ABSTRACT

PROTEIN FUNCTION AND INTERACTIONS IN GAMETOPHYTIC SELF-INCOMPATIBILITY: COLLABORATIVE RECOGNITION OF S-RNASE *IN VIVO*

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My dissertation is based on studies of Gametophytic Self-Incompatibility (GSI), a system that allows plants to reject "self" pollen while accepting "non-self" pollen, thus preventing inbreeding and promoting genetic diversity in populations. In GSI, pollen grains deposited on the stigma of the floral pistil germinate and begin to grow through the transmitting tract tissue of the style. As the pollen tubes grow through the transmitting tract, they import recognition variants of a secreted protein known as the S-locus ribonuclease (S-RNase). If there is a match of recognition specificity between the pollen tube and the imported S-RNase, the S-RNase will degrade pollen-tube RNA, inhibiting protein synthesis & pollen tube growth. Conversely, if there is no match between pollen tube and S-RNase, the action of the S-RNase is inhibited, and the pollen tube continues to grow normally to the ovary.

Inside pollen tubes, non-self S-RNases are recognized by the SCF^{SLF} complex comprising multiple variants of the pollen-recognition protein named SLF, along with three other proteins: SSK1, SBP1 and Cullin-1. I have been using protein-interaction assays (BiFC assays) based on the reconstitution of a fluorescent protein, to study interactions between components of the SCF^{SLF} complex and S-RNase.

Previous studies revealed that multiple SLF genes collaborate during non-self S-RNase recognition. Based on my data, SLF10 and to a lesser extent, SLF1, SLF3, SLF4 and SLF5 showed interaction with different S-RNase constructs. In addition, data in my study suggests that a "bridge" protein may be needed to stabilize proteins interactions between SLF and S-RNase. The work that has been completed will lead to a better understanding of self versus non-self recognition in pollination. An understanding of GSI mechanisms should also lead to the ability to manipulate breeding barriers in agricultural crops such as tomatoes, potatoes and fruit trees.

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PROTEIN FUNCTION AND INTERACTIONS IN GAMETOPHYTIC SELF-INCOMPATIBILITY: COLLABORATIVE RECOGNITION OF S-RNASE *IN VIVO*

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DEDICATION

To my parents, Mr. Zhongyao Qi and Mrs. Jianfen Zong

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LIST OF ABBREVIATIONS

AD – Activating Domain

BD – Binding Domain

BiFC – Bimolecular Fluorescence Complementation

GSI – Gametophytic Self-Incompatibility

HPF/FS – High Pressure Freezing/Freeze Substitution

hpRNA – hairpin RNA

RISC – RNA-Induced Silencing Complex

S-RNase – S-locus-encoded Ribonuclease

SC – Self-Compatibility/-Compatible

SI – Self-Incompatibility/-Incompatible

siRNA – small interfering RNA

SLF – S-Locus-encoded F-box protein

ssRNA – single-strand RNA

TEM – Transmission Electron Microscopy

UAS – Upstream Activating Sequence

Y2H – Yeast Two-Hybrid

CHAPTER 1

INTRODUCTION

> …protected flowers with their own pollen placed on the stigma never yielded nearly a full complement of seed; whilst those left uncovered produced fine capsules, showing that pollen from other plants must have been brought to them, probably by moths. Plants growing vigorously and flowering in pots in the green-house, never yielded a single capsule; and this may be attributed, at least in chief part, to the exclusion of moths. (Darwin 1891)

Ever since Darwin first elaborated his observation of self- and cross-fertilization in Petunia in his book *The Effects of Self and Cross Fertilization in the Vegetable Kingdom* (Darwin 1891), Self-incompatibility (SI) has become an important subject of study. SI is the mechanism according to which plants can reject "self" pollen and therefore prevent inbreeding, while accepting "non-self" pollen. Researchers (Mather 1943; Linskens 1975; de Nettancourt 1977) determined that SI in Petunia was controlled by a single, multi-allelic gene fragment, termed the S-locus, and that recognition and subsequent rejection of self-pollen was governed gametophytically by alleles expressed in pollen, i.e. Gametophytic Self-Incompatibility (GSI) in Petunia. Mutations that inactivated self-incompatibility in pollen had also been identified to be related to centric chromosomal fragments (Brewbaker and Natarajan 1960). In addition, "competitive interaction" in pollen had been proposed to explain the fact that tetraploid plants with diploid heteroallelic pollen were self-compatible (SC). Shivanna and Rangaswamy (1969) had determined that self-incompatibility could be overcome by pollinating immature styles. Ascher (1984) had demonstrated that the strength of the self-incompatibility response could vary

quantitatively, and called this quantitative variation termed “pseudo-self-incompatibility”. Over the past decades, progress achieved in this field has led to a much better understanding at the molecular level of those findings described above.

Further researches resulted in identification of the S-RNase (S-locus-encoded ribonuclease) as the style-expressed recognition component of GSI, functioning together with both the pollen-expressed SLF (S-locus-encoded F-box protein) and a number of other genes that played crucial, supportive or yet undefined roles in the GSI response. Although tremendous progress has been made in understanding the molecular basis of pollen recognition and rejection, many other essential aspects of GSI remain partially unclear. The most commonly accepted model of S-RNase-based incompatibility proposes that both self and non-self S-RNase proteins are imported into extending pollen tubes. In the case of compatible pollination, non-self S-RNases are inhibited from functioning, while in an incompatible pollination, self S-RNases act as ribonucleases and degrade pollen-tube RNA, therefore inhibiting the growth of pollen tubes. Recognition of an S-RNase as self or non-self is determined by the pollen-expressed SLF. What remains puzzling, however, is how the pattern of recognition is determined, in terms of protein interaction(s) between S-RNase and SLF, and/or any other proteins involved in GSI.

