-RNases* retain their cytotoxicity to inhibit the growth of pollen tube (Entani *et al.*, [2014](#)). A molecular study to explain the self-incompatibility response in the *Solanaceae* was conducted by Takayama and Isogai ([2005](#)) who summarized the interaction studies between male and female determinants and built a molecular model to explain self-incompatibility response in the *Solanaceae* (Figure 1.6). It is worth pointing out that *S-RNase* is transferred into the pollen tube (Luu *et al.*, [2000](#)).

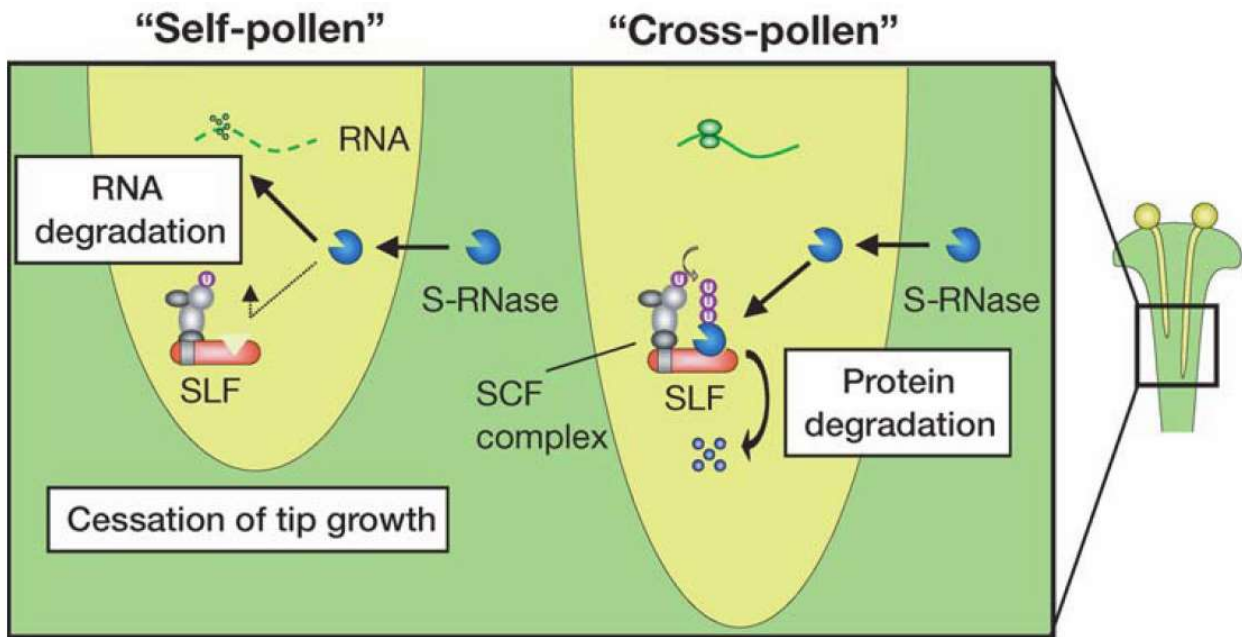


Figure 1.6 **Molecular model of the interaction between S-RNases and SCF^{SLF} complexes in the self-incompatibility of *Petunia*.** When pollen falls on the pistil, S-RNases enter into pollen tube from extracellular matrix of pistil. When SLFs have same S-haplotype as the S-RNases, ubiquitination does not occur, and S-RNase will degrade growth-related RNA and the growth of the pollen tube will be arrested. When SLFs have different S-genotypes from the S-RNases, ubiquitination of S-RNases takes place, as an SCF complex is formed. The SCF complex will subsequently be degraded by the protease. The pollen tube therefore grows normally without influences from S-RNases (Takayama and Isogai, [2005](#)).

In [2011](#), McClure and co-workers proposed a different model to describe how S-RNase degradation occurs in the style. Figure 1.7 shows that different kinds of SLFs proteins exist in pollen tube during the growth of pollen tube. When pistil is compatible with pollen, SLFs proteins (such as SLF1y, SLF2y) will degrade S-RNases proteins. When pistil is incompatible with pollen, there are no corresponding SLFs proteins to degrade S-RNases proteins (SRNx). Therefore, S-RNases proteins will inhibit the activity of pollen RNA, and the growth of pollen tube will be blocked.

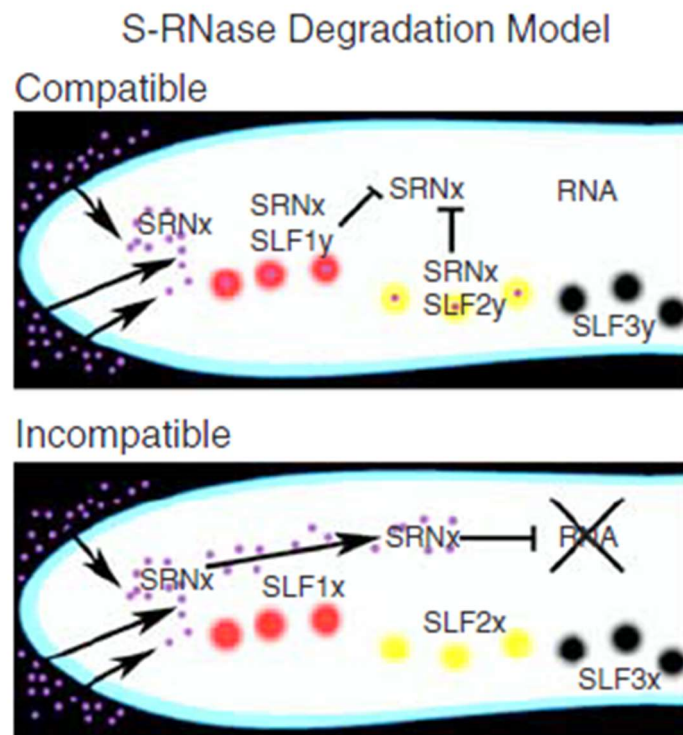


Figure 1.7 **Model for explaining S-RNase degradation.** SRNxs (S-RNase, purple points) exist in pollen tube. When SRNxs are transformed into internal pollen tube, the SLF proteins SLF1y (red points), SLF2y (yellow points) and SLF3y (black points) are starting to recognize SRNxs. Once SRNxs are recognized and combine with SLF proteins, it shows compatibility in pistil. The pollen tube can continue growing. When SRNxs could not combine with SLF proteins, it will degrade RNA in pollen tube, which shows incompatible in pistil (McClure *et al.*, [2011](#)).

Using next-generation sequencing and PCR, multiple *SLFs* were identified in some *S*-haplotypes of *Petunia* (Kubo *et al.*, [2015](#)). According to the report of Kubo and co-workers ([2015](#)), *S_oS_o* homozygote of *Petunia hybrida* has 16 *SLFs*. Before these multiple *SLFs* were identified in *Petunia*, Kubo and co-workers ([2010](#)) considered that different *SLFs* recognise different S-RNases. Hence, they proposed a hypothesis called 'collaborative non-self-recognition model' (Figure 1.8). This model indicated at least three types of *SLFs* that work as the male determinant and each of them recognises a subset of non-self S-RNases respectively or collectively. Kubo indicated that the *SLFs* in each *S*-allele mainly recognize the *S*-

RNase that come from other *S*-alleles. The missing of corresponding *SLF* is the reason why *S*-RNases were remained in self-incompatible haplotypes to degrade RNA and not stop the growth of pollen tube. Fujii and his co-workers (2016) believed that the self-compatible haplotypes are also working with this model, with the *S*-allele contains the corresponding *SLF* in its *S*-allele and the *SLF* will prevent *S*-RNase from degrading RNA in the pollen tube. They also pointed out that the self-compatible plants might be evolved from self-incompatible plants. A self-incompatible plant can become a self-compatible plant by the generation of a new *SLF* to overcome SI.

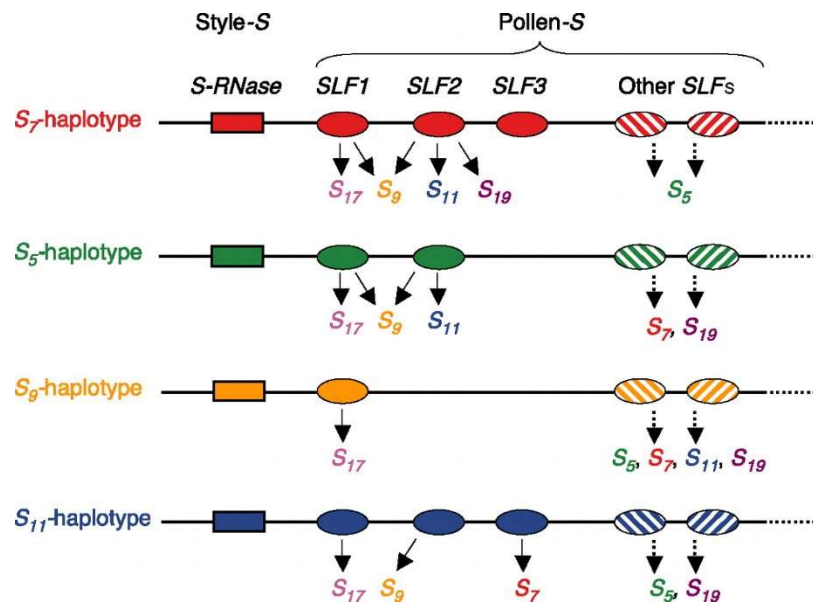


Figure 1.8 **Collaborative non-self-recognition system for S-RNase based self-incompatibility.** Style-S and pollen-S indicate female and male determinants of SI, respectively. Single *S-RNase* constitutes style-S and multiple *SLFs* constitute pollen-S. Each *SLF* can specifically recognize one or more *S-RNases*. For example, pollen-S *SLF1* recognises *S*₁₇-RNase. On the other hand, *SLF1* and *SLF2* could co-recognize *S*₉-RNase in *S*₇-haplotype and *S*₅-haplotype. For recognizing all *S-RNase*, different *SLFs* (*SLF1*, *SLF2*, *SLF3*...other *SLFs*) exist in pollen-S and all non-self *S-RNases* have their specific *SLFs* to recognise (Kubo *et al.*, 2010).

In a recent study, Sun and his colleagues (2018) studied the interaction between SLFs and S-RNases and their role in SI. A CRISPR-Cas9 technology was used to knockout the Skp1 subunit of SCF^{SLF} complex in different varieties of *Petunia inflata*. By observing interaction of the SLF proteins and the S-RNase protein in these transgenic plants, the relationships between the SLF proteins and the S-RNase protein was similar to previous research (Kubo *et al.*, 2010), which has two types of interaction between them: one-to-one (one SLF recognizes one S-RNase) and multiple-to-one (multiple SLFs recognize one S-RNase). The interactions are summarised and presented in Table 1.1 (Sun *et al.*, 2018). Additionally, this analysis also indicated that the polymorphism of *S-RNases* is possibly higher than estimated, the exact number of *SLFs* and one-to-one identification method does not limit the polymorphism of *S-RNases*, because the multiple-to-one identification method allows more recognizable codes (*SLFs* combinations) to exist (Sun *et al.*, 2018).

Table 1.1 A summary of interactions between different S-RNases and SLFs of *Petunia inflata* using CRISPR-Cas9 technology. The '+' or '(+)' indicate there is the reaction of S-RNases and SLFs was observed, the '-' or '(-)' indicate there is no interaction between S-RNases and SLFs observed (Sun *et al.*, 2018).

	S ₂ - RNase	S ₃ - RNase	S ₅ - RNase	S _{6a} - RNase	S ₇ - RNase	S ₁₁ - RNase	S ₁₂ - RNase	S ₁₃ - RNase	S ₁₆ - RNase
S ₂ -SLF1	[-]	[+]	[-]	-	[+]	[-]	+	[+]	-
S ₂ -SLF2	-	-	-	-	+	-	-	-	-
S ₂ -SLF3	[-]	[-]	-	-	-	-	-	-	-
S ₂ -SLF4	[-]	[-]	[+]	[-]	[-]	[-]	[-]	[-]	-
S ₂ -SLF5	[-]	[-]	[-]	[-]	[-]	[-]	[+]	[-]	-
S ₂ -SLF6	[-]	[-]	[-]	[-]	[-]	[-]	[-]	[-]	-
S ₂ -SLF7	-	-	-	-	-	-	-	-	-
S ₂ -SLF8	[-]	[-]	[-]	[+]	[-]	[-]	[-]	[-]	-
S ₂ -SLF9	-	-	-	-	-	-	-	-	-
S ₂ -SLF10	-	-	-	-	-	-	-	-	-
S ₂ -SLF11	-	-	-	-	-	-	-	-	-
S ₂ -SLF12	-	-	-	-	-	-	-	-	-
S ₂ -SLF13	-	-	-	-	-	-	-	-	-
S ₂ -SLF14	-	-	-	-	-	-	-	-	-
S ₂ -SLF15	-	-	-	-	-	-	-	-	-
S ₂ -SLF16	-	-	-	-	-	-	-	-	-
S ₂ -SLF17	-	-	-	-	-	-	-	-	-

1.6 The family *Solanaceae* and the genus *Petunia*

The *Solanaceae* family is an important family of plants for humanity. Many important foods such as potato (*Solanum tuberosum*), eggplant (*Solanum melongena*), tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*) and medicinal plants such as wolfberry (*Lycium*) come from this family (Mueller *et al.*, [2005](#)). Food and Agriculture Organization of the United Nations (FAO) has listed potato as one of the four major food crops in the world (Devaux *et al.*, [2014](#)). As an essential crop, 34.64 kilograms per capita per year of potato were consumed in the world in 2011 (FAO, [2015](#)).

Petunia is a member of the *Solanaceae* family and has a value as an ornamental plant. It is widely considered that *Petunia* originated from South America. The name '*Petunia*' can be traced back to the French '*pétun*', which was the original name of tobacco (Sink, [1984](#)). Because *Petunia* has a close taxonomic relationship with tobacco, '*Petunia*' was produced from '*pétun*' after 1825 and has been used to date (Sink, [1984](#)). As a popular horticultural flower, *Petunia* has two advantages as an ornamental plant. First, *Petunia* is easy to hybridize with other interspecies to obtain various colours. Second, the growing of *Petunia* does not require too much irrigation and attention. In 1944, America suffered a sudden drought, most garden plants died except *Petunia*. This helped make *Petunia* a popular choice for the gardening flower in America after 1944 (Haughton, [1978](#)). According to floriculture crop summary from United States Department of Agriculture, the 2014 annual sale value of *Petunia* exceeded 130 million dollars in America (National Agricultural Statistics Service, [2015](#)).

Moreover, at the same time as being a popular flower for horticulture, *Petunia* plays an important role in the development of research material. *Petunia* is frequently used for studying corolla pigmentation and self-incompatibility at the genetic level. The earliest time that *Petunia* was used as research material in genetic research was 1950s (Gerats and Vandebussche, [2005](#)). Subsequently, more and more researchers chose *Petunia* as plant material, to study floral morphology (Sink, [1973](#); Entani *et al.*, [1999](#)), cytoplasmic male sterility (Laser and Lersten, [1972](#); Nivison and Hanson, [1989](#)), floral scent (Verdonk *et al.*, [2003](#)),

transposon (Koes *et al.*, [1995](#)) and incompatibility (Linskens, [1975](#); Lee *et al.*, [1994](#)). However, the main reason for choosing *Petunia* for floral research is because it has larger flowers compared to most other plants, which means it is easy to control pollination of flower and provide a large amount of tissue material (such as pollen collection) for biological research (Robbins *et al.*, [2000](#); Vandenbussche *et al.*, [2016](#)).

There are over 30 species of *Petunia* in existence. Some species and cultivars such as *Petunia hybrida* and *Petunia inflata* play an important role in molecular and biochemical research (Broothaerts *et al.*, [1990](#); Clark *et al.*, [1990](#); Ai *et al.*, [1992](#); Stehmann *et al.*, [2009](#)). Some important breakthroughs of plant research have used them as the research model (Vandenbussche *et al.*, [2016](#)). For example, the identification of RNAi was done using *Petunia hybrida* as transformation material (Van *et al.*, 1990; Napoli *et al.*, [1990](#)). *Petunia hybrida* is believed to have derived from the hybridization of two *Petunia* species: *Petunia axillaris* and *Petunia integrifolia* (Robbins *et al.*, [2000](#); Bombarely *et al.*, [2016](#)). *Petunia integrifolia* and *Petunia inflata* are difficult to distinguish in detail, because they were classified in two different versions of species described separately (Fries, [1911](#); Smith and Downs, [1966](#)). However, some researchers considered that *Petunia inflata* is a clade of *Petunia integrifolia*, growing in different areas (Ando *et al.*, [2005](#); Bombarely *et al.*, [2016](#)). Because the genetic relationship between *Petunia inflata* and *Petunia integrifolia* is close, more and more researchers believed that *Petunia inflata* can be considered as the male parent of *Petunia hybrida* (Bombarely *et al.*, [2016](#)).

In addition, Bombarely *et al.* ([2016](#)) published the draft genomic sequences of *Petunia axillaris* (a large white flowered *Petunia* that can be self-incompatible either self-compatible) and *Petunia inflata* (a violet flowered *Petunia* that is completely self-incompatible). The publication of the whole genomic sequences of *Petunia inflata* gave this work a chance to study a self-incompatible *Petunia* at the genomic level (Bombarely *et al.*, [2016](#)).

The self-incompatibility of *Petunia* is controlled by a single multi-allelic S-locus as is typical for the *Solanaceae* (Takayama and Isogai, [2005](#)). As indicated above,

the male and female determinants of controlling compatibility are composed as a complex called *S*-allele (Kubo *et al.*, [2010](#)). There are many different *S*-alleles have been identified in *Petunia hybrida*. For example, *Petunia hybrida* has *S*₁-allele (Broothaerts *et al.*, [1990](#)), *S*_v-allele (Robbins *et al.*, [2000](#)), *S*_o-allele (Ai *et al.*, [1992](#)) (also called *S*_{om}-allele by Kubo *et al.*, [2010](#)), and *S*_b-allele (Broothaerts *et al.*, [1991](#)). *Petunia* (2N) has two different *S*-alleles under normal circumstances but when a *Petunia* variety contains two of the same *S*-allele, this variety is homozygous (Levan, [1937](#)). For example, the homozygote that contains two *S*₃-alleles can be called *S*₃*S*₃ homozygous (or refers as *S*₃*S*₃). And so on, when one *Petunia* variety contains two different *S*-alleles called heterozygote, and the naming method is the same as the homozygous. The two *Petunia* varieties used in this study are homozygotes containing two *S*_o-alleles and two *S*_b-alleles respectively. These are referred to as *S*_o*S*_o homozygous and *S*_b*S*_b homozygous. In these two homozygotes, the phenotype of *S*_o*S*_o is self-compatible and the phenotype of *S*_b*S*_b is self-incompatible (or pseudo-self-incompatible).

1.7 Previous studies relevant to this project

As mentioned above, two *S*-alleles of *Petunia hybrida*: the *S*_o-allele and *S*_b-allele were found to have a close relationship. The homozygous haplotype of *S*_o-allele (referred to as *S*_o*S*_o homozygote) is self-compatible, and the homozygous haplotype of *S*_b-allele (referred to as *S*_b*S*_b homozygote) is self-incompatible (Wright, [2004](#)). Wright ([2004](#)) found that after reciprocal cross-pollination, the pollen of *S*_o*S*_o homozygote was compatible with the pistil of *S*_b*S*_b homozygote, but the pollen of *S*_b*S*_b homozygote was incompatible with the pistil of *S*_o*S*_o homozygote (Figure 1.9). Normally, cross-pollination can make SI plants set seeds and show compatibility. But the pollens from SI plants led to incompatibility of SC plants, which means there are some yet unknown reasons that cause this situation.

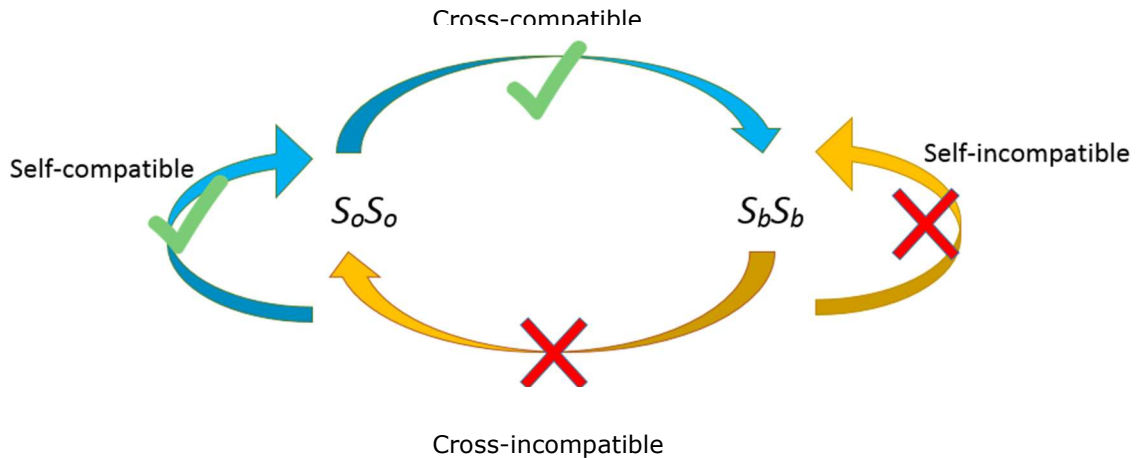


Figure 1.9 **Pollination relationship of S_oS_o homozygote and S_bS_b homozygote.** √: setting seeds after pollination, showed compatibility ×: no seeds set after pollination, showed incompatibility. S_oS_o is self-compatible after self-pollination and S_bS_b is self-incompatible. When the pollen from S_oS_o cross-pollinates S_bS_b , S_bS_b shows compatibility. When the pollen from S_bS_b cross-pollinates S_oS_o , S_oS_o shows incompatibility and does not set seeds (Wright, [2004](#)).

For the purpose of figuring out what caused the differences after reciprocal cross-pollination, primers for the S_o -RNase were used to compare S_o -RNase and S_b -RNase by RT-PCR (the sequence of the S_o -RNase has been published (Ai *et al.*, [1991](#)), the sequence of S_b -RNase was not known), which gave the same size of bands in both of S_oS_o and S_bS_b homozygotes (Czyzyk, [2010](#)). Subsequently, Czyzyk sent the RT-PCR products for sequencing, and the results indicated that the sequences of the S -RNases in these two homozygotes are identical. However, because the sequences of S_o -RNase and S_b -RNase came from the cDNA of RT-PCR, this result still needs to be confirmed by genomic DNA cloning and sequencing.

As the RT-PCR product sequences of S_o -RNase and S_b -RNase were found to be 100% identical, to further understand the phenotype, the expression level analysis of S -RNases was carried out (Czyzyk, [2010](#)). The pollen from 5 stages of buds of S_oS_o and S_bS_b were collected for expression analysis. QRT-PCR analysis indicated

that the *S-RNases* are expressed at a similar level both in S_oS_o and S_bS_b homozygotes (Czyzyk, [2010](#)) (Figure 1.10).

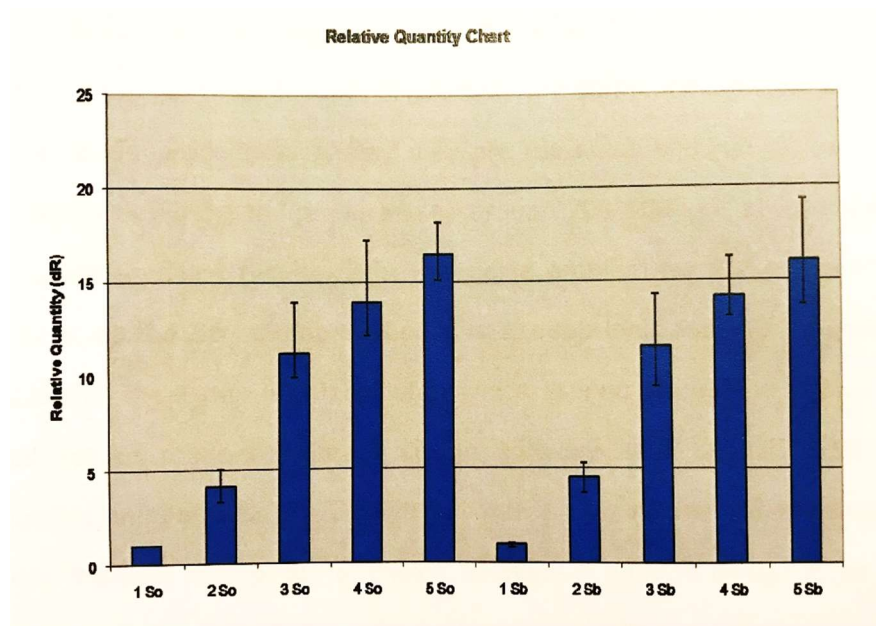


Figure 1.10 **The Relative expression level of S_o -RNase and S_b -RNase.** The y-axis represents the relative quantity of expression, the x-axis represents the samples that come from four stages of stamens. In the x-axis numbers "1-5" represent the five stages of RNA extracted from anthers. The So and Sb represent the S_o -RNase and S_b -RNase, respectively (Czyzyk, [2010](#)).

All the above findings indicate that the female determinant (*S-RNase*) does not have significant differences in sequence or expression level in the S_oS_o and S_bS_b . Therefore, this suggests that the male determinants (*SLFs*) might be the key genes that distinguish these homozygotes in terms of self- and cross-incompatibility. Fortunately, Kubo and co-workers ([2015](#)) chose S_{om} -haplotype of *Petunia hybrida* for the *SLF* sequence analysis. The S_{om} -haplotype is same variety of *Petunia* hybrids with S_oS_o homozygote used in this project, just different in the naming. PCR and next-generation sequencing ensured all of *SLFs* were identified from S_{om} -haplotype. Therefore, any *SLFs* differences between S_oS_o and S_bS_b can be found via RT-PCR or qRT-PCR.

For the second part of this project, there are only two *SLFs* expression analysis papers that has been published to date from Kao and co-workers ([2004](#)) and Hua and co-workers ([2007](#)), which indicated that *SLFs* express in pollen tube, pollen and anther of *Petunia* by RNA gel blot analysis and RT-PCR. However, there are no published literature to show the expression pattern of *SLFs* using GUS or GFP analysis. With the publication of whole genome sequence of *Petunia axillaris* and *Petunia inflata* (Bombarely *et al.*, [2016](#)), this provided an opportunity to study the promoter regions of *SLFs* and understand how the *SLFs* family expression is coordinated.

AIMS OF THIS PROJECT

The first part of this project:

To further study the S_oS_o homozygote and S_bS_b homozygote of *Petunia hybrida* to better understand the phenotypic differences

- To investigate the male determinants (*SLFs*) expression differences between S_oS_o and S_bS_b by RT-PCR
- To investigate the male determinants (*SLFs*) expression level differences between S_oS_o homozygote and S_bS_b homozygote by collecting pollen from four stages buds and running qRT-PCR
- To investigate the male determinants (*SLFs*) sequences differences between S_oS_o homozygote and S_bS_b homozygote by genomic PCR and sequencing

The second part of this project:

Promoter studies in *Petunia inflata* using genomic sequence data

- To compare and study the sequences of *SLF* promoters and *SLF* coding regions of *Petunia inflata* and *Petunia hybrida* by alignment
- To study how the expression of *SLF* family of *Petunia inflata* is coordinated by cloning selected promoters into a GUS fusion vector
- Transformation into *Petunia hybrida* 'Mitchell' to study the site and timing of expression in anther tissues

CHAPTER 2 MATERIALS AND METHODS

2.1 Seed sowing and growth conditions

All seeds were sown in the glasshouse F3 of the University of Nottingham in Sutton Bonington campus with the following conditions: temperature: 20°C for 24 hours; photoperiod with supplementary lighting (400 Watt horticulture flowering pressure sodium bulb lamp) in the winter: 16 hours day/ 8 hours dark, and no supplementary lighting in the summer; venting thermostat: 31°C. The seeds were sown in small 8 cm pots containing Levington F2+S seed and modular compost plus sand (major constituents: sphagnum moss peat; electrical conductivity: 201-290 μ S; pH: 5.3-6.0; amount of fertilizer added mg/L: N144, P73, K239). Each seedling was transplanted to independent 8 cm small pots, after the seedlings grew to 2-3 cm height. When the seedlings grew to ~10 cm height, they were transplanted to 13 cm/2 L pots containing specific *Petunia* mix-compost (60% Levington M3 pot and bedding compost [major constituents: sphagnum moss peat; electrical conductivity: 280-380 μ S; pH: 5.3-6.0; amount of fertilizer added mg/L: N204, P104, K339], 20% perlite and 20% vermiculite).

2.2 Plant genetic stocks

For studying the breakdown of SI in *Petunia hybrida*, two cultivars were used in this work: S_oS_o homozygous for the S_o -allele (S_oS_o) and S_bS_b homozygous for the S_b -allele (S_bS_b). *Petunia hybrida* seed stock N483 (Family ID used in the research group of Tim Robbins, heterozygote or homozygote containing S_b -allele) and N493 (S_oS_o homozygote) used in this project were derived from stocks studied by previous PhD students Paul Wright and Rafal Czyzyk. The S_b -allele in N483 was obtained from Dr. Wim Broothaerts originally (University of Leuven). The parental genotypes of N483 family are S_bS_v (♀) X S_bS_b (♂). S_bS_b homozygous is self-incompatible, which is hard to produce seeds by self-pollination (Czyzyk, [2010](#)). For overcome SI to store S_b -allele, another self-incompatible homozygous

contained S_v -allele was used to cross-pollinate with S_bS_b homozygous (Czyzyk, [2010](#)).

In this project, the seeds of N483 family were sown first. When the germinated plants of N483 grow up, a self-pollination was performed. The seeds obtained from self-pollinated N483 were sown, and 12 seeds successfully germinated. Then, these progenies of N483 were screened and four of them were selected. These four plants (N483.1, N483.8, N483.9 and N483.10) were self-pollinated, their progenies were named as N502, N503, N504, N505. N512 is the progeny of N493 after self-pollination. The S_o -allele in N493 was originally obtained from Free University of Amsterdam. The plant's parental genotype and family ID are shown in Table 2.1. A diagram is shown in Figure 2.1 to indicate the relationship among families in this project.

Table 2.1 **Parental genotype and family ID of *Petunia hybrida* stock.**

Family ID used in the research group of Tim Robbins	Parental genotype
N483	$S_bS_v \times S_bS_b$
N502	$S_bS_b \otimes$
N503	$S_bS_b \otimes$
N504	$S_bS_b \otimes$
N505	$S_bS_b \otimes$
N493	$S_oS_o \otimes$
N512	$S_oS_o \otimes$

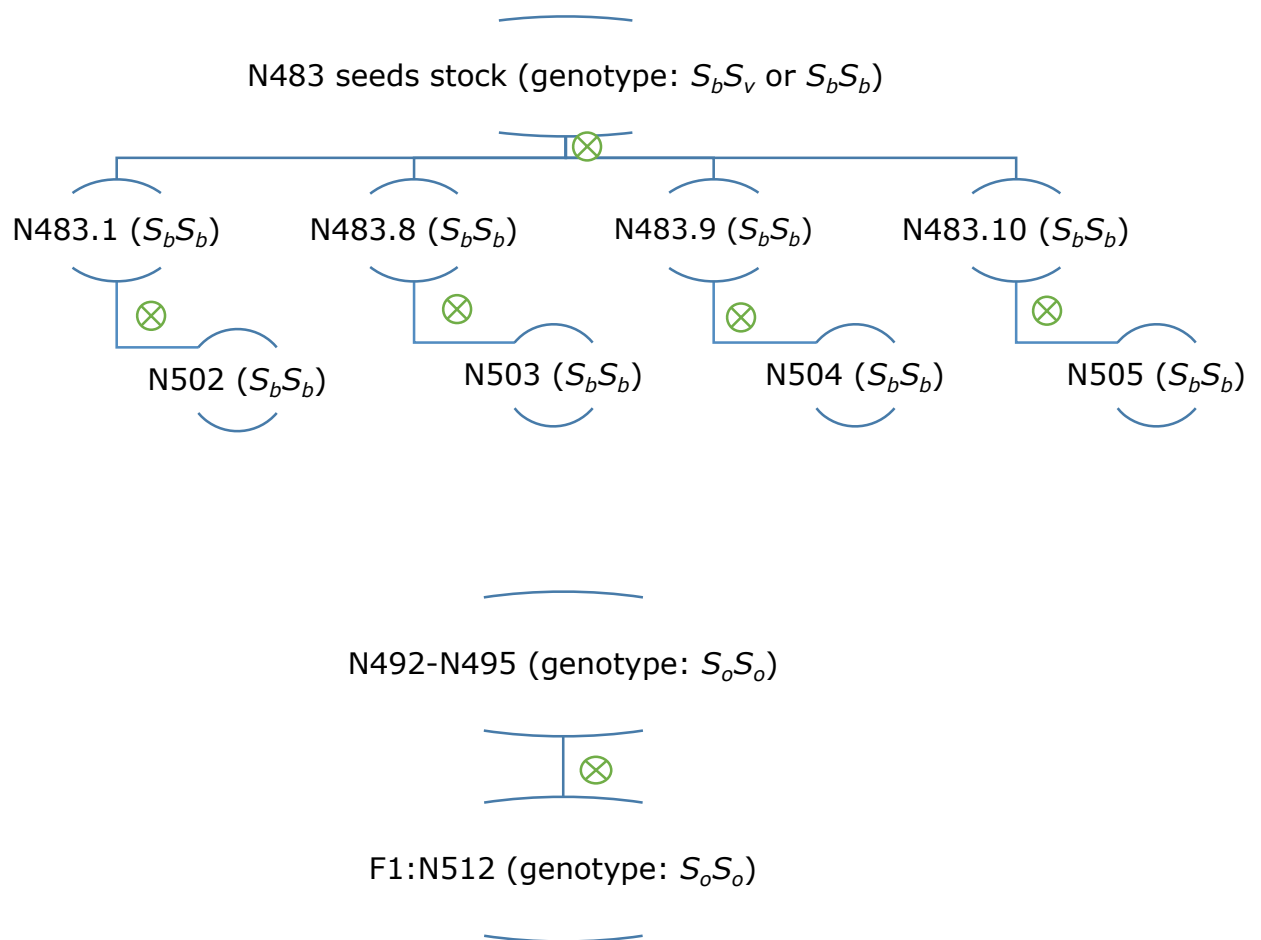


Figure 2.1 **Pedigree diagram of the relationships among families of *Petunia hybrida* used in this project.**

For studying the *SLF* promoter, *Petunia inflata*. S_6 stock was obtained from Thomas L. Sims (Northern Illinois University, U.S.A) and originally came from Francesca Quattrocchio (University of Amsterdam). This stock is the progeny of the original plant material used in genome sequencing of *Petunia inflata* (Bombarely *et al.*, [2016](#)). The plant parental genotype and family ID is shown in Table 2.2.

Table 2.2 **Parental genotype and family ID for *Petunia* stocks used in *SLF* promoter study.**

Family ID used in Tim's group	Parental information provided from Tom Sims
N496	<i>S</i> ₆ homozygous of <i>Petunia inflata</i>

2.3 DNA and RNA extraction

2.3.1 Genomic DNA extraction

A GenElute™ plant genomic miniprep kit (SIGMA-ALDRICH) was used for genomic DNA extraction. Two fresh leaf discs were taken from each plant using the cover of a 1.5 ml Eppendorf tube. Liquid nitrogen was used to freeze leaf tissue, which was quickly grinded using a sterilized micro-pestle. The rest of the extraction process was performed following the instructions from the manufacturer. All DNA samples were labelled, concentration was determined using a NanoDrop™ machine and stored at -20°C.

2.3.2 Plasmid DNA extraction

A GenElute™ plasmid miniprep kit (SIGMA-ALDRICH) was used for plasmid DNA extraction. 1 ml of an overnight *E.coli* culture (cultured with LB medium containing antibiotic) was transferred to a 1.5 ml micro-centrifuge tube and centrifuged at 13,000 rpm for 1 min, the procedure was repeated until no culture left behind. The rest of the extraction process was performed following the instructions from the manufacturer. All plasmid DNA samples were labelled, concentration was determined using a NanoDrop™ machine and stored at -20°C.

2.3.3 RNA extraction

A RNeasy® plant mini kit (QIAGEN) was used for all RNA extractions in this project. RNA extraction was performed following the instructions from the manufacturer of the RNeasy® plant mini kit. For pollen RNA, 25 anthers of 5 flowers from the same plant were collected in a 1.5 ml micro-centrifuge tube using disinfected tweezers, and snap frozen in liquid nitrogen. The plant tissue was grinded using sterile micro-pestle until tissue became a fine powder. Then, the rest of RNA extraction was performed following the instructions from the manufacturer of the RNeasy® plant mini kit. The RNA sample was labelled, and the quality of RNA was checked by agarose gel. All samples were stored at -80°C.

2.4 Primers used or designed for the purpose of this project

For S-allele genotyping, the sequence of *S_o-RNase*, *S_b-RNase* and *S_v-RNase* primers were obtained from Ai *et al.* ([1992](#)), Broothaerts *et al.* ([1991](#)) and Robbins *et al.* ([2000](#)), respectively (for details see appendix 4).

For the primers for 16 *SLFs* RT-PCR, the primer design for *SLF* gene-specific RT-PCR was based on the alignment of all 16 *SLF* sequences of the *S_{om}*-haplotype (Kubo *et al.*, [2015](#)). The *SLF* sequences of *S_{om}*-haplotype were downloaded from NCBI (<https://www.ncbi.nlm.nih.gov/>). The alignment was made by PRALINE multiple sequence alignment (<http://www.ibi.vu.nl/programs/pralinewww/>). Figure 2.2 shows the least similar regions of alignment. The sequences of *SLF* primers were selected from the least similar regions to generate *SLF* gene specific primers. Because the sequences of *SLFs* are highly similar, also for maximally amplifying the *SLF* coding regions, the forward primers of *SLFs* were designed at around 360 bp site (5'-3') and the reverse primers were designed at around 860 bp site (5'-3') (for details see appendix 4). All primers were compared with each *SLF* sequence to make sure they were specific. It is worth pointing out that *SLFs* do not contain the intron (Sims and Robbins, [2009](#)), therefore, the designing of *SLF* primers does not need to consider it. When primers were designed for qRT-

PCR of all 16 *SLFs*, the designing method for qRT-PCR was similar to the designing for RT-PCR, but slightly different in the target sequence length for qRT-PCR is less than 200 bp (for details see appendix 4).