Distribution and Mechanism of GSI

Igic and Kohn (2001) estimated that gametophytic self-incompatibility occurs in up to three quarters of eudicot families. As in *Petunia hybrida*, the most widely distributed mode of GSI is based on the interaction of style- and pollen-expressed allelic proteins which are encoded by a single, multi-allelic S-locus (Fig. 1) which encodes two recognition functions pollen-S

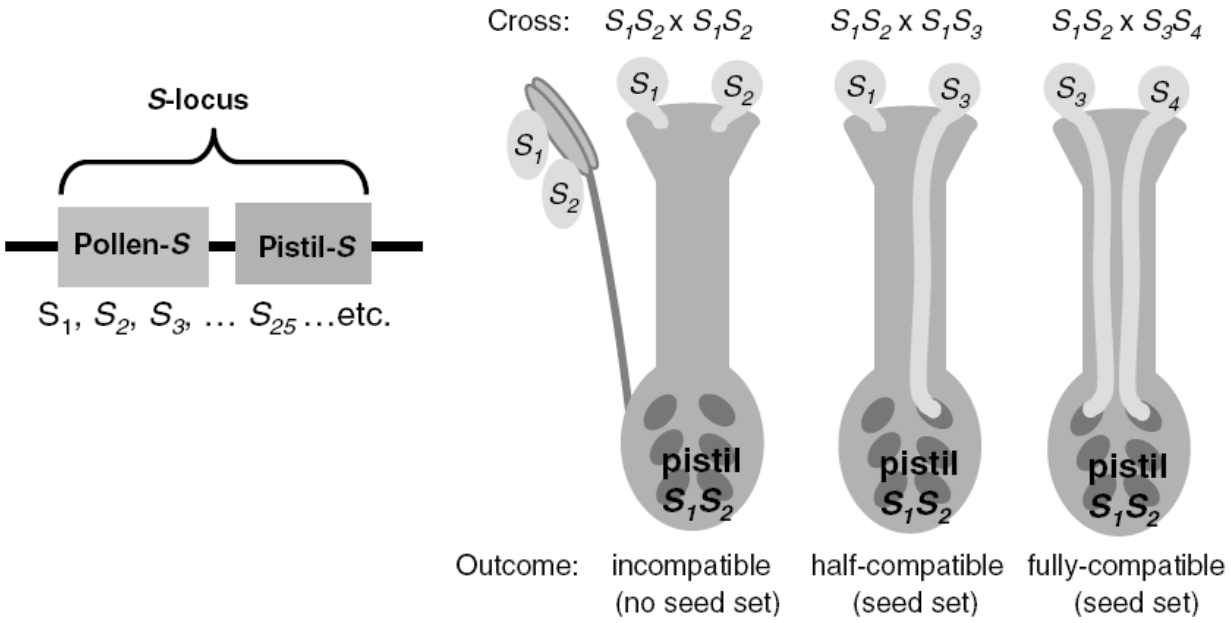


Figure 1: Multiallelic S-locus governing GSI response. GSI is based on the interaction of style- and pollen-expressed allelic proteins, which are encoded by a single, multi-allelic S-locus containing pollen-S and pistil-S. If S_2 pollen is deposited on an S_1S_2 pistil, pollen-S_2 matches the S_2 gene in the pistil, leading to an incompatible cross and no seed set; If S_3 pollen is deposited on an S_1S_2 pistil, pollen-S_3 does not match either pistil-S gene, resulting in a compatible pollination and seed set.

(expressed in pollen/pollen tube) and pistil-S (expressed in style). Together, a matching Pistil-S and Pollen-S comprise an S-locus haplotype. Recognition and rejection of pollen rely on whether there a match of S-haplotypes between the growing pollen tube and the style. If the S-haplotype expressed in the pollen ("pollen-S") matches one of the two S-haplotypes expressed in the style, i.e. an incompatible cross, the pollen-tube growth is significantly inhibited in the transmitting tract in the style. In this case, fertilization rarely occurs and therefore normally no seeds are produced. On the contrary, if there is no match between the S-haplotype expressed in the pollen and those expressed in the style, i.e. a compatible cross, pollen tubes continue to grow until they

reach the ovary, leading to fertilization and production of seeds (Fig. 1). Generally, compatible versus incompatible crosses can be distinguished by the existence of seed capsules. In compatible crosses, large seed capsules that sometimes contains up to a few hundred seeds are observed. In a fully incompatible cross, no seed capsules are formed and thus no seeds produced. An alternative method of distinguishing whether a cross is compatible or incompatible is tracking pollen tubes by fluorescence microscopy. A stain (aniline blue) is applied to stain callose (a β-1,3 glucan found in pollen tubes), and gives fluorescence with UV illumination. Normally in incompatible crosses, most of the pollen tubes are found to stop extending in the upper third of the style, while in compatible crosses pollen tubes grow out the end of the style and eventually reach the ovary (Fig. 2).