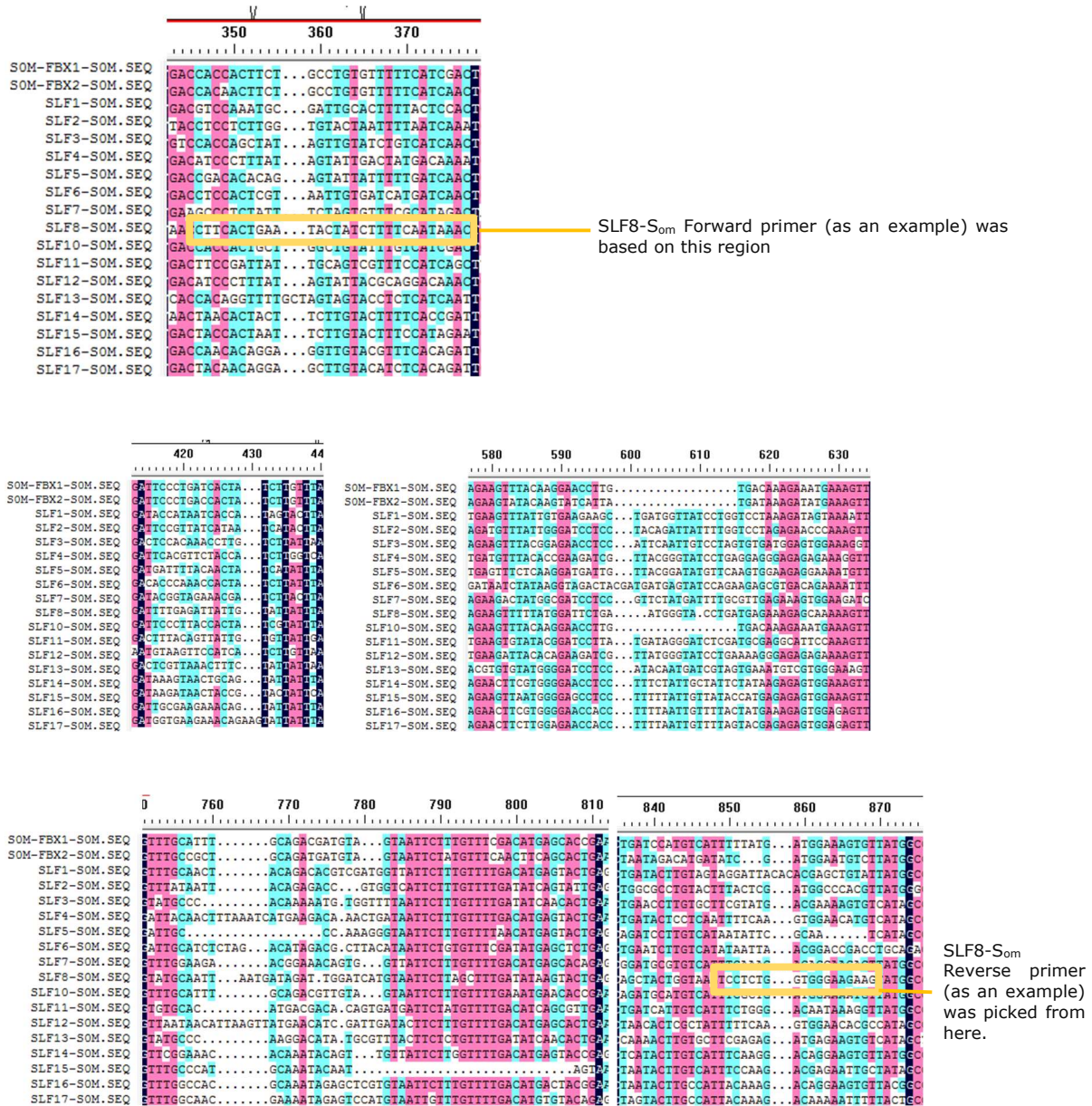


Figure 2.2 The alignment of all *SLFs* of *S_{om}*-haplotype using PRALINE multiple sequence alignment to enable primer design. SLF8-Som Forward primer (5'-CTTCACTGAATACTATCTTTTCAATAAAC-3') and Reverse primer (5'-CTTCTTCCCACCAGAGGA-3') were designed based on the sequence area shown boxed in yellow.

To confirm that the sequence data downloaded from *Petunia inflata* genome database (<https://solgenomics.net/>) can be used in *Petunia inflata* S_6 stock obtained from Thomas L. Sims (Northern Illinois University, U.S.A), three *SLFs* (*SLF3-S₆*, *SLF13-1-S₆*, *SLF14-1-S₆*) were picked, and their primers were designed (*SLF3-S₆* for rt-pcr, *SLF13-1-S₆* for rt-pcr and *SLF14-1-S₆* for rt-pcr) using Primer 3 (<http://primer3.ut.ee/>) (details see appendix 4). For amplifying *SLF* promoter fragments, three pairs of primers (*SLF3pro*, *SLF13-1pro* and *SLF14-1pro*) were designed based on the whole genome sequence of *Petunia inflata* S_6 using Primer 3 like before. Lastly, in order to detect whether *SLFpromoter:GUS* construct successfully be built with the correct direction, also to detect the construct in transgenic plants, another three pairs of primers (Test *SLF3*, Test *SLF13-1* and Test *SLF14-1*) (for details see appendix 4).

2.5 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was used to test the genotype of DNA from plants, and to confirm the constructs of transformed colonies and to amplify the fragment needed for the cloning. There are several types of PCR tests that were used in this project.

2.5.1 Genomic PCR for testing *S*-alleles

The genomic PCR was used to test the specific *S*-allele for a plant following DNA extraction. Genomic PCR reactions were prepared in a total of 25 μ l volume which contained 5 μ l of 5X MyTaq reaction buffer (Bioline), 1 μ l of 10 mM forward primer, 1 μ l of 10 mM reverse primer, 0.1 μ l of 5 U/ μ l MyTaq DNA Polymerase (From Bioline Company), 2 μ l of DNA sample and 15.9 μ l sterile water. 5X MyTaq reaction buffer comprises 5 mM dNTPs, 15 mM $MgCl_2$, stabilizers and enhancers. Genomic PCR amplification was performed in a Verity 96 well thermal cycler (Applied Biosystems) under the following cycling conditions: for S_o and S_b primer, an initial 3 mins denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 30 secs, annealing with S_o primer at 57°C for 30 secs, extension at 72°C for 1 min,

then a final extension at 72°C for 5 mins and hold at 10°C were used. For S_v primer, an initial 2 mins denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing with S_v primer at 48°C for 30 secs, extension at 72°C for 1 min, a final extension at 72°C for 5 mins and hold at 10°C were used. All PCR products were tested by agarose gel electrophoresis. If the PCR product size is less than 500 bp, a 2% agarose gel will be used for testing. If the PCR product size is over 1500 bp, the concentration of agarose gel will be 1%. Other sizes of PCR products are tested in 1.5% agarose gel.

2.5.2 Genomic PCR using Phusion® high-fidelity DNA polymerase

In order to amplify long templates with high accuracy before cloning, genomic PCR using Phusion® high-fidelity DNA polymerase (Thermo scientific), was used in the preparation of fresh PCR product for cloning. Genomic PCR reactions were prepared in a total 25 µl volume which contained 5 µl of 5X high-fidelity Phusion® reaction buffer, 1 µl of 10 mM dNTPs (Bioline), 1 µl of 10 mM forward primer, 1 µl of 10 mM reverse primer, 0.25 µl of 2 U/µl Phusion high-fidelity DNA polymerase, 2 µl of DNA sample, 0.75 µl of 100% DMSO and 14 µl of sterile water. Genomic PCR amplification was performed in a Verity 96 well thermal cycler (Applied Biosystems) under the following cycling conditions: for the primers named as So-F2 and V26-R5, an initial 30 secs denaturation at 98°C, followed by 35 cycles of denaturation at 98°C for 10 secs, annealing with S_o primer temperature at 59°C for 30 secs, extension at 72°C for 30 secs, then a final extension at 72°C for 7 mins and hold at 10°C were used. For the primers to amplified *SLF* promoter fragment (SLF3pro Forward, SLF3pro Reverse etc.), an initial 30 secs denaturation at 98°C, followed by 35 cycles of denaturation at 98°C for 10 secs, annealing with promoter primer at tested annealing temperature for 30 secs, extension at 72°C for 30 secs, and then a final extension at 72°C for 7 mins and hold at 10°C were used. The PCR products were checked by agarose gel electrophoresis with 1% agarose gel.

2.5.3 Colony PCR

The purpose of colony PCR is to confirm whether DNA fragment is successfully ligated into vector. Colony PCR reactions were prepared in a total 25 µl volume which contained 5 µl of 5X MyTaq reaction buffer, 1 µl of 10mM forward primer, 1 µl of 10mM reverse primer, 0.1 µl of 5 U/µl MyTaq DNA Polymerase, 2 µl plasmid DNA (total 100ng) and 16.9 µl sterile water. Colony PCR amplification was performed in a Verity 96 well thermal cycler (Applied Biosystems) under the following cycling conditions: an initial 2 mins denaturation at 94°C, followed by 25 cycles of denaturation at 94°C for 1 min, annealing with at 45°C for 30 secs, extension at 72°C for 1 min, then a final extension at 72°C for 7 mins and hold at 10°C were used. All PCR products were tested by agarose gel electrophoresis with 1.5% agarose gel.

2.5.4 Reverse transcription polymerase chain reaction (RT-PCR)

2.6.4.1 RNA quality test

The RNA quality test was used for testing that the RNA was not degraded. Three µl of RNA and 2 µl of 5X loading buffer was mixed gently by pipette and loaded into 1.5% agarose gel. Agarose gel was run at 90V for 1 hour.

2.5.4.2 The cDNA synthesis for RT-PCR

The super script III reverse transcriptase kit (Invitrogen) was used in cDNA synthesis of positive RT-PCR. 1 µg of RNA (variable volume of RNA added when synthesized cDNA), 1 µl of 0.5 µg/µl oligo (dT), 1 µl of 10mM dNTP mix (dNTP, dGTP, dCTP and dTTP at natural pH) and (11 µl minus RNA volume) µl of sterile water were added into a tube and gently mixed by pipette. After the mixture was incubated at 65°C for 5 mins, the tube was placed on ice for 2 mins. One µl of 5X first-stand buffer, 1 µl of 0.1M DTT, 1 µl of 60 U/µl RNase OUT (RNase inhibitor; Invitrogen) and 1 µl of 200 U/µl super script III reverse transcriptase were added into the tube. Then, the mixture was incubated at 50°C for 1 hour, and heated to 70°C for 15 mins. For cDNA synthesis of negative RT-PCR, same volume RNA and

(20 µl minus RNA volume) µl of sterile water were added into a tube. Last, the cDNA was stored at -80°C.

2.5.4.3 RT-PCR

In order to confirm that the cDNA samples were not contaminated by DNA, a cDNA quality test using house-keeping gene elongation factor 1 alpha (EF1a) (details see appendix 4) was carried out before RT-PCR. Quality test was prepared in a total of 12 µl volume which contains 2.5 µl of 5X MyTaq reaction buffer, 0.5 µl of 10mM EF1a forward primer, 0.5 µl of 10mM EF1a reverse primer, 0.1 µl of 5 U/µl MyTaq DNA Polymerase, 1 µl of cDNA and 7.95 µl of sterile water. cDNA quality test was performed in a Verity 96 well thermal cycler (Applied Biosystems) under the following cycling conditions: an initial 6 mins denaturation at 94°C, followed by 25 cycles of denaturation at 94°C for 30 secs, annealing at 59°C for 30 secs, extension at 72°C for 30 secs, then a final extension at 72°C for 5 mins and hold at 10°C were used. The RT-PCR products were checked by agarose gel electrophoresis with 1.5% agarose gel.

For the comparison of *SLFs* between *S_oS_o homozygote* and *S_b-haplotype*, RT-PCR was used in this project. RT-PCR reactions were prepared in a total of 12 µl volume which contains 2.5 µl of 5X MyTaq reaction buffer, 0.5 µl of 10mM forward primer, 0.5 µl of 10mM reverse primer, 0.1 µl of 5 U/µl MyTaq DNA Polymerase, 1 µl of cDNA and 7.95 µl of sterile water. RT-PCR amplification was performed in a Verity 96 well thermal cycler (Applied Biosystems) and same RT-PCR program except different *SLF*-specific annealing temperatures. All RT-PCR products were checked by agarose gel electrophoresis with 1.5% agarose gel.

2.5.4.4 Quantitative real time RT-PCR (qRT-PCR)

The qRT-PCR in this project was performed using the Light Cycler 480 real-time PCR system and Maxima SYBR Green/ROX qPCR master mix (2X) from Thermo Scientific™ Company. To begin with, a qRT-PCR was performed for testing primer efficiency. The master mix for *SLFs* primers and one control EF1a primer was made up respectively by 0.5 µl of 10 mM forward primer, 0.5 µl of 10 mM reverse primer,

2 µl of water and 5 µl of Maxima SYBR Green/ROX qRT-PCR master mix. Then, the master mix was added into the real-time PCR white plate with 2 µl of 4 gradients of diluted cDNA: undiluted, 1/5 diluted, 1/25 diluted and 1/125 diluted. For obtaining accurate results, the cDNA samples used for qRT-PCR were collected from three independent *S_bS_b* plants. Each reaction using one cDNA sample was set up in triplicate. The qRT-PCR program performed for testing primer efficiency was started by a preheat 95°C for 10 minutes, and then 45 cycles of 95°C for 30 seconds, annealing temperature of each *SLFs* for 30 seconds, and 72°C for 1 minute were performed. The housekeeping gene *EF1a* was used as the control group and normalization. The primer efficiency and standard curves of *SLFs/EF1a* were calculated by pyQPCR (qRT-PCR data analysis software, download from <http://pyqpcr.sourceforge.net/>).

According to the results of best primer efficiency, the annealing temperature that obtained 90%-110% primer efficiency was considered as the best annealing temperature for qRT-PCR (Taylor *et al.*, [2010](#)). Each *SLF* primer pair (for primer details see appendix 4) was used in real-time qRT-PCR with *EF1a* as control. Each reaction was made up by one unit of master mix of the same composition and volume as described above and 2 µl of 1/25 diluted cDNA. The qRT-PCR program to test expression of each *SLF* was the same as described for testing primer efficiency. As previously described, the cDNA for each reaction was taken from three biological replications, and the reaction under the same conditions was repeated three times to minimize experimental errors. All qRT-PCR data were collected at the end of the whole qRT-PCR program. The collected data was analysed using pyQPCR software.

2.5.5 CAPS analysis using CP100 primers with a digestion of *Hinf*I enzyme

The CAPS analysis using CP100 primers (for details see appendix 4) used the same condition as described in section 2.5.1, but the primer annealing temperature was set at 63°C. Then 10 µl of PCR product for each sample was digested by *Hinf*I enzyme by incubating at 37°C for 120 minutes after adding 2.5 µl of conversion buffer 2 (restriction buffer from Advanced Biotech), 0.5 µl of *Hinf*I enzyme and 7

µl of water. The digested products were checked by agarose gel electrophoresis with 2% agarose gel.

2.6 Double delta Ct analysis

For analysing the qRT-PCR data by a statistical analysis, double delta Ct analysis was performed, following a analysis methods website (<https://bitesizebio.com/24894/4-easy-steps-to-analyze-your-qpcr-data-using-double-delta-ct-analysis/>). Four average of the Ct values were calculated for each stage of pollen collection firstly: 1) the average of the Ct values for the gene being evaluated (using S_oS_o cDNA and *SLFs* primers, refers as TSLF); 2) the average of the Ct values for the gene being set as control of 1) (using S_bS_b cDNA and *SLFs* primers, refers as CSLF); 3) the average of the Ct values for housekeeping gene being tested (using S_oS_o cDNA and EF1a primers, refers as TEF1a); 4) the average of the Ct values for housekeeping gene set as control of 3) (using S_oS_o cDNA and EF1a primers, refers as CEF1a).

Then the differences of each test group and control group were calculated between 1) and 3) (TSLF-TEF1a), 2) and 4) (CSLF-CEF1a). The differences between test group and control group then subtracted (TSLF-TEF1a)- (CSLF-CEF1a) to get a double delta Ct value. Lastly, an inverse variance of double delta Ct value was performed to show the gene expression fold differences.

2.7 PCR purification

Before cloning or sequencing, a purification of any PCR product is necessary. There are two methods of PCR purification that have been used in this project.

2.7.1 PCR product purification

PureLink PCR purification kit Invitrogen was used to purify the single PCR product. PCR purification was performed following the instructions from the manufacturer. The purified DNA was stored at 4°C for immediate use or at -20°C for long-term storage.

2.7.2 Gel purification

When agarose gel showed multiple bands under UV light, gel extraction was used to extract a PCR product of specific size. GenElute™ Gel Extraction Kit (SIGMA-ALDRICH) was used in this project. The agarose gel was minimally cut under UV light and inserted into a 1.5 ml micro-centrifuge tube. Then, the protocol of agarose gel extraction was performed following the instructions from the manufacturer. All PCR products not used immediately were stored at -20°C.

2.8 Cloning of genomic PCR products

2.8.1 TOPO® Cloning reaction

For building entry vector containing the *SLF* promoter region, a pCR®8/GW/TOPO® cloning kit (ThermoFisher Scientific) was used in this project. pCR®8/GW/TOPO® cloning reaction was performed following the instructions from the manufacturer: the '*SLF* promoter' was initially amplified using Phusion high-fidelity DNA polymerase, which used the same method as described in section 2.5.2. Then, PCR product was purified by gel purification (for more details see section 2.7.2). Subsequently, 2 µl of fresh purified PCR product, 1 µl of salt solution, water and 1 µl of TOPO vector (Total volume 6 µl) were gently mixed in a 1.5 ml micro-centrifuge tube to conduct TOPO cloning reaction. Whole TOPO cloning reaction was incubated for 5 mins at room temperature (22°C to 23°C). During TOPO cloning reaction was conducting, a vial of 25 ml one shot® chemically competent *E.coli* (TOP10) was taken from -80°C freezer and thawed in ice. After 5 mins, 2 µl of the

TOPO® cloning reaction was added to the thawed *E.coli* and gently mixed. The tube with mixture (*E.coli* and TOPO® cloning reaction) was incubated on ice for 30 mins. Then, the tube with mixture was heat-shocked in a 42°C water bath for 30 secs without shaking and then immediately transferred into ice. After 1 min cool down in ice, 250 µl of S.O.C. medium (room temperature, medium was provided with kit, medium contains 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose) was added into tube and the tube was shaken horizontally (200 rpm) in a 37°C shaking incubator for 1 hour. Solid LB medium (15 g/L agar added) was microwaved for 10 mins. After the LB medium cooled down, 200 µl 50 mg/ml spectinomycin stock was added for 100ml LB medium. To ensure at least one plate will have well-spaced colonies and maximum colony be produced, 20 µl and 40 µl S.O.C. medium were spread on different LB plates respectively. 25 µl transformation was spread on pre-warmed LB plates that contained 100 µg/ml spectinomycin. The LB plates were placed in a 37°C non-shaking incubator overnight.

2.8.2 LR reaction

For building a destination vector containing *SLFpromoter:GUS* construct, Gateway™ LR Clonase™ II Enzyme Mix kit (ThermoFisher Scientific) was used in this project. LR reaction was performed following the instructions from the manufacturer: to begin with, entry clone (pEVSLF3.3 etc.) and destination clone (pGWB3, obtained from Dr. Ranjan Swarup) were thaw in ice. Then, 1 µL of entry clone (after plasmid DNA extraction, 50 ng/µL), 1 µL of destination vector (100 ng/µL), 2 µL of TE buffer (pH 8.0) and 1 µL of LR Clonase™ II enzyme mix were gently mixed in a Eppendorf PCR tube as the LR reaction mixture. The mixture was incubated at 25°C overnight. After reacting overnight, 1 µL of Proteinase K solution was added into mixture for terminating the LR reaction and the tube was incubated at 37°C for 10 minutes. 1 µL of LR reaction was transformed into 50 µL of One Shot™ OmniMAX™ 2 T1 Phage-Resistant Cells (ThermoFisher Scientific, cell was placed in a 2 ml tube). After incubating on ice for 30 minutes, the transformation was heat-shocked in a 42°C water bath for 30 secs without shaking and then immediately transferred into ice. 250 µl of S.O.C. medium (room temperature) was added into the 2 ml reaction tube and the tube was shaken

horizontally (200 rpm) in a 37°C shaking incubator for 1 hour. 20 µl and 100 µl of the transformation was spread onto LB agar plates (containing 50 µg/ml kanamycin) respectively. Last, these LB agar plates were incubated in a 37°C non-shaking incubator overnight.

2.9 Transformation of *Agrobacterium* and *Petunia hybrida*

2.9.1 Transformation of *Agrobacterium* by electroporation

ElectroMAX™ *Agrobacterium tumefaciens* LBA4404 competent cells from Invitrogen™ Life Technologies Company were used in the transformation. After thawing on ice, 20 µl of LBA4404 cells and 1 µl of destination vector were added to a pre-chilled 1.5 ml Eppendorf tube and gently pipetted using pre-chilled pipette tips. Then the cells/DNA mixture was transferred into the bottom of a pre-chilled 0.1cm electroporation cuvette. Electroporation was performed for 1 second using the following program: 2000 V, 200 Ω and 25 µF. 1 ml of YM medium (see appendix 2) was added to the cuvette and mixed well with cells, then mixture was transferred into a new Eppendorf tube and incubated in a 28°C shaking incubator for 3 hours. For optimal isolation of colonies, the culture was divided into three different volumes (50 µl, 150 µl, and 800 µl) and applied onto the YM plates containing 50 µg/ml Kanamycin, 50 µg/ml Streptomycin and 25 µg/ml Rifampicin (see appendix 2 and appendix 3). The plates were labelled and incubated at 28°C for 48-56 hours.

2.9.2 Transformation of *Petunia hybrida* leaf disks using *Agrobacterium*

About 100 young leaves were collected from one-month old healthy *Petunia hybrida* (W115) and soaked in 10% bleach for 20 minutes to sterilize the leaf surface. After the leaves were washed by three changes of sterile distilled water, they were transferred in a sterilized container in the laminar flow hood. To ensure working in a sterilized environment, the leaves were cut into approximately 1 cm

X 1 cm disks near the lighted burner. Then, the leaf disks were placed in a sterilized container with some sterile water to help them stay hydrated.

In order to confirm that the explants cutting and transformation operation were sterile, a control group was set to compare with the transformed group. Then, 10 healthy leaves were cut into same size as described above and were divided into two parts, 10 of leaf disks were directly placed on CCM without any antibiotics and another part was placed on CCM containing 100 µg/ml kanamycin. The other leaf disks were immersed in the *Agrobacterium* culture for 15 minutes for infection, then the leaf disks were placed on sterilized filter papers to remove excess culture. When the leaf disks were not too wet or not too dry, they were placed on the co-cultivation medium (CCM) (recipe see appendix 2).

All plates were placed in the growth room with 25°C/16 hours daytime and 25°C/8 hours nighttime for 3 days. After 3 days the leaf disks for control were transferred in the fresh CCM, and the other infected leaf disks were transferred onto the regeneration and selection medium (RSM) (see appendix 2) containing 100 µg/ml Kanamycin and 200 µg/ml Carbenicillin. All plates were replaced by fresh CCM and RSM every two weeks, respectively. Once the shoots from explants grew until 2 cm high, they were transferred into the rooting media (RM) (see appendix 2) containing 100 µg/ml Kanamycin and 200 µg/ml carbenicillin.

When the rooting of shoots was in a good condition and grew to about 2-3 cm, these shoots were gently transferred to the small pots with specific *Petunia* mix-compost (60% Levington M3 pot and bedding compost, 20% perlite, and 20% vermiculite) and grown in the growth cabinet with 20°C for 24 hours and 16 hours day-time/8 hours night-time. The supplementary lighting and temperature in the growth cabinet are 600 Watt horticultural LED lamp and 25°C. For allowing shoots to slowly adapt to the new environment, all pots were covered with a 10cm clean and clear inverted plastic bag to reduce evaporation. The plastic bag was gradually cut one corner by one corner, and then removed when transgenic plant was fully adapted to the new environment after three or four weeks.

2.10 GUS assay

The fresh stamens, pistils and pollen grains were collected and placed in 1.5ml Eppendorf tubes. Pre-chilled fixative (90% acetone) was added into the tubes and tissues were fixed for 15 minutes. After the fixative was removed from tubes, the ice-cold staining buffer without X-Gluc (see appendix 4) was added into tubes and then wash tissue several times. X-Gluc staining buffer was then infiltrated into the tissues for 15 minutes under a vacuum environment, the tissues were then incubated in darkness at 37°C overnight.

After the X-Gluc staining buffer was gently removed by pipette, distilled water was added into tubes to wash tissue several times and acetic acid/methanol (1 volume/ 3 volumes) was used to gently wash again by pipette. Two extra washes: 70% ethanol and distilled water were performed before observation. For storing the tissue, 100% ethanol was added until it covered all tissues.

CHAPTER 3 COMPARISON OF 16 SLFs

EXPRESSION BETWEEN S_oS_o AND S_bS_b

HOMOZYGOTES OF *PETUNIA HYBRIDA*

3.1 Introduction

3.1.1 Pseudo-self-incompatibility (PSC)

In general, most self-incompatible plants have a normal SI mechanism to prevent the pollination from self-pollen, which means self-incompatibility should not produce seeds in principle. But some self-incompatible plants are not absolutely self-incompatible. Sometimes they are in an intermediate state between self-incompatible and self-compatible. Thus, pseudo-self-incompatibility (PSC) was used to describe this phenomenon (Flaschenreim and Ascher, [1979](#)). More specifically, PSC represents that some self-incompatible plants are unable to refuse pollen even if they have the functional SI determinants when self-pollination happens (Ascher, [1984](#)). PSC exists widely in many self-incompatible plants (Levin, [1996](#)), such as *Petunia integrifolia* (Dana and Ascher, [1985](#)) and Olive (Alagna *et al.*, [2019](#)). Usually, PSC is often linked with SC for the reason of similar phenotypes. In fact, there are some differences that were observed in their phenotypes (Levin, [1996](#)). Some researchers found that SC plants can produce plump seed sets after self-pollination. But the PSC plants can obtain two kinds of seed sets after self-pollination: plump seed set contains plentiful seeds and slender seed set without seeds inside (Ascher, [1984](#); Levin, [1996](#); Czyzyk, [2010](#))

There are several different causes of PSC in SI plants. The first one is caused by mutations affecting pollen or stigma function, this kind of mutation may be caused by becoming polyploid through spontaneous mutation. Self-incompatible plants normally reject pollen in the stigma or the pollen tube stops growing when it reaches one-third of style. However, because of the mutation in the pollen, stigma or affecting pollen tube growth in the flower, the breakdown of SI leads to a failure of pollen rejection (Flaschenriem and Ascher, [1979](#)). The second type of PSC is

considered that part breakdown of SI happened in flower, but has no direct relations with the mutation of pollen, stigma or pollen tube (Clark *et al.*, [1990](#)). The third type of PSC is caused by environmental factors or developmental changes (Takahashi, [1973](#); Breton *et al.*, [2016](#)). For example, the flowers of *Campanula rapunculoides* are self-incompatible when they have just flowered, but they will become self-compatible after flowers opened a while, therefore, *Campanula rapunculoides* is considered to be PSC (Richardson *et al.*, [1990](#); Good-Avila and Stephenson, [2003](#); Good-Avila *et al.*, [2008](#)).

For the explanation of how PSC works so far, some researchers speculated that it is caused by the mutation of the genes in *S*-locus caused by the environmental effects (Takahashi, [1973](#)), or the interaction of these genes influencing the normal function, resulting in flower reduction or even in loss of the ability to reject their pollen (Stevens and Kay, [1989](#)). In addition, it has been suggested that PSC may possibly be caused by the mutation of the downstream genes in the SI pathway, or a potential extra *S*-allele exists in PSC plants that results in the breakdown of SI (Murase *et al.*, [2004](#); Baldwin and Schoen, [2017](#)). However, how PSC works at the molecular level is still not confirmed yet. This study involved PSC in *Petunia*, which may provide some further evidence to understand PSC.

One of the judging criteria to define whether a plant is pseudo-self-incompatible, self-compatible or self-incompatible, is the percentage of self-incompatible seed set as a proportion of all pollinations (Flaschenreim and Ascher, [1979](#); Litzow and Ascher, [1983](#)). *Petunia integrifolia* is usually considered to be self-incompatible (Dell'Olivo *et al.*, [2011](#)). Dana and Ascher studied wild *Petunia integrifolia* in [1985](#), by collecting their seeds from the wild, breeding, screening and observing them after self-pollination. 80% samples based on population were shown to be self-incompatible. Similarly, a screening of *Petunia hybrida* *S_b*-haplotype stocks was performed, and over 80% of flowers on an independent plant showed no seed produced after self-pollination. It can be, therefore, suggested that this *S_b*-haplotype is self-incompatible (Wright, [2004](#); Czyzyk, [2010](#)). In addition, Litzow and Ascher ([1983](#)) showed that more generations can cause an increase in proportion of seed-in set in PSC plants. This is because the phenotype of PSC plants might be controlled by some combinations or numbers of unconfirmed

genes in S-locus. More generations can increase the sample number to select the plants needed for research.

3.1.2 CP100 maker to identify different S-alleles

In general, the *S-RNases* from different S-alleles have different sequences, for example, the sequences of *S-RNases* from S_o -allele and S_3 -allele are different, thus using the sequences of *S-RNases* to design primers can simply differentiate them (Sharef, [2017](#)). In previous research Wright ([2004](#)), Czyzyk ([2010](#)) and Robbins *et al.* ([2000](#)) found that the sequences of S_o -*RNase* and S_b -*RNase* are identical, which contradicts their phenotypes. For better distinguishing these two *Petunia* S-alleles, Wright and Robbins (unpublished) used an RFLP marker called CP100 to discriminate between them.

CP100 is a restriction fragment length polymorphism (RFLP) marker first found in potato, which was discovered to be located near the *S-RNase* in S-locus of potato (Gebhardt *et al.*, [1991](#)). Using CP100 marker, it was found that the restriction fragment of S-allele also can be obtained from *Petunia hybrida*, due to the similarity of potato and *Petunia* at the sequence level (Gebhardt *et al.*, [1991](#)). Subsequently, S-locus and CP100 were found that closely locates at chromosome III of *Petunia* (ten Hoopen *et al.*, [1998](#)). Therefore, 48 varieties of *Petunia hybrida* can be differentiated by RFLP using CP100 marker (Harbord *et al.*, [2000](#)). Robbins (unpublished) used the cDNA of CP100 marker as a DNA probe to develop a cleaved amplified polymorphic sequence marker (CAPS). After cloning and sequencing of the CP100 region from a *Petunia hybrida* genomic library (Robbins, unpublished), primers were designed based on the sequences of these restriction fragments that can be used to easily distinguish *Petunia* S-alleles. Thus, CP100 RFLP analysis method was used to discriminate and confirm *Petunia* stocks at genetic level by Wright and Czyzyk ([2004](#); [2010](#)), and this is an accurate and convenient analysis method. There are several restriction sites with polymorphism that were identified in the CP100 fragments between S_oS_o and S_bS_b . These enzymes can help the CP100 fragments to be digested into different pieces of DNA fragments. This helps to distinguish different cultivars of *Petunia hybrida* (Wright, [2004](#); Czyzyk, [2010](#)). For example, the *HinfI* enzyme can cut the PCR product of

S_bS_b using CP100 marker into two different sizes of fragments: one is 700 bp and one is 300 bp, but the CP100 fragments of S_oS_o cannot be cut. It is worth pointing out that CP100 is near S-locus, but they do not share sequences of S-locus (ten Hoopen *et al.*, [1998](#)). Therefore, the CP100 fragment is different from any gene in S-locus.

3.2 Results

3.2.1 Testing S-allele genotype in *Petunia hybrida* genetic stock

For obtaining S_bS_b homozygote, the seeds of N483 (genotype: S_bS_v or S_bS_b) obtained from Czyzyk ([2010](#)) were sown first, but there is only one seed germinated. For obtaining more plant materials, a self-pollination for N483 was carried out, and the seeds obtained after self-pollination were sown. 12 progenies of N483 have survived and have been numbered as follow: N483.1, N483.2, N483.3, N483.4, N483.5, N483.6, N483.7, N483.8, N483.9, N483.10, N483.11 and N483.12.

As a start point of genetic analysis of S_o -allele and S_b -allele genotype in *Petunia hybrida*, after the seedlings were established, a genomic PCR using S_v -RNase, S_b -RNase and S_o -RNase primers was used to confirm the S-allele genotype. The results of agarose gel after electrophoresis are shown in Figure 3.1. When the genomic PCR was carried out, S_b -RNase primers provided from Czyzyk were found to be not working (not shown in results). After failing to test S_b -RNase primers annealing temperature at a series of gradient PCR and generate fresh S_b -RNase primers, also considering the sequences of S_b -RNase and S_o -RNase are 100% identical (Wright, [2004](#) and this study). S_o -RNase primers were used in this genomic PCR instead of S_b -RNase primers. Figure 3.1 (A) shows that all progenies have a strong band at ~800 bp with the S_o -RNase primer. Figure 3.1 (B) shows that there are no bands of size ~500 bp with S_v -RNase primer, with the exception of positive control (S_vS_v DNA stock provided by Sharef) that shows a strong band of size ~500 bp. Therefore, the S-allele genotype of N483.1-N483.12 are

confirmed as S_bS_b , and it is also confirmed that their DNA and RNA can be used for PCR and RT-PCR.

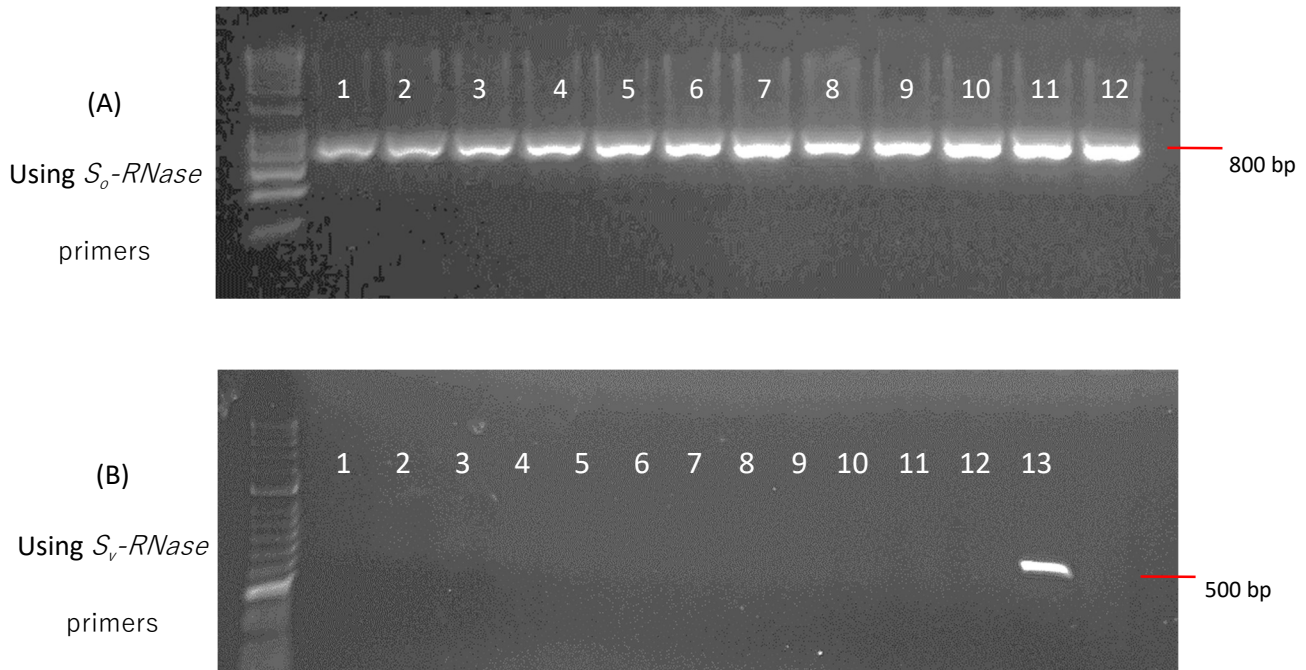


Figure 3.1 **S-allele genotype analysis for *Petunia hybrida* family N483 using S_o -RNase primers (A) and S_v -RNase primers (B).** Lane 1- Lane 12 in (A) represent the PCR results using S_o -RNase primers and the cDNA of different S_oS_o plant materials: N483.1, N483.2, N483.3, N483.4, N483.5, N483.6, N483.7, N483.8, N483.9, N483.10, N483.11, N483.12. Lane 1 – Lane 12 in (B) represent the PCR gel results using S_v -RNases primers and the cDNA same arrange with (A), Lane 13 in (B) is the positive control which used the cDNA of S_vS_v homozygote proved by Sharef.

3.2.2 Selection of self-incompatible S_bS_b *Petunia hybrida* genetic stock

According to the previous studies, although SI can cause plants to lose the ability to self-fertilize, this capability is not 100% able to happen successfully (Flaschenreim and Ascher, [1979](#)). There are still situations where self-incompatible plants can self-fertilize and produce seeds (Dana and Ascher, [1985](#); Alagna *et al.*, [2019](#)). In order to obtain both the ideal SI genotype and phenotype in S_bS_b stock for later experiments, self-pollination (10 mature flowers per plant)

was carried out for all 12 S_bS_b homozygotes (N483.1-N483.12). If 10 self-pollinated flowers produced 6 (60%) or more full seed capsules and no or less shriveled capsules, this means this plant is self-compatible. If over 8 (80%) shriveled capsules are produced on one plant, this plant could be confirmed as self-incompatible. If 10 self-pollinated flowers grow more than one (10%) shriveled capsule and less than 6 (60%) full capsules, it means this plant is pseudo self-compatible (PSC) (Dana and Ascher, [1985](#); Levin, [1996](#)). Figure 3.2 shows the shriveled capsule observed and full seed capsule after self-pollination.



Figure 3.2 **The pattern of shriveled and full seed capsules observed in this project.** Bar =1 cm.

Table 3.1 shows the level of self-compatibility of each S_bS_b homozygote, which indicates that there is no plant that showed completely self-incompatible phenotype among all S_bS_b homozygotes. Czyzyk ([2010](#)) previously reported that the S_bS_b shows to be completely self-incompatible after self-pollination. Considering the genotype of all plants was confirmed as S_bS_b via PCR described above, it is highly possible that these S_bS_b homozygotes had become PSC in phenotype. The best way to obtain ideal SI phenotype S_bS_b homozygotes was sowing the seeds of pseudo self-compatible plants from this round of self-pollination and selecting the ideal SI plants from them. According to the criteria

for judging SC/SI/PSC, N483.1, N483.8, N483.9 and N483.10 belong to pseudo self-compatible (PSC). The progenies of these PSC plants have the potential to show complete self-incompatibility. Therefore, seeds were collected and sown for these 5 PSC plants to select ideal SI plants. Because the most of capsules are filled, N483.2, N483.3, N483.4 and N483.5 are not good plant materials for later experiments. N483.7, N483.11 and N483.12 sprouted but did not flower for testing self-incompatibility. Therefore, all plants that were self-compatible and had no flower were discarded.

Table 3.1 The seed set observed for 12 S_bS_b stock plants after self-pollination. 10 flowers were self-pollinated per plant. PSC: pseudo self-compatibility, SC: self-compatibility, NT: no flower for testing. The plant shows PSC is highlighted in blue.

Plant ID	Genotype	The number of filled capsules per plant	The number of semi-filled capsules per plant	The number of empty capsules per plant	SC/SI/PSC/NT
N483.1	S_bS_b	3	6	1	PSC
N483.2	S_bS_b	9	0	1	SC
N483.3	S_bS_b	10	0	0	SC
N483.4	S_bS_b	8	1	1	SC
N483.5	S_bS_b	10	0	0	SC
N483.6	S_bS_b	6	4	0	SC
N483.7	S_bS_b	0	0	0	NT
N483.8	S_bS_b	5	2	3	PSC
N483.9	S_bS_b	5	4	1	PSC
N483.10	S_bS_b	6	0	4	PSC
N483.11	S_bS_b	0	0	0	NT
N483.12	S_bS_b	0	0	0	NT

The first self-progeny of N483.1, N483.8, N483.9 and N483.10 were named as new family ID: N502, N503, N504 and N505, respectively. About 50 seeds of each PSC self-progenies were sown. Because of the individual difference among the seeds, even the growth situation of them is the same, still some of their seeds have not germinated. When the survived F_1 progenies were mature, a new round

of self-pollination was carried out to test for SI. From 19 self-pollinated progenies (other plants had no flower for testing), N502.1, N502.22, N502.25, N502.32, N504.2 and N505.5 had over 7 shriveled capsules in one plant (Table 3.2). Therefore, these 6 plants are self-incompatible and are the ideal S_bS_b stock for later experiments. Other plants with pseudo-self-compatible or self-compatible phenotype were discarded.