Gametophytic self-incompatibility has been well studied not only in Petunia, but also in a number of other horticultural and agronomic species such as those in the Solanaceae [*S. tuberosum* (Potato), *S. lycopersicum* (Tomato), *N. alata* (Tobacco)], Rosaceae [*M. domestica* (Apple), and *P. dulcis* (Almond)] and Plantaginaceae [*A. majus* (Snapdragon)] (Igic and Kohn 2001).Therefore, a thorough understanding of the mechanism of self-incompatibility at the molecular level should result in the ability to manipulate such inbreeding barriers in economically and agriculturally significant crops. Although the topic of this dissertation focuses on the GSI system in Petunia, a large part of our current understanding of GSI also comes from studies involving other genera of the Solanaceae, such as *Nicotiana*, and *Solanum*, as well as from Rosaceae (*Prunus*) and Plantaginaceae (*Antirrhinum*). So in sections below, besides research completed in Petunia, the discoveries from other plants will also be mentioned appropriately.

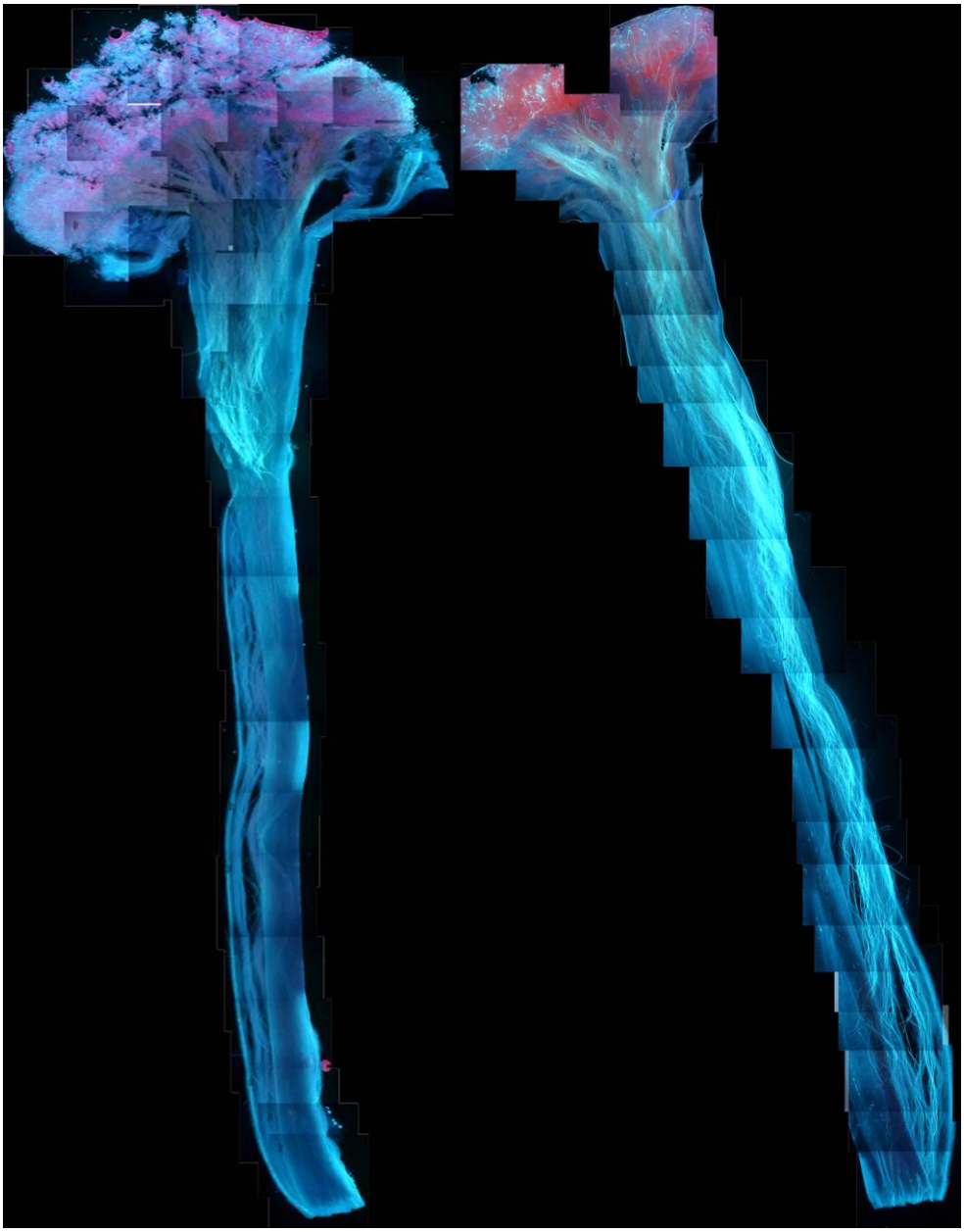


Figure 2: Micrographs of pollen tubes. Both figures show styles containing fluorescent pollen tubes. Each image of a complete style comprises of dozens of separate images of partial styles. The image on the left shows an incompatible pollination in which pollen tubes stop growing in the upper third of the style. The image on the right image shows a compatible pollination in which pollen tubes reach the end the style.

Biochemical Genetics of GSI

Although Darwin (1891) first described the fundamental feature of self-incompatibility in Petunia, it was not until 1933 that Harland and Atteck found that pollen recognition was in fact controlled by a gametophytic mechanism. In terms of the pollinating process in GSI plants, pollen grains are deposited directly on the surface of the stigma, germinate, and create pollen tubes that will extend through the extracellular matrix of the transmitting tract of the style. Pollen tubes expressing alleles of SLF (pollen-S) that are different, or "non-self" in other words, from the S-RNase alleles expressed in the style (i.e. a compatible pollination) grow at a higher rate relative to incompatible pollen tubes. Incompatible pollen tubes may also show morphologically abnormal features such as increased deposits of callose and swollen tips (de Nettancourt 1977). In GSI, recognition of pollen as compatible versus incompatible, is determined by the haploid genotype of the individual pollen grain as "non-self" versus "self". Recognition is a pollen-tube by pollen-tube phenomenon, and is therefore cell-autonomous.

Current models for GSI system propose a cytotoxic mechanism for pollen-tube degradation. That is during an incompatible pollination, S-RNase imported into pollen tubes functions and degrades pollen-tube RNA, therefore inhibiting protein synthesis and reducing pollen-tube growth. This cytotoxic model is supported by the findings that S-RNase is imported into pollen tubes before functioning, that ribonuclease activity is required for pollen-tube degradation, and that degradation of pollen-tube RNA is correlated with incompatibility. It is also observed, however, that incompatible pollen tubes can be repaired by grafting experiments (Lush & Clarke 1996), and that polysomes remain intact in incompatible pollen tubes (Walles & Han 1998), which may indicate that GSI system is more than a simple cytotoxic model.

A generally accepted fact is that the inhibition of S-RNase activity inside pollen tubes leads to a compatible pollination (McClure & Franklin-Tong 2006; Hua et al. 2008; Sims & Robbins 2009; Chen et al. 2010; Kubo et al. 2010). Various evidence supporting such inhibition model includes the analysis of pollen part mutants (Golz et al. 1990, 2001; Tsukamoto et al. 2005), the observation that both self and non-self S-RNases are imported into pollen tubes (Luu et al. 2000; Goldraij et al. 2006), a loss-of-function study showing that down-regulation of SSK1 (a component of a SCF^{SLF} E3 ubiquitin ligase model, see section below) results in loss of compatibility (Zhao et al. 2010), and the findings of "competitive interaction" experiments (Sijacic et al. 2004; Kubo et al. 2010).

Genes and Proteins Involved in GSI

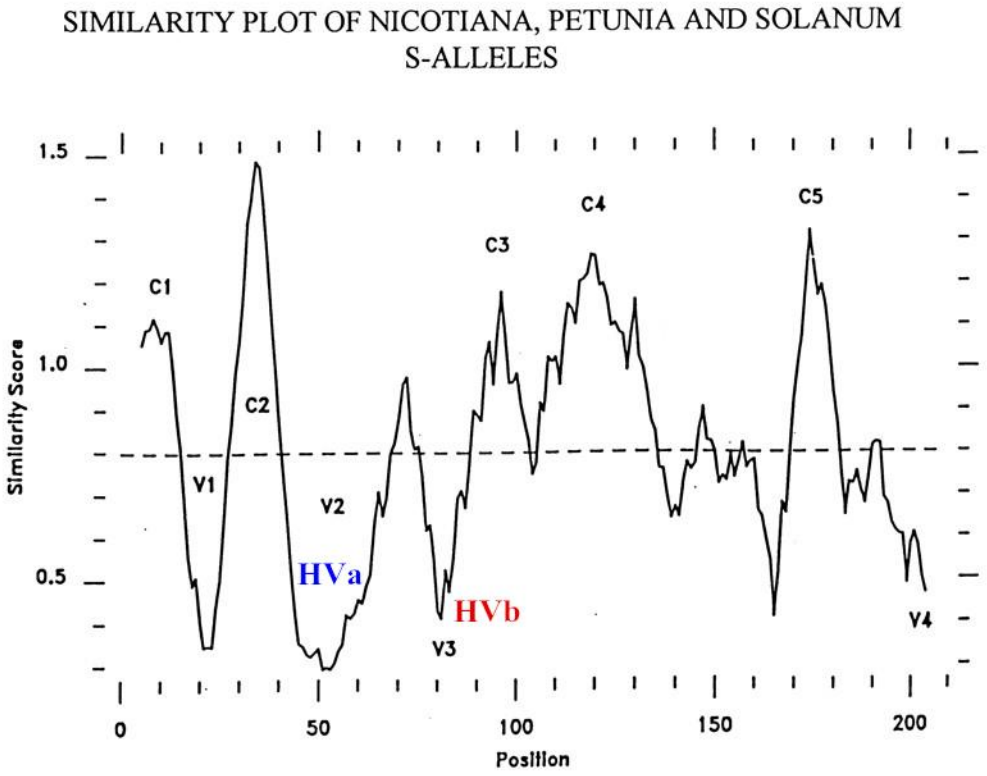
To date, a few different genes have been identified and demonstrated to play crucial or presumed roles in GSI. Two primary genes, S-RNase and SLF (SFB in Rosaceae), have been verified as pistil-S and pollen-S genes. Recent data shows that previously identified SLFL (SLF-like) genes are actually true SLF genes, and SLF proteins may recognize S-RNase in a collaborative fashion (Kubo et al. 2010). Four other non-S-locus genes, HT-B, 120 KDa, SSK1 and Cullin-1 have also been found to be significantly involved during pollen rejection or acceptance. Another gene, SBP1, is known to interact with both S-RNase and SLF, and may be a component of a SCF^{SLF} E3 ubiquitin ligase complex. Important characteristics of each of these genes and proteins will be summarized below.