Table 3.2 **Seed set observed for 19 progenies selecting for SI.** 10 flowers were self-pollinated per plant. PSC: pseudo self-compatibility, SI: self-incompatibility, SC: self-compatibility. Self-incompatible plants are highlighted in blue.

Plant ID	Genotype	The number of filled capsules per plant	The number of semi-filled capsules per plant	The number of empty capsules per plant	SC/SI/PSC
N502.1	S_bS_b	0	1	9	SI
N502.14	S_bS_b	4	2	4	PSC
N502.16	S_bS_b	2	4	4	PSC
N502.18	S_bS_b	3	6	1	PSC
N502.22	S_bS_b	0	1	9	SI
N502.23	S_bS_b	4	4	2	PSC
N502.25	S_bS_b	1	1	8	SI
N502.28	S_bS_b	4	3	3	PSC
N502.31	S_bS_b	2	5	3	PSC
N502.32	S_bS_b	1	2	7	SI
N504.2	S_bS_b	0	2	8	SI
N504.3	S_bS_b	0	8	2	PSC
N505.4	S_bS_b	4	4	2	PSC
N505.5	S_bS_b	1	0	9	SI
N505.6	S_bS_b	5	2	3	PSC
N505.15	S_bS_b	8	2	0	SC
N505.16	S_bS_b	0	4	6	PSC
N505.17	S_bS_b	5	2	3	PSC
N505.21	S_bS_b	4	1	5	PSC

3.2.3 The genotype confirmation of 6 screened S_bS_b using CAPS-CP100 analysis

For confirm the genotype of plant materials, CAPS-CP100 analysis is necessary to test the 6 screened self-incompatible S_bS_b . In order to better compare the CAPS-CP100 analysis, 12 seeds of S_bS_b (N512) were sown. When both of ideal S_bS_b and S_oS_o had grown until mature, the DNA was initially extracted from the leaves of 5 S_oS_o and all screened S_bS_b plants. CAPS-CP100 analysis was performed, and the result of CAPS analysis is shown in the Figure 3.3. All 6 screened S_bS_b (N502.1, N502.22, N502.25, N502.32, N504.2 and N505.5) showed that DNA fragments were digested to two DNA fragments (700 bp and 300 bp) by *HinfI* enzyme, but the DNA fragments from S_oS_o were not digested. Therefore, it verifies 100% that the genotype of 6 screened plant materials is S_bS_b .

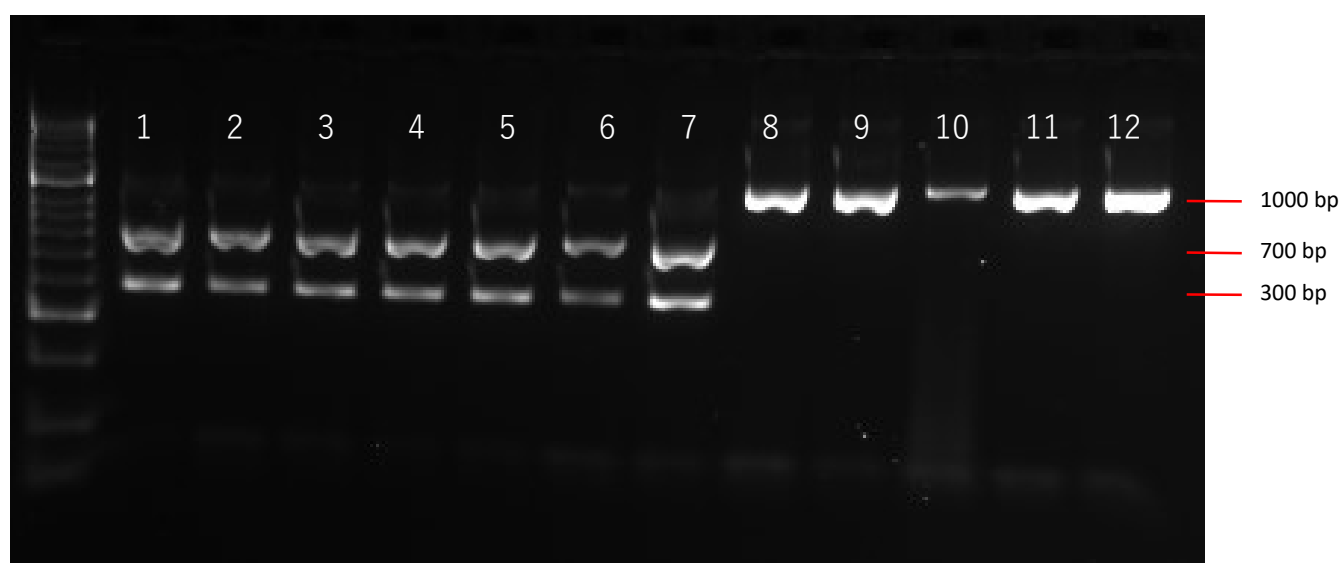


Figure 3.3 **CAPS analysis using CP100 marker with the digestion by *HinfI* enzyme.** Lanes 1-7 represent 6 screened S_bS_b plants: N502.1B, N502.1C, N502.22, N502.25, N502.32, N504.2 and N505.5. Lanes 8-12 represent 5 S_oS_o plants: N512.5, N512.6, N512.7, N512.8 and N512.9.

3.2.4 Expression comparison of 16 *SLFs* between S_oS_o homozygote and S_bS_b homozygote of *Petunia hybrida*

After the plant materials were prepared well, expression comparison of 16 *SLFs* between S_oS_o and S_bS_b was planned. According to the previous study, the male determinant (*SLFs*) might be the key genes that distinguish these haplotypes at the phenotypic level (Wright, 2004; Czyzyk, 2010). A series of RT-PCR were initiated using specific *SLF* primers in an attempt to identify any expression differences of *SLFs* between S_oS_o and S_bS_b .

With a series of RNA extraction, cDNA synthesis, primer designing, the comparisons of *SLF* expression levels in mature pollen were carried out by RT-PCR using gene-specific *SLF* primers. In each case the expression level was compared using cDNA from S_bS_b and S_oS_o homozygotes. A total of 16 *SLF* primer pairs (SLF1- S_{om} , SLF2- S_{om} , SLF3- S_{om} , SLF4- S_{om} , SLF5- S_{om} , SLF6- S_{om} , SLF7- S_{om} , SLF8- S_{om} , SLF10- S_{om} , SLF11- S_{om} , SLF12- S_{om} , SLF13- S_{om} , SLF14- S_{om} , SLF15- S_{om} , SLF16- S_{om} and SLF17- S_{om}) were used in RT-PCR (for primer details see appendix 4). The results of RT-PCR comparison of all 16 *SLFs* are shown in Figure 3.4, which indicate that 15 *SLFs* are transcribed in both S_oS_o and S_bS_b . Interestingly, the primers for SLF8- S_{om} gave a product in S_oS_o but not in S_bS_b . This may indicate that *SLF8* is transcribed in S_oS_o but not in S_bS_b . A summary of RT-PCR results is shown at Table 3.3. The results of RT-PCR also show some differences in band intensity (expression level) between S_oS_o and S_bS_b . Some *SLFs* have higher expression in S_bS_b than S_oS_o : *SLF1*, *SLF2*, *SLF6*, *SLF10*, *SLF12* and *SLF15*. *SLF13* shows opposite pattern with higher expression in S_oS_o than S_bS_b .

Table 3.3 **Summary of expression data for all 16 *SLFs* between S_oS_o homozygote and S_bS_b homozygote.** + means this *SLF* gene is transcribed in the mature stamens of this haplotype; - means this *SLF* is not transcribed in this haplotype.

	S_oS_o homozygote	S_bS_b homozygote
SLF1-Som	+	+
SLF2-Som	+	+
SLF3-Som	+	+
SLF4-Som	+	+
SLF5-Som	+	+
SLF6-Som	+	+
SLF7-Som	+	+
SLF8-Som	+	-
SLF11-Som	+	+
SLF12-Som	+	+
SLF13-Som	+	+
SLF14-Som	+	+
SLF15-Som	+	+
SLF16-Som	+	+
SLF17-Som	+	+



Figure 3.4 **Comparison of expression of all 16 *SLFs* between *S_oS_o* homozygote and *S_bS_b* homozygote using synthesized cDNA of anther.** All water controls in these RT-PCR were negative, and the housekeeping gene *EF1a* in these RT-PCR were positive (not shown in results).

3.2.5 Another round of expression comparison of *SLF8* between S_oS_o and S_bS_b homozygotes of *Petunia hybrida*

The results described above indicate that *SLF8* is transcribed in S_oS_o but not in S_bS_b . In order to confirm the accuracy of these results, a new *SLF8* primers (*SLF8-S_{om} 2*, for details see appendix 4) were designed, based on different sequence regions of *SLF8-S_{om}*. At the same time, the selection of S_bS_b was finished. After testing optimal annealing temperature by gradient PCR, a new round of RT-PCR using new *SLF8* primers (*SLF8-S_{om} 2*) and ideal plant materials was performed. The RNA of S_bS_b was extracted from N502.1. The results of RT-PCR (Figure 3.5) show that *SLF8* does exist in both, S_oS_o and S_bS_b .

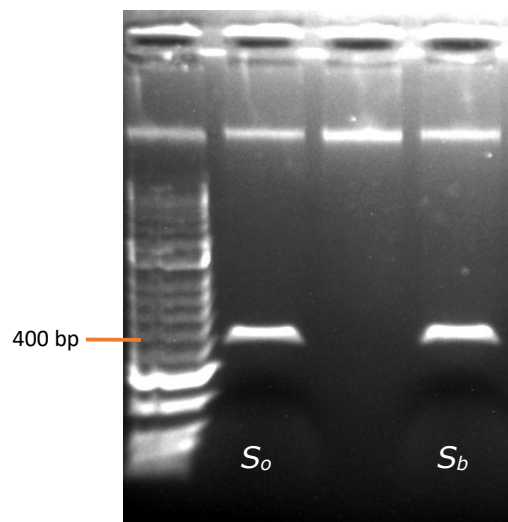


Figure 3.5 **Comparison of expression of *SLF8* between S_oS_o and S_bS_b homozygotes using ideal plant materials and new designed *SLF8* primers (*SLF8-S_{om} 2*).** The water controls in This RT-PCR was negative, and the housekeeping gene *EF1a* in this RT-PCR was positive (not shown in results).

3.3 Discussion

3.3.1 Selecting completely self-incompatible S_bS_b of *Petunia hybrida*

In previous studies (Broothaerts *et al.*, [1991](#); Wright, [2004](#); Czyzyk, [2010](#)) the S_bS_b of *Petunia hybrida* was self-incompatible. However, from the result of Table 4.1, some plants showed a partial breakdown of self-incompatibility (self-incompatibility and self-compatibility appear in SI plant after self-pollination). As described before, this phenomenon is called pseudo self-compatibility. In order to obtain completely self-incompatible S_bS_b homozygotes, another round of self-pollination was carried out to select S_bS_b homozygotes. Completely self-incompatible (SI) S_bS_b homozygotes were retained and pseudo self-compatible (PSC) and self-compatible (SC) S_bS_b homozygotes were discarded. In fact, the genotypes of S_bS_b homozygotes were the same no matter what phenotype (PSC, SI or SC) they manifested. However, S_bS_b homozygotes with self-incompatible phenotype can provide more reliable results when comparing with S_oS_o homozygotes at the expression level.

3.3.2 Testing S -allele genotype in *Petunia hybrida*

In section 4.2.1, two genomic PCR using S_v -*RNase* and S_o -*RNase* primers were used to identify the S -genotype of plant materials (N483.1-N483.12). The reason why S_o -*RNase* primers were used to identify S_b -*RNase* gene in *Petunia hybrida* stocks is because the S_b -*RNase* primers from a previous Ph.D. student Rafal Czyzyk ([2010](#)) were not working and the sequence of S_o -*RNase* was proven to be identical with S_b -*RNase*. The PCR results showed that the S -genotype of 12 plants (N483.1-N483.12) are S_bS_b , whereas the self-pollination test indicated that these 12 plants are SC or PSC instead of SI. According to previous studies from Rafal Czyzyk, both S_vS_v homozygote and S_bS_b are self-incompatible. The S_v -allele was not expected to appear in the material plants used for this project. Therefore, it should be confirmed whether these PCR results are reliable.

In a previous study, Rafal Czyzyk also had shown that Cleaved Amplified Polymorphic Sequences (CAPS) analysis with CP100 primers (an RFLP marker

derived from a region closely linked with S-locus in potato) could distinguish S_o -*RNase* and S_b -*RNase*. It is worthwhile to use CAPS analysis for confirming S-allele genotype of existing screened S_bS_b homozygotes. The CAPS analysis results shown in Figure 3.3 indicated that the genotypes of all S_bS_b stocks have been confirmed as S_bS_b , and the genotypes of all S_oS_o stocks have been confirmed as S_oS_o . This gave the confidence to conduct the next experiments using these plants because they have an ideal phenotype and genotype.

3.3.3 Different results between two rounds of *SLF8* RT-PCR

The RT-PCR was repeated in two rounds for testing the expression of *SLF8*. In the first round of RT-PCR, the RT-PCR result of *SLF8* showed in Figure 3.4, indicated that *SLF8* may only exist in S_oS_o . At that time, it was considered that *SLF8* is as partial gene duplication that exists in S_oS_o . Therefore, the breakdown of SI in S_oS_o is a kind of 'competitive interaction', and is caused by partial gene duplication (*SLF8*). However, when a new *SLF8* primer was designed, a new round of RT-PCR using ideal plant material was performed and showed that *SLF8* does exist in both, S_oS_o and S_bS_b . The differences between these two rounds of RT-PCR are the plant material and *SLF8* primers. It is possible that ideal plant material N502.1 may provide more accurate results, or the primers have problem. A qRT-PCR can give more information about it.

3.3.4 The comparison of expression for all 16 *SLFs* between S_oS_o homozygote and S_bS_b homozygote

To begin with, All *SLFs* showed the bands on both of S_bS_b and S_oS_o homozygote. The strength of bands between different *SLFs* shows some differences. However, it cannot suggest that the different *SLFs* have different expression strengths. This is because these RT-PCR were carried out in an inconsistent reaction environment. Different reaction environments, such as annealing temperature or agarose gel electrophoresis, all possibly affected the RT-PCR results.

However, the different strength of bands between S_bS_b and S_oS_o may give us some information. The RT-PCRs of *SLF1*, *SLF2*, *SLF6*, *SLF10*, *SLF12* and *SLF15* indicate they have strong band in S_bS_b homozygote and weak band in S_oS_o homozygote. Conversely, *SLF13* shows higher expression in S_oS_o homozygote than S_bS_b homozygote. The *SLF* expression results of RT-PCR are not reliable enough for testing expression level of each *SLF*, although some differences of expression level between S_oS_o homozygote and S_bS_b homozygote are possible. Thus, qRT-PCR using *SLF* primers is necessary to improve our understanding of the expression level of each *SLF*. This will also provide more information on all *SLFs* expression in S_bS_b homozygotes and S_oS_o homozygotes.

3.3.5 The difference between S_oS_o and S_bS_b homozygotes is not caused by an missing *SLF*

Most S-alleles are functional in the wild species of *Petunia* hybrid, and S_o -allele arose as a mutation of S_b -allele (Broothaerts *et al.*, [1991](#); Ai *et al.*, [1992](#); Robbins *et al.*, [2000](#)). With the results provided by Czyzyk ([2010](#)) and this report, the sequences of *S-RNases* between these two homozygotes are 100% identical and the expression levels are similar. In this project, the primers designed for RT-PCR are based on the *SLFs* sequence of S_oS_o . The RT-PCR results showed these primers are able to amplify the *SLFs* of S_bS_b . If their sequences are totally different, the RT-PCR results of S_bS_b would not give results. Therefore, it is possible that the *SLFs* sequences of these two homozygotes are also identical.

Previous PhD student Czyzyk suggested that the differences between the S_oS_o homozygote and S_bS_b homozygote are possibly caused by the phenomenon of “competitive interaction”. This is observed with diploid hetero-allelic pollen in a self-compatible tetraploid in *Petunia* (Sims and Robbins, [2009](#)). The breakdown of self-incompatibility happen in the pollen of self-incompatible plants is called as competitive interaction (Huang *et al.*, [2008](#)). After doubling the genome of a self-incompatible diploid, the pollen of this tetraploid will lead this tetraploid to show as self-compatible after self-pollination (Sijacic *et al.*, [2004](#)). It is pointed out that the duplication of whole genome is not necessary to cause the breakdown of SI,

and the breakdown of SI can be caused by the partly gene duplication (Pandey, [1965](#); Golz *et al.*, [2001](#)).

Tsukamoto and co-workers ([2005](#)) found that the duplication of an *SLF* gene was relevant to the breakdown of pollen function in the wild species *Petunia axillaris*. This duplication was detected by Southern blot analysis. Czyzyk ([2010](#)) used the same method to test for a duplication of *SLF* gene that distinguishes the S_oS_o homozygote and S_bS_b homozygote of *Petunia hybrida*. The original surmise from Czyzyk was that a duplicated *SLF* gene is present in the S_oS_o homozygote. However, the results indicated that there are no obvious duplications of an *SLF* gene found in either S_oS_o homozygote or S_bS_b homozygote of *Petunia hybrida*, which apparently excludes the possibility that the phenotype differences between S_oS_o homozygote and S_bS_b homozygote are caused by the duplication of *SLF* gene.

However, since these early studies more *SLFs* have been identified from *Petunia hybrida* and *Petunia axillaris* (Kubo *et al.*, [2015](#); Bombarely *et al.*, [2016](#)), indicating that the results from Tsukamoto *et al.* ([2005](#)) and Czyzyk ([2010](#)) could be expanded with more *SLFs*. Tsukamoto *et al.* ([2005](#)) and Czyzyk ([2010](#)) only studied *SLF1* in *Petunia axillaris* and *Petunia hybrida*, therefore, investigating other *SLFs* whether duplicate in S_oS_o homozygote will provide more evidence to explain the differences between S_oS_o homozygote and S_bS_b homozygote.

When the results of the first round of RT-PCR were coming out, I had conjectured that whether there is an *SLF* missing in S_bS_b or an extra *SLF* exists in S_oS_o (*SLF8*). I thought *SLF8* was the duplicated *SLF* that would be able to explain all. However, the second round of RT-PCR (the RT-PCR of *SLF8*) proved to me that I was wrong. All *SLFs* are expressed in both of these two homozygotes, and there is no missing *SLF* or extra *SLF* in there. Therefore, a round of qRT-PCR is necessary for the next experiment. It can provide some evidence to explain whether the differences are caused by gene duplication, and also to support the results of the second round of RT-PCR of *SLF8*.

CHAPTER 4 QUANTITATIVE ANALYSIS OF SLF

GENE EXPRESSION LEVELS IN S_oS_o AND S_bS_b

HOMOZYGOTES OF *PETUNIA HYBRIDA*

4.1 Introduction

4.1.1 The competitive interaction can lead the breakdown of SI

In general, most SI plants in the wild are diploid (Lewis, [1979](#)), but if the diploid is induced to mutate to tetraploid by colchicine, a breakdown of SI of these plants will occur. This phenomenon is called competitive interaction and was first used to describe the phenomenon between the two types of pollen: diploid heterozygous and homozygous pollen of *Prunus avium* (Crane and Lawrence, [1931](#)) and *Pyrus communis* (Crane and Thomas, [1939](#)) can cause the SI of diploid cherry and European pear was hampered. Subsequently, the pistil of tetraploid *Petunia axillaris* was found to be unable to reject the diploid heterozygous self-pollen (Stout and Chandler, [1941](#)). Further research provided more details to describe this phenomenon. The tetraploid of heterozygous *Petunia axillaris* produced three kinds of diploid pollen, two homozygous types and one heterozygous pollen. The diploid homozygous and haploid pollens gave the same results both failing to self-fertilize with its pistil. However, the diploid heterozygous pollen is able to self-fertilize with its pistil (Figure 4.1) (Emery *et al.*, [1960](#); Golz *et al.*, [2000](#)). In brief, the diploid heterozygous pollen gains the ability to cause the breakdown of SI.

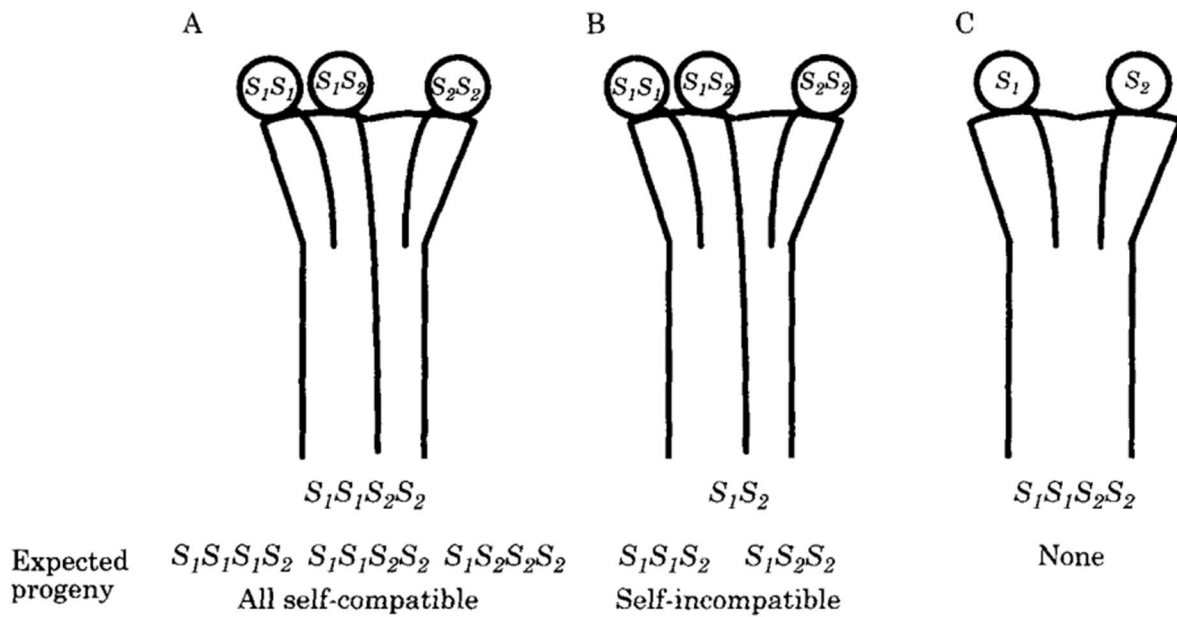


Figure 4.1. **The schematic diagram of competitive interaction as occurs in most of gametophytic self-incompatible plants.** The genotype of diploid is S_1S_2 , with polyploid breeding, the genotype of tetraploid becomes $S_1S_1S_2S_2$. The genotypes of self-pollen derived from tetraploid have three types: S_1S_1 , S_2S_2 and S_1S_2 . Only heterozygous pollen S_1S_2 causes the breakdown of self-incompatibility in both tetraploid and diploid (S_1S_2) plants (A and B). However, as opposed to the heterozygous pollen from tetraploid, the pollen from diploid is unable to cause the breakdown of self-incompatibility in tetraploid (C) (Golz *et al.*, 2000).

Several SI plants were observed to have competitive interaction, such as *Malus pumila* and *Pyrus* (De Nettancourt, 2001), *Prunus cerasus* (Lansari and Iezzoni, 1990), *Nicotiana alata* (Golz *et al.*, 2001), *Lycopersicon esculentum* (Santos *et al.*, 1997) and *Petunia axillaris* (Tsukamoto *et al.*, 2005). However, not all self-incompatible plants of GSI have competitive interaction. For example, the tetraploid of *Oenothera* is self-incompatible as it is a diploid, no matter whether diploid heterozygous pollen is used to pollinate its pistil (Lewis, 1943). Different species will emerge different level of competitive interaction. For instance, *Leguminosae* and *Solanaceae* have different capability to set seeds when competitive interaction occurs. The main reason for these differences among species is the different background rather than the superiority of one S-locus to another (Golz *et al.*, 2000).

Usually, the competitive interaction is observed when pollen from tetraploid pollinated the diploid pistil. On the contrary, when pollen from diploid pollinated the tetraploid pistil, the competitive interaction did not occur (Figure 4.1 B and C) (Lewis, [1943](#)). This phenomenon is because the pollen from diploid is haploid, and its phenotype is the same as diploid homozygous pollen from tetraploid, both of them are unable to cause the breakdown of self-incompatibility in most of the plants. All these studies of competitive interaction provided many clues to help the corroboration of male determinants (*SLFs*) in pollen (Sims and Robbins, [2009](#)).

4.1.2 The duplication of a partial genome could lead to the breakdown of SI

In fact, the duplication of the whole genome is not necessary for the occurrence of competitive interaction. Golz and his colleagues ([1999](#)) mutated some *Nicotiana glauca* via radiation, rendering the self-incompatible tobacco self-compatible. Significantly, in all of the mutants in which the pollen function was inhibited, became unable to refuse self-pistil. After observing the pollen of these mutants, one extra chromosome was found in these pollen mutants. Using DNA blot analysis to study this extra chromosome, it was shown that this extra chromosome has a close relationship with S-locus. Further studies indicated that the duplicated DNA fragment in this extra chromosome was pollen male determinant (*SLFs*), whereas the female determinant (*S-RNase*) was not replicated (Golz *et al.*, [2001](#)). This finding can be pointed to as evidence that the duplication of *SLFs* can cause the breakdown of SI in *Nicotiana glauca*. According to this result, the competitive interaction caused by duplication of *SLFs* can be considered as the second inhibitor in the SI of tetraploid, which can control self-incompatibility process (Sims and Robbins, [2009](#)).

All the mutations described above were obtained from the artificial approach. However, competitive interaction also exists in the wild. Tsukamoto and co-workers ([2003](#)) studied 19 populations of *Petunia axillaris*, and they observed one special population of *Petunia axillaris* in Uruguay called U83. Some plants from this population of *Petunia axillaris* were self-incompatible, but most of them are

self-compatible. Studying these self-compatible *Petunia axillaris* led to a finding that all of them contained either of two *S*-gene (SI linked gene named as S_{c1} and S_{c2}). One of *S*-genes S_{c2} was found to share identical pollen-*S* sequence with another *S*-allele (named as S_{17} -allele in another population U1), and yet pollen containing S_{c2} lost the self-incompatibility function. Moreover, they found that as long as the plants contained S_{c2} , they were definitely self-compatible. When *SLFs* was discovered as the male determinant of self-incompatibility in pollen and successfully cloned (Sijacic *et al.*, [2004](#)), the *SLF* in S_{17} -allele was cloned and sequenced, homozygote $S_{c2}S_{c2}$ was investigated via the sequenced using the sequence of *PaSLF17*. The results indicated that the S_{c2} -allele carried two genes in pollen: one gene has an identical sequence with *PaSLF17* and another extra one gene called *PaSLF19*. The presence of these genes led all pollens contained S_{c2} to lose the function of self-incompatibility, and all progenies containing S_{c2} became self-compatible (Tsukamoto *et al.*, [2005](#)).

4.1.3 The previous studies to investigate whether competitive interaction is the key to explain the difference between S_oS_o and S_bS_b

It was found that an additional *S*-allele in *Petunia axillaris* could lead the SI to be hampered (Tsukamoto *et al.*, [2005](#)). Czyzyk and Robbins ([2010](#)) had surmised that the self-compatibility of S_oS_o is caused by the duplication of *S*-allele or partial *S*-allele. They restrictively digested the *S-RNases* and *SLF1* in S_oS_o and S_bS_b using Southern blot analysis which is the same method used in the publication of Tsukamoto and his colleagues ([2003](#); [2005](#)). No obvious duplications of *S-RNases* and *SLF1* were observed in these two homozygotes. This indicates that it is possible that the competitive interaction is not the reason causing the different SI in S_oS_o and S_bS_b we look for. However, it is too early to negate this surmise because more *SLFs* were identified in S_oS_o . We need a quantitative experiment to test and verify the RT-PCR results described at the last chapter. Moreover, the comparison of the expression level of *SLFs* between S_oS_o and S_bS_b is not performed yet. A round of qRT-PCR is also worth to carry out to see whether there is a difference of *SLFs* expression level between S_oS_o and S_bS_b . The results of qRT-PCR can help us to understand whether the *SLF* genes have duplications in these two

homozygotes, by comparing the results of qRT-PCR from this project and the results of Southern blot analysis from Czyzyk ([2010](#)).

4.2 Results

4.2.1 New primer design for qRT-PCR of 16 *SLF* genes

For detecting the expression level of all *SLF* genes in a S_oS_o homozygote (S_oS_o) and S_bS_b homozygote (S_bS_b), a series of qRT-PCR experiments were planned. The primers used in the initial RT-PCR experiments for comparing *SLF* expression between S_oS_o and S_bS_b homozygotes of *Petunia hybrida* are not suitable for qRT-PCR study. Thus, new primers to amplify short lengths of sequence (~100 bp) were designed for qRT-PCR analysis. All primers have been compared with all *SLF* sequences for specific checking. The annealing temperature of primers was tested by gradient PCR, and the annealing temperature summary of primers is shown in appendix 4.

4.2.2 qRT-PCR for testing *SLF* expression levels in mRNA from S_oS_o and S_bS_b homozygotes

As mentioned in section 4.3, five completely SI S_bS_b homozygotes were screened and three of them were used for mRNA extraction material of S_bS_b homozygotes. Three S_oS_o homozygotes named as N512.1, N512.2, N512.3 were used as RNA extraction material of. In order to study in detail on the difference between S_oS_o and S_bS_b homozygotes, anthers of *Petunia* were collected from four stages of bud development. The classification of four stages of buds was as follows: buds between 10 mm-20 mm long belong to first stage, second stage containing buds between 20 mm-30 mm long and third stage buds length are between 30 mm-40mm. The length of buds which is over 40mm but not opened were classified into fourth stage. Because the anther's maturation situation and sample-contaminated level of the opened flower were difficult to control, the RNA from opened flower was not considered as a new stage. Stages were named as b1-b4

for S_bS_b homozygotes and o1-o4 for S_oS_o homozygotes. RNA was extracted from the anther of buds by RNeasy® plant mini kit (QIAGEN) and cDNA was synthesized for RT-PCR. All synthesized cDNA samples were tested via a 25 cycle RT-PCR utilizing the housekeeping gene EF1a; All cDNA samples were confirmed as no DNA was degraded and no RNA was contaminated (data not shown).

An initial qRT-PCR was performed utilizing the Roche LightCycler 480 (qRT-PCR machine) and ThermoFisher science SYBR green master mix kit to test primer efficiency using diluted S_oS_o homozygote cDNA. The dilution of cDNA was set up at 4 levels, containing 100% undiluted cDNA, 20%, 4% and 0.8% diluted cDNA. In order to be consistent with previous qRT-PCR experiments of *S-RNases* (Rafal Czyzyk, [2010](#)), a housekeeping gene was utilized (*Elongation Factor 1a*, *EF1A*) and the primers had good performance in qRT-PCR experiments performed by Ashtekhwaz Sharef ([2017](#)) and Rafal Czyzyk ([2010](#)). The method of qRT-PCR is described in section 2.5.4.4. A summary of primer pairs efficiency is shown in Table 4.1. The efficiency of primer pairs should be in 90% to 110% for best accuracy (ThermoFisher, [2016](#)) All primer efficiencies shown in Table 4.1 are within this range, thus, all primer pairs can be used in next experiments.

Table 4.1 Efficiency of 16 SLFs primer pairs and the housekeeping gene *EF1a* as the positive control

Primer Pair	Efficiency (%)	R ²
SLF1-Som (For qPCR)	95.58%	0.997
SLF2-Som (For qPCR)	95.63%	0.993
SLF3-Som (For qPCR)	100.02%	0.996
SLF4-Som (For qPCR)	90.66%	0.997
SLF5-Som (For qPCR)	96.18%	0.994
SLF6-Som (For qPCR)	92.31%	0.989
SLF7-Som (For qPCR)	103.43%	1.000
SLF8-Som (For qPCR)	91.78%	0.997
SLF10-Som (For qPCR)	103.20%	1.000
SLF11-Som (For qPCR)	94.76%	0.999
SLF12-Som (For qPCR)	96.31%	0.995
SLF13-Som (For qPCR)	103.96%	0.999
SLF14-Som (For qPCR)	94.51%	0.995
SLF15-Som (For qPCR)	99.39%	0.997
SLF16-Som (For qPCR)	92.04%	0.997
SLF17-Som (For qPCR)	96.13%	0.994
EF1a	105.76%	0.996

After obtaining the best primer efficiency of each primer pair, a series of qRT-PCR were performed using the 16 pairs of *SLF* gene-specific primers and *EF1a* primers (for details see appendix 4), respectively. Each reaction was performed with two sets. One set used specific *SLF* primers and another used *EF1a* primers for normalization. The four stages of cDNA of three *S_oS_o* homozygotes and three *S_bS_b* homozygotes were set up for both sets. For the purpose of obtaining reliable data, the tissues from each stage of pollen were collected from three independent *Petunia* with the same genotype. Then, RNA extraction and cDNA synthesis were performed. All reactions were performed in triplicate or duplicate in the same conditions. More specifically, each reaction used the same cDNA, and the same primers were repeated three times in the same round of qRT-PCR.

After normalizing the data with the housekeeping gene, the expression levels of 16 *SLF* genes were compared between *S_oS_o* homozygotes and *S_bS_b* homozygotes, as shown in Figure 4.3. The stage 1 of *S_oS_o* homozygote was used as the calibrator for other samples and it was appointed a value of 1. Also, a statistical analysis were performed using double delta Ct analysis (for analysis method sees section 2.6) to evaluate the *SLFs* expression fold change between *S_oS_o* and *S_bS_b* homozygotes, as shown in Table 4.2. The values in Table 4.2 show the expression fold comparison of the *SLFs* in *S_oS_o* and *S_bS_b*. The *SLFs* expression level in *S_bS_b* was set as the comparison unit. For example, the expression fold value of *SLF1* at stage 4 is 1.73 this indicates that the expression level of *SLF1* in *S_oS_o* is 1.73 times higher than in *S_bS_b* at stage 4.

Table 4.2 **The *SLFs* expression fold change between S_oS_o and S_bS_b homozygotes using double delta Ct analysis.** The significant figure was remained two numbers after decimal point.

<i>SLFs</i> name	<i>SLF</i> Expression Fold value in $S_oS_o : S_bS_b$	<i>SLF</i> Expression Fold value in $S_oS_o : S_bS_b$	<i>SLF</i> Expression Fold value in $S_oS_o : S_bS_b$	<i>SLF</i> Expression Fold value in $S_oS_o : S_bS_b$
<i>SLF1</i>	0.16	1.59	0.92	1.73
<i>SLF2</i>	17.38	2.42	2.30	2.01
<i>SLF3</i>	36.89	1.00	2.92	2.06
<i>SLF4</i>	2.27	2.41	0.75	2.51
<i>SLF5</i>	1.36	1.83	2.62	1.53
<i>SLF6</i>	0.98	1.18	0.95	2.16
<i>SLF7</i>	2.50	2.07	0.99	2.40
<i>SLF8</i>	0.57	1.26	1.25	1.67
<i>SLF10</i>	0.92	1.18	0.15	1.54
<i>SLF11</i>	1.43	0.92	1.77	1.37
<i>SLF12</i>	1.37	2.64	2.92	1.05
<i>SLF13</i>	1.60	2.23	0.69	1.92
<i>SLF14</i>	2.77	2.95	0.68	1.56
<i>SLF15</i>	0.94	1.15	1.02	2.56
<i>SLF16</i>	1.06	1.41	0.63	2.90
<i>SLF17</i>	3.20	2.49	0.63	2.32

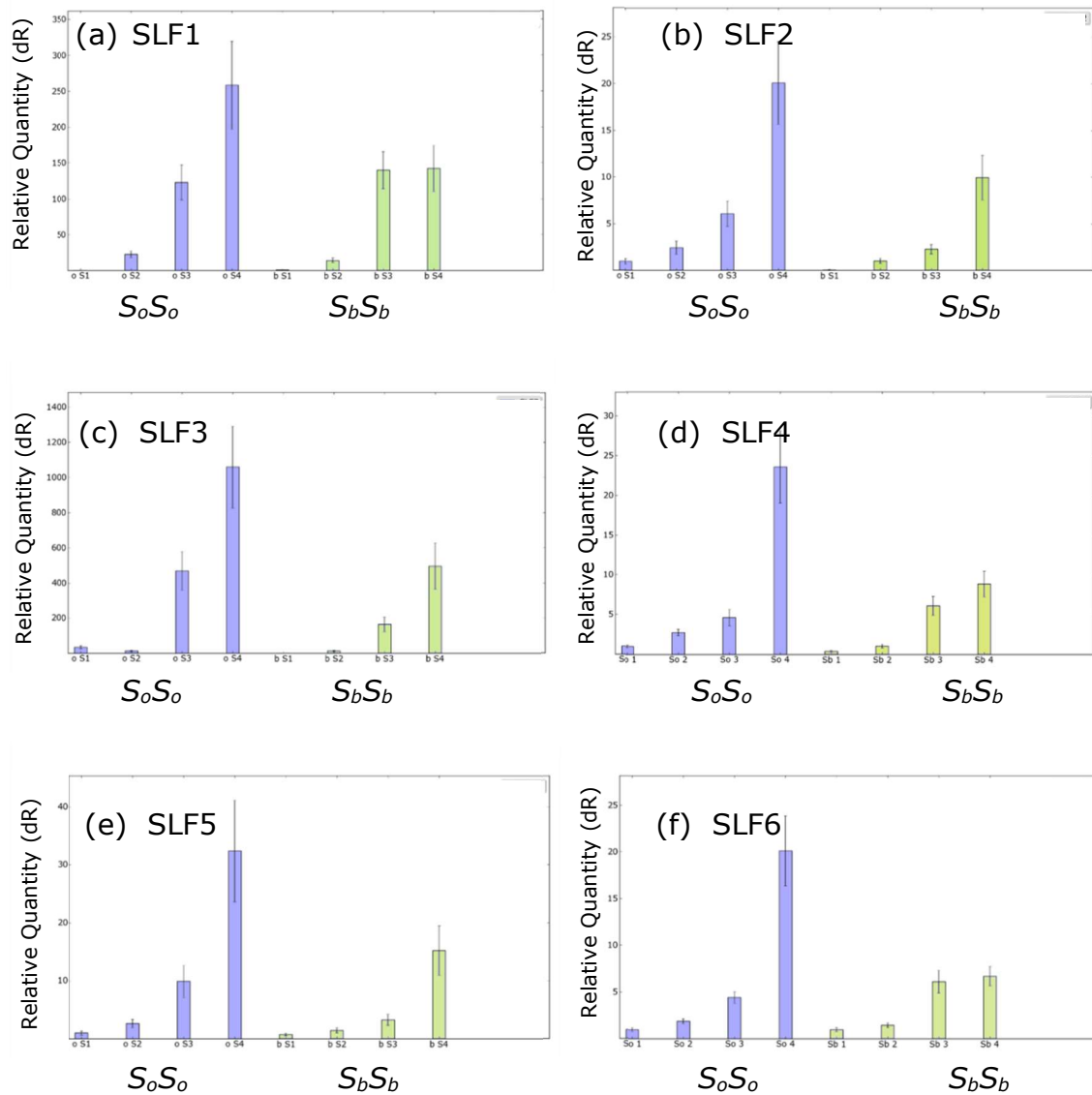


Figure 4.3.1 **The relative expression level of some *SLF* genes in S_oS_o (purple) and S_bS_b (green) homozygotes.** The y-axis represents the relative quantity of expression, the x-axis represents the sample coming from four stages of stamens, the purple bars are S_oS_o and the green bars are S_bS_b . (a)-(f) represent the expression level comparisons using different *SLFs* primer: (a) SLF1; (b) SLF2; (c) SLF3; (d) SLF4; (e) SLF5; (f) SLF6. The symbols o and b represent the cDNA from S_oS_o homozygote and S_bS_b homozygote, respectively. S1, S2, S3 and S4 represent the four stages of RNA extracted from anthers. The o S1 (stage 1 of S_oS_o homozygote) is used as the calibrator, appointed a value of 1. *EF1a* was used as a housekeeping gene to normalize all qRT-PCR.

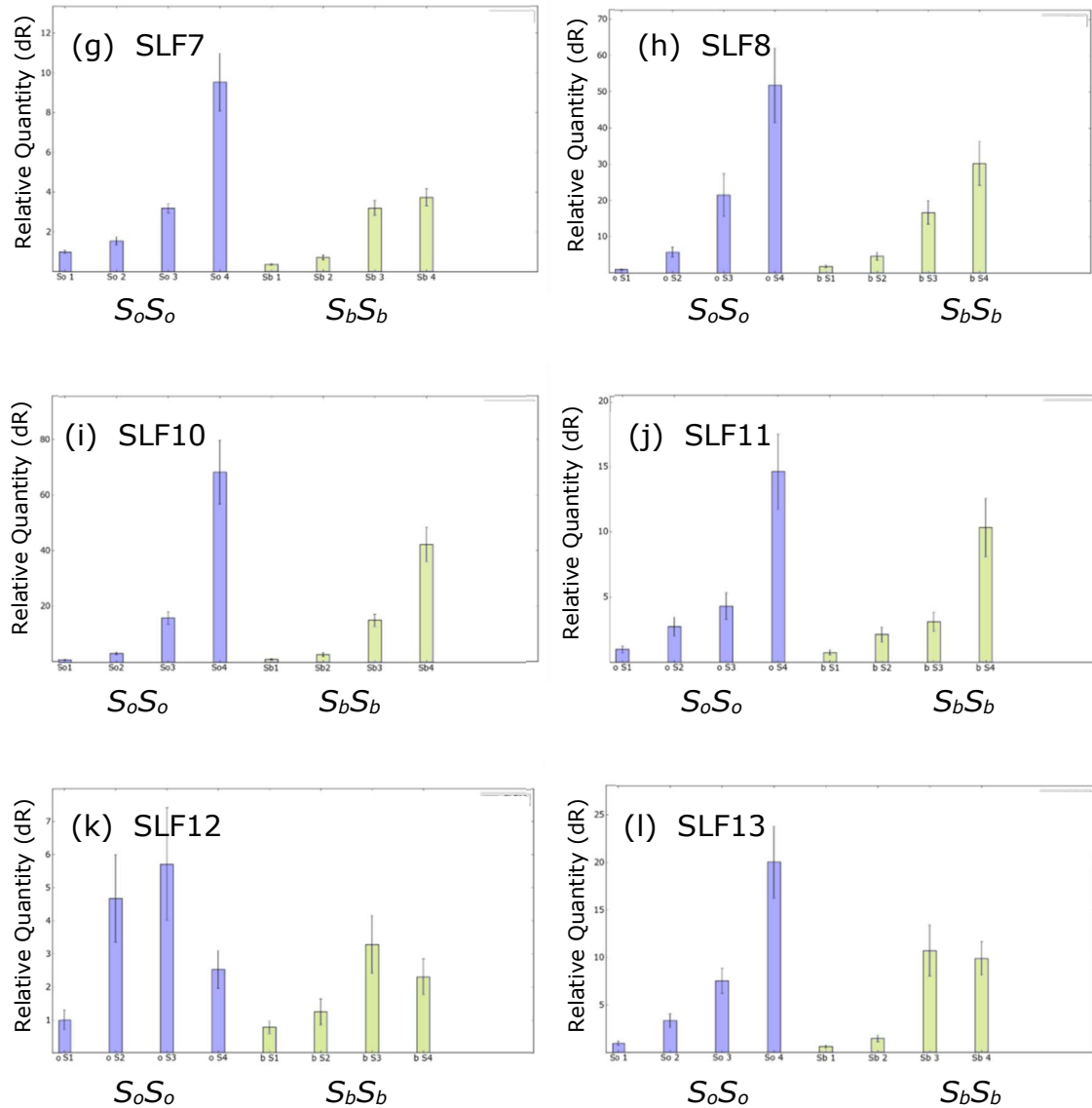


Figure 4.3.2 **The relative expression level of some *SLF* genes in S_oS_o (purple) and S_bS_b (green) homozygotes.** The y-axis represents the relative quantity of expression, the x-axis represents the sample coming from four stages of stamens, the purple bars are S_oS_o and the green bars are S_bS_b . (g)-(l) represent the expression level comparisons using different *SLFs* primer: (g) SLF7; (h) SLF8; (i) SLF10; (j) SLF11; (k) SLF12; (l) SLF13. The symbols o and b represent the cDNA from S_oS_o homozygote and S_bS_b homozygote respectively. S1, S2, S3 and S4 represent the four stages of RNA extracted from anthers. The o S1 (stage 1 of S_oS_o homozygote) is used as the calibrator, appointed a value of 1. *EF1a* was used as a housekeeping gene to normalize all qRT-PCR.

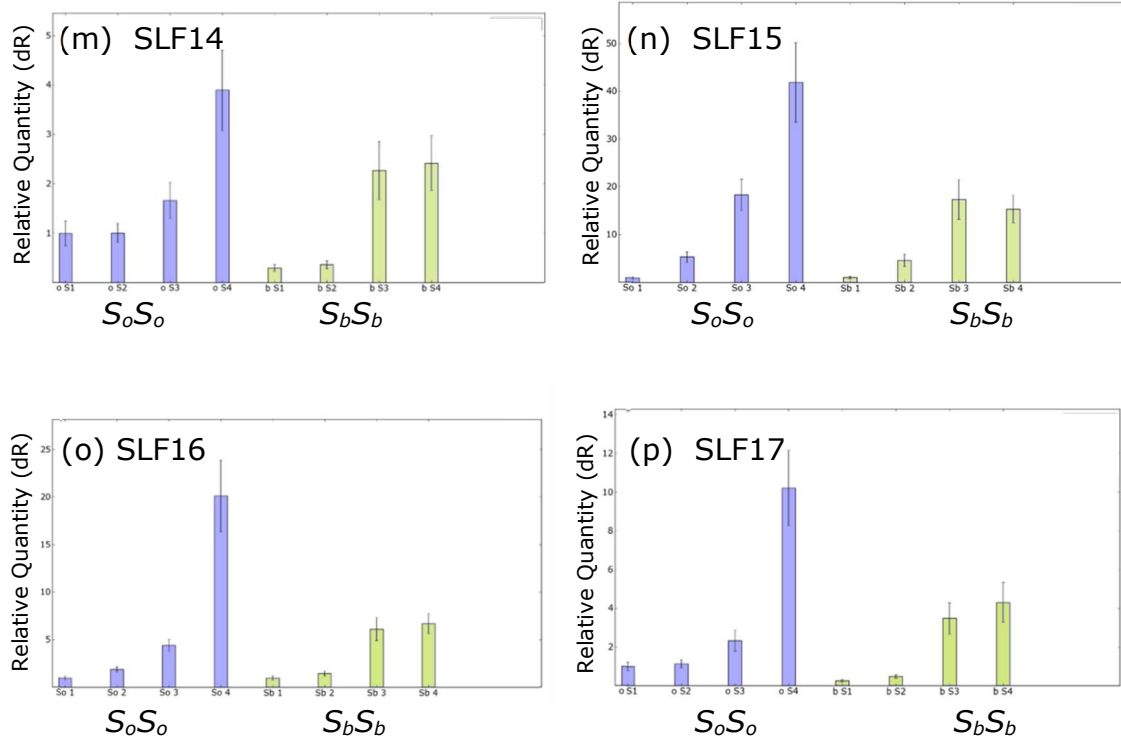


Figure 4.3.3 **The relative expression level of some *SLF* genes in S_oS_o (purple) and S_bS_b (green) homozygotes.** The y-axis represents the relative quantity of expression, the x-axis represents the sample coming from four stages of stamens, the purple bars are S_oS_o and the green bars are S_bS_b . (m)-(p) represent the expression level comparisons using different *SLFs* primer: (m) SLF14; (n) SLF15; (o) SLF16; (p) SLF17. The symbols o and b represent the cDNA from S_oS_o homozygote and S_bS_b homozygote respectively. S1, S2, S3 and S4 represent the four stages of RNA extracted from anthers. The o S1 (stage 1 of S_oS_o homozygote) is used as the calibrator, appointed a value of 1. *EF1a* was used as a housekeeping gene to normalize all qRT-PCR.

The four bars on the left in all figures (4.3a-4.3p) illustrate the trend in *SLF* expression level during anther development in S_oS_o homozygotes. The expression level of all *SLFs* in S_oS_o and S_bS_b shows an increase trend from stage 1 to stage 4 except *SLF12*. Specially, *SLF12* showed a different trend compared with the others, an increase to reach a peak at stage 3 and steep decline to stage 4. The expression level of stage 4 was significantly lower than stage 3, and even lower than stage 2 in *SLF12* of S_oS_o . The S_bS_b follows a similar trend, but the *SLF12* is expressed less at stage 2 than stage 4.

The four bars on the right in all figures show the corresponding *SLF* expression levels in S_bS_b homozygotes. There are several trends to note in these 16 *SLFs*: some of them (*SLF2*, 3, 5, 8, 10 and 11) show a significant increase like most of the *SLFs* in the S_oS_o homozygotes with sharp increase and reach the high point at stage 4. However, not all *SLFs* in S_bS_b homozygote reach the peak at stage 4, some *SLFs* (*SLF1*, 4, 6, 7, 13, 14, 15, 16 and 17) showed a sharp climb from stage 2 to stage 3, and a slight rise or fall from stage 3 to stage 4. In the Figure 4.3 (k) for *SLF12*, the trend in S_bS_b homozygotes is similar to that for S_oS_o homozygotes, but the decline degree of expression level from Stage 3 to Stage 4 was abnormal and lower than the chart of *SLF12* in S_oS_o homozygote.

Comparing all *SLFs* expression levels at stage 4 between S_oS_o and S_bS_b in Table 4.2 and Figure 4.2, most of *SLFs* (*SLF2*, 3, 4, 6, 7, 13, 15, 16 and 17) in S_oS_o is significantly higher expressed than S_bS_b at the stage 4, as twice express as high as S_bS_b ; some of them (*SLF1*, 5, 8, 10, 11 and 14) have nearly 50% more expression in S_oS_o than S_bS_b . For the expression level at stage 1, stage 2 and stage 3 of S_oS_o and S_bS_b , most of them show no significant difference between these two homozygotes except *SLF2* (Stage 2 and 3), *SLF3* (Stage 3), *SLF4* (Stage 1 and 2), *SLF5* (Stage 2 and 3), *SLF7* (Stage 1 and 2), *SLF12* (Stage 2 and 3), *SLF13* (Stage 2), *SLF14* (Stage 1 and 2) and *SLF17* (Stage 1 and 2) in S_oS_o are twice as many as in S_bS_b . The expression level stage 1 in *SLF2* and *SLF3* shows a great difference between S_oS_o and S_bS_b (17 times and 36 times difference, respectively). In addition, *SLF12* of S_oS_o on average expressed two times more than the *SLFs* of S_bS_b at the stage 2 and stage 3, but the stage 4 shows no difference on the expression level between S_oS_o and S_bS_b .

4.2.3 Partial sequencing of 16 *SLFs* mRNA from S_oS_o and S_bS_b homozygotes

In order to determine whether there are differences at the genomic level for the 16 *SLFs* from these two homozygotes, sequencing comparisons of 16 *SLF* mRNA were carried out. The *SLF* gene sequences of S_oS_o have already been published by Kubo *et al.* (2015) as S_{om} -haplotype, but S_bS_b has not been reported. The best approach to compare the sequences of *SLFs* between these two homozygotes is to sequence all *SLFs* in S_bS_b . The sequencing method used in Kubo's group (2015) was next generation sequencing (NGS). However, in this project, partial sequencing, a fast method, was chosen to identify any differences between the *SLFs* RNA sequences between S_oS_o and S_bS_b .

As mentioned in section 1.4.3, the coding region of *SLFs* has two functional domains: a conserved and a hypervariable regions. The hypervariable region was considered as the most distinctive in *SLF*. According to the alignment of 16 *SLFs* of S_oS_o homozygote, the least similar regions of alignment were located at 350 bp-870 bp. Thus, there are more possibilities to find bases different in these regions. Moreover, 16 pairs of *SLFs* primers described at section 3.2.4 and 3.2.5 had already been designed from these regions. Thus, a round of genomic PCR using these specific-designed primers used in the RT-PCR were performed. Each homozygote used the three bio-replications, which are the same as with qRT-PCR. All PCR products were sent to Eurofins Company for sequencing, and all samples were sequenced from both directions using their specific forward and reverse primers. Excluding the unreliable sequences caused by sequencing and considering the sequencing region is the FBA domain of *SLFs* (Li *et al.*, 2017), the results can be illustrated as the sequences of all S_oS_o homozygotes products, which are identical with all S_bS_b homozygotes products (the sequencing results are not shown). A summary of the sequencing results is shown at Table 4.3.

Table 4.3 Summary of the alignment data between sequenced *SLFs* and original *SLFs* data of S_oS_o

Sequencing products name	Total score of sequencing	Sequencing coverage on original <i>SLFs</i> data of S_{om} -haplotype	Max identity of sequencing results in same <i>SLF</i>
SLF1 using S_oS_o genomic DNA (referred to as 'SLF1 in S_oS_o ' on follows)	458 bp	39.14%	100%
SLF1 in S_bS_b	458 bp	39.14%	
SLF2 in S_oS_o	448 bp	45.67%	100%
SLF2 in S_bS_b	439 bp	44.75%	
SLF3 in S_oS_o	294 bp	25.39%	100%
SLF3 in S_bS_b	294 bp	25.39%	
SLF4 in S_oS_o	400 bp	32.92%	100%
SLF4 in S_bS_b	412 bp	33.91%	
SLF5 in S_oS_o	449 bp	38.47%	100%
SLF5 in S_bS_b	445 bp	38.13%	
SLF6 in S_oS_o	519 bp	43.91%	100%
SLF6 in S_bS_b	519 bp	43.91%	
SLF7 in S_oS_o	457 bp	38.76%	100%
SLF7 in S_bS_b	469 bp	39.61%	
SLF8 in S_oS_o	457 bp	38.57%	100%
SLF8 in S_bS_b	460 bp	38.81%	
SLF10 in S_oS_o	450 bp	39.47%	100%
SLF10 in S_bS_b	451 bp	39.56%	
SLF11 in S_oS_o	459 bp	39.13%	100%
SLF11 in S_bS_b	458 bp	39.05%	
SLF12 in S_oS_o	430 bp	36.37%	100%
SLF12 in S_bS_b	411 bp	34.77%	
SLF13 in S_oS_o	454 bp	38.90%	100%
SLF13 in S_bS_b	453 bp	38.82%	
SLF14 in S_oS_o	449 bp	38.38%	100%
SLF14 in S_bS_b	443 bp	37.86%	
SLF15 in S_oS_o	440 bp	37.74%	100%
SLF15 in S_bS_b	440 bp	37.74%	
SLF16 in S_oS_o	460 bp	39.02%	100%
SLF16 in S_bS_b	461 bp	39.10%	
SLF17 in S_oS_o	471 bp	40.36%	100%
SLF17 in S_bS_b	471 bp	40.36%	

4.3 Discussion

4.3.1 New data supports the results of second round of RT-PCR of *SLF8*

It was considered that the missing of *SLF8* in S_bS_b and competitive interaction are the possible reason to explain the differences between S_oS_o and S_bS_b . However, the second round of RT-PCR of *SLF8* using new designed primers and ideal plant material suggested it was wrong. The results of qRT-PCR and genomic sequencing also support the result from the second round of RT-PCR of *SLF8*: *SLF8* exists and is expressed in S_bS_b .

In fact, the missing band of *SLF8* in the first round of RT-PCR may be caused by several reasons show as follows. First, the cDNA used in RT-PCR of *SLF8* could be problematic. The plant material used in the first round of RT-PCR was unselected S_bS_b . As could be seen in Table 3.1 and 3.2, some S_bS_b showed PSC after self-pollination. The reason why a SI plant shows PSC is still unknown to date. But use of unselected S_bS_b may cause the missing band on the first round RT-PCR of *SLF8*. Second, the *SLF8* primer (SLF8-Som) used in the first round of RT-PCR may have some problems. The problem may be that 'SLF8-Som' primers are contaminated or degraded. This is because a BSc student Temi Daike (BSc student 2017/18) repeated the pollen collection and RT-PCR with 16 *SLFs* primers. She also found that 'SLF8-Som' primers were unable to give her an RT-PCR result. Therefore, the primer problem may cause the missing band in *SLF8* as well. However, the qRT-PCR result of *SLF8* shows that *SLF8* is expressed in S_bS_b with 100% accuracy. The result of the first round RT-PCR of *SLF8* should be considered as a mistake in the experiment, and should not be used as the foundation of final conclusion.

4.3.2 The sequencing and qRT-PCR results of *SLFs* in S_oS_o and S_bS_b

About 25% - 45% of *SLFs* sequences in S_oS_o and S_bS_b were found to be 100% identical, as summarized in Table 4.3. The different sequencing coverages on original *SLFs* data of S_{om} -haplotype are limited to the primers. Also, in order to sequence and identify all *SLFs* in S_bS_b , it is best to use the same methods to identify *SLFs* in S_oS_o as were used by Kubo and co-workers (2015). They used next-generation sequencing to identify all *SLFs*, but it took a long time. Therefore,

the primers for sequencing used are the same as the primers used in the RT-PCR, but this time used in the genomic PCR. Therefore, not whole *SLFs* sequences in *S_oS_o* and *S_bS_b* were sequenced.

As shown in Figure 5.1 and Figure 5.4, these primers were designed from the most distinguishing region in each *SLF*, which is called FBA domain (Li *et al.*, [2017](#)). If the *SLFs* have a different sequence, the FBA domain should be the region where differences appear. The sequencing results using these primers led to the results that we expected. This is because when Wright ([2004](#)) cross-pollinated the pollen of *S_bS_b* to the pistil of *S_oS_o*, the incompatibility was observed in the *S_oS_o*, which indicates that the pollen of *S_bS_b* and the pistil of *S_oS_o* should be carried the same *S*-allele. According to the previous studies about *S*-allele, carrying the same *S*-allele means this two haplotypes contain same *S*-RNase and *SLFs*. The results of *S*-RNases and *SLFs* sequencing gave the evidence to prove it. Despite the fact that the sequencing regions of *SLFs* were not covered completely by *SLFs* sequence, it can be predicted that the *SLFs* sequences in *S_oS_o* and *S_bS_b* are identical because near half sequence of *SLFs* was shown to be identical. This gives us confidence, especially they located at the FBA domain of these *SLFs*.

The qRT-PCR results shown in Figure 4.3 are informative and are worth discussing. *SLF12* has a different change trend when comparing with others. The *SLFs* expression at stage 4 should be higher than stage 3's. Kubo and his co-workers ([2015](#)) proposed that the *SLFs* are all expressed and work together to identify some *S*-RNases, therefore the expression of *SLF12* may be inhibited by some transgenic factor, or possible it is not necessary for degrading *S_o*-RNase or *S_b*-RNase. When pollen is mature enough at stage 4, it will be ready to pollinate the pistil once the flower opens. The peak expression of *SLF12* seems to be earlier than other *SLFs*, which means *SLF12* is expressed earlier than other *SLFs*. The earlier expression will lead *SLF12* to be insufficient to work when pollen lands on the pistil. Therefore, *SLF12* may not be necessary for SI in both *S_oS_o* and *S_bS_b*. The result of *SLF3* indicates that *SLF3* is expressed more strongly in both *S_oS_o* and *S_bS_b* when compared with other *SLFs*. The reason causes it is that *SLF3* might play an important role during SI. More expression of *SLF3* is useful during SI in *S_oS_o* and *S_bS_b*.

However, *SLF12* and *SLF3* are not the reason we look for to explain the differences between S_oS_o and S_bS_b . Although *SLFs* are expressed in four stages, there is no doubt that the expressions levels of most of *SLFs* at Stage 4 are the most interesting finding. The results from double delta Ct analysis and the diagram of relative expression level, all indicated that the expression levels of *SLFs* in fourth stage of S_oS_o and S_bS_b show a significant differences: all *SLFs* except *SLF12* in S_oS_o have 1.5-fold or two-fold expression than in S_bS_b . All above information can be used to deduce several hypotheses to explain the differences between S_oS_o and S_bS_b , as elaborated below.

4.3.3 Implications for the possible basis of phenotypic difference of S_oS_o and S_bS_b homozygotes

The qRT-PCR relative quantity chart of all *SLFs* show that nearly all 16 *SLFs* have almost double expression level at Stage 4 in S_oS_o compared to S_bS_b . In addition, the partly sequencing results of all *SLFs* in these two homozygotes indicate that the sequences of *SLFs* are highly probably the same. Thus, according to all evidence we have now, there are three assumptions to explain the different phenotypes between S_oS_o and S_bS_b homozygotes.

1) Assumption 1

The differences of *SLF* gene expression levels are due to gene duplication of *SLFs* in S_oS_o . The competitive interaction considered that duplication of *SLFs* might cause the breakdown of S-RNase based self-incompatibility (Tsukamoto *et al.*, [2003](#)). As can be seen from the Figure 4.3, some *SLFs* in S_oS_o have almost 2-fold higher expression in stage 4 than S_bS_b . The *SLFs* and their promoter region in S_oS_o were possibly mutated and duplicated. However, it is hard to confirm that there are duplications that exist in S_oS_o , unless a genomic DNA southern blot analysis is used to prove it.

Assuming that the duplication of *SLFs* exist in S_oS_o , and the sequences of *SLFs* in S_oS_o and S_bS_b , the hypothesis in the light of this clue can be explained as following: S_oS_o and S_bS_b share the same parents, therefore it is possible that they have

similar *S*-allele. Self-compatibility or self-incompatibility of *S_oS_o* and *S_bS_b* is controlled by the interaction of two factors: *S*-RNases and SLFs (Ai *et al.*, [1992](#); Broothaerts *et al.*, [1991](#); Sims and Robbins, [2009](#)). As far as the comparison of *S*-RNases is concerned, *S_oS_o* and *S_bS_b* were shown to have identical *S*-RNase genomic DNA sequences (Czyzyk, [2010](#); this thesis), and the *S*-RNase expression level of *S_oS_o* was shown to be not obviously different from *S_bS_b* in pistils (Czyzyk, [2010](#)). Hence the differences between these two closely related homozygotes are not due to *S*-RNase.

On the investigation of *SLFs* sequences and expression, the identical sequences of the most variable and discriminate region in all *SLFs* of both *S_oS_o* and *S_bS_b* illustrated that the sequences of *SLFs* in *S_oS_o* and *S_bS_b* are identical, as far as sequenced about 40-50% of gene. However, the comparisons of expression level of *SLFs* were different from the expression comparison of *S*-RNases. Most of *SLFs* from *S_oS_o*, showed approximately 2-fold or 1.5-fold higher expression as *S_bS_b*, which could generate a speculation: *S_oS_o* contains duplicated *SLFs* encoding region and promoter region in *S*-allele compared with *S_bS_b* that causes higher expression of *SLFs* in *S_oS_o* (Figure 4.4.1). Combining with the definition of competitive interaction: duplication of the partial genome can cause the SI process to be hampered (Tsukamoto *et al.*, [2005](#)). The SC of *S_oS_o* is possibly caused as the results of the breakdown of SI, as a result of all or most of *SLFs* and their promoter (or even possible is a part of *S*-allele) duplication. Notwithstanding this hypothesis being able to explain the different SI phenotypes between *S_oS_o* and *S_bS_b*, there are questions remaining Competitive interaction is usually specific to heteroallelic pollen that combines different *S*-haplotypes. As shown in Figure 4.1, only heteroallelic pollen can lead to the breakdown of SI in the diploid pistil, and homoallelic pollen still is self-incompatible for pistil, even if the pollen of *S_oS_o* is duplicated, it is still homoallelic. Therefore, whether *S_oS_o* belongs to the special case like the detection from Tsukamoto and his co-workers ([2005](#)), or it has another explanation, more evidence is required to support or reject this hypothesis.

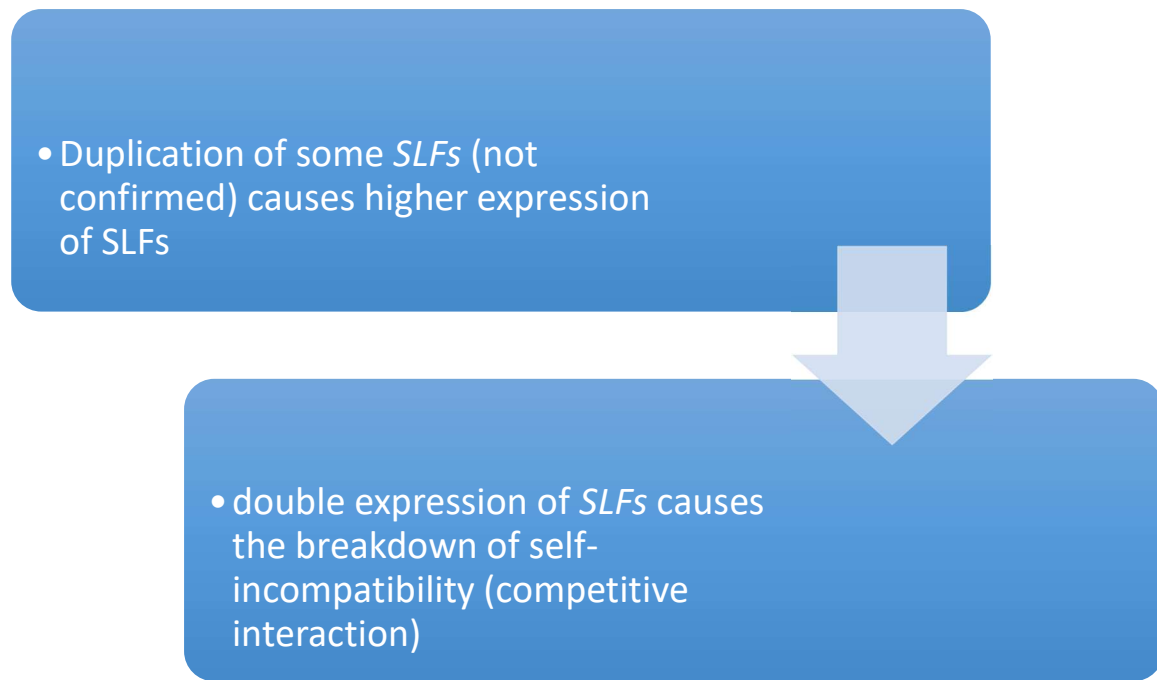


Figure 4.4.1 **A hypothesis to explain the different phenotype between S_oS_o and S_bS_b .**

2) Assumption 2

Competitive interaction may be the reason why S_oS_o shows self-compatibility but S_bS_b is self-incompatible. The duplication of *SLFs* is not the only possible explanation for more expression of *SLFs* in S_oS_o than S_bS_b . The expression of genes is controlled by many factors, one speculation is that there is a trans-acting factor that exists in pollen that reduces the expression of *SLFs* in S_bS_b . This potential trans-acting factor plays a role to regulate the promoter of *SLFs* in S_bS_b and causes *SLFs* in S_bS_b to have lower expression than in S_oS_o (Figure 4.4.2). Usually, the molecular amount of S-RNases and *SLFs* in *Petunia* has a 'threshold' to be balanced in the pollen tube. For example, if the molecular ratio of S-RNases and *SLFs* is 1:1 in a self-compatible plant, all S-RNases will be ubiquitinated and degraded. Remaining growth-related RNA will stimulate the pollen tube to continue grow and complete the fertilization, which shows self-compatibility in the phenotype of S_oS_o . If the molecular ratio of S-RNases and *SLFs* in pollen tube is 2:1, the *SLFs* are not sufficient to target *all* S-RNases, rendering S-RNases that have a higher protein amount than *SLFs*. Then, remained S-RNases stop the pollen tube growing, which possible happening in S_bS_b . A schematic diagram of molecular

response of SLF and S-RNase was made to explain this hypothesis and are shown with a more detailed explanation in general discussion (Chapter 7). Despite the fact that there is no current evidence to prove that this trans-acting factor exists in S_oS_o , this hypothesis is the most reasonable for now to explain the different phenotype between S_oS_o and S_bS_b comparing with other hypothesis.

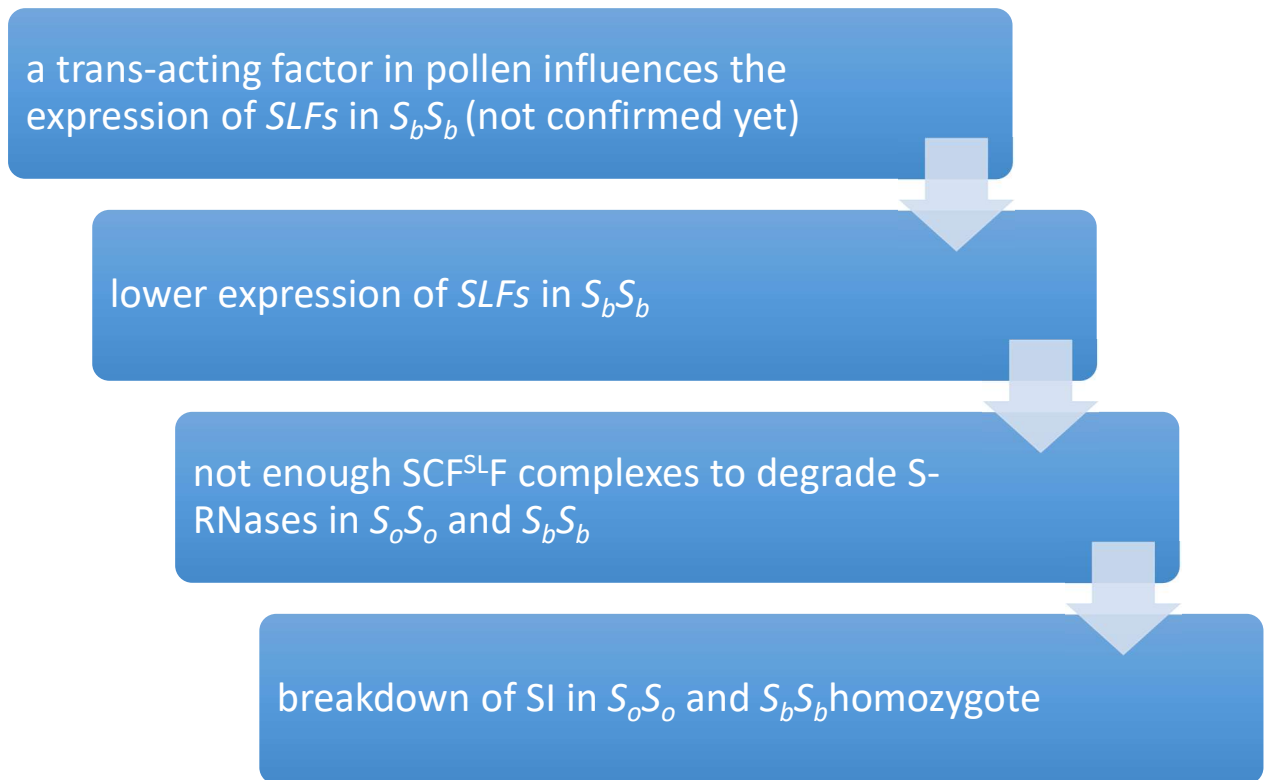


Figure 4.4.2 **One of hypotheses “threshold balance” to explain the different phenotype between S_oS_o and S_bS_b homozygotes.**

3) Assumption 3

The third assumption is that there is a different *SLF* we have not detected in S_bS_b yet, which causes the SI in S_bS_b . We have not sequenced whole *SLFs* sequences of S_bS_b , thus it is possible that one or more *SLFs* are different between S_oS_o and S_bS_b , and there is a mutation that we have not detected. For instance, a mutation that appears in the outside sequenced region could lead to aberrant *SLF* protein, this aberrant *SLF* protein could lead to the degradation of the S_o -RNase and S_b -RNase. However, it is also possible that there is a new *SLF* that exists in S_bS_b ,

which is totally different from any *SLFs* of S_oS_o . Thus no *SLFs* primers designed based on S_oS_o *SLFs* sequences are able to detect it. Only the methods which identified all *SLFs* of S_oS_o can prove its presence. According to the recognition model from Kubo and his co-workers (2015), the SI in self-compatible plants is caused by the absence of a corresponding *SLF*. Therefore, it is possible that the difference in S_bS_b and S_oS_o is caused by this different *SLF*: S_oS_o have the corresponding *SLF*, S_o -RNase thus can be degraded, the corresponding *SLF* of S_bS_b changes and becomes a new *SLF* that unable to degrade S_o -RNase and S_b -RNase anymore. This is why S_bS_b shows self-incompatibility (Figure 4.4.3). However, the assumption of 'new *SLF*' could not explain the different expression levels between S_oS_o and S_bS_b . Moreover, there is no sequence data of full *SLFs* in S_bS_b obtained by NGS or published. Therefore, this is the least-evidenced hypothesis between these three hypotheses.

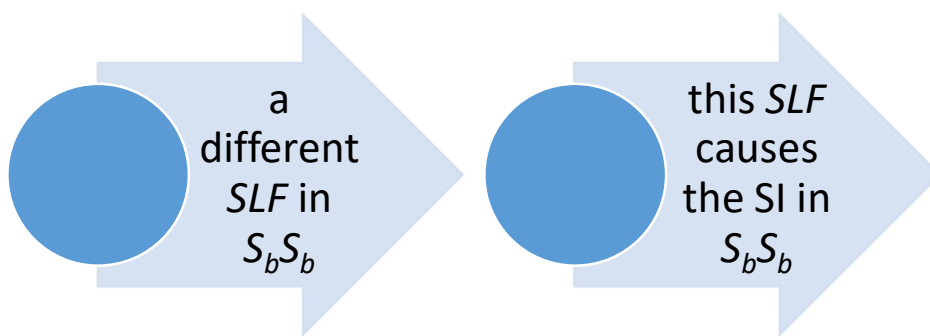


Figure 4.4.3 **One of hypotheses to explain the different phenotype between S_oS_o and S_bS_b homozygotes.**

Overall, the “threshold balance” hypothesis is the most reasonable explanation for now. The potential trans-acting factor in pollen is still not identified to date, but the presence of it is possible because of the different expression level of *SLFs* between S_oS_o and S_bS_b homozygotes. To date, there are no publications to explain the phenotype differences between S_oS_o and S_bS_b homozygotes, and the data obtained in this project (RT-PCR analysis, qRT-PCR analysis and partial cDNA sequencing of 16 *SLFs* in S_oS_o and S_bS_b) are the latest research progress in this field.

4.3.4 The contribution of this project is to support previous evolution theory of S-RNase based SI

This work not only finds a breakthrough to explain why S_oS_o pollen can make S_bS_b pistil to become cross-incompatible, but also provides a strong evidence to support the previous evolution theory in S-RNase based SI proposed by Fujii and his co-workers ([2016](#)). Fujii and his co-workers believed that the SC plants might have been evolved from SI plants. In their model, SI plants originally have not contained a SLF to degrade self S-RNase. With the evolution during many generations, a new SLF came out to degrade self S-RNase, which causes the breakdown of SI. According to the finding of this project, this model can be explained as follows. As two cultivars of *Petunia hybrida*: S_oS_o and S_bS_b , they have the same parents: *Petunia axillaris* and *Petunia inflata*. S_oS_o and S_bS_b share the same sequence of S-RNase, very possibly the same sequence of SLFs and similar expression levels of S-RNase, but have different expression level of SLFs. It can be assumed that S_bS_b is SI plants is before evolution, and S_oS_o is the SC plants is after evolution. With the evolution, the higher expression levels of SLFs causes the breakdown of SI in S_oS_o (not generating new SLFs to degrade S_o -RNase and S_b -RNase). Therefore, the pollen of S_oS_o can cause the pistil of S_bS_b to fail to set seeds. The identification time of S_b -allele (Broothaerts *et al.*, [1991](#)) and S_o -allele (Ai *et al.*, [1992](#)) may also provide a bit of information. This evidence will be published soon to help us to understand more about the evolution in S-RNase based SI.

CHAPTER 5 CHARACTERIZATION OF SLF EXPRESSION IN *PETUNIA INFLATA* USING SLFPROMOTER:GUS CONSTRUCTS

5.1 Introduction

5.1.1 *GUS* gene as one of reporter genes used in transgenic research

A reporter genes are genes that encode products that can quickly determine whether the foreign gene has been successfully transferred into target cells, organs or tissues and detect its expression activity (Gallagher, [2012](#)). A unique characteristics of reporter gene is that the gene product should not have a similar function to any product from the untransformed plant (Jefferson, [1987 a](#)). A simple characteristic is that the product of reporter gene needs to be easily observed (Jefferson *et al.*, [1987 b](#)). In general, the reporter gene can be used in the following situations: the detection of gene promoter activity (Wang *et al.*, [2005](#)), separation of some protein (Garbarino and Belknap, [1994](#)), and as the marker of transformation (Liu *et al.*, [2013](#)).

The *GUS* gene was originally isolated from *E.coli*, and this gene encodes a hydrolase β -glucuronidase (GUS) (Jefferson, [1987 b](#)). This hydrolase reacts with 5-bromo-4-chloro-3-indoyl- β -D-glucuronic acid (X-Gluc) and generates a blue precipitation in cells, that can be observed under natural light (Prell *et al.*, [2002](#)). There is no endogenous GUS activity detected in most plant cells, and neither detected in most of fungi or bacteria (except *E.coli*) thus far (Jefferson, [1989](#)). This is why the *GUS* gene is widely used as a reporter gene of plants, bacteria, and fungi. It is especially widely used in the transformation experiments to study the expression of foreign genes (Gallagher, [2012](#)). In addition, the 3' end of the *GUS* gene can be connected with other genes, and this fusion gene can be normally expressed and have GUS activity (Gallagher, [2012](#)). This provides useful conditions for studying foreign gene expression in cells or tissues. The GUS protein is stable in transgenic plant cells or extracts. Its half-life in plants can reach half-

day and sometimes even several days (De Ruijter *et al.*, [2003](#)). Additionally, *GUS* is also resistant at higher temperatures than normal reporter genes. Overall, *GUS* has many advantages as a reporter gene, it is a good choice for promoter studies in most plants.

5.1.2 Previous expression studies of self-incompatibility related genes or other pollen genes

The methods to locate the expression position of *SLFs* in this project can be compared with previous SI-related gene expression studies of *Petunia* or related species. In the early studies in the research field of *Petunia* gene expression position analysis, RT-PCR has been used as a convenient and direct method. Three decades ago, Clark and his co-workers ([1990](#)) initially cloned and sequenced *S-RNase*. They extracted RNA from different tissues of *Petunia*, such as leaves, petals, styles, and anthers. These RNA samples, coming from different tissues, were cloned and then hybridized in the slot blotter equipment. They found that *S-RNases* only expressed in style because the bands were observed on the obtained gel pic from slot blotter when using style RNA rather than other RNA.

The combination of two research methods: quantitative real-time PCR assays and GFP/GUS analysis was always a popular choice for the expression analysis in *Petunia*. For example, Liu *et al.* ([2011](#)) studied the expression analysis of a gene called *ERF* (*Ethylene-responsive element-binding factor*). They choose to study the expression of *ERF* in two ways. One way was to compare the expression differences among several groups of genes via quantitative real-time PCR assays. Another way was to clone the target gene coding region sequence into a pGreen vector. This vector contained a *35S* promoter and a *GFP* fusion, and then this transformed vector was transformed into onion leaves. Finally, they observed the expected subcellular localization of target gene expression at epidermal cells under fluorescence.