S-RNase: the Style-Recognition Component

Clark et al. (1990) and Ai et al. (1990) first reported cDNA sequences for S-proteins in *Petunia hybrida* and *P. inflata*. These protein sequences were highly similar to the T2-type of ribonucleases (RNase) discovered in fungi, which resulted in the term "S-RNase". As an S-locus encoded ribonuclease, S-RNase is featured by its ability to selectively inhibit the growth of self-pollen tubes in the style. The expression of S-RNase gene peaks at late stage of the development of the pistil (Clark et al. 1990), and produces a secreted protein that accumulates in the transmitting tract of the style and reaches high levels (Anderson et al. 1989; Ai et al. 1990). A number of studies (Anderson et al. 1989; Ai et al. 1990; Clark et al. 1990; Ioerger et al. 1991; Xue et al. 1996; Ishimizu et al. 1998) of sequence analysis of S-RNase genes identified from various sources demonstrated a regular pattern of five highly conserved domains and two hypervariable domains. (Fig. 3), HVa and HVb (Ioerger et al. 1991; Clark et al. 1990), which are adjacent to each other. Conserved domains C2 and C3 contain histidine residues where the catalytic site of the ribonuclease is formed (Ida et al. 2001).

Gain-of-function experiments (Lee et al. 1994), in which the coding sequence of the S_3-RNase of *Petunia inflata* was transferred to a plant having the S_1S_2-genotype, demonstrated that transgenic plants expressing the S_3-RNase proteins at a similar level to endogenous S-RNases had obtained the ability to reject S_3 pollen. Lee et al. (1994) also applied gene down-regulation of the S_3-RNase in S_2S_3-genotype plants. Plants with significantly suppressed expression of S_3-RNase were not able to reject S_3 pollen. In a later study, McCubbin, Chung and Kao (1997) transferred a variant of S_3-RNase of *Petunia inflata*, designated H93R (i.e. $S_{3\text{-H93R}}$), into an S_2S_3 background. The transgenic plant S_2S_3 ($+S_{3\text{-H93R}}$) showed a dominant-

A.



B.

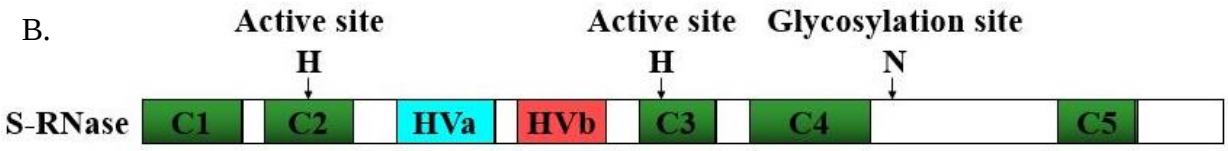


Figure 3: Conserved and variable domains of S-RNase. (A) Amino acid sequence alignment plot for eighteen Solanaceae S-RNase alleles using PlotSimilarity. The middle dotted line represents the average similarity score across the entire sequence. Peaks above the dotted line are conserved regions and valleys below are variable regions. The hypervariable regions V2 (HVa) and V3 (HVb) are thought to determine the recognition specificity of S-RNase (Sims 1993). (B) Schematic figure of domains within the S-RNase protein.

negative phenotype that only affected the S_3 allele. That is, when a S_2S_3 ($+S_{3\text{-H93R}}$) plant was self-pollinated, the transgenic plant was self-compatible. Crosses by pollen from other plants, however, indicated that the plant with dominant-negative phenotype had lost the ability to reject S_3 pollen but still retained the ability to reject S_2 pollen, i.e. the $S_{3\text{-H93R}}$ allele only interrupted the normal function of the S_3 allele. Additionally, there is one common aspect of the above experiments in that only the style recognition was modified, whereas pollen-part recognition was not affected, leading to the conclusion that the "pollen-S" protein was encoded by a separate gene from the S-RNase.

The ribonuclease activity of S-RNase was found to be the direct cause of pollen rejection (McClure et al. 1990). Researchers labeled pollen-tube RNA *in vivo* by watering plants with prepared ^{32}P-orthophosphate solution, and observed that pollen-tube RNA was degraded in incompatible pollinations while not degraded in compatible pollinations. Later studies (Huang, Lee, Karunanandaa, Kao, Ryo etal. 1994) confirmed that the ability of S-RNase to reject self-pollen could be eliminated by mutation of a histidine to an asparagine at the active site, i.e. removing the catalytic ribonuclease activity of the S-RNase. In addition, based on the fact that S-RNase proteins are glycoproteins, researchers (Woodward et al. 1989, Karunanandaa et al. 1994) also tested elimination of the glycosylation sites of S-RNase proteins, which resulted in no effect on the ability of S-RNase to reject self-pollen.

Non-S-Locus Stylar Proteins

Although in the style, the S-RNase plays a critical role in recognition and cytotoxicity, i.e. degrading pollen-tube RNA, other style-expressed proteins are also necessary to a successful self-incompatibility response, including HT-B and the 120 KDa protein.

In transgenic experiments involving different species of *Nicotiana*, Murfett et al. (1996) found that expression of the S-RNase gene in transgenic SC *Nicotianna plumbaginifolia* was inadequate for self-pollen rejection, while expression of the S-RNase gene in (*N. plumbaginifolia* X SC *N. alata*) hybrid plants resulted in successful self-pollen rejection. This indicated that some factor(s), other than S-RNase, must exist in *N. alata* that are not expressed in *N. plumbaginifolia*, and that the factor(s) must be required for self-pollen rejection. In a later study (McClure et al. 1999), a protein termed HT was isolated from self-incompatible *N. alata*, and down-regulation of HT in transgenic plants demonstrated the loss of ability to reject self-pollen, even though S-RNase was expressed normally in styles. O'Brien et al. (2002) extended this work by confirming two isoforms of HT, named HT-A and HT-B. Antisense experiments in transgenic plants showed that down-regulation of HT-B resulted in a conversion from SI plants to SC plants. Down-regulation of HT-A, however, had no effect on the SI response. In addition, the significance of HT-B to self-pollen rejection was also demonstrated from another point by analyzing different SC and SI species of tomatoes. Kondo et al. (2002a, b) showed that both S-RNase and HT-B proteins from SC tomato species are defective at different levels. The S-RNase gene in SC cultivated tomatoes could not be amplified from its genome, which suggested deletion of S-RNase gene most likely occurred. Transgenic tomato plants, in which the introduced functional S-RNase genes expressed at high levels, failed to gain the ability to reject self pollen.