On some researches of *SLFs*, Kubo and his co-workers ([2015](#)) carried out an *SLF* expression research that was reported in the supplementary of their paper. They

planned to find the expression location pattern of *SLF*. However, the sequences of whole-genome or *SLF* promoter of *Petunia* were not published yet. Therefore, they used the promoter of a pollen specific gene called *LAT52* from tobacco (*Nicotiana tabacum*) instead of *Petunia SLF* promoter. The plasmid vector *pRI909* which was attached to the *LAT52-promoter* was constructed and been used for illustrating the expression location of *SLF3* from one haplotype of *Petunia hybrida*. Finally, clear blue proteins were discovered in the pollen tube under fluorescence microscopy.

Furthermore, these two research methods not only been studied in the expression analysis of SI related genes in *Petunia*, but also other pollen genes from other species have similar applications. Nguyen and his co-workers ([2016](#)) studied the expression of a rice pollen gene called *rice microspore-preferred gene 1 (RMG1)*. They divided the experiment into two parts to analyse the expression of *RMG1*. The first part of expression analysis built a construct with *LAT52* promoter (*LA52promoter:GFP-GUS* fusion) via two vectors: enter vector *pDONR201* and destination vector *pKGWFS7*. Another part is an expression analysis comparison of eight genes via qPCR similar manipulation with this project. Studying the expression of *RMG1* from these two parts, renders the final result as comprehensive and direct. Overall, the above research summary demonstrated that qRT-PCR and GFP/GUS transgenic expression analysis are the most popular choices in the expression research of pollen related-genes and *Petunia* genes. QRT-PCR was used to study differential gene expression comparisons. GFP/GUS transgenic expression analysis was also used to find expression location of genes.

The importance of this part of this project is because the whole genome sequence of *Petunia inflata* (one of *Petunia* species, the one parent of *Petunia hybrida*) was first published by Bombarely and co-workers ([2016](#)). This makes using *Petunia* own *SLF* promoter sequence to study *SLF* expression feasible. In fact, this is not the first time to study the expression location of pollen-specific gene in the past decades. The previous studies of pollen-specific gene expression location mainly used the promoter sequence of tomato pollen gene called *LAT52* to transfer into Tobacco, *Arabidopsis* and *Petunia hybrida* to observe the expression location of *SLF* (Eady *et al.*, [1994](#); Moore, [2006](#)). In addition, Kubo and co-workers ([2015](#))

used the promoter of *LAT52* to fuse different *SLFs* of *Petunia* and transferred construct into *Petunia*, identified the occurrence of SI via observing the expression of *SLFs*. In this project, using *Petunia*'s own *SLF* promoter sequences will give more precise and more reliable results to illustrate the *SLFs* expression location of *Petunia* via GUS analysis. In addition, the combination of qRT-PCR and *GUS* transgenic expression analysis can provide more information about the *SLFs* expression in *Petunia*.

5.1.3 Previous homology analysis of *SLFs* in *Petunia*

Kubo and co-workers ([2015](#)) identified 16-20 *SLFs* from different *Petunia hybrida* and *Petunia axillaris*. They used the cDNA sequence of *SLFs* to build the neighbour-joining phylogenetic trees via MEGA5, and found that some *SLFs* have close relationships with each other. Also, after Bombarely and co-workers ([2016](#)) identified all *SLFs* in *Petunia inflata*, they built a neighbour-joining phylogenetic tree to investigate the homology of *SLF* coding regions in *Petunia inflata* (Figure 5.1). *SLFs* were grouped into different types with high homology. In Figure 5.1, some *SLFs* of *Petunia inflata* such as *SLF7-1* with *SLF7-2*, *SLF13-1* with *SLF13-2*, *SLF11-1* with *SLF11-2*, *SLF14-1* with *SLF14-2*, *SLF5-1* with *SLF5-2*, have high similarity.

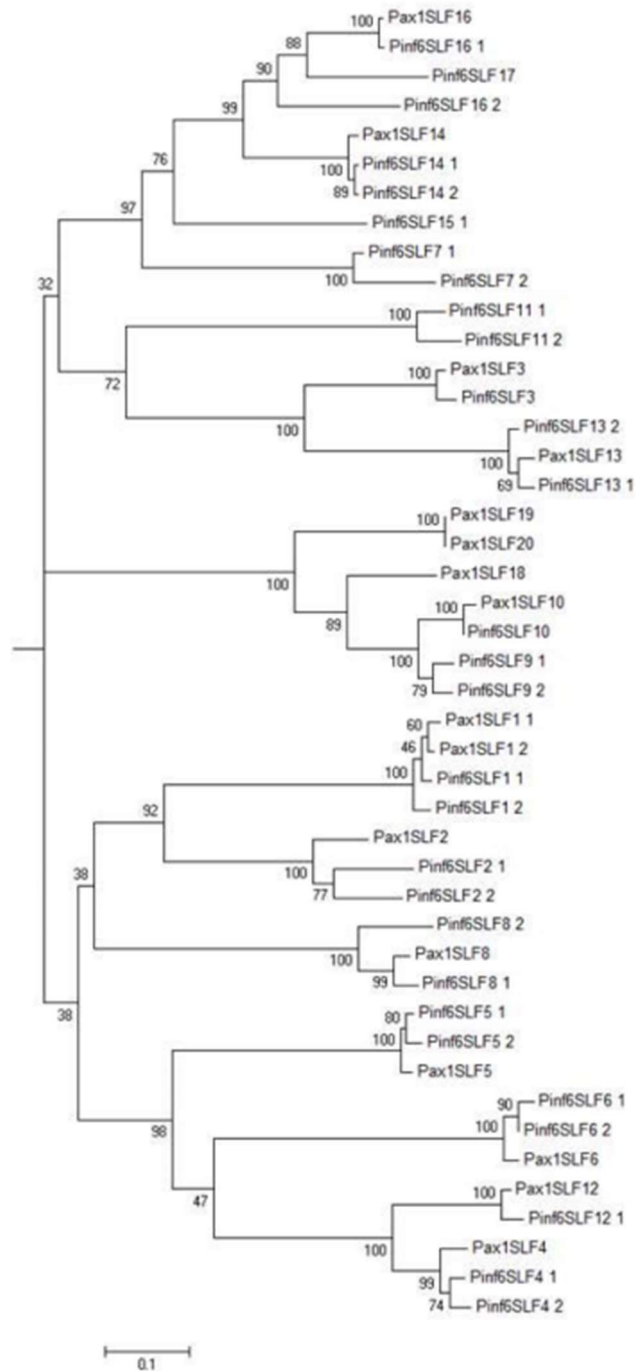


Figure 5.1 **Neighbour-joining phylogenetic trees of cDNA sequences of all SLFs identified in *Petunia axillaris*. N and *Petunia inflata* S₆.** The bar for each tree shows the number of nucleotide replacements per site. Numbers on each branch illustrates the bootstrap values (100 percentage at total) with 1,000 repetitions (Bombarely *et al.*, [2016](#), Supplementary note 11).

5.2 Results

Kao and co-workers ([2004](#)) used RNA gel blot analysis to reveal that *SLFs* express in pollen and anther in *Petunia*. Also, Hua and co-workers ([2007](#)) used RT-PCR to prove it. In order to use GUS analysis to directly locate the expression of *SLFs* in *Petunia*, a series of *SLFs* selection, construction of *SLFpromoter:GUS* were performed to achieve the aims. Because the whole genome sequence of *Petunia hybrida* is not published yet, the published genome sequence of *Petunia inflata* was used to study *SLF* expression location by GUS analysis.

5.2.1 Comparison of *SLF* promoters in *Petunia inflata*

Inspired by the ideas from Bombarely and co-workers ([2016](#)) described in section 5.1.3, in order to confirm the grouping methods for *SLFs* is also suitable to grouping *SLF* promoters and also to select the ideal *SLFs* to study their expression, a similar method to Bombarely and co-workers was used to build neighbour-joining phylogenetic trees. The *SLF* promoters regions were obtained from the *Petunia* genome database (<https://solgenomics.net/>) and transferred into Snapgene format. The size of the upstream region of the start codon of *SLFs* was selected to be 2000 bp as a safe region to contain the whole promoter of *SLFs*, following the suggestion from Rockman and Wray ([2002](#)). In fact, the downloaded sequences of *SLF* promoter should not be called as “promoter” because it is not the precise promoter of *SLFs*. The upstream of the *SLFs* coding sequence was just chosen as the candidate region of *SLF* promoters. However, to simplify the naming of the upstream region of the *SLFs* coding sequence with a short substitute, the term “*SLF* promoter” is used. The neighbour-joining phylogenetic trees were made by MEGA-X, and results are shown in Figure 5.2. In Figure 5.2, most *SLF* promoters show no significant homologies with each other.

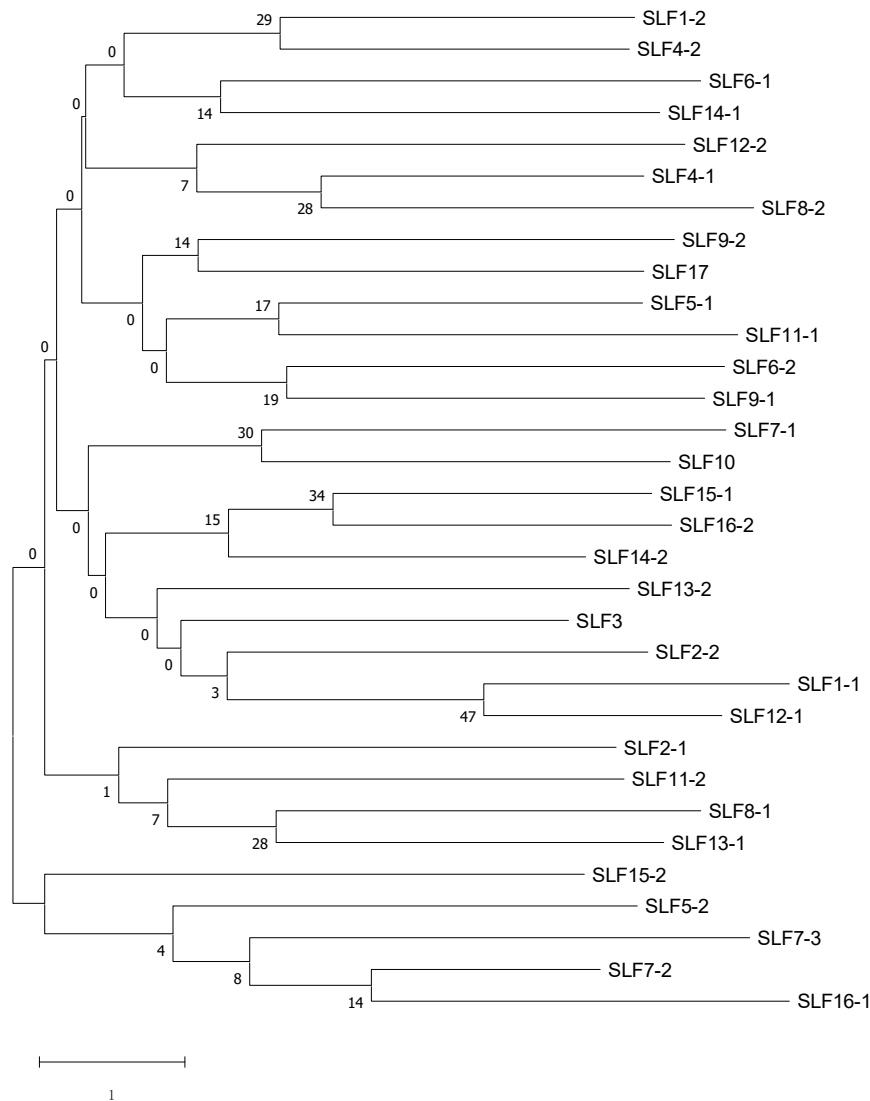


Figure 5.2 **Neighbour-joining phylogenetic trees of genomic DNA sequences of *SLF* promoters in *Petunia inflata*.** The scale bar at the bottom right corner is the number of nucleotide replacements per site. Numbers on each branch illustrates the bootstrap values (100 percentage at total) with 1,000 repetitions.

5.2.2 Comparison of *SLF* coding regions in *Petunia inflata* and *Petunia hybrida*

Petunia inflata and *Petunia hybrida* are two closely related species of *Petunia*. However, different numbers of *SLFs* were identified from these two species. In order to find the homology of these *SLFs*, another neighbour-joining phylogenetic tree was built. The cDNA sequences of 16 *SLFs* in *S₀S₀* homozygote of *Petunia hybrida* were obtained from NCBI (Kubo *et al.*, [2015](#)). MEGA-X was used for creating the neighbour-joining phylogenetic tree, and the result is shown in Figure 5.3. Figure 5.3 indicates the similarity of cDNA sequences of *SLFs* in *Petunia inflata* and *S₀S₀* homozygote of *Petunia hybrida*. Some *SLFs* of *Petunia inflata* and *Petunia hybrida* shows high homology, such as *SLF6-1* in *Petunia inflata* with *SLF6* in *Petunia hybrida*, *SLF13-1* and *SLF13-2* in *Petunia inflata* with *SLF13* in *Petunia hybrida*, *SLF1-1* in *Petunia inflata* with *SLF1* in *Petunia hybrida*. More analysis on how to select *SLFs* for promoter studies is provided in the Discussion section of this chapter.

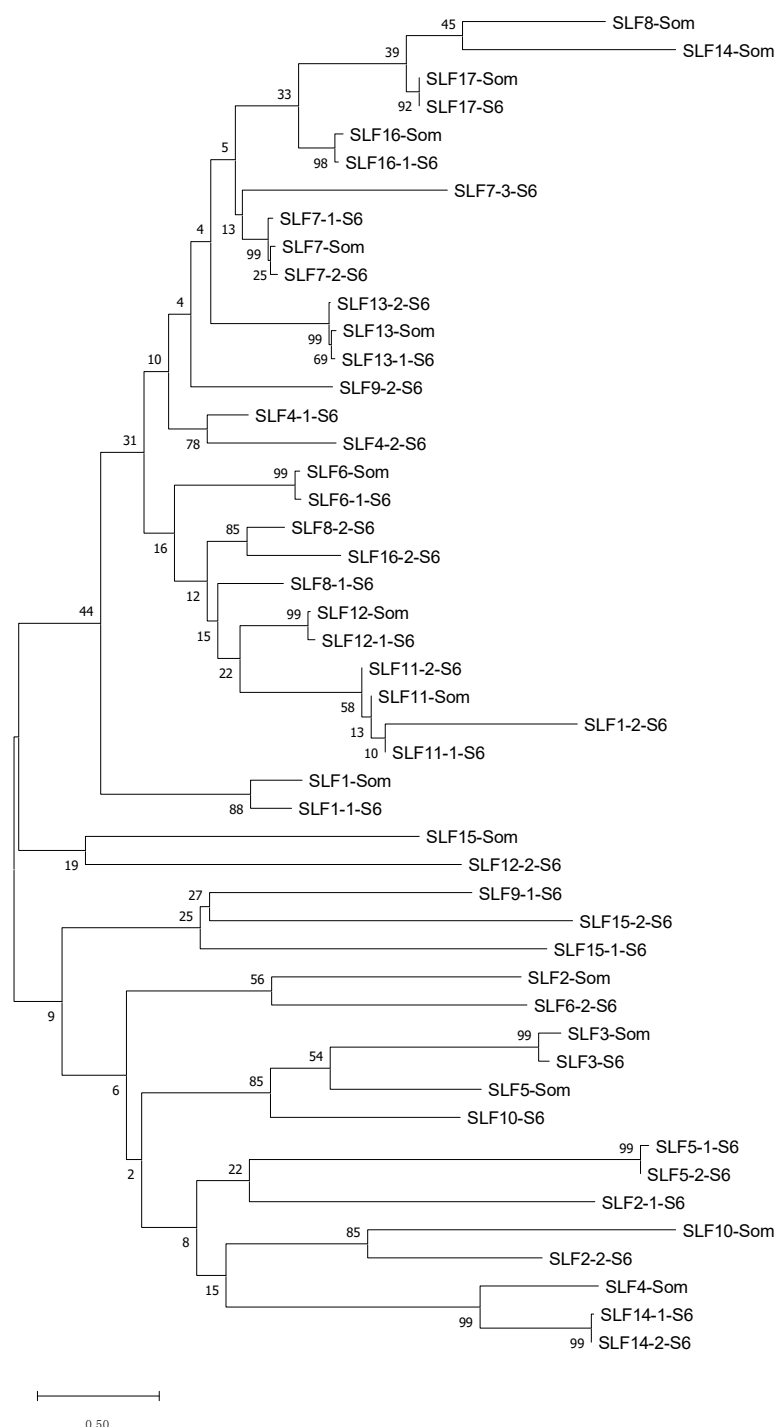


Figure 5.3 **Neighbour-joining phylogenetic tree of cDNA sequences of *SLFs-S₆* (*SLFs* of *Petunia inflata*) and *SLFs-S_{om}* (*SLFs* in *Som*-haplotype of *Petunia hybrida*).** The scale bar at the bottom right corner is the number of nucleotide replacements per site. Numbers on each branch illustrates the bootstrap values (100 percentage at total) with 1,000 repetitions. The *SLF* sequences named with '-S₆' refers the *SLFs* of *Petunia inflata*, and '-S_{om}' indicates this *SLF* sequence belongs to *Petunia hybrida*.

5.2.3 Expression analysis of selected *SLFs* in *Petunia inflata* using RT-PCR

To enable *SLF promoter:GUS* fusion analysis to test the *SLF* expression pattern one variety of *Petunia inflata* called *S₆* was chosen to provide *SLF* promoter sequences based on the published sequence (Bombarely *et al.*, [2016](#)). The *Petunia inflata S₆* stock was obtained from Thomas L. Sims (Northern Illinois University, one of the main participants in the sequencing consortium of *Petunia*). The seeds of *S₆* stock were sown and grown in the glasshouse with the same growth conditions described in section 2.1.

When the *S₆* plants grew until mature, the DNA extraction from young and healthy leaves using GenElute™ plant genomic miniprep kit (SIGMA-ALDRICH) was utilized to prepare fresh *Petunia inflata* DNA for promoter cloning. Because the *SLFs* sequence data of *Petunia inflata* is RNA sequence, in order to confirm that the *Petunia inflata S₆* seeds obtained from Dr. Sims could be used for promoter analysis, RNA extraction was carried out using RNeasy® plant mini kit (QIAGEN) when plants started flowering. According to the previous studies (Kubo *et al.*, [2010](#); Kubo *et al.*, [2015](#)), the main expression position of *SLFs* is in the pollen. Therefore, the anther of *Petunia inflata S₆* was collected to extract RNA. The RNA samples were reverse transcribed to cDNA and stored at -80°C for the next experiments.

An RT-PCR using cDNA of *Petunia inflata* and new-designed primers (*SLF3-S6* for RT-PCR, *SLF13-1-S6* for RT-PCR and *SLF14-1-S6* for RT-PCR, for primer details see appendix 4) was carried out, and three clear bands were obtained (see Figure 5.4). This indicates that the selected three *SLFs*: *SLF3*, *SLF13-1* and *SLF14-1* are actively transcribed in the pollen of *Petunia inflata* and can be used in the following transformation experiments as the selection of *SLF* promoter. Additionally, the stock obtained from Sims can be now confirmed as *Petunia inflata S₆*.

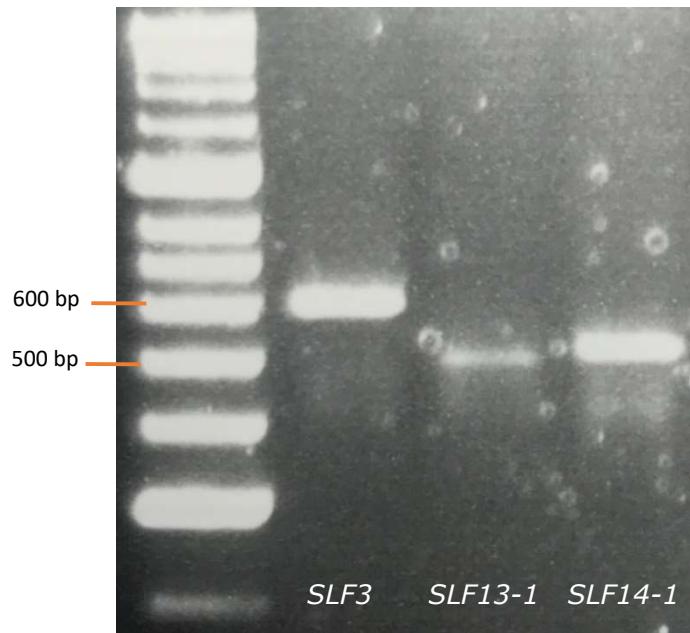


Figure 5.4 **Amplification of *SLF3*, *SLF13-1* and *SLF14-1* in the pollen cDNA of *Petunia inflata* by RT-PCR.**

5.2.4 Transformation of *SLF* promoter fragments into pCR8 for entry vector

5.2.4.1 Primer design for amplifying *SLF* promoter regions and manually screening for promoter elements

In order to provide the *SLF* promoter fragments for transformation, three pairs of *SLF* promoter region primers were designed based on the whole genome sequence of *Petunia inflata* *S*₆ using Primer 3 (for primer details see appendix 4). As the *SLF* genes in *Petunia inflata* show a high degree of similarity (Egea-Cortines and Weiss, 2016 the supplementary 12, Bombarely *et al.*, [2016](#)), the specificity of primers was assessed with an alignment of all 29 *SLF* promoter regions using Clustal Omega, respectively (the alignment data are not shown). The alignment results indicate that these three pairs of primers are specific in the *SLF* promoter regions. However, because of the technical deficiency of BLAST, the specificity of these three pairs of primers were not assessed in whole genome data of *Petunia inflata* *S*₆. Additionally, some common pollen promoter elements were screened in the upstream of the *SLFs* coding sequence. For example, a promoter element of a

pollen-specific gene *LAT52*: *POLLEN1LELAT52* (5'-AGAAA-3') was identified and shown that it is a necessary promoter element for the expression of *LAT52* (Bate and Twell, [1998](#)). A total of 10 potential *POLLEN1LELAT52* elements were observed in the upstream of the *SLF3* coding sequence (Figure 5.5). The other two upstream of the *SLFs* coding sequences shared similar promoter element screening results (data are not shown). There are some other pollen elements such as *5659BOXLELAT5659* (5'-GAAWTTGTGA-3', W=A/T) (Twell *et al.*, [1991](#)) that were also screened in the upstream regions of the *SLFs* coding sequence, but no elements were identified (data are not shown). After confirming the specificity of primers in downloaded promoter sequences data was acceptable, the optimal annealing temperature of all primers was tested using My Taq DNA polymerase in gradient PCR (for the annealing temperatures see appendix 4). Gradient PCR reaction for each *SLF* promoter primer was performed in one run of PCR. The method of gradient PCR is the same as described in section 2.5.1, but different in having the annealing temperature gradient setting in one PCR. These confirmed-optimal annealing temperatures were used in providing *SLF* promoter fragments to build entry vectors.

5.2.4.2 Transformation of *SLF* promoter fragments into pCR8 for construction of entry vectors

In order to build entry vectors for studying *SLF* promoter regions, the pCR®8/GW/TOPO® cloning kit (for details see section 2.7.1) has been used in this project. The selected *SLF* promoter fragments (*SLF3*, *SLF13-1*, and *SLF14-1*) were amplified using genomic PCR with Phusion® high-fidelity DNA polymerase and genomic DNA of *Petunia inflata* S₆. The *SLF3*pro, *SLF13-1*pro and *SLF14-1*pro forward and reverse primers, were used in the genomic PCR (for primer details see appendix 4). In order to increase the concentration of PCR product, PCR reaction for each *SLF* promoter had 6 replications in one run of PCR. The PCR products were run in one gel for the purification of PCR later (Figure 5.6). The PCR program was set the same as described in section 2.5.2. The expected size of the PCR products was about 2000 bp. As illustrated in Figure 5.5, there are multiple bands shown on the agarose gel (Figure 5.6 a, b). For obtaining the expected PCR products to build the entry vector, all target bands of ~2000 bp were excised from agarose gels under UV light. The agarose gel of *SLF14-1*pro has no multiple bands (Figure 5.6 c). However, to unify the experimental operation in three of biological replications, the agarose gel of *SLF14-1*pro was processed in the same gel cutting operation. A gel extraction using the GenElute™ gel extraction kit was performed to purify the excised gel bands. Then, an extra PCR to add terminal A bases at both ends of the promoter fragments was carried out after gel purification. The purified PCR products were ligated into pCR®8/GW/TOPO® vector (refer to the methods), and transformed into One Shot™ TOP10 chemically competent *E.coli*. The transformed *E.coli* cells were applied onto the LB plates containing 100 µg/ml spectinomycin overnight. Once colonies grew in the plates, single colonies were picked and cultured overnight in LB medium containing the same concentration of spectinomycin until the culture was saturated. Then, colony PCR was performed to confirm the embedded promoter fragments in these colonies (data now shown). One colony of pEVSLF3 (colony name: pEVSLF3.3), one colony of pEVSLF13-1 (colony name: pEVSLF13-1.1) and four colonies of pEVSLF14-1 (colony name: pEVSLF14-1.1, pEVSLF14-1.2, pEVSLF14-1.3 and pEVSLF14-1.4) were confirmed as successfully cloned.

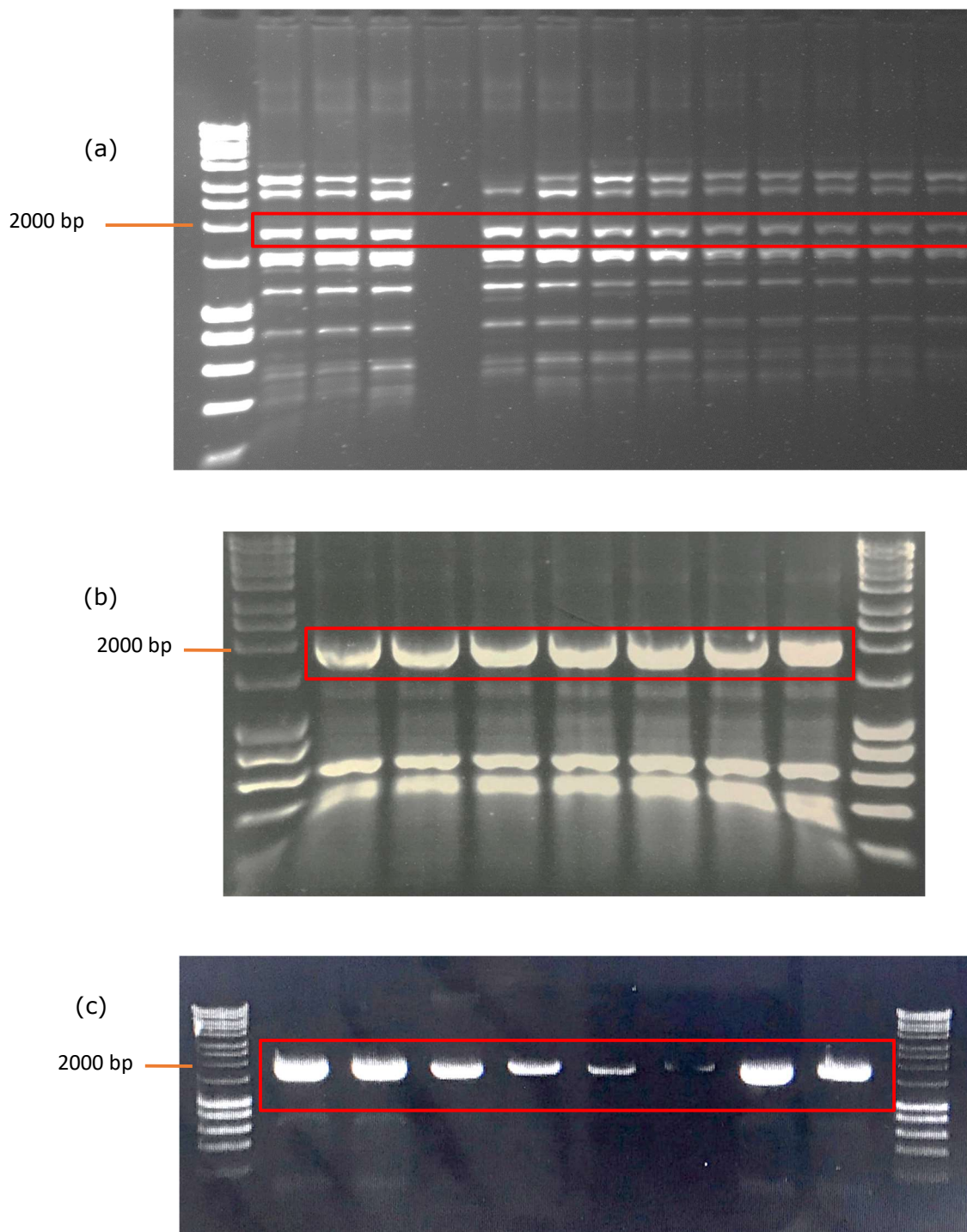


Figure 5.6 **Genomic PCR for *SLF3* (a), *SLF13-1* (b) and *SLF14-1* (c) promoters for cloning into entry vector.** The PCR primers used were SLF3pro forward and reverse primers (a), SLF13-1pro forward and reverse primers (b), SLF14-1pro forward and reverse primers (c). The gel was cut in the region of ~2000 bp (red boxes) for purification. The different lanes of each gel are the replications of each PCR reactions.

5.2.4.3 Sequencing to confirm the inserted promoter direction of pEVSLF3, pEVSLF13-1 and pEVSLF14-1

In order to confirm the PCR fragments of '*SLF* promoter' were successfully cloned into entry vector with the correct direction, a sequencing was planned after colony PCR. The GenElute™ plasmid miniprep kit was used for the plasmid DNA extraction of pEVSLF3.3, pEVSLF13-1.1, and all the pEVSLF14-1 colonies. All plasmid DNA samples were sent to sequencing. To confirm the sequencing results, the sequence of pCR®8/GW vector was found on the website of SnapGene ([https://snapgene.com/resources/plasmid_files/ta_and_gc_cloning_vectors/pCR_8_GW_TOPO_\(linearized\)/](https://snapgene.com/resources/plasmid_files/ta_and_gc_cloning_vectors/pCR_8_GW_TOPO_(linearized)/)) and entered into SnapGene software to create the schematic diagram of pCR®8/GW vector (see appendix 1). This schematics in appendix 1 shows the information on the site of the M13 forward and reverse primers in the vector.

Figure 5.7 shows the sketch map of the alignment between one part of pEVSLF3.3 (a)/ pEVSLF13-1.1 (b)/ pEVSLF14-1.4 (c) sequenced using M13 forward primer and the entry vector models made up by the sequences of pCR®8/GW and SLF3 promoter (a)/SLF13-1 promoter (b)/SLF14-1 promoter (c). The partial sequences of pEVSLF3.3, pEVSLF13-1.1 were continuous from attL1 site to *SLF* promoter front section, which indicates that the entry vector pEVSLF3.3, pEVSLF13-1.1 for promoter study have been considered as correctly transformed as the correct direction. Additionally, pEVSLF14-1.4 has been confirmed as correctly transformed for the same reason with pEVSLF3.3 and pEVSLF13-1.1. However, pEVSLF14-1.1, pEVSLF14-1.2, pEVSLF14-1.3 were found as transform-failed. Entry vector pEVSLF3.3, pEVSLF13-1.1 and pEVSLF14-1.4 were stored at -80°C for next stage experiments.

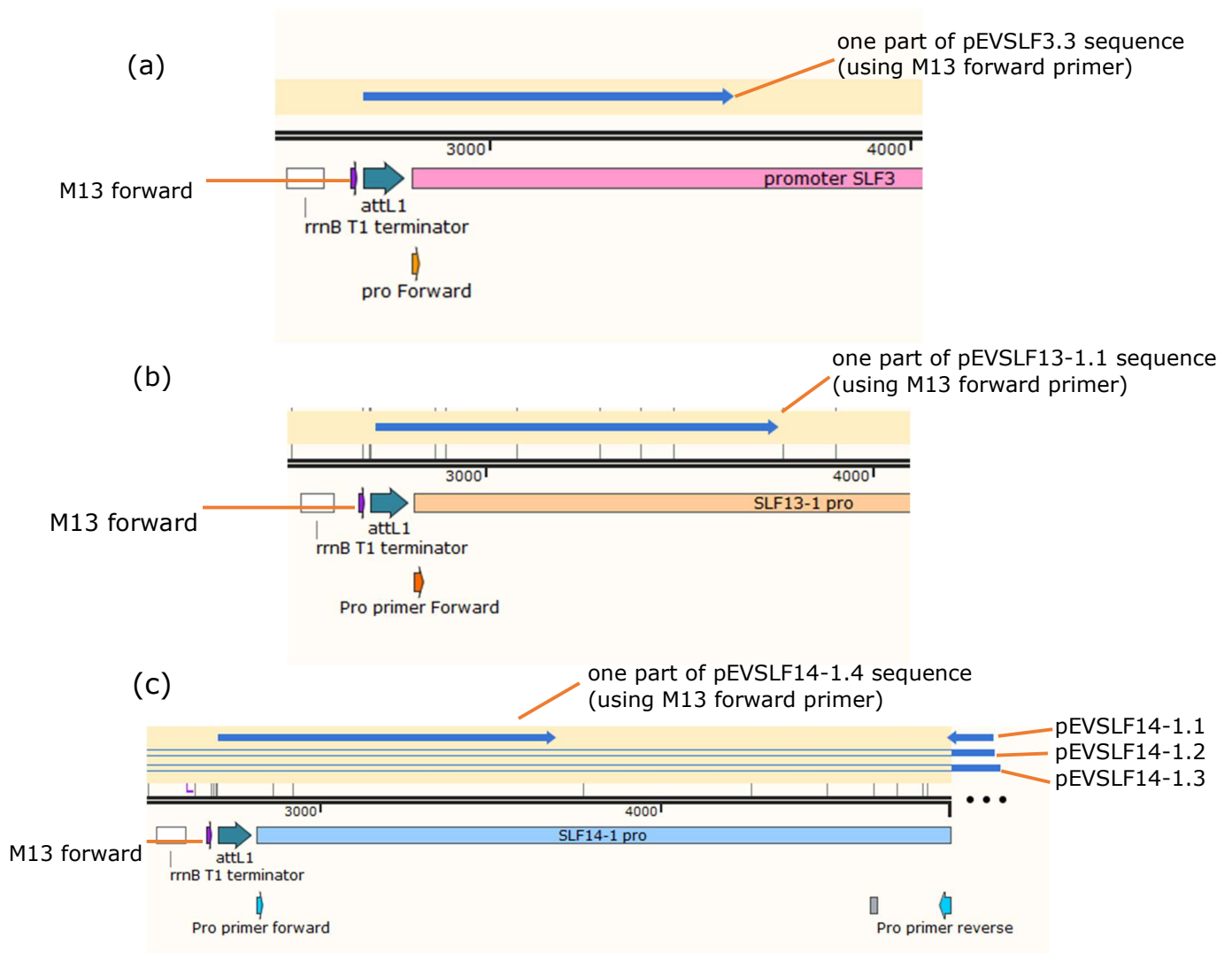


Figure 5.7 Sequence alignments of one part of pEVSLF3.3 (a)/ pEVSLF13-1.3 (b)/ pEVSLF14-1.1 to pEVSLF14-1.4 (c) sequence using M13 forward primer and pCR®8/GW + SLF3/ SLF13-1/ SLF14-1 promoter vector. Entry vector + promoter region was ligated in computer using SnapGene; pCR®8/GW map was download from Thermo Fisher Scientific website. (a) Blue arrow indicates sequencing results obtained for pEVSLF3.3. Purple arrow illustrates M13 forward site and pink area indicates *SLF3* promoter. (b) Blue arrow indicates sequencing results obtained for pEVSLF13-1.1. Orange arrow illustrates M13 forward site and yellow area indicates *SLF13-1* promoter. (c) Light blue arrow illustrates M13 forward site and light blue area indicates *SLF14-1* promoter.

5.2.5 Transformation of entry vector into pGWB3 to create *SLFpromoter:GUS* constructs

5.2.5.1 Primer design to select destination vector containing *SLFpromoter:GUS* construct

Before transforming *SLF* promoter fragments from the entry vectors into the destination vector *pGWB3*, the sequence for *pGWB3* was downloaded from GenBank (GenBank number: AB289766.1) and viewed in SnapGene. For creating the vector map containing the construct, the promoter fragments of *SLF3*, *SLF13-1* and *SLF14-1* were pasted into *pGWB3* vector map in SnapGene, and the diagrams of each destination vector used in this project were created (see appendix 1).

As the distance between M13 forward and reverse sites in *pGWB3* is over 4000 bp (see appendix 1), using M13 forward and reverse primers to select the destination vector that contains the *SLFpromoter:GUS* construct is unrealistic. In order to select destination vectors containing the target construct, 3 new pairs of primers (Test *SLF3*, Test *SLF13-1* and Test *SLF14-1*) were designed at the end of each *SLF* promoter and the beginning of the *GUS* fusion marker sequence (for the primer locations in destination vector map see appendix 1; for the primer details see appendix 4). It is worth pointing out that these 3 primer pairs were designed for testing the presence of the correct *SLFpromoter:GUS* construct and not for testing the full length of each promoter.

5.2.5.2 Gateway cloning of the entry vector into pGWB3 for building *SLFpromoter:GUS* constructs

After confirming that the inserted promoters in entry vectors have the correct orientation, *SLF3/SLF13-1/SLF14-1* promoter fragments were transformed from the entry vector pEVSLF3/ pEVSLF13-1/ pSLF14-1 into destination vector *pGWB3* by LR reaction. The LR reactions followed the protocol from the Gateway™ LR Clonase™ II Enzyme Mix kit (ThermoFisher Scientific). After the reactions, the *pGWB3-SLFpro* vectors were transformed into One Shot™ OmniMAX™ 2 T1 Phage-Resistant *E.coli*, applied on LB agar medium and incubated overnight. Single colonies were cultured in LB medium containing 100 µg/ml kanamycin and grown overnight until the culture was saturated. The correctly transformed colonies with intact *SLF* promoters were identified by colony PCR using promoter-specific test primers (for primer details see appendix 4). As the distance between T3F and T3R, T13F and T13R, T14F and T14R sites are 459 bp, 680 bp, and 585 bp respectively, these are the expected size of the PCR product (see appendix 1). After the selection of colony PCR, colonies DVSLF3.1 (destination vector SLF3.1), DVSLF13-1.1 to DV13-1.6, DV14-1.1 gave PCR products close to the expected size as shown in Figure 5.8, which indicated that these colonies have been correctly transformed.

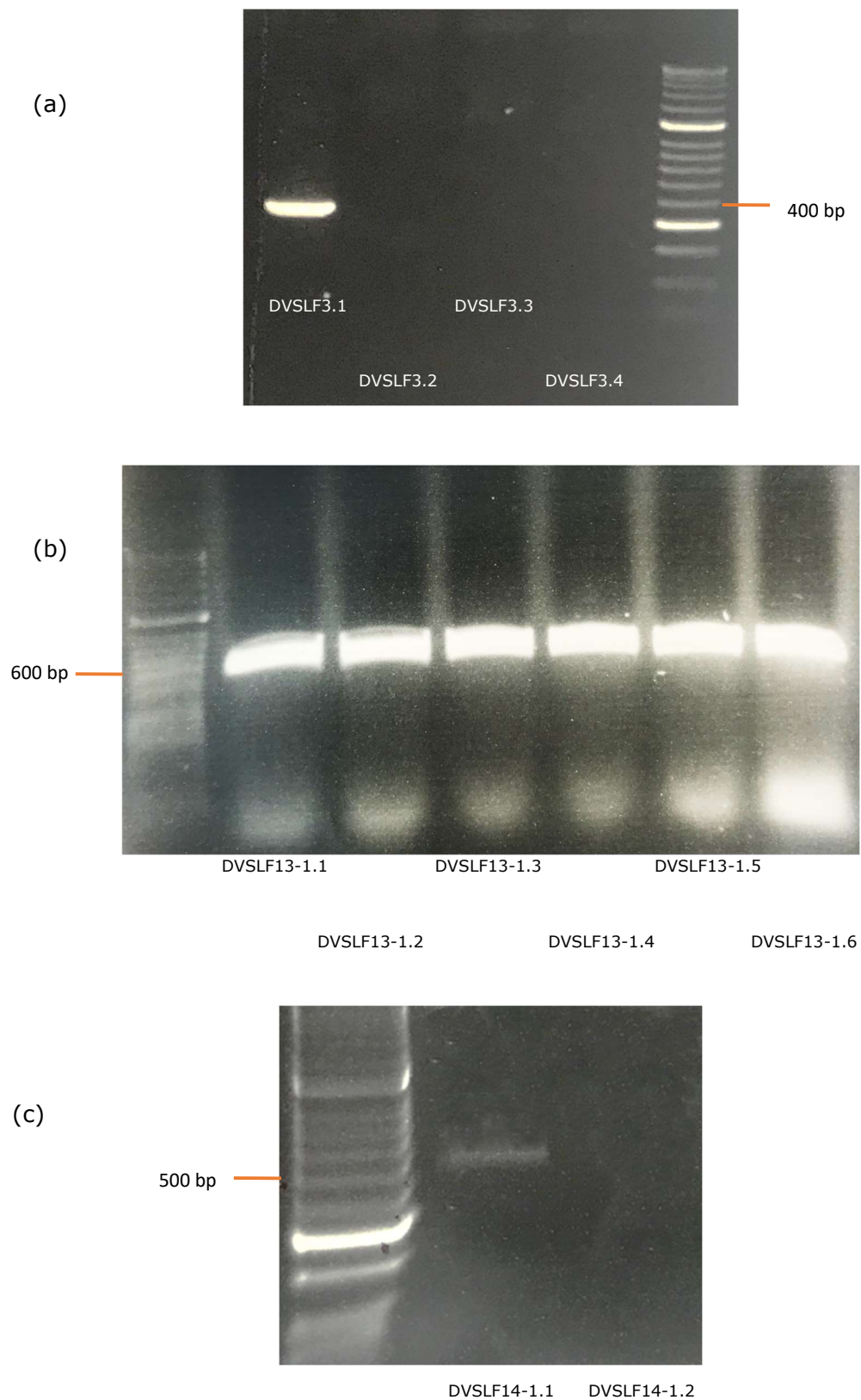


Figure 5.8 **Colony PCRs to test putative transformants containing *SLF promoter:GUS* constructs.**

5.2.5.3 Sequencing to confirm whether destination vector contains *SLFpromoter:GUS* constructs

Six colonies of DVSLF13-1 showed the same size of band in colony PCR, DVSLF13-1.3 was selected to be the representative of DVSLF13-1 for sequencing. The GenElute™ plasmid miniprep kit was used for the saturated cultures DVSLF3.1, DVSLF13-1.3, and DVSLF14-1.1. As can be seen from the schematics of DVSLF3/13-1/14-1 (see appendix 1), M13 Forward and Reverse primers cannot provide sufficient information about *SLFpro:GUS* construct in the destination vector. Thus, these plasmid DNA samples were sent for sequencing using the specific primers for confirming *SLFpro:GUS* construct T3F/T3R, T13F/T13R, T14F/T14R, respectively. Figure 5.9 shows the sketch maps of sequence alignments of one part of DVSLF3.1/ DVSLF13-1.3/ DVSLF14-1.1 sequences and pGWB3 vector map containing *SLFpro:GUS*.

All the selected destination vectors (DVSLF3.1, DVSLF13-1.3, DVSLF14-1.1) show the expected sequences and correct promoter-inserted direction. Thus, destination vectors DVSLF3.4, DVSLF13-1.3, and DVSLF14-1.1 have been considered as correctly transformed and were stored at -80°C for next stage experiments.

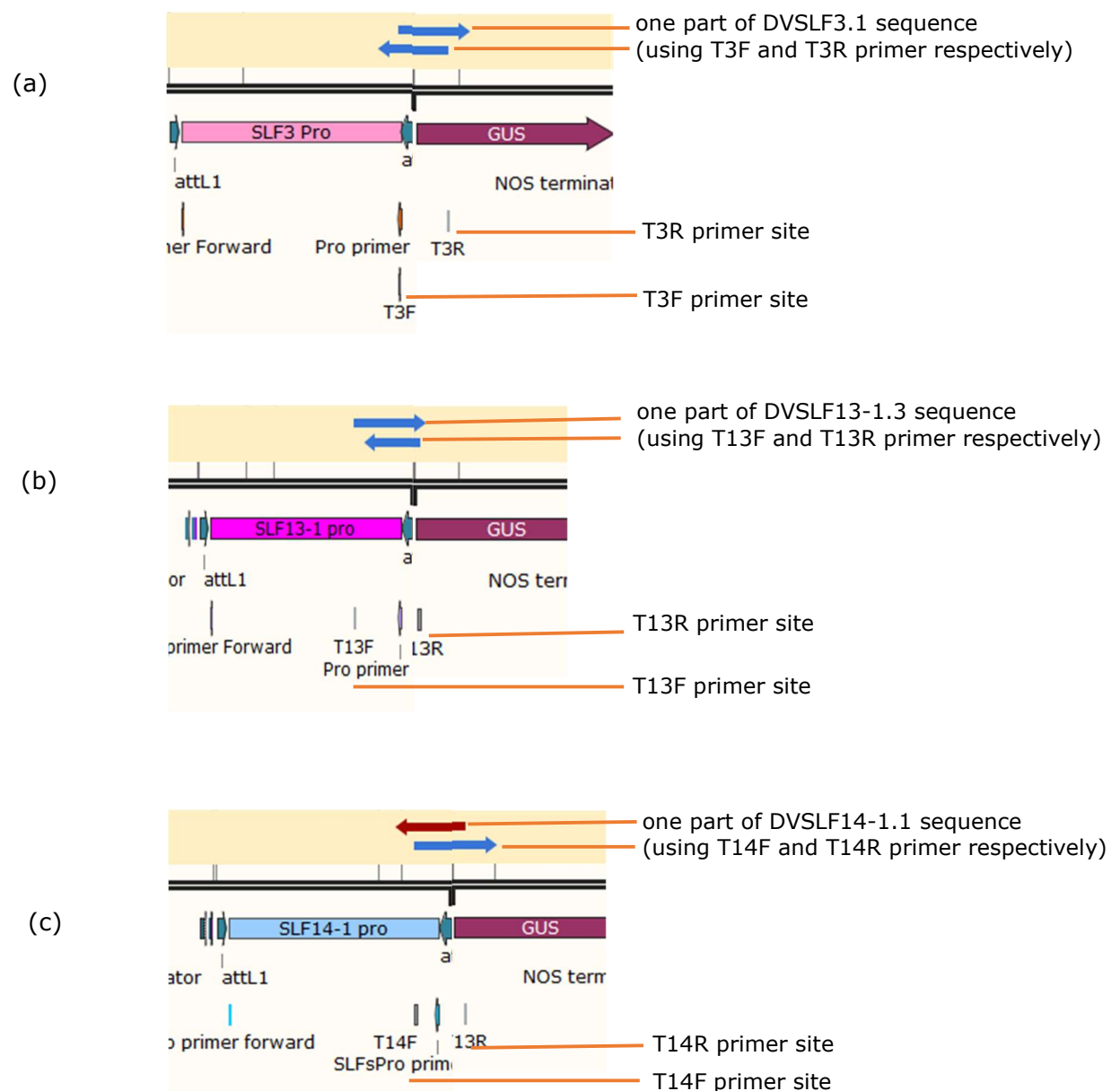


Figure 5.9 **Sequence alignments of one-part sequence of DVSLF3.1 (a)/DVSLF13-1.3 (b)/ DVSLF14-1.1 (c) and pGWB3 vector map containing SLF promoter.** (a) Blue arrow indicates sequencing results of DVSLF3.1. Light pink area illustrates *SLF3* promoter. (b) Blue arrow indicates sequencing results of *SLF13-1.3*. Pink area illustrates *SLF13-1* promoter. (c) Blue arrow indicates sequencing results of DVSLF14-1.1. Light blue area illustrates *SLF14-1* promoter.

5.3 Discussion

5.3.1 Comparison of *SLF* coding regions and *SLF* promoters in *Petunia inflata*

Some *SLFs* in *Petunia inflata* are highly homologous. Bombarely and co-workers ([2016](#)) indicated that 29 *SLFs* in *Petunia inflata* could be grouped as 6 clades via phylogenetic analysis. Clade 1: *SLF7*, *SLF14*, *SLF15*, *SLF16*, *SLF17*. Clade 2: *SLF3*, *SLF11*, *SLF13*. Clade 3: *SLF9*, *SLF10*. Clade 4: *SLF1*, *SLF2*. Clade 5: *SLF8*. Clade 6: *SLF4*, *SLF5*, *SLF6*. After applying the same grouping method with Bombarely and co-workers in the phylogenetic tree for *SLF* promoters, no obvious link was found when comparing these two phylogenetic trees of *SLF* coding regions and *SLF* promoters. The evolution of *SLF* promoters seems to be faster than the evolution of *SLF* coding regions. However, the *SLF* promoter analysis via phylogenetic tree still needs better methods to ameliorate. This is because the size of upstream of *SLFs* coding region was set as 2000 bp when obtaining the sequencing data from Sol Genomics Networks (the website where storing *Petunia* whole sequence data), but this 2000 bp is not precise promoter region, but just an estimated region to contain the *SLF* promoter. Therefore, this result is not so representative and only can be considered as a forecast result. However, if there are *SLF* promoter regions are identified in the future, the similar comparison will provide a strong result.

5.3.2 The comparison of *SLF* coding regions between *Petunia inflata* and *Petunia hybrida*

As mentioned above, Bombarely and co-workers ([2016](#)) identified 29 *SLFs* in *Petunia inflata*, and Kubo and co-workers ([2015](#)) identified 16 *SLFs* in *S_{om}*-haplotype of *Petunia hybrida*. Comparing the *SLFs* in *Petunia hybrida* and *Petunia inflata*, *Petunia inflata* have more *SLFs*. *Petunia inflata* is the male parent of *Petunia hybrida* (Bombarely et al., [2016](#)). Over 50 and 10 *S*-alleles were identified in *Petunia inflata* and *Petunia hybrida*, respectively, considering that *Petunia hybrida* only inherits some part of *S*-alleles from *Petunia inflata* (Robbins et al.,

[2000](#)). Thus, it is reasonable that the *SLFs* number of *Petunia hybrida* is lower than that of the *Petunia inflata*. In addition, the *SLFs* of *Petunia inflata* show high similarity in sequences, such as *SLF7-1* and *SLF7-2*, *SLF13-1* and *SLF13-2*. Bombarely and co-workers explained this situation as *SLF* gene duplication. Because of the near sequences between them (like *SLF7-1* and *SLF7-2*), it is better not to use both of them for promoter study.

5.3.3 Extending the sequence analysis of *SLF* coding regions to the *SLF* promoters

The second part of this project is to study the *SLFs* expression pattern with *GUS* fusion construct. There are 29 *SLFs* that were identified from *Petunia inflata* (Bombarely *et al.*, [2016](#)). However, it is arduous to study the promoter of all 29 *SLFs* in this project, thus a selection of optimal *SLF* candidates was chosen.

There are five reasons for choosing *SLF3*, *SLF13-1* and *SLF14-1* as the best candidates for studying *SLF* promoter location. Firstly, these three *SLFs* of *Petunia inflata* are closely linked with one of *SLFs* in *Petunia hybrida* (Figure 5.3). *SLF3-S6* is shown to have high homology with *SLF3-Som* with high reliability (99/100 credibility). *SLF13-1-S6* is shown to have high homology with *SLF13-Som* with middle-range reliability (69/100 credibility). *SLF14-1-S6* is shown to have high homology with *SLF14-Som* with high reliability (99/100 credibility). Secondly, these three *SLFs* of *Petunia inflata* have low homology when comparing their genomic DNA sequences. This ensures that representative results will be provided in the expression analysis of *SLF* promoter. Thirdly, there is no missing base at the upstream of these three *SLFs* in the *Petunia* genome database. About 2000 bp size of upstream base region was used in previous *Petunia* expression studies (Derolles and Gardner, [1988](#); Gargul *et al.*, [2017](#)). In order to maximize transformation of complete *SLF* promoters into transgenic plants, 2000 bp size of upstream base region was selected as the best size of promoter for transformation. However, after *SLF* promoters of *Petunia inflata* were downloaded from the database, some *SLF* promoter regions were found to have missing bases problems, such as *SLF12-1* upstream 274 bp, *SLF15-1* upstream 745 bp and *SLF16-1*

upstream 284 bp. Therefore, these *SLF* promoters are not ideal for promoter transformation.

Then, *SLF14* was grouped in clade 1, *SLF3* and *SLF13* were grouped in clade 2 by Bombarely and co-workers (2016). The result of *SLF14* will be compared with *SLF3* or *SLF13*, as the different clades. The results of *SLF3* will be able to be compared with *SLF13*, as the same clade. This will make results have more informative. Last, these three *SLFs* of *Petunia inflata* contain over 15 pollen-specific activators in the selected promoter regions. Bate and Twell (1998) indicated that in the region between the site -52 to -72 exists an important pollen-specific activator for *SLF* promoter in tomato (another important member in *Solanaceae*), which contains two expression control elements 'AGAAA' and 'TCCACCATA'. About 6 expression control elements were found in the alignments of these three *SLF* promoter regions. To summarise, *SLF3*, *SLF13-1* and *SLF14-1* were considered as best *SLFs* for transformation in this project.

5.3.4 The identification of the upstream of *SLF* coding region and the choice of DNA polymerase

At the beginning of building the constructs for this project, the DNA polymerase used in the PCR of promoter fragments was My Taq DNA polymerase (Bioline). The product advantages of this DNA polymerase are that it is inexpensive and the master mixture from this kit is easy to use when preparing the PCR master mix. Usually, this polymerase is used for the PCR for which base-fidelity is not a priority. At the beginning of the design of the promoter primer, the base-accuracy is sufficient for determining annealing temperature. High fidelity of DNA polymerase is not necessary at this stage of the experiment. When the annealing temperatures of primers were investigated (for details see appendix 4), amplifying promoter fragments were performed for cloning into the entry vector. The "normal" fidelity DNA polymerase was not suitable for obtaining high fidelity promoter fragments anymore. In the past experiments, we found that the PCR products using My Taq DNA polymerase gave missing bases, bases error leading to mutation. These experiment errors are not allowed to happen in this cloning, because the base

mutation of the 'real' *SLF* promoter is able to cause failure of GUS analysis. The size of the potential promoter region was set as the 2000 bp in this project, which is a 'safe' size for ensuring the *SLF* promoter is included and cloned into the vector. Actually, some prerequisite elements of promoter (cis-elements) were commonly identified in the promoter of tomato pollen gene (*LAT52*, *LAT56*, and *LAT59*) and eight rice pollen gene, such as *POLLEN1LELAT52* (5'-AGAAA-3') and *5659BOXLELAT5659* (5'-GAAWTTGTGA-3', W=A/T) (Bate and Twell, [1998](#); Twell *et al.*, [1991](#)). The deletion of them can cause the function-loss of pollen genes (Nguyen *et al.*, [2016](#)). After aligning these two cis-elements with the upstream regions of start codon of *SLF3*, *SLF13-1* and *SLF14-1*, and also according to the suggestion from the previous promoter study (Rockman and Wray, [2002](#)), the size about 2000 bp was considered as the safest one to contain the real *SLF* promoter. Therefore, any bases in this region should be guaranteed is amplified correctly. However, it should be noted that even that there is a lot of evidence showing that the 2000 bp is the safest size to contain the promoter like described before, it still unable to be guaranteed that the ~2000 bp upstream region of *SLF*'s start codon truly contains the promoter of SLFs until it is identified.

In order to achieve the certainty described above at the maximum extent, using high fidelity DNA polymerase is the best choice. The PCR for obtaining promoter fragments in this project used Phusion® high-fidelity DNA polymerase (Thermo Scientific), which can drastically reduce the mutation probability of nucleotide sequence in PCR amplification (Frey and Suppmann, [1995](#)). However, there is still exists a small possibility for mutation. For example, there is an experiment in early stages of this project that showed this possibility. In this experiment, one mutation was found in the sequencing results of the genomic DNA of *S_o-RNase* and *S_b-RNase* using high-fidelity polymerase (data not shown). The sequencing result of this mutation shows that some bases were missing or wrong during amplifying. This example indicates that a sequencing to confirm amplified DNA fragment bases sequence after cloning is necessary. Fortunately, the sequencing results showed the three entry vectors pEVSLF3.3, pEVSLF13-1.1 and pEVSLF14-1.4 we built did not contain mutations (sequencing results are not shown in this thesis). This gives the first assurance for the next LR reaction and cloning.

5.3.5 The multiple bands (after using high-fidelity DNA polymerase)

There were multiple bands in the electrophoresis gel picture when amplifying the promoter region for building entry vector, while single band is expected. The PCR was run in once when using each '*SLF* promoter' primers, 6 replications were used to increase the PCR product amount. The reaction conditions are qualified, because 6 replications gave the same results. For this reason gel purification was used to obtain target fragments. The root cause is that the '*SLF* promoter' primers are not specific. In fact, the reasons for using unspecific primers are explained as follows: after the designing of primer, the My Taq DNA polymerase was used to test primer annealing temperature, and the only single band appears on the gel pic. However, when the DNA polymerase was replaced with high-fidelity DNA polymerase, the accuracy of PCR was greatly improved, and different size of DNA fragments were amplified. This could explain why multiple bands have appeared in the gel when using high-fidelity DNA polymerase. On the other side, another reason that could cause the appearance of multiple bands is that the designed primers were unable to check primer specificity. As an example, when whole genome was sequenced, Arabidopsis could use Primer 3 (Primer design website) with its genome database to check primers specificity after designing them (Han and Kim, [2006](#); Ye *et al.*, [2012](#)). But the *Petunia* primers could not be manipulated in the same way, because the genome of *Petunia* is stored at another website (Sol Genomics Website, <https://solgenomics.net/tools/blast/>) which could not align any sequences below 50 bp, rendering the specificity of primers not possible to be checked. Therefore, the only way was to purify the PCR products and then send the entry vectors for sequencing for ensuring the cloned promoter sequence was the correct one. In fact, the sequencing of entry vector gave us a satisfactory result because there is no mutation that was found in the entry vectors (data not shown).

Furthermore, a series of failures appeared when connecting *SLF* promoter fragments with pCR™8/GW/TOPO® vector. According to the sequences and troubleshooting in the user guidebook of the entry vector used in this project, two terminal A bases at 3' and 5' ends were necessary for PCR products to connect

with the entry vector (Invitrogen Company, [2012](#)). Usually, the PCR product ends will add several terminal A bases if using My Taq DNA polymerase (Brownstein *et al.*, [1996](#)), but if using high-fidelity DNA polymerase, the two ends of PCR product have no extra terminal A bases which is “blunt” (Chester and Marshak, [1993](#)). Therefore, an extra PCR to add some terminal A bases was necessary after gel purification. If only terminal A bases are added, the promoter DNA fragment using high-fidelity DNA polymerase can normally attach entry vector following gateway methods.

CHAPTER 6 TRANSFORMATION OF *PETUNIA*

HYBRIDA WITH *SLF3PROMOTER:GUS* CONSTRUCT

6.1 Introduction

Usually as a transgenic model plant, *Arabidopsis* is the best choice in plant research. But because *Arabidopsis* do not belong to S-RNase based SI, thus, it is not the ideal plant material choice for transformation of this project. In fact, *Arabidopsis* was used as transformation material to study the expression location of pollen specific gene *LAT52* of tomato, which had successfully obtained transgenic plants in the past (Eady *et al.*, [1994](#)). *Brassica* belongs to sporophytic self-incompatibility, and also have been used as a popular selection of transformation material (Nasrallah *et al.*, [1991](#); Ping *et al.*, [2018](#); Scandola and Samuel, [2019](#); Lian *et al.*, [2019](#)). However, the determining factors of *Brassica* SI is different with S-RNase base SI system we are studying. In the S-RNase based SI system, tobacco, potato and tomato were used as transformation material in the past as well, which led to generation of useful results (*Nicotiana*: Moore *et al.*, [1990](#); Suzuki *et al.*, [1996](#); Chung *et al.*, [1999](#); Lin *et al.*, [2019 b](#); *Solanum tuberosum*: Sheerman and Bevan, [1988](#); *Solanum lycopersicum*: Li *et al.*, [2015](#); Zhao *et al.*, [2015](#); Ye *et al.*, [2018](#)). However, *Petunia* has several advantages, including: shorter life period, lower requirements of the growth environment, easy to transform, and the callus of *Petunia* is easier to grow shoots. Thus, *Petunia* is the best choice of transformation for this project when compared to other species.

Petunia has many cultivars to choose from as transformation hosts, *Petunia hybrida* cv 'Mitchell' (also called W115 line, referred to as *Petunia* W115 here) was one of the most popular choices for transformation (Ausubel *et al.*, [1980](#)). This cultivar originally comes from hybrid of *Petunia axillaris* (♀) and the hybrid (♂) of *Petunia axillaris* and *Petunia hybrida* cv *Rose of Heaven* (Verdonk *et al.*, [2005](#)). *Petunia* W115 is more outstanding in the growth condition and tissue culture when compared to other *Petunia* species or cultivars. Also, an important merit of W115 is its sensitive to the antibiotic kanamycin. The explant of wild type W115 is unable to grow in medium containing kanamycin (Yue *et al.*, [2017](#); Wu *et al.*, [2019](#); Lin

et al., [2019 a](#)), which helps selection of the transgenic plants before moving to the soil. In addition, *Petunia* W115 is self-compatible, rendering it is easy to obtain seeds via self-pollination.

The transformation host used in this project was *Petunia* W115, the method of transformation was following that of previous Ph.D. students at University of Nottingham (Moore, [2006](#); Sharef, [2017](#)). The details of transformation (see section 2.9.1) used promoter *LAT52* of tomato connected with *GUS* fusion to see where *LAT52* is expressing (Moore, [2006](#)). Sharef ([2017](#)) used *LAT52* promoter to connect *Antirrhinum SLF* constructs, and *SLFs* function was studied in their transgenic plants and progenies. But it is worth noting that the W115 was not used in the project of Sharef. Instead, the S_3S_v heterozygotes were used (a self-incompatible *Petunia* cultivar containing S_3 -allele and S_v -allele). Their experiments were successful and received expected results, this indicates their methods and the choice of *Petunia* W115 are feasible.

In [2016](#), Bombarely and his co-workers first published the draft genome sequences of *Petunia*. This made using *Petunia* own promoter to build *GUS* fusion possible, despite of some DNA sequences of *SLF* promoter still not being fully sequenced (some *SLFs* were not selected as promoter analysis because some sequences of potential promoter region are not been sequenced yet). Therefore, the experiments described below used the destination vector *PGWB3-proSLF3* mentioned before, and utilized agrobacterium to transfer promoter *SLF3:GUS* fusion into *Petunia* W115 and to observe the expression pattern of *SLF3* after GUS analysis. The expression pattern of *SLF3* can be used to detect the initial expression position of *SLFs* in *Petunia*.

6.2 Results

6.2.1 Seed germination, transformation and tissue culture

Petunia hybrida self-compatible line 'Michelle' W115 (W115 is the Free University of Amsterdam seed stock code) was chosen as the transformation plant material in this project, and the seed stocks were obtained from Professor Ronald Koes (Free University of Amsterdam). The timing of sowing seeds was one month before the transformation, because the leaves of one-month young plants are the best material for transformation (McCormick, [1991](#)). The seeds of *Petunia hybrida* W115 were sown in the soil and growth environment was same as for growing S_oS_o and S_bS_b homozygotes of *Petunia hybrida*, described at section 2.1.

After confirming that the destination vector contains *SLF3promoter:GUS* construct by sequencing, the destination vector was transferred from *E.coli* to *Agrobacterium* cells via electroporation. A series of transformation of *Petunia hybrida* leaf disks using *Agrobacterium*, selecting transgenic plants using Kanamycin were performed to transfer the construct into W115. At last, 13 transgenic plants and several wild types of W115 were transferred into the greenhouse for quick growth.

6.2.2 Genetic PCR to test transgenic plants candidates

The transgenic plants were grown until they were approximately 20 cm high. In order to check that these kanamycin-resistant transgenic plants contained the construct, a GUS PCR using the T3F and T3R primers designed before (for primer sequences see appendix 4) and the DNA extracted from each transgenic plant (for methods see section 3.5) was performed. The DNA samples were extracted from 13 transgenic plants named as *proSLF3:GUS-A1*, *proSLF3:GUS-A2*, *proSLF3:GUS-A3*, *proSLF3:GUS-A4*, *proSLF3:GUS-A5*, *proSLF3:GUS-A6*, *proSLF3:GUS-A7*, *proSLF3:GUS-A8*, *proSLF3:GUS-A9*, *proSLF3:GUS-A10*, *proSLF3:GUS-A11*, *proSLF3:GUS-A12*, *proSLF3:GUS-A13*, and one W115 wild type. The PCR results showed that all transgenic plants contained the target construct, except *proSLF3:GUS-A10* and wild type control, which gave no PCR product (data now

shown). *ProSLF3:GUS-A10* was abandoned and will not be considered as the experimental material for future experiments.

6.2.3 Phenotype comparisons of transgenic plants and wild type

Following two months of growth, most of the transgenic plants started flowering. The flowers from five different growth stages of the transgenic plants were collected to observe the phenotype (Figure 6.1 A). The flowers from the same growth stages of wild type were also collected for comparison (Figure 6.1 B). In fact, the five growth stages of flowers there were no significant differences in the outside phenotype of sepals and petals between transgenic plant and wild type.

Because the external phenotypes of sepals and petals have no conspicuous differences, mature stamens and pistil in flowers from the fifth stage were collected and compared. There were no phenotypic differences in stamens and pistils between transgenic plants (Figure 6.2 A) and wild type control (Figure 6.2 B), except the different length caused by individual differences or age of the flower.

Most of the transgenic plants and wild type control have no differences in phenotype. Only one slight phenotypic difference is the petals of transgenic *proSLF3:GUS-A6*, *proSLF3:GUS-A8* (Figure 6.3 F and H) was undulating when comparing with wild type control (Figure 6.3 A), and the flowers edges of some transgenic plants were not smooth, which shows a waviness (Figure 6.3 B, C, E, F and H).



Figure 6.1 **Different growth stage of flowers from transformant *proSLF3:GUS-A6* (A) and a W115 control plant (B).**



Figure 6.2 **Contrast of stamens and pistils from the opened flowers of transgenic *proSLF3:GUS-A6* (A) and a wild type control (B).**

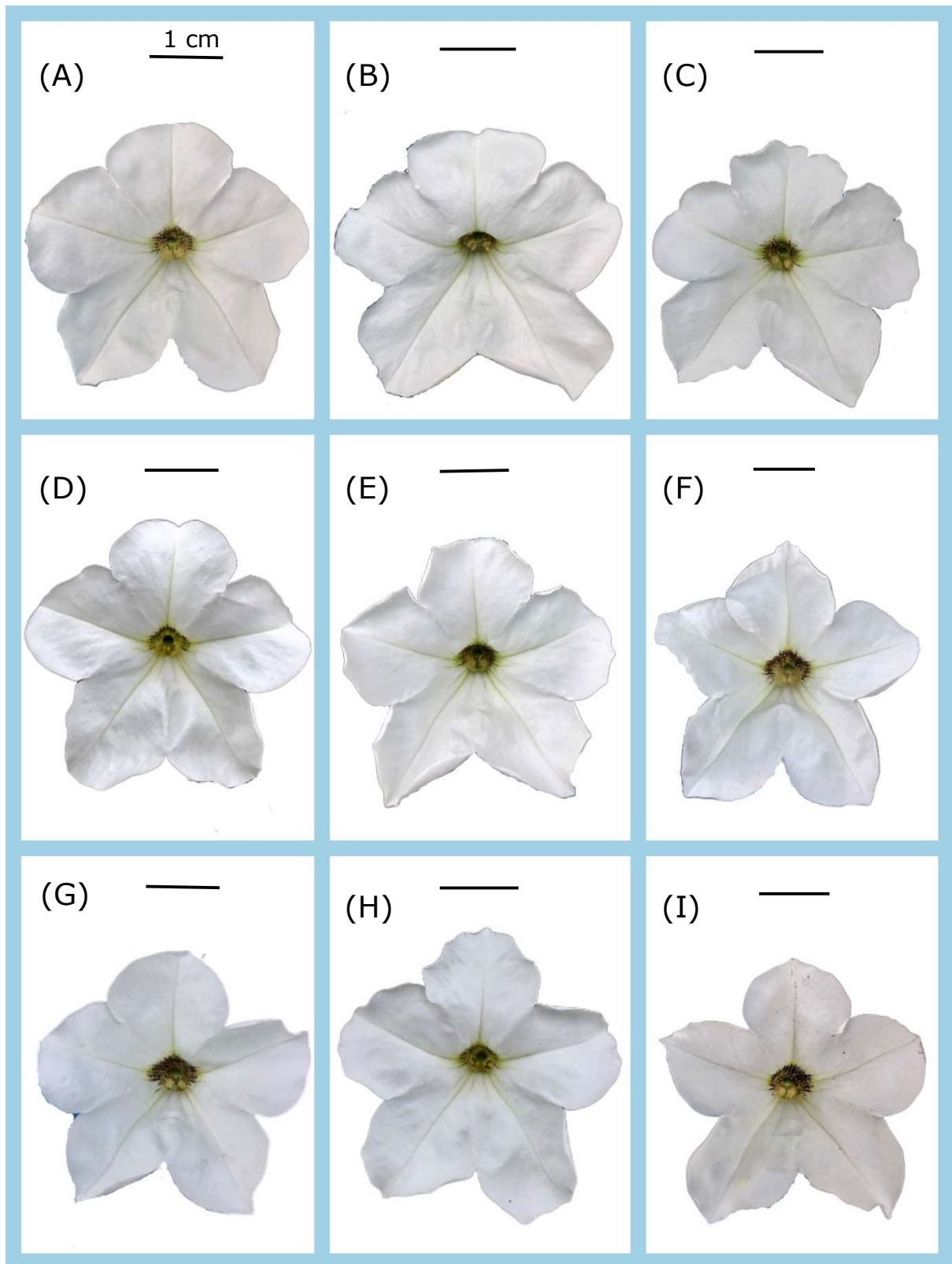


Figure 6.3 **Flowers collected from wild type control (A) and transgenic plants (B-I).**

6.2.4 GUS analysis of transgenic plants and wild type.

Kao and co-workers ([2004](#)) indicated that *SLFs* of *Petunia* is expressed in pollen and anther. It was also suggested that *SLFs* is not expressed in ovary, style, and leaves of *Petunia* (Hua *et al.*, [2007](#)). For confirming this result using GUS analysis, pollens, stamens and pistils were collected from the four stages of transgenic plants and wild type control mentioned before. These collected tissues were processed following the methods described at section 2.10 to soak in the staining buffer that added X-Gluc. The blue colour on Figure 6.4 indicates the expression location of *SLF3* in stamens after the X-Gluc and β -glucuronidase interacted for 20 hours. There is no blue precipitation shown in the pistil of transgenic plant and wild type (data not shown). The GUS analysis indicates that there is no visible blue be shown in the stamens of stage 1 and stage 2 (Figure 6.4 A and B), but the stamens at stage 3 and stage 4 show obvious blue colour in anther, and all blue colour concentrates in the anther (Figure 6.4 C and D). According to the previous definition, stage 4 of flowers is the length not exceeding 40 mm but still not open. In order to observe what happened after pollen release from anther, a new stage of pollen was collected and named stage 5. Stage 5 of pollen was collected from the opening flower, which pollen exposed but still attaches on anther. Figure 6.4 E shows stage 5 of stamen, and there is no observed blue appearing in the stamen, but the tip of anther still has a little blue remaining. It is possible to infer that *SLF* expresses in pollen because the stamen of wild type has no blue colour (Figure 6.5). Figure 6.6 shows the dyed pollens obtained from the fifth stage observed under a microscope. The blue precipitations can only be observed in the centre of pollen, but not in pollen wall and generative cell.

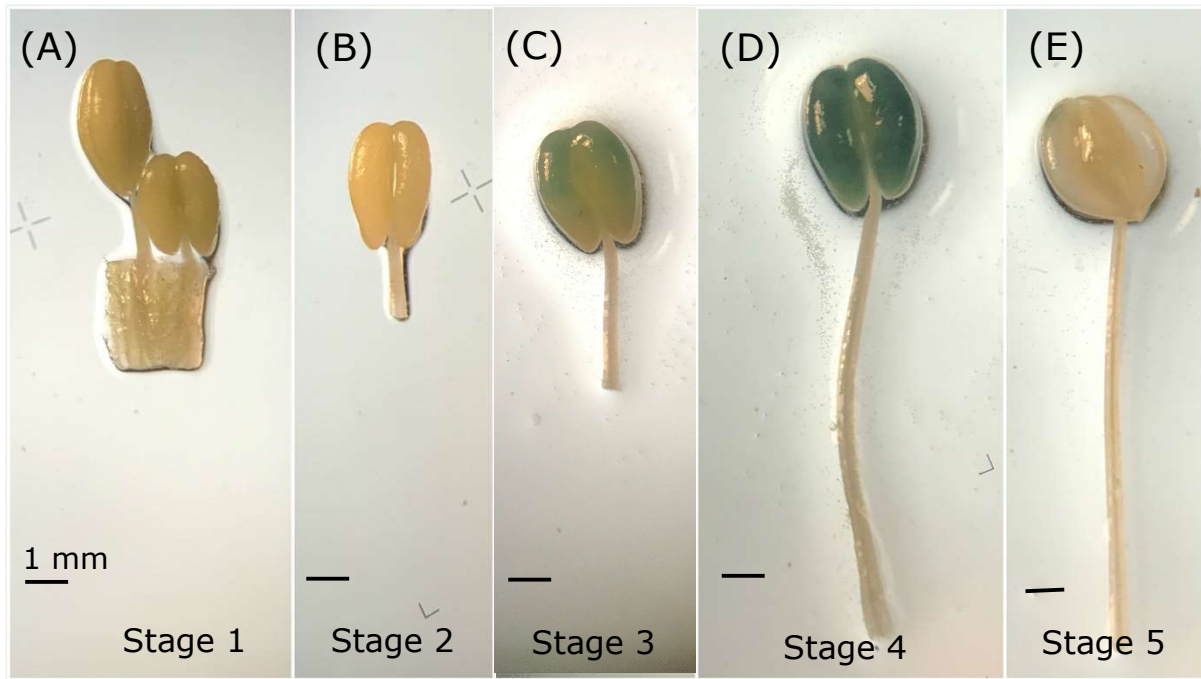


Figure 6.4 **GUS analysis of transgenic immature anthers at the different growth stages collected from *proSLF3:GUS-A7*.** (A) — (D) Stage1 — Stage4 of transgenic immature anthers; (E) Transgenic mature anther after releasing most of the pollen.

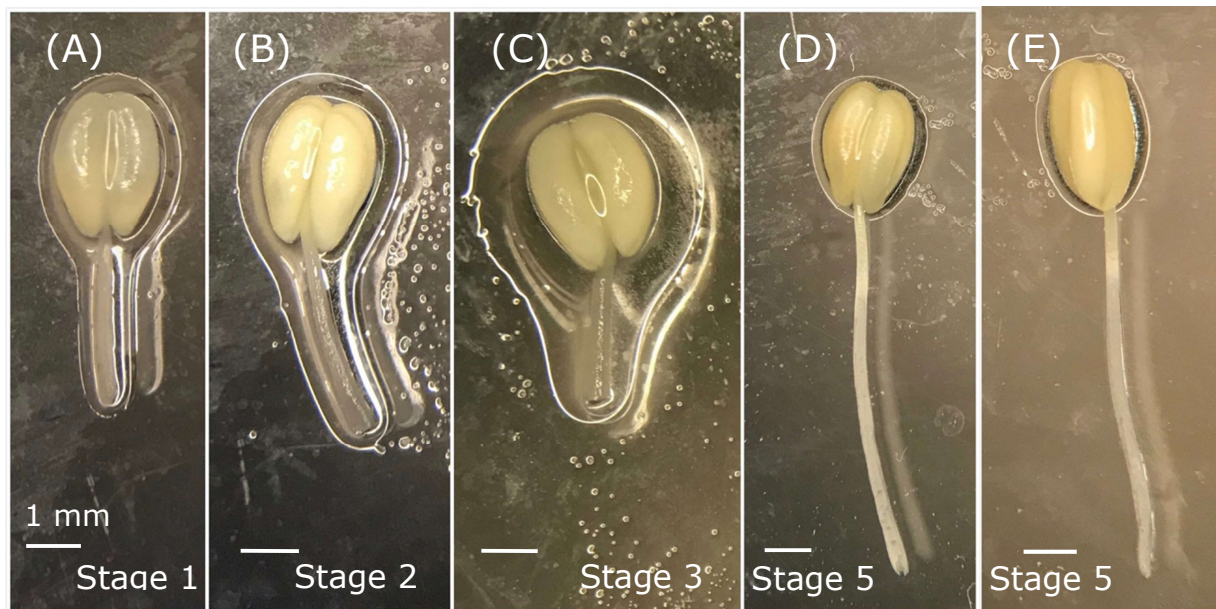


Figure 6.5 **GUS analysis of wild type immature anthers at the different growth stages.** (A) — (E) Stage 1— Stage 5 of wild type immature anthers.

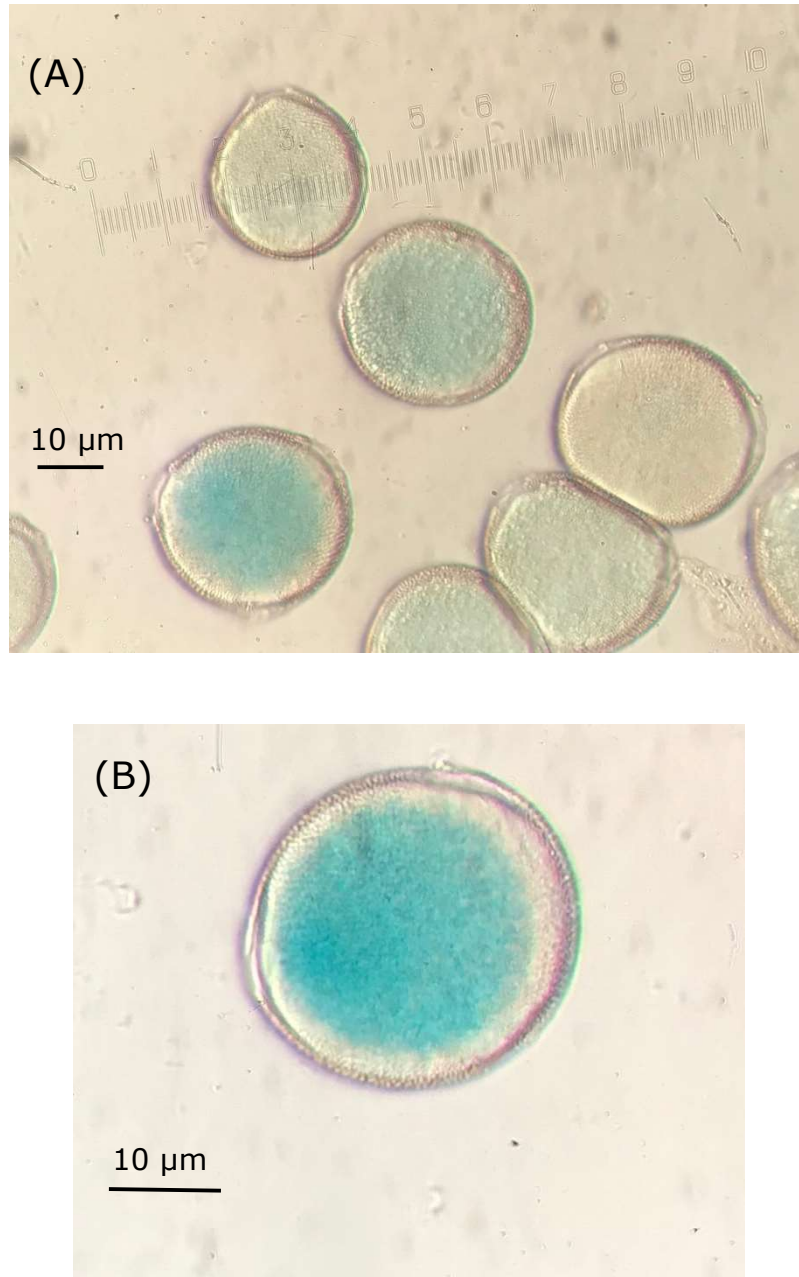


Figure 6.6 **GUS assay of *SLF3* promoter transformant pollen from *proSLF3:GUS-A6*.** (A) Pollens collect from stage 5 of *proSLF3:GUS-A6*. (B) Independent pollen observed under 100X microscopy.