Additionally, the HT-B genes in SC cultivated tomatoes could not be fully transcribed due to a stop codon in its open reading frame, which resulted in production of an abnormal protein.

Another style-expressed protein in addition to the S-RNase is the 120 KDa protein. Cruz-Garcia et al. (2005) hypothesized that S-RNase may be imported into pollen tubes as a complex that included more than one proteins. In a recent study, Goldraij et al. (2006) demonstrated that S-RNase imported into pollen tubes wass sequestered in a vaculolar compartment that was bound by the 120 KDa protein. The 120 KDa protein was also found to be required for self-pollen rejection in antisense experiments. Down-regulation of the120 KDa protein by RNA interference in *Nicotiana plumbaginifolia* X *N. alata* hybrids led to the inability to reject pollen carrying the same S allele.

SLF: the Pollen-Recognition Component

The first gene clone that turned out to be pollen-S (Lai et al. 2002) was published sixteen years after the first cDNA encoding S-RNase was identified (Anderson et al. 1986). The improvement in techniques of cloning DNA libraries with large inserts and sequencing long DNA fragments enabled identification of pollen-S as the S-locus F-box gene. Prior to such technological progress, previous studies were still able to predict the expected pollen-S in the following aspects: (1) plants with defective style- or pollen-expression were often found to retain fully functional GSI in the non-defective tissue, indicating that the style-recognition component (S-RNase) and the pollen-recognition component (expected pollen-S) were encoded by separate gene sequences. This indication was later supported by transgenic experiments, as described above, in which gain-of-function or loss-of-function methods only affected the specificity of S-

RNase but had no effect on the specificity of the pollen-recognition component (2) It was assumed that genes encoding S-RNase and pollen-S are physically linked or located in a chromosomal region that is suppressed from recombination (due to the observation that the S-RNase gene and pollen-S gene rarely recombine). (3) Pollen-S gene must be expressed in pollen with a pollen-specific pattern. (4) Physical interaction(s) between S-RNase and pollen-S was/were strongly implicated based on the models of GSI mechanism. (5) Pollen-S was expected to be similarly polymorphic as S-RNase, in which polymorphism occurred mostly in the hypervariable regions, due to the fact that the entire S-locus was suppressed from recombination.

To date, most evidence suggests a cytotoxic model for inhibition of pollen-S. That is the S-RNase terminates the elongation of pollen tubes by degrading pollen RNA and therefore inhibiting protein synthesis (Sims 2005). In compatible crosses, the ribonuclease activity of S-RNase is inactivated, although the exact pattern of such inactivation has not yet been fully deciphered. The other hypothesis was that S-RNase was not imported into pollen tubes and thus inhibited from acting inside pollen tubes. This was, however, proved not the case by direct observation of pollen tubes via transmission electron microscopy (TEM) and immunogold labeling of S-RNase, resulting in the observation that both compatible and incompatible S-RNases were imported into pollen tubes (Luu et al. 2000). In a more recent study, Goldraij et al. (2006) demonstrated that S-RNases imported into pollen tubes in *Nicotiana* pollen were sequestered in a vacuolar compartment in compatible pollinations and at the early stage of pollen-tube development in incompatible pollinations.

Early observations (Crane and Lewis 1942; Lewis and Modlibowska 1942; Brewbaker and Natarajan 1960; de Nettancourt 1977), when pollen-S remained elusive, predicted the existence of the pollen-expressed inhibitor of S-RNase via “competitive interaction” in tetraploid

plants. It was found that tetraploid heterozygous styles were able to reject haploid pollen with only one and matching S allele, whereas these same styles were not able to reject diploid, heteroallelic pollen containing two different S alleles. These results suggested that the non-self-pollen-S-allele of the two interacted with S-RNase competitively and inhibited its ribonuclease activity. This phenomenon, which causes breakdown of GSI on the pollen side was then termed "competitive interaction". In later transgenic experiments competitive interaction approaches also provided proof that S-locus F-box genes are in fact pollen-S.

Lai et al. (2002) identified a pollen-expressed F-box gene, named AhSLF, by screening a BAC library from *Antirrhinum hispanicum.* AhSLF was isolated as a 63 kb clone, and was located about 9 kb away from the S-RNase gene. McCubbin et al. (2000a) and Wang et al. (2003, 2004) also screened a BAC library but from S_2S_2 *Petunia inflata* and discovered one of 50 genes isolated from a region containing the S_2-RNase was in fact a pollen-expressed F-box gene, termed $PiSLF_2$ (Wang et al. 2004; Sijacic et al. 2004). $PiSLF_2$ was subsequently verified as pollen-S in transgenic experiments relying on the phenomenon of competitive interaction in pollen. Sijacic et al. (2004) transformed S_1S_1 *Petunia inflata* with a PiSLF2 gene construct. Transgenic plants, which expressed both the endogenous F-box gene PiSLF1 and the transgene PiSLF2, were found to be self-compatible, leading to the conclusion that self-incompatibility broke down because transgene PiSLF2 expressed in pollen competitively interacted with S_1-RNase. In addition, such breakdown in self-incompatibility only occurred in the pollen and did not affect the stylar ability to reject incompatible pollen. That is, pollen from the transgenic plant S_1S_1 (+PiSLF2) was compatible on wildtype S_1S_1 plants, while S_1 pollen from wildtype S_1S_1 plant was incompatible on the transgenic plant S_1S_1 (+PiSLF2), i.e. rejected in styles.