6.3 Discussion

6.3.1 *Petunia inflata* and *Petunia hybrida* were used in GUS analysis

In this project, *Petunia inflata* was used as the provider of *SLF* promoter region, and *Petunia hybrida* was used as a plant material to express *SLF*promoter:*GUS* reporter. In fact, this is not the first time to use one species and transform it into another species. As mentioned before, Eady and his co-workers ([1994](#)) transferred the promoter *LAT52* (tomato pollen gene) into Tobacco and *Arabidopsis*. Moore ([2006](#)) also did similar experiment but transferred *LAT52* into *Petunia hybrida*. Both of them have shown us the feasibility of expressing one species' gene in another species. In this project, *Petunia inflata* and *Petunia hybrida* have a very close relationship, as *Petunia inflata* is one parent of *Petunia hybrida*. Therefore, it is feasible to choose these two species as plant materials. In addition, the genome of *Petunia hybrida* has not yet been published, using the *SLF* promoter of *Petunia hybrida* is not feasible for now. The genome of *Petunia inflata* is the best way to achieve the aims of this project. For the choice of transgenic plant material, *Petunia hybrida* "W115" is a popular choice and has a good record as transgenic host (Ausubel *et al.*, [1980](#); Moore, [2006](#); Sharef, [2017](#)). Transferring the construct built using genomic DNA of *Petunia inflata* into *Petunia hybrida* can minimize the problems caused by species differences. This is also the first time in research using *SLF* promoter genomic DNA of *Petunia* to transform in *Petunia*, which makes a contribution in the *SLF* expression of *Petunia*.

6.3.2 Phenotype comparisons between transgenic plants and wild type

The undulating petals were observed in the flowers of two transgenic plants *proSLF3:GUS*-A6, *proSLF3:GUS*-A8 (Figure 6.3 F and H). Other transgenic plants show no significant differences when compared with wild type. This phenomenon was also observed by a previous Ph.D. Moore ([2006](#)). When she used *LAT52* promoter connected with *GUS* fusion and transformed in W115, two of six transgenic plants were found to have visibly overlapping petals in flowers. However, the overlapping petals in flowers were shown to be controlled by a group of genes named *MADS-box genes* (Van Der Krol and Chua, [1993](#); Rijpkema *et al.*,

[2006](#)) rather than the *SLF* genes. Thus, the observed undulating petals can be considered as the mutations in Moore's study and this study. Moreover, this phenotype might be a heritable epigenetic one.

6.3.3 GUS assay used in this project

As a gene that can be stably inherited to the offspring, *GUS* has always been a good choice to show the position of gene expression (Gallagher, [2012](#)). It is also widely recognized that the GUS assay has the advantages of stability, easy storage, and convenient observation (De Ruijter *et al.*, [2003](#); Gallagher, [2012](#)). In this project, the method of GUS assay is learned from Wenzhe Yin who is a postdoctoral fellow in our laboratory.

During GUS assay, the 90% acetone as fixative was added to coagulate proteins in pollen cells and maintain the original structure of cells. After the fixative was washed using distilled water and the ice-cold staining buffer without X-Gluc, X-Gluc staining buffer was added into Eppendorf tubes to flood whole tissue under a vacuum environment. The tissues were then incubated in darkness at 37 °C incubator overnight. After the blue precipitates were detectable in the tissues, the ethanol was used to remove staining buffer. The X-Gluc staining buffer contains 1 M of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and Na_2HPO_4 solution, 0.5 M of EDTA, 0.5 M of $\text{K}_3[\text{Fe}(\text{CN})_6]$ solution, 1 M of $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$ solution, 1 M of X-Gluc and 10% Triton X-100. $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, Na_2HPO_4 , $\text{K}_3[\text{Fe}(\text{CN})_6]$ and $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$ solutions that provide necessary reaction environment for X-Gluc and GUS protein. EDTA works as preventing the influence of ferrous ion on GUS. Triton X-100 was used to make sure X-Gluc enters the cell and interacts with GUS protein. Also, the reason why infiltrating tissues in X-Gluc staining buffer under a vacuum environment is that the vacuum environment will push out the air around the tissue, so the staining buffer can better interact with the tissues in each place. Therefore, based on the above reaction environment, the GUS in tissues was able to hydrolyse X-Gluc to the blue precipitate observed in Figure 6.6.

6.3.4 The reason why blue precipitation appeared in pollen

The pollen GUS staining results demonstrated that only some pollens show blue precipitations in their cells, but other pollens have no blue precipitation or only a light blue colour appears (Figure 6.6). This phenomenon was potentially caused by several possible factors. Firstly, some pollens might not be fully dyed at the staining stage. The size of *Petunia* pollen is small which usually only have 0.1 mm (Figure 6.6 A and B). This is possible that staining buffer thus could not dye every pollen grain well, or these pollens need more time to dye because of low expression of *SLF3*. Secondly, *SLF3* is not expressed sufficiently, or it is possible that some β -glucuronidase had degraded at the time when anther was collected. In short, some pollens show very little or no blue when comparing to other pollens. It is also confirmed by qRT-PCR described at section 4.2.2 that the expression of *SLF3* is under temporal control in pollen. The expression period of *SLF* increased significantly from stage 3, reached a peak at stage 4, and decreased slightly at stage 5. This is consistent with the previously described trend of change explained via qRT-PCR. In addition, it is also possible that the different expression level of *SLF3* in pollen is related to T-RNAs in the pollen.

6.3.5 The expression location of *SLF3* in anther and pollen

Stamen at stage 5 has no obvious blue precipitate (Figure 6.4 E) and this is most likely caused by the deficit of pollen in anther at this stage. Moreover, this also indicates that *SLF* is fully expressed in pollen rather than the anther and filament of the stamen. The expression of *SLF* in stamen might follow this pathway: the pollen starts maturing from stage 1 to stage 3, then it will be mature enough at stage 4 and *SLF* expression reaches the highest peak when pollen matured. When flowers ready for opening, anthers also open to release pollen. When pollens are all spread onto pistil by insects, anther will be empty. This can explain why figure 6.4 E has no easily visible blue dye on stamen. (Goldberg *et al.*, [1993](#))

From Figure 6.6, it appears *SLF3* might be expressed in the vegetative nucleus, vacuole or cytoplasm, rather than pollen exine or intine and generative cell. Nevertheless, it is hard to determine if *SLF* is expressed at tube nucleus, vacuole or tube cell, because the restriction of GUS reporter gene and also the pollen has not been germinated. But for now, based on current experimental results, *SLF* is more likely to be expressed in the vegetative nucleus and stored in the cytoplasm.

6.3.6 The analysis of *SLFs* expression position

Since the experimental problems happened when transforming the destination vector from *E.coli* to *Agrobacterium*, the other two constructs (*SLF13-1pro:GUS* and *SLF14-1pro:GUS*) have failed to transform into W115. There was only one construct (*SLF3pro:GUS*) successfully transformed, which caused the deficiency of a comparison of independent transgenic plants and made the GUS analysis result not sufficiently scientifically reliable. However, whether the results in this project are reliable can be assessed by comparison with the results from previous related pollen specific gene-GUS transgenic studies. *LAT52* is a good choice to achieve comparison, as it is one of gametophyte specific genes. *LAT52* (*Late Anther Tomato 52*) is one of the group genes identified in the tomato pollen, which is expressed after pollen mitosis I (Twell *et al.*, [1989](#)). The function of *LAT52* was considered as the necessary factor to mediate the pollen hydration and the growth of pollen tube, which specifically interacts with a pollen-specific receptor kinases LePRK2 (Muschietti *et al.*, [1994](#); Tang *et al.*, [2002](#)). Eady and co-workers ([1994](#)) used the promoters of pollen specific gene of tobacco and tomato to build a *promoter:GUS* fusion and express in *Arabidopsis*, and their results are similar with our results that found blue colour on pollen. Moore ([2006](#)) used the promoter of *LAT52*, fused with the *GUS* reporter gene and transferred into *Petunia* W115. She also observed blue pollen similar to Figure 6.6. Therefore, as a pollen gene, *SLF3* expresses in pollen is reliable.

For the *SLF* expression study of *Petunia*, Hua and his co-workers ([2007](#)) collected the RNA from the anthers from different stages and other tissues like ovary, style, and leaves of *Petunia inflata*. RT-PCR was performed to test the expression position of *SLFs*. They found that the *SLFs* have a strong expression on mature

pollen grains, pollen tubes and later stage of anthers, but no expression was found in the ovary, style, and leaf. The results of RNA gel blot analysis also supported it (Kao *et al.*, [2004](#)). For now, both of *SLF* expression analysis from Kao *et al.* ([2004](#)) and Hua *et al.* ([2007](#)) is confirmed in this project by GUS analysis. We also found strong expression in the mature pollen and mature anther, and no expression was found in the style of transgenic plants (data now shown). Also, the *SLFs* of *Antirrhinum* were observed that expressed in the pollen grains, microspores, and tapetum by detecting hybridization signals (Lai *et al.*, [2002](#)). However, except pollen grains that were found to have blue colour, the microspores and tapetum of anthers did not have *SLF* expression in this project.

The qRT-PCR results of *SLFs* indicate that the expression of *SLFs* starts from stage 1, and shows a significant increase at stage 3, then reaches the strongest expression at stage 4. This trend is the same as shown in the GUS analysis. In GUS analysis, it is visible that the stamens of stage 3 and stage 4 showed blue. Also, the blue in stage 3 is obviously weaker than in stage 4. Both results of qRT-PCR and GUS analysis of *SLFs* support each other.

All discussions above indicate that although our *SLF* transgenic plants lack of a comparison of independent transgenic plants, it can be proved that the results of this experiment are reliable. Thus, based on the above analysis, if more *SLF-GUS* analyses are performed, the expected results will most probably be similar to the results in this project and previous studies. To summarize, gene expression results using the *SLF* promoter of *Petunia* showed that *SLF* was expressed only in pollen and not in other tissues of the stamen. More specifically, *SLF3* might be expressed on the vegetative nucleus, vacuole or cytoplasm.

6.3.7 The biological contribution of this work in *SLFs* of *Petunia*

As the project is the first GUS analysis using the *SLF* promoter of *Petunia* and transferring it into *Petunia* itself, the most direct contribution a confirmation that the *SLF* of *Petunia* expresses in the pollen. In order to investigate the interaction between *SLFs* and S-RNase, the previous studies from Kubo and co-workers ([2010](#); [2015](#)) used the *LAT52* promoter instead of real *SLF* promoter to construct

structure (*LAT52promoter:SLF:GFP*). As described before, they successfully detected the interaction location of SLFs and S-RNase is in the pollen tube. It was proved that *SLFs* of *Petunia* is expressed in pollen, rather than ovary, style, and leaves (Kao *et al.*, [2004](#); Hua *et al.*, [2007](#)). The GUS analysis of *SLF* expression in this project suggests that the *SLFs* is fully expressed in pollen before SLFs interact with S-RNase in pollen tube.

As mentioned above, the process in S-RNase based SI happened in the pollen tube. Before the pollen tube began to grow, as shown in the Figure 6.4, the male determinant *SLF* began to express in the pollen of the stamens. The expression of *SLF* was basically not observed in the stamen in the first stage (no blue precipitate can be seen), then the clear blue precipitate appears in the stamens in the third and fourth stage. These results conform to the expression level changes detected by qRT-PCR in Figure 4.3.1. This is because the expression of *SLF* shows an exponential growth during the pollen development. After the pollen is fully mature, the anthers split to release the pollen (the fourth stage). Then, the *SLF* begins to enter the pistil as the pollen tube grows, and will recognize and interact with the S-RNase from the pistil to complete the SI process. The results from this project provided a strong evidence that *SLF* is expressed in pollen, not in pistil.

Incidentally, this work also demonstrates that it is possible to use '*SLF3* promoter' to perform experiments similar to those done by Kubo and co-workers ([2010](#); [2015](#)). The '*SLF3* promoter' has been proven to work well in transformation experiment, and the 2000 bp upstream of the *SLFs* coding sequence contains the real *SLF3* promoter. It worth to try using '*SLF3* promoter' in this project to drive the expression of other *SLF* genes. For example, building a construct *SLF3promoter:SLF:GFP* to detect the interaction of all SLFs and S-RNases in the pollen tube will be interesting and worth to try. Also, there are no publications to show *SLF* expression using '*SLF* promoter' of *Petunia* and GUS analysis, the GUS analysis is new and can be published.

CHAPTER 7 GENERAL DISCUSSION, CONCLUSION AND FUTURE WORK

Self-incompatibility (SI) is a mechanism of some plants to protect the genetic diversity, by rejecting the interaction between self-pollen and pistil to produce seeds (Sims and Robbins, [2009](#); Li *et al.*, [2017](#)). It has been shown that SI has a different molecular response pathway in different plants (Takayama and Isogai, [2005](#)), such as SI of some common crops and garden plants in the families *Brassicaceae*, *Solanaceae* and *Rosaceae* (Takayama and Isogai, [2005](#)).

Petunia, one genus of the *Solanaceae*, was chosen as the plant material used in this project. The SI process of *Petunia* is considered to be controlled by two important factors: the male determinant (*SLFs*) and female determinant (*S-RNase*), which all located in one S-locus (Kubo *et al.*, [2015](#)). During pollination and fertilization, once the self-pollen falls on the stigma, the SCF^{SLF} complex starts to identify *S-RNase* in the pollen tube. Because the *SLFs* of SCF^{SLF} complex from self-pollen comes from the same S-allele with *S-RNase*, the SCF^{SLF} complex is unable to target the *S-RNase*. Therefore, the *S-RNase* remains in the pollen tube and is able to degrade RNA to stop the growth of pollen tube. If the cross-pollen falls on the stigma, the *SLFs* from cross-pollen recognize the *S-RNase* that come from different S-alleles. The SCF^{SLF} complex will then target the *S-RNases* (ubiquitination). The *S-RNases* will be degraded, and the RNA will remain, allowing the pollen tube to continue to grow (Franklin-Tong and Franklin, [2003](#)).

However, SI does not always occur in self-incompatible plants, sometimes the breakdown of SI is observed leading for plants to lose the ability to reject self-pollen (Tsukamoto *et al.*, [2003](#)). In fact, there are many potential reasons to cause this phenomenon. Sometimes it is because the determinants controlling SI have mutated and lost functions. The mutations may not only happen in the male and female determinants but also in other factors of SI process, for example, the non-S-specific factor called *HT-B* that is expressed together with *S-RNases*. Mutation of *HT-B* influences the SI function of pistils to render the plant self-compatible

(McClure *et al.*, [1999](#); O'Brien *et al.*, 2002; Kondo *et al.*, [2002](#); McClure, [2006](#)).

The duplication of part of the genome or of whole genome will often lead to competitive interaction and the collapse of the SI process (Golz *et al.*, [2000](#); Golz *et al.*, [2001](#); Sims and Robbins, [2009](#)). In addition, some self-incompatible plants have been discovered that still can grow limited seeds after self-pollination in nature, and this is called pseudo-self-incompatibility (PSC) (Flaschenreim and Ascher, [1979](#), Robbins *et al.*, [2000](#)). Unfortunately, there is still no reasonable explanation at the molecular level about PSC.

7.1 The comparison of *S-RNases* and *SLFs* in S_oS_o and S_bS_b homozygotes

7.1.1 The comparison of *S-RNases* in S_oS_o and S_bS_b homozygotes

The S_oS_o homozygote (referred to as S_oS_o) of *Petunia hybrida* is self-compatible and the S_bS_b homozygote (referred to as S_bS_b) is self-incompatible. Despite these two homozygous coming from the same parents (*Petunia axillaris* ♀ X *Petunia integrifolia* ♂), they are two varieties that contain two different *S*-allele. Interestingly, 20 amino acids at the N-terminal sequence of S_o -allele and S_b -allele were found to be the same (Robbins *et al.*, [2000](#)). In addition, the pollen of S_bS_b was found to be cross-incompatible with the pistil of self-compatible S_oS_o and was unable to produce seeds (Wright, [2004](#)). As described above, usually, the self-incompatibility is there to prevent the pollination by self-pollen and preserve the pollination by non-self-pollen. S_o -allele and S_b -allele are different *S*-allele detect by different group, but the pollen of S_oS_o homozygote seems to have the same SI ability with S_bS_b to make the pistil of S_bS_b failing to fertilize.

Since SI in petunia was found to be mediated by the *SLFs* and *S-RNases* present in *S*-allele, a comparative study of *S-RNases* and *SLFs* between these two homozygotes has been planned, with the aim of finding an explanation for the phenotypic difference between them. Wright ([2004](#)) and Czyzyk ([2010](#)) first chose

to study the *S-RNases* from these two homozygotes. They first sequenced the RT-PCR products of *S_o-RNase* and *S_b-RNase*, the RNA sequences of *S_o-RNase* and *S_b-RNase* were found to be identical. In the subsequent comparison of expression levels of *S_o-RNase* and *S_b-RNase* using qRT-PCR (Czyzyk, [2010](#)), it was found that *S_o-RNase* and *S_b-RNase* have similar temporal expression levels in immature flower and mature flower.

In this project, to obtain more accurate and publishable DNA sequences, *S_o-RNase* and *S_b-RNase* were amplified using the genomic DNA samples of *S_oS_o* and *S_bS_b* homozygotes, which were obtained from Rafal Czyzyk and cloned into pCR™ 2.1-TOPO® vector and sequenced. The sequencing data confirmed that the genomic DNA of *S_o-RNase* and *S_b-RNase* are 100% identical (data not shown). From the data described above, it is clear that the *S-RNases* of these two homozygotes have identical DNA and RNA sequences, and also have indistinguishable expression levels at different growth stages of flowers. Therefore, the *S-RNases* of *S_oS_o* and *S_bS_b* do not provide an explanation for the phenotypic difference. It has been previously reported that part or whole *S*-gene duplications also cause the failure of SI. Therefore, Czyzyk ([2010](#)) speculated whether it is possible that the *S*-gene duplication is the key reason causing the difference between these two homozygotes. He checked whether the *S-RNases* are duplicated in *S_oS_o* and *S_bS_b* by Southern blot analysis. However, the results of Southern blot analysis demonstrated that no duplications have been found in the *S-RNases* when comparing the two homozygotes (Czyzyk, [2010](#)).

7.1.2 The comparison of *SLFs* in *S_oS_o* and *S_bS_b* homozygotes

Since the female determinant of SI, the *S-RNase*, shows no differences in *S_oS_o* and *S_bS_b*, the direction of research was turned to the male determinants, the *SLFs*. In this project, based on the 16 *SLF* sequences of the *S_o*-haplotype published by Kubo and his colleagues ([2015](#)), 16 pairs of *SLF* primers were designed and the RT-PCR was performed using the primers and RNA extracted from the stamens of *S_oS_o* and *S_bS_b*. The RT-PCR results revealed that all 16 *SLFs* were expressed in these two homozygotes and also the *SLFs* in these two homozygotes are indistinguishable in terms of the size of the RT-PCR products. In fact, the first

round of RT-PCR results showed interesting findings: *SLF8* is not expressed in S_bS_b . This may be the key to explain the differences between S_oS_o and S_bS_b . If *SLF8* indeed does not exist in S_bS_b , the *SLF8* in S_oS_o is possible to be a new *SLF* or a duplication of other *SLFs*. According to the definition of competitive interaction, a duplication of SI related gene could cause the breakdown of SI. This is why the S-RNase in S_oS_o and S_bS_b share an identical sequence and expression level change, but they have different self-compatibility. However, after redesigning a pairs of new *SLF8* primers, and using selected ideal S_oS_o and S_bS_b plants as plant material, the second round of RT-PCR proved that *SLF8* does exist in both S_oS_o and S_bS_b . Therefore, this surmise has been discarded.

For comprehending more accurately the expression levels of the 16 *SLFs* at different stages of flowering, four different stages of flower development were studied, similar to the previous qRT-PCR tests of *S-RNases* (Czyzyk, [2010](#)). Stamens were collected to extract RNA, synthesize cDNA, and then a series of qRT-PCR were carried out. According to the qRT-PCR results, the 16 *SLFs* can be divided into three categories, as described below. The first group is the *SLFs* with an expression level in S_oS_o that is nearly twice as high as in S_bS_b (*SLF2*, *SLF3*, *SLF4*, *SLF6*, *SLF7*, *SLF13*, *SLF15*, *SLF16* and *SLF17*). The second group is the *SLFs* with an expression level in S_oS_o that is significantly higher than in S_bS_b but does not reach twice as high level (*SLF1*, *SLF5*, *SLF8*, *SLF10*, *SLF11* and *SLF14*). The third group is those for which the *SLF* expression level of S_oS_o and S_bS_b are similar, but they have different growth trends compared with other *SLFs* (*SLF12*). As a special group, *SLF12* is shown to reach the peak of expression level at the third stage comparing with others. However, the time that the pollen lands on the stigma is after the fourth stage. Premature expression of *SLF* is unnecessary for SI. Therefore, it is considered that *SLF12* may not be necessary for SI in both S_oS_o and S_bS_b .

In a complementary study, Czyzyk also checked duplication of *SLF1* from these two homozygotes (only *SLF1* was identified in S_oS_o homozygote at that time), with no duplications of *SLF1* found when comparing them. However, the qRT-PCR results of *SLF1* indicated that the expression level of *SLF1* in S_oS_o is significantly higher than S_bS_b (this study), which suggests that higher *SLF1* expression is not

related to gene duplication or mutations (competitive interaction). Certainly, if more Southern blots prove that other *SLFs* have not been duplicated in S_oS_o compared to S_bS_b , then this inference will have more shreds of evidences to prove it. With the partial sequencing and aligning the 16 *SLFs*, the results suggest the *SLFs* sequences are identical in S_o and S_b haplotypes. Due to there are no complete 100% sequence alignments to prove they are 100% identical. According to the fact that these partial sequencing regions of *SLFs* are located in the FBA domain (F-box associated domain) of *SLFs*, which is the main region for *SLFs* to distinguish each other, different *SLFs* coming from different *S*-alleles should have differences in this region. The amino acid sequence comparison studies from Hua *et al.* (2007), and the neighbor-joining phylogenetic trees of *SLFs* from Kubo *et al.* (2015) also provide some evidence to support this inference. Therefore, it can be speculated that the DNA sequences of *SLFs* of S_oS_o and S_bS_b are identical. If all of 16 *SLFs* including promoter sequences can be sequenced in future research, this inference will be more certain.

From the data of *SLF* gene comparison presented, the DNA sequences can be determined to be identical between the two haplotypes. In addition, the result of *SLF1* Southern blot analysis indicated that there was no evidence to suggest the SI breakdown of S_oS_o is caused by the duplication of *SLFs* (Czyzyk, 2010). Therefore, the main difference between these two homozygotes is that some of the *SLFs* have significant differences in the expression levels at some stages. Based on these results, it is speculated that the differences between S_oS_o and S_bS_b homozygotes are caused by the different expression level of *SLFs* at stage 4. Considering the expression level of *SLFs* in S_bS_b to be standard, some *SLFs* being over-expressed in S_oS_o . Alternatively some *SLFs* are less-expressed in S_bS_b if the *SLFs* expression level in S_oS_o is considered to be standard. This may be regulated by some potential trans-acting factors.

If we consider the *SLFs* over-expression in S_oS_o as the explanation for the phenotypic difference. When self-pollination of S_oS_o occurs, overexpressed *SLFs* formed more SCF^{SLF} complexes. Excessive SCF^{SLF} complexes lead the *S_o-RNases* to be all degraded or remained not enough to degrade growth-related RNA, which caused the collapse of SI response in S_oS_o . This model can also explain why the

pollen from S_bS_b will be cross-incompatible with the pistil of S_oS_o . This is because the molecular level of SLFs from pollen of S_bS_b is lower by about two-fold than the S_o -RNases, assuming a fixed amount of S_o -RNases. Therefore, the SLFs and SCF^{SLF} complexes from S_bS_b are unable to identify and degrade all S_o -RNases in the pollen tube. This causes some S_o -RNases to remain active and degrade the RNA, rendering pollen tube stop growing. Figure 7.1 shows a schematic diagram of the hypothesis based on the data presented. This diagram illustrates the different molecular levels of SLFs and S-RNases between S_oS_o and S_bS_b . The relative molecular ratio of SLFs in S_oS_o and S_bS_b is assumed to be 2:1, and the relative molecular ratio of S-RNases in S_oS_o and S_bS_b is presumed to be 1:1.

Also, this model can be understood with another way. The expression of *SLFs* in S_oS_o is at normal level, but the expression of *SLFs* in S_bS_b is insufficient. This may be due to the regulation of a trans-acting factor from pollen, or that the *SLF* promoters of S_bS_b are mutated. Insufficient SLF leads the S_bS_b to have not enough SCF^{SLF} complex be formed if assume S_b -RNases stay in a fixed amount. There are no enough SCF^{SLF} complexes to break down all S_b -RNases. Therefore, there are some residual S_b -RNases are remained to degrade the pollen tube growth related RNA, and lead the pollen tube of S_bS_b stop to grow that shows self-incompatible. Furthermore, it can be speculated that the expression of *SLFs* in S_bS_b may be unstable due to the unstable regulation of the potential trans-acting factors. The low expression of *SLFs* in some flowers causes S_b -RNases to remain in the pollen tubes, the pollen tubes therefore stop growing and no seeds are produced. In contrast, the normal expression of *SLFs* in some S_bS_b flowers results in a low level of S_b -RNases remaining in the pollen tubes, so the growth of pollen tubes is not affected and the seeds are generated. This explanation is consistent with the observation in S_bS_b screening, which should be all self-incompatible at this round of self-pollination. However, it has been found that pseudo-self-incompatibility had appeared in some S_bS_b (the proportion of SI can be increased by screening).

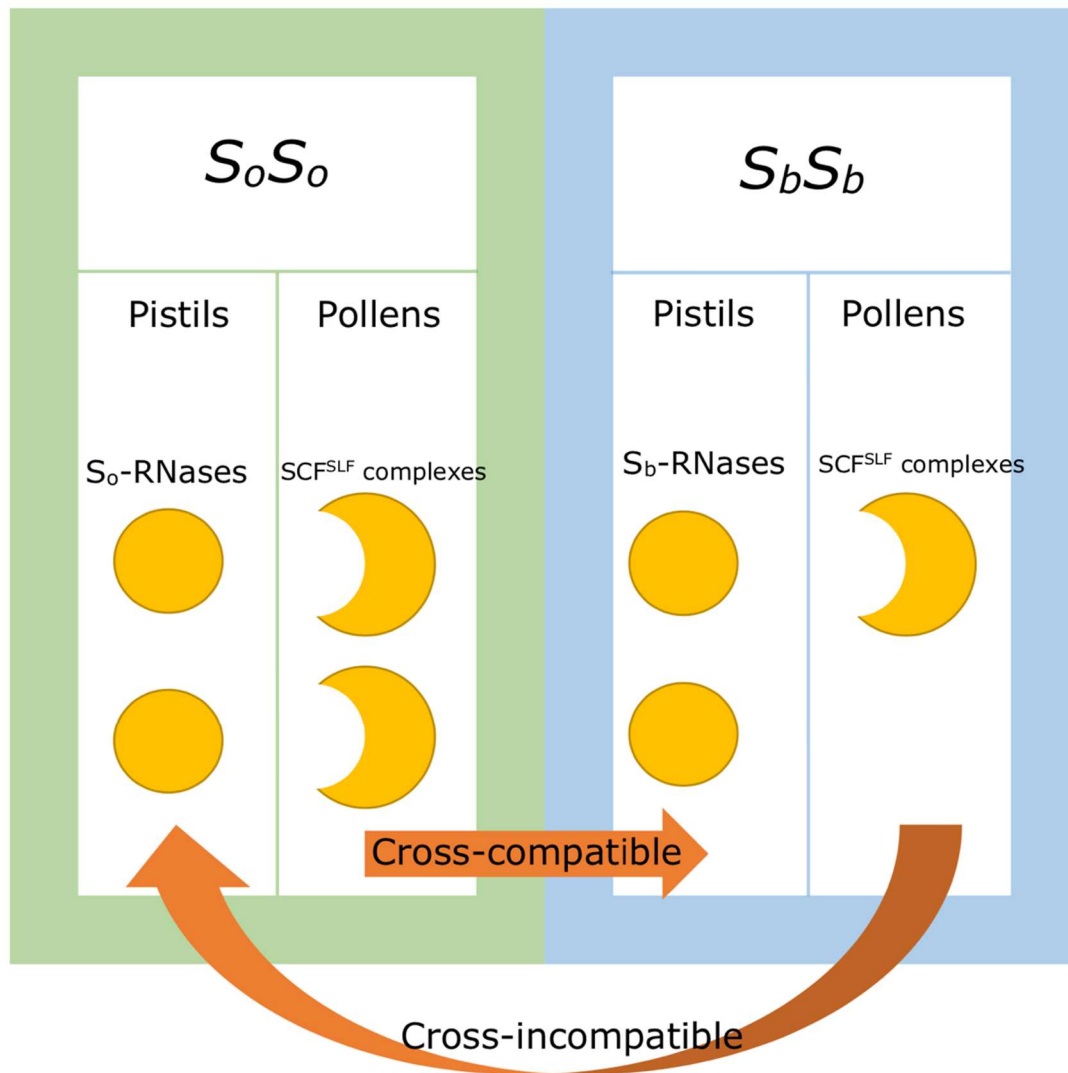


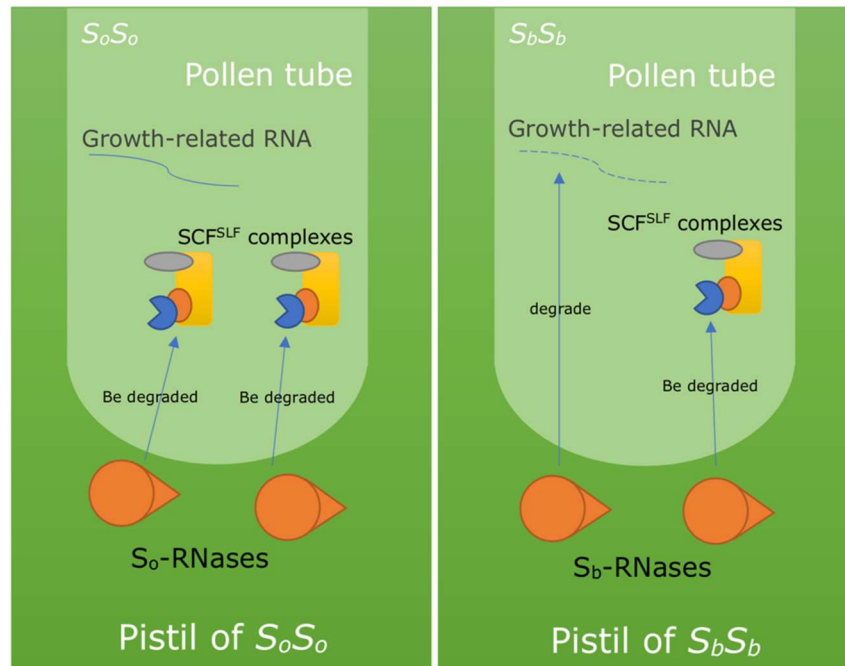
Figure 7.1 **A hypothesis to explain the self and cross incompatibility differences between S_oS_o and S_bS_b homozygotes of *Petunia hybrida*.** The circle represents S-RNases, and the half-moon represents SCF^{SLF} complex. The molecular ratio of SCF^{SLF} complex to S_o -RNase in S_oS_o is twice that of S_bS_b .

Figure 7.2 demonstrates another schematic diagram showing what occurs in the pollen tube at the molecular level when self-pollination and cross-pollination takes place. When self-pollination occurs, the molecular ratio of SLFs and S_o -RNases in S_oS_o is 1:1, therefore no S_o -RNases remain to degrade RNA. However, the molecular ratio of SLFs and S_b -RNases in S_bS_b is 1:2, thus, there is residual S_b -RNases to degrade RNA and stop pollen tube growing. When cross-pollinating

these two homozygotes, the molecular ratio of SCF^{SLF} complexes from S_oS_o pollen and the S_b -RNases can be considered is 1:1, the ubiquitination leads to no S_b -RNases to remain and degrade RNA. When cross-pollination occurs with S_oS_o , the amount of S_o -RNase molecules is double that of the SCF^{SLF} complexes, this causes residual S_o -RNases that can degrade RNA and stop pollen tube to grow.

According to previous theories from Kubo's group (Kubo *et al.*, [2015](#); Fujii *et al.*, [2016](#)), the SC plants might have evolved from SI plants. The results from this work support this and supply more information about this evolution theory. Fujii and co-workers ([2016](#)) indicated that after generating a new *SLF* in SI plants for mutation or some reason, SI plants will evolve into SC plants to ensure more progenies have been produced. In this project, as one of SC plants, S_oS_o has a higher expression of *SLFs* except *SLF12* when comparing with S_bS_b . In addition, S_oS_o and S_bS_b have identical *SLFs/S-RNases* sequence and similar expressions of *S-RNases*. Therefore, it is possible that higher expression of *SLFs* in S_oS_o is the result of evolution from S_bS_b . This evolution results in a SI plant (S_bS_b) becoming a SC plant (S_oS_o). This finding has not been published yet, thus, the results of this project will help us to understand more about the theory of evolution between SI plants and SC plants.

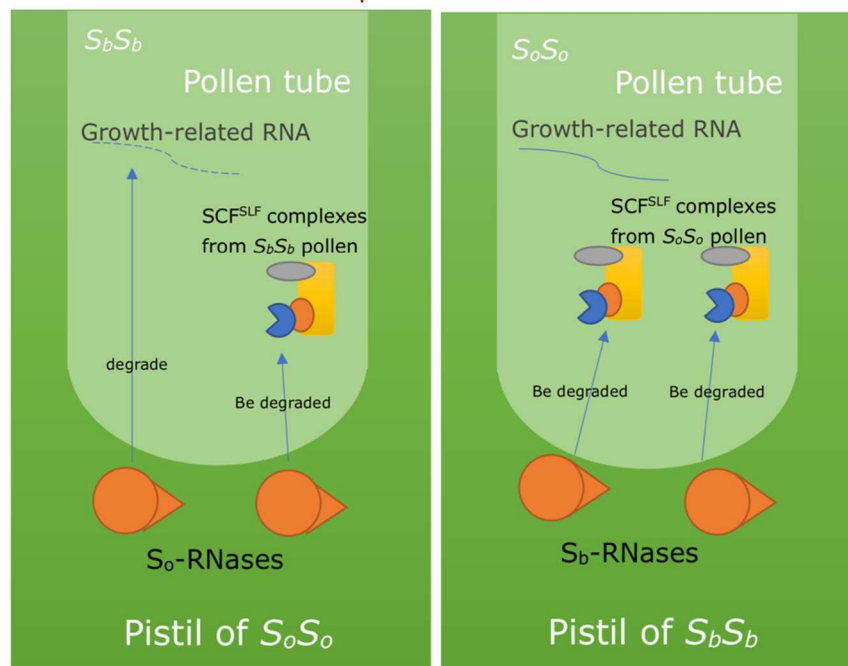
Self-pollination



The growth of pollen tube growing: (✓)

(×)

Cross-pollination



The growth of pollen tube growing: (×)

(✓)

Figure 7.2 **A schematic diagram of a hypothesis for events taking place in the pollen tube of S_0S_0 and S_bS_b during self-pollination and cross-pollination.** The orange circles with a point are S-RNases, the blue, orange, yellow and grey complexes are SCF^{SLF} complexes, the blue forms are SLFs. The dotted line indicates the degraded growth-related RNA and the arrow shows the interaction between SCF^{SLF} complexes, S-RNases and RNA.

7.2 Promoter studies of *Petunia inflata* SLF genes

The second part of the aim of this project, studying *Petunia inflata* SLF gene promoters, has been only partially completed. Before this work started, there were only few publications that indicated the expression location of SLFs in *Petunia* by RNA gel blot analysis and RT-PCR. SLFs were found only to be expressed in pollen, pollen tube and anther (Kao *et al.*, [2004](#); Hua *et al.*, [2007](#)). For firstly detecting the expression pattern of SLFs using GUS analysis, a series of SLFs selection, SLF promoter:GUS construction, transformation and GUS analysis were performed.

Due to the whole genome sequence or SLF promoter sequence of *Petunia hybrida* were unpublished, the second part of the aim of this project was based on the draft genome sequence of *Petunia inflata* published by Bombarely and his co-workers ([2016](#)). For finding the SLFs homology between *Petunia inflata* and *Petunia hybrida*, the neighbour-joining phylogenetic trees of SLFs using the data of *Petunia inflata* and *Petunia hybrida* was built. The result of homology analysis illustrates that some SLFs have high homology in these two varieties, and some closely related SLFs even have high homology such as SLF3 in *Petunia inflata* with SLF3 in *Petunia hybrida*. This directly affects the subsequent choice of SLF to study promoter function. However, the SLF gene promoter candidate regions were found to have less homology than SLF gene. This is because the region of promoters is not confirmed yet. Only picking approximately 2000 bp regions to use may cause inaccurate results.

After the screening based on neighbour-joining phylogenetic trees, SLF3, SLF13-1, and SLF14-1 of *Petunia inflata* were selected as SLFs for promoter:GUS (β -glucuronidase) fusion analysis. The reason for not using GFP (green fluorescent protein) as a reporter protein is that the previous study of tomato specific gene LAT52 found the pollen gene only express in the pollen of *Petunia hybrida* (Moore, [2006](#)), using GUS will be easier to observe. After constructing the entry vectors and destination vectors, only SLF3promoter:GUS fusion was successfully transformed into *Petunia hybrida* cv 'Mitchell' (W115). W115 is a good choice for transgenic host as it has close relationship with *Petunia inflata*. The GUS study of the SLF3 promoter activity found that it was only expressed in the stamens of

flower and specifically it was shown to be expressed only in the pollen. In more detail, *SLF3* was mainly expressed in the vegetative nucleus, vacuole or cytoplasm of pollen, but it is unclear which compartment of pollen it expresses at. According to previous studies all *SLFs* are expressed in pollen at the same time (Li *et al.*, [2016 b](#)). Despite the fact that *SLF13-1* and *SLF14-1* were not eventually transferred to *Petunia hybrida*, it can be predicted that their expression position is no different with *SLF3*.

The GUS analysis results of *SLFs* can correspond to the expression change by qRT-PCR. Both of them show that *SLFs* have the strongest expression in pollen at the fourth stage. In addition, *SLFs* in pollen show exponential growth from the first stage to the fourth stage. When pollen is mature enough to reach the fifth stage, *SLFs* will lead the pollen tubes to enter pistil from pollen and then to meet the S-RNases in pollen tube to process SI recognition. This process is in line with the observations of previous researchers (Kao *et al.*, [2004](#); Hua *et al.*, [2007](#)). As a pollen S-gene, *SLFs* are only expressed in pollen and only react with S-RNases in the pollen tube. This is why *SLFs* was only found in pollen, pollen tube and anther, rather than ovary, style, and leaves. There are no publications to use '*SLF* promoter' of *Petunia* to study *SLFs*, thus, this part will be published in the future.

7.3 Future work

Currently, the *SLF* gene sequences of *S_bS_b* are based on the *SLF* sequences of *S_bS_b* that were used to design primers. In the future work, if the *SLFs* of *S_bS_b* can be fully sequenced like *S_oS_o*, it will be more certain whether there is a difference in the DNA sequence between them. Then, if this is cloned and sequenced like *S_o-RNase* and *S_b-RNase* performed in this project, this will be more accurate. However, the best way to sequence the *SLFs* of *S_bS_b* is using next generation sequencing (NGS) like Kubo's works ([2015](#)). In addition, the reason for the *SLF* gene expression levels in *S_oS_o* being higher than *S_bS_b* has not been found yet. It is only speculated that there is a potential regulatory gene. In future experiments, the function study of other factors in SI response can be carried out, for example, knockdown or overexpression of factor that may affect the *SLF* gene expression of *S_bS_b*. It is speculative that this trans-acting factor should express in the pollen

of S_bS_b . This is because the pollen of S_bS_b made the pistil of S_oS_o become cross-incompatible. Therefore, there is a possible trans-acting factor in the pollen of S_bS_b to adjust the expression of *SLFs*. However, based on the studies I learned, I did not find which gene meets the requirements. The protein analysis of *SLFs* in both S_oS_o and S_bS_b may be a good direction to study the differences between S_oS_o and S_bS_b . Moreover, building *35S:SLF* constructs can be used to test whether transformed S_bS_b can become self-compatible when they have similar expression level of *SLFs* according to the observation we have got so far. Additionally, in order to determine which members of the *SLF* family are required to convert S_bS_b to SC, the '*SLF3* promoter' can be used to build a *SLF3promoter:SLF* construct and transformed into S_bS_b , which will be a good work to explore. For the study of the expression position of *SLFs*, it is regrettable that in this project only the *SLF3* promoter was transferred into W115. If 3-5 *SLF* promoter transformations can be completed, this will make the results more convincing. Also, an experiment similar to that carried out by Kubo *et al.* ([2010](#); [2015](#)) will be interesting. In order to detect the interaction of each *SLF* and S-RNase, Kubo and co-workers used *LAT52* promoter to build a *LAT52promoter:SLF:GFP* construct. If *LAT52* promoter is replaced by '*SLF3* promoter', this may provide interesting results.

7.4 General conclusion

The phenotypic difference between S_oS_o and S_bS_b homozygotes is that S_oS_o is self-compatible and S_bS_b is self-incompatible. Their S-RNases, the female determinant of SI response, are 100% identical in terms of the genomic DNA sequences and they have similar expression levels. For the male determinant *SLFs*, the DNA sequences of *SLFs* are considered identical thus far, and all are expressed in the stamens of S_oS_o and S_bS_b based on RT-PCR. However, qRT-PCR has revealed some significant differences in expression levels. Most of *SLFs* in S_oS_o showed a significantly higher expression level when compared with S_bS_b , and the expression level of 9 *SLFs* in S_oS_o are double that of S_bS_b . This may be caused by the regulation of the *SLF* gene family or by another trans-acting factor. The increased

expression of *SLF* genes in S_oS_o is the most likely explanation for the different phenotypes of S_oS_o and S_bS_b .

The GUS analysis indicated that *SLF3* of *Petunia inflata* expressed mainly in pollen of stamens. In more detail, *SLF3* expression was observed in the vegetative nucleus, vacuole or cytoplasm of pollen, but it is unclear which compartment of pollen it specifically expresses at. Both qRT-PCR and GUS analysis showed that SLFs express in pollen and the strength of expression is an exponential growth during the maturation of pollen.

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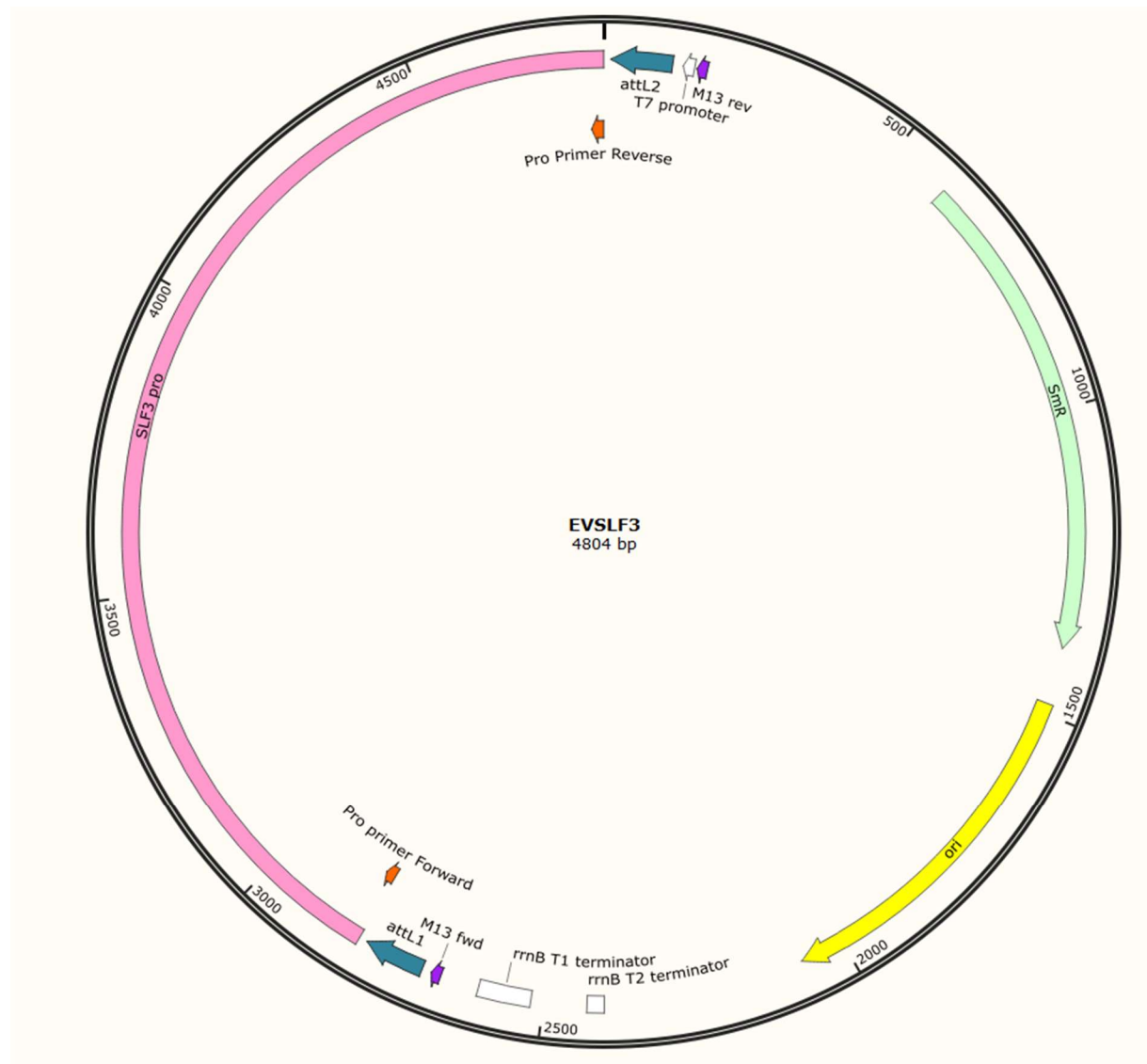
Zhao P, Zhang L and Zhao L (2015). Dissection of the style's response to pollination using transcriptome profiling in self-compatible (*Solanum pimpinellifolium*) and self-incompatible (*Solanum chilense*) tomato species. *BioMed Central Plant Biology*, 15, 119.

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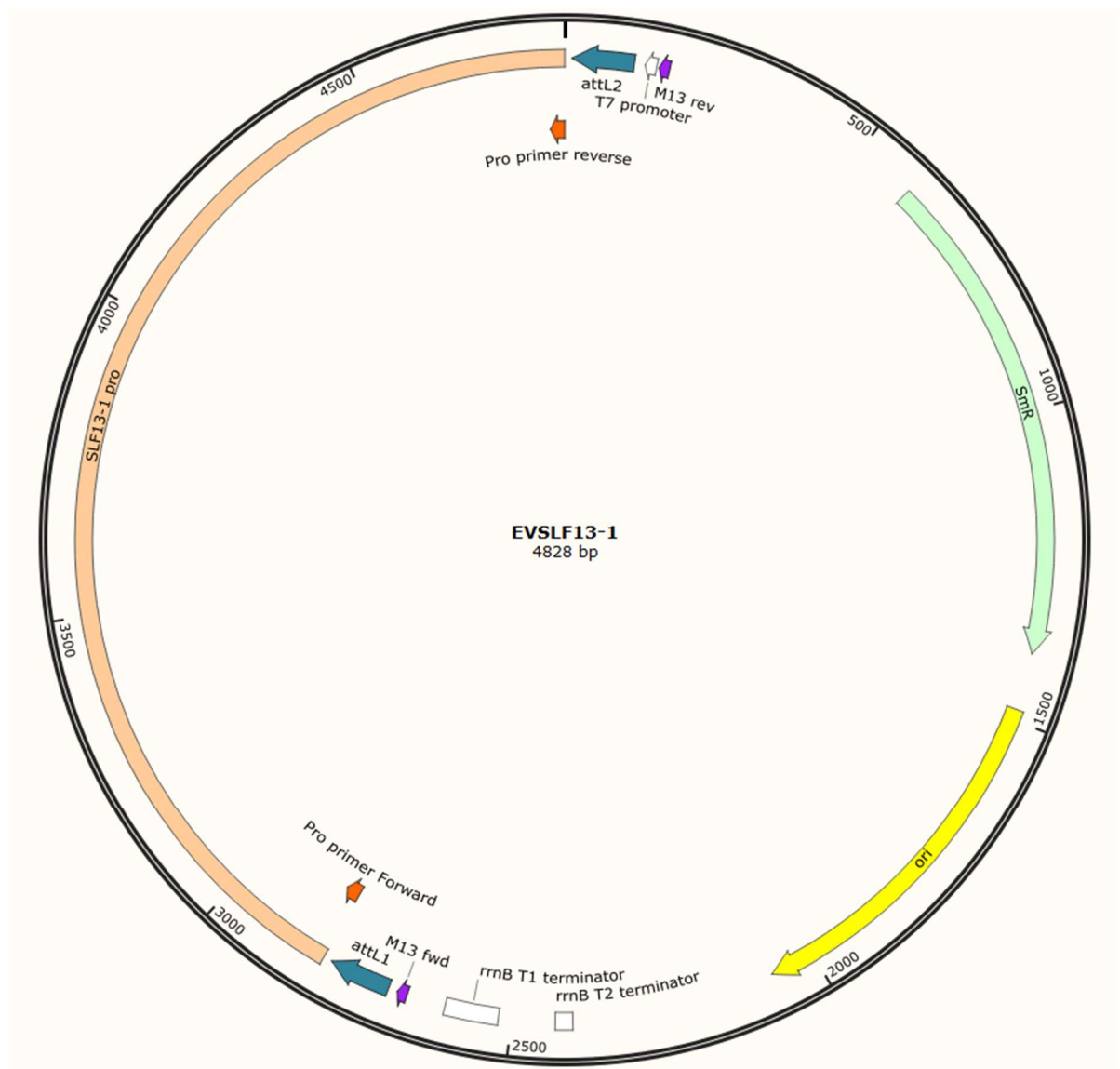
APPENDICES

Appendix 1 T-DNA maps

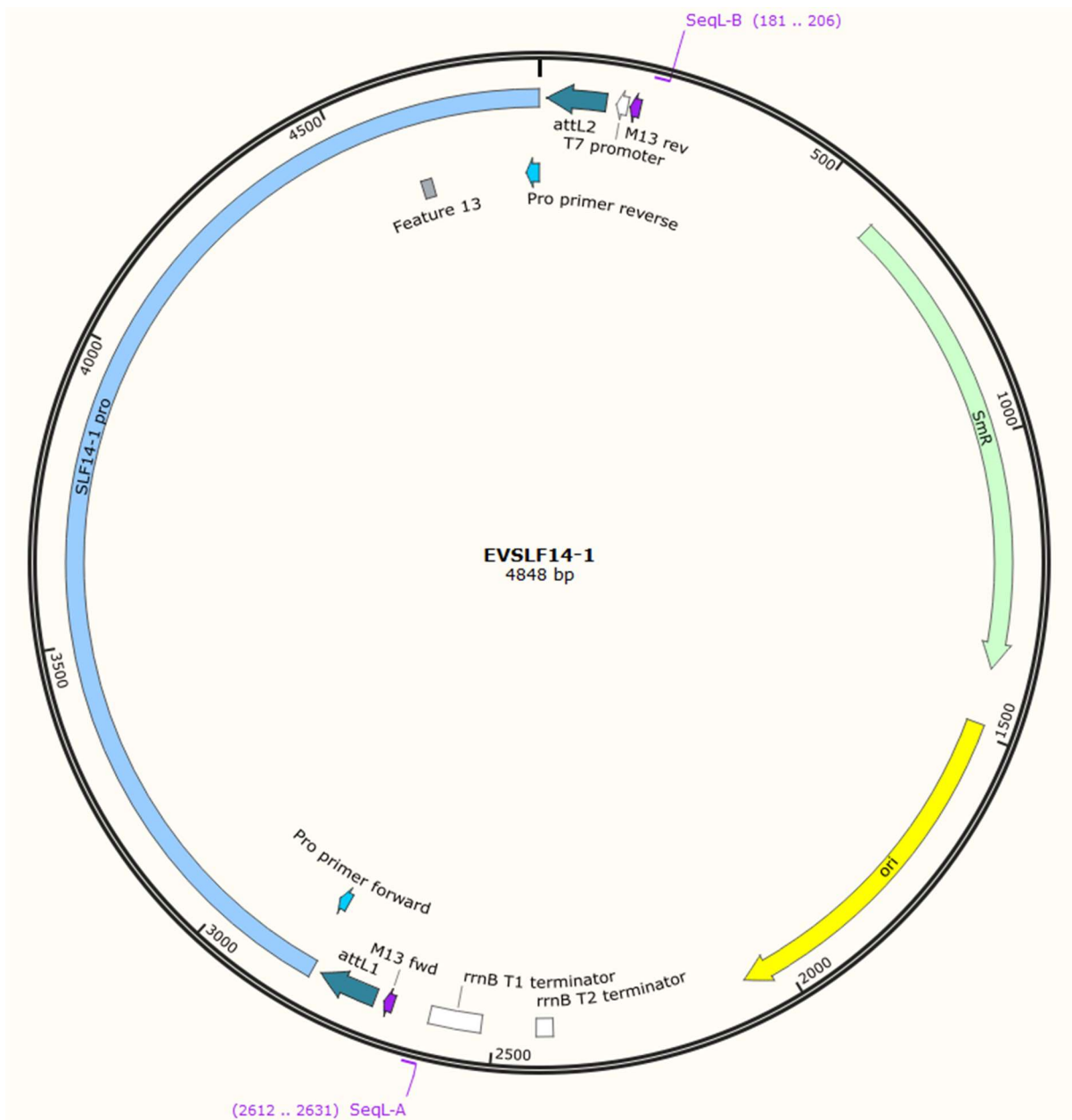
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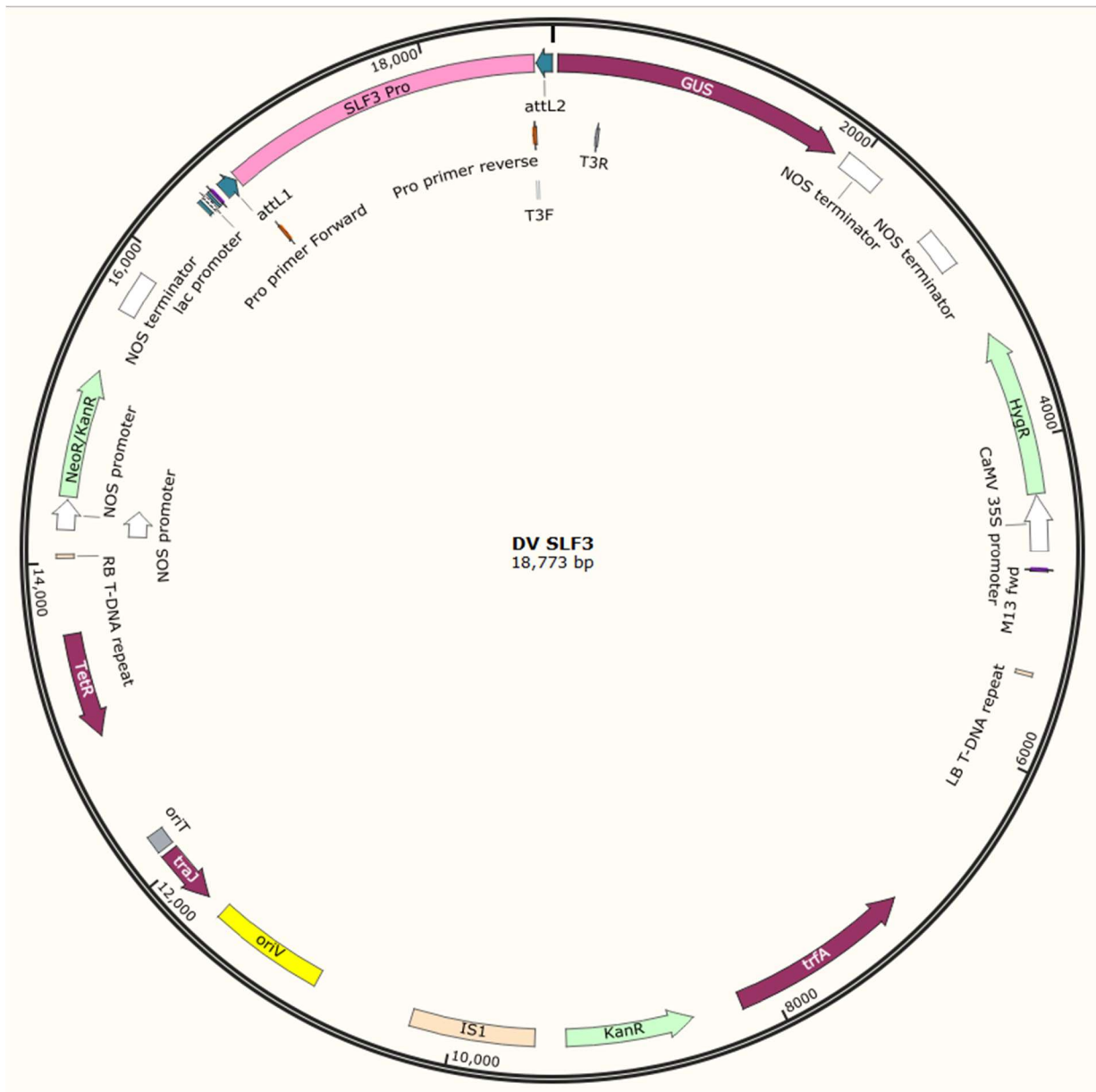
EVSLF13-1



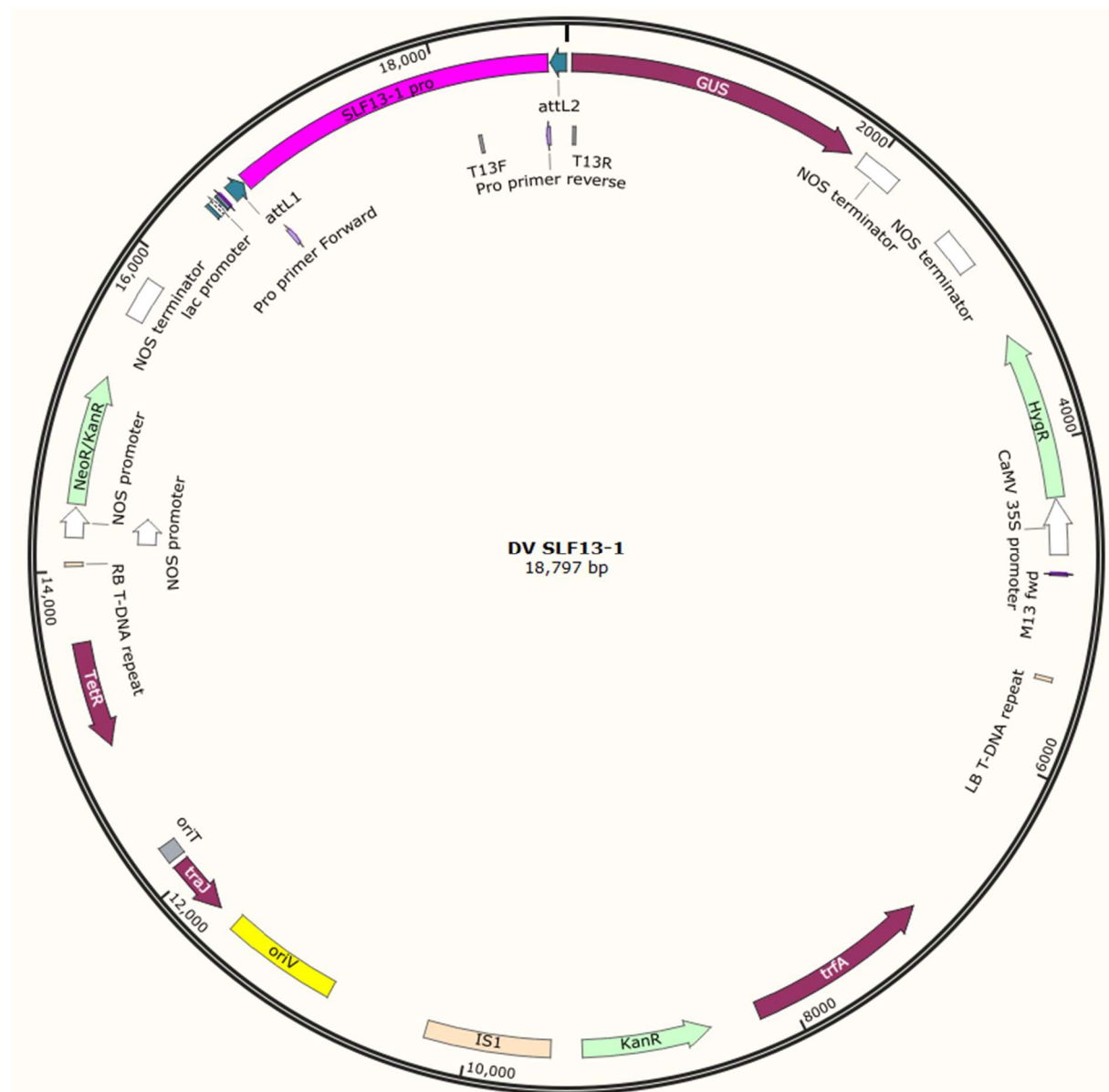
EVSLF14-1



DVSLF3



DVSLF13-1



DV SLF14-1
18,817 bp

Genetic elements and features shown on the map:

- SLF14-1 pro**: 18,000 bp promoter region.
- attL1**: Restriction site for attL1.
- Pro primer forward**: Primer for SLF14-1 pro.
- attL2**: Restriction site for attL2.
- T14F** and **T13R**: SLF14-1 pro primer reverse sites.
- GUS**: GUS gene.
- NOS terminator**: NOS terminator.
- 2000**: 2,000 bp segment.
- HygR**: Hygromycin resistance gene.
- CamV 35S promoter**: CamV 35S promoter.
- pmv31TM**: pmv31TM gene.
- LB T-DNA repeat**: LB T-DNA repeat.
- 1600**: 1,600 bp segment.
- trfA**: trfA gene.
- 1800**: 1,800 bp segment.
- IS1**: IS1 element.
- 10,000**: 10,000 bp segment.
- oriV**: oriV origin of replication.
- 12,000**: 12,000 bp segment.
- oriT**: oriT origin of transfer.
- traj**: traj gene.
- 14,000**: 14,000 bp segment.
- TetR**: TetR gene.
- RB T-DNA repeat**: RB T-DNA repeat.
- 1,400**: 1,400 bp segment.
- NOS promoter**: NOS promoter.
- NeoR/KanR**: Neomycin/Kanamycin resistance gene.
- 1,000**: 1,000 bp segment.
- NOS terminator**: NOS terminator.
- lac promoter**: lac promoter.
- 16,000**: 16,000 bp segment.

Appendix 2 Medium recipes

TBE buffer:

108g Tris base

55g Boric acid

9.12g EDTA pH 8

Loading Buffer:

0.25% bromophenol blue

0.25% xylene cyanol FF

40% sucrose

LB broth for 1 litre:

10g Peptone

5g Yeast extract

10g NaCl

Make up to 1 litre with distilled water

Sterilized mixture

LB Agar Plates:

10g Peptone

4g Yeast Extract

10g NaCl

15g Agar

Distilled water is used to make 1 litre then autoclaved to dissolve agar and sterilize medium.

The medium is poured into plates under sterile hood. Then left for 20 minutes to solidify and covered the plates.

Co-cultivation medium (CCM) for 1 Litre:

4.4g Murashige and Skoog Basal Salt Mixture

30g sucrose

Distilled water is used to make 1 litre

Adjust pH to 5.8 with KOH and HCL

8g plant tissue culture agar was added then autoclaved to dissolve agar and sterilize medium

The medium is poured into plates after adding 1000 μ l BAP 1 μ g/ml (stock concentration 1 mg/ml) and 10 μ l NAA 0.01 μ g/ml (stock concentration 1 mg/ml)

when the medium cool down to about 45 degree under a sterile hood. Then left for 20 minutes to solidify and covered the plates.

Regeneration and selection medium (RSM) for 1 litre:

4.4g Murashige and Skoog basal salt mixture

30g sucrose

Distilled water is used to make 1 litre

Adjust pH to 5.8 with KOH and HCl

8g plant tissue culture agar was added then autoclaved to dissolve agar and sterilize medium

The medium is poured into plates after adding 1000 μ l BAP for 1 μ g/ml (stock concentration 1 mg/ml), 10 μ l NAA for 0.01 μ g/ml (stock concentration 1 mg/ml), 1000 μ l Kanamycin for 100 μ g/ml (stock concentration 100mg/ml) and 1000 μ l Carbenicillin for 200 μ g/ml (stock concentration 200mg/ml) when the medium cool down to about 45 degree under a sterile hood. Then left for 20 minutes to solidify and covered the plates.

Rooting medium (RSM) for 1 litre

4.4g Murashige and Skoog basal salt mixture

30g sucrose

Distilled water is used to make 1 litre

Adjust pH to 5.8 with KOH and HCl

8g plant tissue culture agar was added then autoclaved to dissolve agar and sterilize medium

The medium is poured into plates after adding 1000 µl Kanamycin for 100 µg/ml (stock concentration 100mg/ml) and 1000 µl Carbenicillin for 200 µg/ml (stock concentration 200 mg/ml) when the medium cool down to about 45 degree under a sterile hood. Then left for 20 minutes to solidify and covered the plates.

YM medium for 1 litre:

0.4 g Yeast extract

10 g Mannitol

0.1 g NaCl

0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

0.5 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$

Distilled water is used to make 1 litre

Adjust pH to 5.8 with KOH and HCl

Sterilized mixture

Appendix 3 Antibiotic and recipes

Ampicillin (50mg/ml stock concentration)

0.5 g Ampicillin

10 ml sterile water

Filter sterilised

Store at -20°C

Kanamycin (50mg/ml stock concentration)

0.5 g Kanamycin

10 ml sterile water

Filter sterilised

Store at -20°C

Spectinomycin (100mg/ml stock concentration)

1.0 g Spectinomycin

10 ml 100% Ethanol

Filter sterilised

Store at -20°C

Carbenicillin (200mg/ml stock concentration)

2.0 g Carbenicillin

10 ml sterile water

Filter sterilised

Store at -20°C

Rifampicin (50mg/ml stock concentration)

0.5 g Rifampicin

10 ml 100% DMSO

Filter sterilised

Store at -20°C

6-Benzylaminopurine (BAP) (stock concentration 1 mg/ml)

0.1g 6-Benzylaminopurine

20 ml 0.1mol/L NaOH solution to dissolve and 80 ml sterile water

Filter sterilised

Store at -20°C

1-naphthaleneacetic acid (NAA) (stock concentration 1 mg/ml)

0.1 g 1-naphthaleneacetic acid

100 ml sterile water

Filter sterilised

Store at -20°C

Appendix 4 List of primers or enzymes used in this study

N o.	Primer Name	Sequence (5' to 3')	Annealing temperature (°C)	Expect PCR product Size (bp)	Function	Source		
1	S ₀ Forward (So-F2)	GGTTATTGCAAAATTAAGGG	57	735	To amplify S ₀ -RNase	Ai <i>et al.</i> (1992)		
	S ₀ Reverse (V26-R5)	ATGTTCTCTCTTCGAAGTTCGCG						
2	S ₀ Forward	CTGTACTCGTCCCTTTTGG	58	~800	To amplify S ₀ -RNase	Czyzyk (2010)		
	S ₀ Reverse	TCTTTCTGGTCATGATGTTCT						
3	S _v Forward	GGACGAAGCTGATTGAAGGG	48	~500	To amplify S _v -RNase	Robbins <i>et al.</i> (2000)		
	S _v Reverse	CGATTTTCATATATTGGC						
4	Elongation Factor 1a (EF1a) Forward	CCTGGTCAAATTGGAAACGG	59	~100	For as a control gene in PCR, RT-PCR and qRT-PCR	Czyzyk (2010)		
	Elongation Factor 1a (EF1a) Reverse	CAGATCGCCTGTCAATCTTGG						
5	CP100 primer Forward	CAAAGCGATTTACCAGTTTAgAAATC	63	1021	For analysis CAPS-CP100	Wright (2004) Czyzyk (2010)		
	CP100 primer Reverse	GATGAGGACTCACAAACAGTGGTGA						
6	<i>Hinf</i> I enzyme	C'ANTC (enzyme digestion site) CTNA'G	37	-				
7	SLF1-S _{0m} Forward	AAATGCGATTGCACTTTTACTCC	59	498	For RT-PCR of 16 <i>SLFs</i>	This study		
	SLF1-S _{0m} Reverse	CATAATACAGCTCGTGTGTA ATCCT						
8	SLF2-S _{0m} Forward	CTCCTCTTGGTGTACTAATTTTAATCA	60	489				
	SLF2-S _{0m} Reverse	CGTGGGCCATCGAGT						

9	SLF3-S _{om} Forward	CACCAGCTATAGTTGTATCTGTCATC	60	363		
	SLF3-S _{om} Reverse	CGTGGGCCATCGAGTA				
10	SLF4-S _{om} Forward	TCACGTTCTACCATCTTGG	57	442		
	SLF4-S _{om} Reverse	TATGACATGTTCCACTTGAAA				
11	SLF5-S _{om} Forward	CGACACACAGAGTATTATTTTGATCA	62	489		
	SLF5-S _{om} Reverse	TATGATTGCGAATATTATGACAAGG				
12	SLF6-S _{om} Forward	ACTCGTAATTGTGATCATGATCAAC	62	578		
	SLF6-S _{om} Reverse	CCACAGGAATCTCGGGCT				
13	SLF7-S _{om} Forward	CCTCTATTTCTAGTGTTCGCATAGAC	63	500		
	SLF7-S _{om} Reverse	CATAACTCTTCCTGTCTTTGAAATGAC				
14	SLF8-S _{om} Forward	CTTCACTGAATACTATCTTTCAATAAAC	58	505		
	SLF8-S _{om} Reverse	CTTCTTCCCACCAGAGGA				
15	SLF10-S _{om} Forward	GCTGGCTGTATTGTCATCG	62	475		
	SLF10-S _{om} Reverse	CATAACATTTTCATCAGCGA				
16	SLF11-S _{om} Forward	TTCCGATTATTGCAGTCGTTT	60	500		
	SLF11-S _{om} Reverse	CATAACCTTTATTGTCCAGAAATG				
17	SLF12-S _{om} Forward	ATCCCTTTATAGTATTACGCAGGACAA	64	506		

	SLF12-S _{om} Reverse	GGCGTGTTCACCTTGAAAAA				
18	SLF13-S _{om} Forward	GGTTTTGCTAGTAGTACCTCTCATCAAT	63	492		
	SLF13-S _{om} Reverse	TCTCATCTCTCGAAGCACAAAGT				
19	SLF14-S _{om} Forward	ACTACTTCTGTACTTTTCACCGAT	58	499		
	SLF14-S _{om} Reverse	CATAACACTTCCTGTCCTTGA				
20	SLF15-S _{om} Forward	CTACCACTAATTCTGTACTTTCCATAGAA	61	475		
	SLF15-S _{om} Reverse	GCAATTCTCGTCTTGAAATG				
21	SLF16-S _{om} Forward	GGAGGTTGTACGTTTCACAGAT	60	498		
	SLF16-S _{om} Reverse	GTAACACTTCCTGCTTTGTAATGG				
22	SLF17-S _{om} Forward	CAGGAGCTTGACATCTCACAGA	62	503		
	SLF17-S _{om} Reverse	AGTAAAAATTTTGTCTTTGTAATGGC				
23	SLF8-S _{om} 2 Forward	CGCCTAGCCCTTTTGT	62	428	For checking the RT-PCR result of <i>SLF8</i> (The second round of RT-PCR of <i>SLF8</i>)	
	SLF8-S _{om} 2 Reverse	AGCAAATCAACGTCAGAGACTC				
24	SLF1-S _{om} (For qPCR) Forward	CAACTACAGACACGTCGATGG	58	106	For qRT-PCR of 16 <i>SLFs</i>	
	SLF1-S _{om} (For qPCR) Reverse	TACAGCTCGTGTGTAATCC				
25	SLF2-S _{om} (For qPCR) Forward	ATAATTACAGAGACCGTGGTC	61	95		
	SLF2-S _{om} (For qPCR) Reverse	CGTGGGCCCATCGAGTC				

26	SLF3-Som (For qPCR) Forward	TGAACCTTGTGCTTCGTAT	58	99		
	SLF3-Som (For qPCR) Reverse	CTACTTTCCTCCTTGGATCA				
27	SLF4-Som (For qPCR) Forward	CTTTAAATCATGAAGACAACTG	54	111		
	SLF4-Som (For qPCR) Reverse	TATGACATGTTCCACTTGAAA				
28	SLF5-Som (For qPCR) Forward	GGATGATTGTTACGGATATGTTCAAGTGG	63	134		
	SLF5-Som (For qPCR) Reverse	GAGAACAAAGGTACCCAAATATGGT				
29	SLF6-Som (For qPCR) Forward	AACGTTTTCTGTACTCAT	52	78		
	SLF6-Som (For qPCR) Reverse	GCGTCTATGTCTAGAGATG				
30	SLF7-Som (For qPCR) Forward	GAAGAACGGAACAGTGGTT	57	104		
	SLF7-Som (For qPCR) Reverse	CATAACTCTTCCTGTCTTTGA				
31	SLF8-Som (For qPCR) Forward	CCTCTGGTGGGAAGATATG	62	93		
	SLF8-Som (For qPCR) Reverse	TAGACGGATCCATCTCGGAATC				
32	SLF10-Som (For qPCR) Forward	TCGCTGATGGAAATGTTATG	59	93		
	SLF10-Som (For qPCR) Reverse	TTTCTGTTGGACTACTAGGCAT				
33	SLF11-Som (For qPCR) Forward	CACATGACGACACAGTGATG	59	106		
	SLF11-Som (For qPCR) Reverse	CATAACCTTTTATTGTCCCAGA				
	SLF12-Som (For qPCR) Forward	AATAACATTAAGTTATGAACATCGATTG	60			

34	SLF12-Som (For qPCR) Reverse	TATGGCGTGTTCCACTTGA		116		
35	SLF13-Som (For qPCR) Forward	CCCAAGGACATATGCGTTTAC	63	105		
	SLF13-Som (For qPCR) Reverse	TATGACACTTCTCATCTCTCGA				
36	SLF14-Som (For qPCR) Forward	CGGAAACACAAATACAGTTGTT	60	106		
	SLF14-Som (For qPCR) Reverse	CATAACACTTCCTGTCCTTGA				
37	SLF15-Som (For qPCR) Forward	GCCCATGCAAATACAATAGT	60	76		
	SLF15-Som (For qPCR) Reverse	GCAATTCTCGTCTTGA				
38	SLF16-Som (For qPCR) Forward	GCCACGCAAAATAGAGCTCG	58	108		
	SLF16-Som (For qPCR) Reverse	CGTAACACTTCCTGTCTTTGT				
39	SLF17-Som (For qPCR) Forward	GCAACGAAAAATAGAGTCCATGTA	59	108		
	SLF17-Som (For qPCR) Reverse	AGTAAAAATTTTGTCTTTGTAATGG				
40	SLF3-S6 for rt-pcr Forward	GGAAGTGCCTGAACCTTG TG	59	606	To confirm published <i>SLFs</i> cDNA sequences match with the <i>Petunia inflata</i> materials	
	SLF3-S6 for rt-pcr Reverse	GCGATCCTTCCAAATTGCCA				
41	SLF13-1-S6 for rt-pcr Forward	ACGTGTGTATGGGGATCCTC	59	514		
	SLF13-1-S6 for rt-pcr Reverse	TTGGGAACTTGCAATTGTCG				
42	SLF14-1-S6 for rt-pcr Forward	GGCACATCTCATTGGTTCGG	59	549		
	SLF14-1-S6 for rt-pcr Reverse	ACCAGCATCATCAACGAGGA				

43	SLF3pro Forward	CGTAGCCTTTACCAGTTGCC	58.0	1988	To amplify <i>SLF</i> promoter fragment from <i>Petunia inflata</i>
	SLF3pro Reverse	TGCTGCTTTTGTTCATTATCCCA			
44	SLF13-1pro Forward	TTTGAGAGAAAGAAAAAGAGAAGA	59.0	2012	
	SLF13-1pro Reverse	GTTATTACACACAATCCTTGAAC TG			
45	SLF14-1pro Forward	GGAAAAGGTCTTTTGCATCTC	59.0	2032	
	SLF14-1pro Reverse	TCAAAGAAACCTAACTCTAAAAAAC C			
46	Test SLF3 Forward (T3F)	TGAACAAAAGCAGCAAGGGC	60	459	To confirm <i>SLF</i> promoter::GUS construct
	Test SLF3 Reverse (T3R)	TGACCCACACTTTGCCGTAA			
47	Test SLF13-1 Forward (T13F)	TCTGACCACTGCCTCGAACT	60	680	
	Test SLF13-1 Reverse (T13R)	TCACGGGTGGGGTTTCTAC			
48	Test SLF14-1 Forward (T14-1F)	GCTGCATGCATTACCCTTTCC	60	485	
	Test SLF14-1 Reverse (T14-1F)	TCCCACCAACGCTGATCAAT			

Appendix 5 GUS staining buffer

For 10 ml staining buffer (pH: 7.8):

68.3 μ l 1 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ solution

931.7 μ l 1 M Na_2HPO_4 solution

20 μ l 0.5 M EDTA

100 μ l 0.5 M $\text{K}_3[\text{Fe}(\text{CN})_6]$ solution

100 μ l 1 M $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$ solution

100 μ l 1 M X-Gluc

500 μ l 10% Triton X-100

Water to volume