Similar transgenic experiments were also conducted by Qiao et al. (2004) via transforming S_3S_3 *Petunia hybrida* with AhSLF-S_2 pollen –F-box gene as well as the S_2-RNase gene from *A. hispanicum*. Transgenic plants, containing both complete genes and expressing both at comparable level as endogenous F-box and S-RNase genes, were self-compatible and also compatible with pollen from wildtype S_3S_3 *Petunia hybrida*. This could also be predicted based on competitive interaction between different pollen-S genes expressed in the same pollen grain. Given the results from Sijacic et al. (2004) and Qiao et al. (2004), it has been clear that the F-box gene is responsible for self-pollen rejection on the pollen side and encodes pollen-S protein of GSI. Results from Qiao et al. (2004) additionally demonstrated the conservation of pollen-S genes since AhSLF-S_2 pollen –F-box only shared 30% amino-acid similarity with endogenous *P. hybrida* SLF genes.

SBP1: the Pollen-Expressed S-RNase-Binding Protein

Sims and Ordanic (2001) intended to isolate pollen-expressed proteins that interacted with the S-RNase, and therefore screened a yeast two-hybrid library from mature pollen of *Petunia hybrida* with a bait construct of the N-terminal part of the *P. hybrida* S_1-RNase. A gene named PhSBP1 (i.e. *Petunia hybrida* S-RNase binding protein) was found to bind to N-terminal but not C-terminal regions of the S-RNase. Sequencing results indicated that PhSBP1 contained a C-terminal RING-HC protein domain, which has been known to be related to E3 ubiquitin ligases (Fig. 4) and suggests a potential role for ubiquitination in GSI. Meanwhile, F-box protein is known as a component of SCF-type (i.e. Skp1, Cullin, F-box containing) E3 ubiquitin ligase, which ubiquitinates target proteins and degrades them via the 26S proteosome. Other than Skp1,

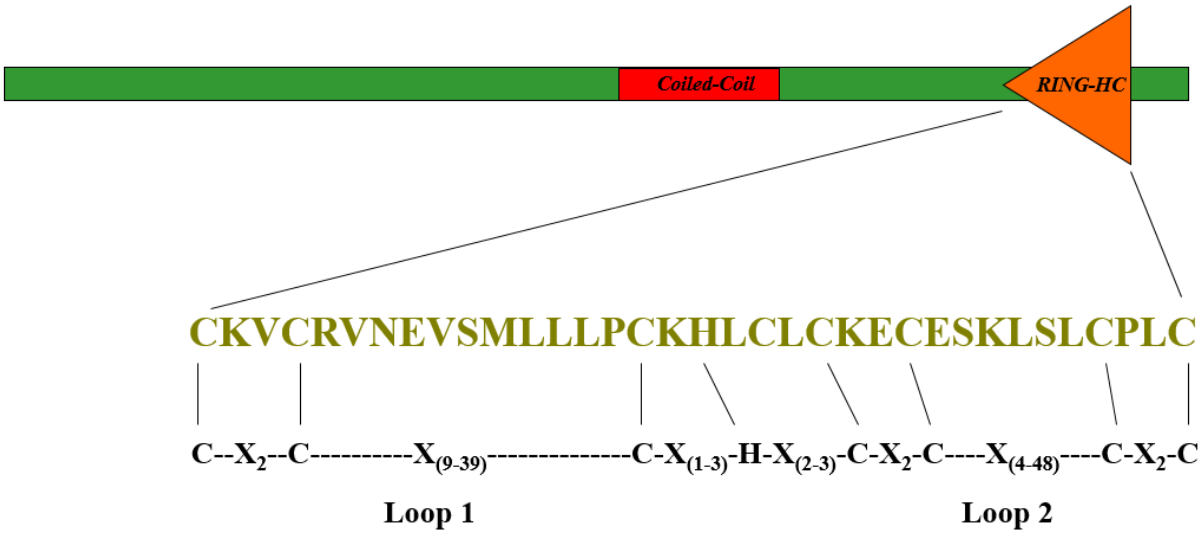


Figure 4: PhSBP1 protein-domain structure. PhSBP1 contains a C-terminal RING-HC protein domain, which is known to be related to E3 ubiquitin ligases and suggests a potential role for ubiquitination in GSI.

Cullin and F-box proteins, the SCF complex contains a RING protein RBX1 (Cardozo and Pagano 2004; Schwechheimer and Villalobos 2004). In later studies, O'Brien et al. (2004) and Hua & Kao (2006) isolated SBP1 orthologues from *Solanum chacoense* (i.e. ScSBP1) and *Petunia inflata* (i.e. PiSBP1), and confirmed the interactions between SBP1 and S-RNase proteins. In addition, SBP1 was demonstrated to have E3 ubiquitin ligase activity *in vitro* (Hua and Kao 2006; Sims, unpublished), and was also shown to interact with SLF *in vitro* (Hua and Kao 2006; Sims et al. 2010). To date, however, little effort has been made to discover the pattern of protein interaction between SBP1 and numerous SLF variants *in vivo*.

SSK1: a Skp1-Like Protein

Huang et al. (2006) screened a yeast two-hybrid library with AhSLF-S_2 as bait and identified a Skp1-like protein, termed AhSSK1 which interacted with AhSLF and was subsequently found to interact with another component of SCF-type E3 ubiquitin ligase, CUL1. In a later study (Zhao et al. 2010) cloned the orthologue of AhSSK1 from *Petunia hybrida* (PhSSK1), and showed that it interacted with PhSLF-S_{1L} and PhSLF$_{3L}$. Additionally, down-regulation of PhSSK1 in transgenic *Petunia hybrida* led to significantly lowered fertility of compatible pollen, i.e. loss of ability to recognize and inhibit the ribonuclease activity of S-RNase after SSK1 was down-regulated.

SCF^{SLF} E3 Ubiquitin Ligase Complex

Given the results from experiments described above, a putative SCF^{SLF} model including specific protein-protein interactions is considered the most reasonable mechanism by which S-RNase proteins are recognized as self versus non-self. The model can be explained as follows: pollen grains, both self and non-self, are deposited on the stigmatic surface during pollination. They germinate and produce pollen tubes that start extending in the transmitting tract of the style, where they meet with S-RNase. Both self and non-self S-RNases are imported into pollen tubes. In styles, S-RNase of a different haplotype from that expressed in pollen will be recognized as non-self by a SCF^{SLF} E3 ubiquitin ligase complex, followed by ubiquitination and eventually degradation via the 26S proteasome. S-RNase recognized as self will not be targeted for ubiquitination leading to retention of ribonuclease activity, and will act to degrade pollen-tube RNA, thus inhibiting protein synthesis and terminating the elongation of pollen tubes.

This putative SCF^{SLF} complex, however, does not contain exactly the same components as the typical SCF-type complex, which has Skp and Cullin proteins. Hua and Kao (2006) found no binding occurred between SKP1 proteins and PiSLF. In similar experiments (Huang et al. 2006), interaction between SKP1 in *Antirrhinum* and AhSLF was not observed. As described above in section, "SSK1," Huang et al. (2006) isolated a SKP1-like protein, AhSSK1, and confirmed its binding to AhSLF and CUL1 (i.e. Cullin protein). Huang et al. (2006) also proposed that AhSSK1 may act as a bridge between AhSLF and CUL1. In addition, the typical SCF-type complex contains a small RING domain protein termed RBX1. No interaction was detected between RBX1 (from *Petunia inflata*) and either SLF or CUL1 (Hua and Kao 2006). On the contrary, Li et al. (2014) identified RBX1 as a part of SCF-type complex by using SLF as a bait in co-immunoprecipitation followed by mass spectrometry. In addition, both Sims (unpublished) and Hua & Kao (2006), however, found that SBP1 interacted with an E2 ubiquitin conjugating protein PhUBC1 in vitro, and that SBP1 interacted to SLF *in vitro* (Hua and Kao 2006; Sims et al. 2010). Together, the SCF^{SLF} E3 ubiquitin ligase complex may function in gametophytic self-incompatibility with components including SLF, SBP1 (or RBX1), CUL1, and SSK1 (replacing SKP1). This complex is now the most widely accepted as the model of recognizing and inhibiting non-self S-RNase in GSI.

For clarity, protein interactions involved in the SCF^{SLF} E3 ubiquitin ligase complex are summarized as follows: (1) S-RNase interacts with SLF and with SBP1 as well. Pull-down assays using style extracts showed SLF preferentially interacted with non-self S-RNase while not interacting with self S-RNase (Hua and Kao 2006). In addition, yeast two-hybrid assays also showed similar preference of non-self versus self interactions (Sims et al. 2010). (2) SBP1 interacts with S-RNase, SLF, Cullin-1 and PhUBC1, an E2 ubiquitin-conjugating enzyme (Sims

and Ordanic 2001; O'Brien et al. 2004; Hua and Kao 2006; Sims et al. 2010; Sims unpublished). (3) SSK1 interacts with SLF and Cullin-1 (Huang et al. 2006; Zhao et al. 2010). Together, the SCF^{SLF} E3 ubiquitin ligase model is presented below (Fig. 5).

Vacuolar Sequestration of S-RNase

Another model of recognition and inhibition of non-self S-RNase is the vacuolar-sequestration model (Fig. 6). As described in the section "Non-S-Locus Stylar Proteins," two style-expressed proteins, HT-B and the 120 KDa protein, have been demonstrated to be correlated with self-incompatibility (McClure et al. 1999; Hancock et al. 2005). Goldraij et al. (2006) observed that S-RNase, HT-B and the 120 KDa protein were all imported into both compatible and incompatible pollen tubes that were extending. In compatible pollination, it appeared that S-RNase was sequestered in a vacuolar compartment bounded by the 120 KDa protein, and that HT-B was degraded in compatible pollen tubes. In incompatible pollinations, however, such vacuolar compartments broke apart at the late stage of pollination, resulted in release of cytotoxic S-RNase into cytoplasm. HT-B remained intact in incompatible pollen tubes whereas 120 KDa protein was no longer present.

Given all researches accomplished so far, two models have been proposed. Since in the sequestration model it is still elusive what the role of SLF might be, and how an S-RNase that is sequestered in a vacuole interacts with SLF, current data tends to support ubiquitination as the key factor in the GSI response and as the consequence of being recognized as non-self S-RNase.

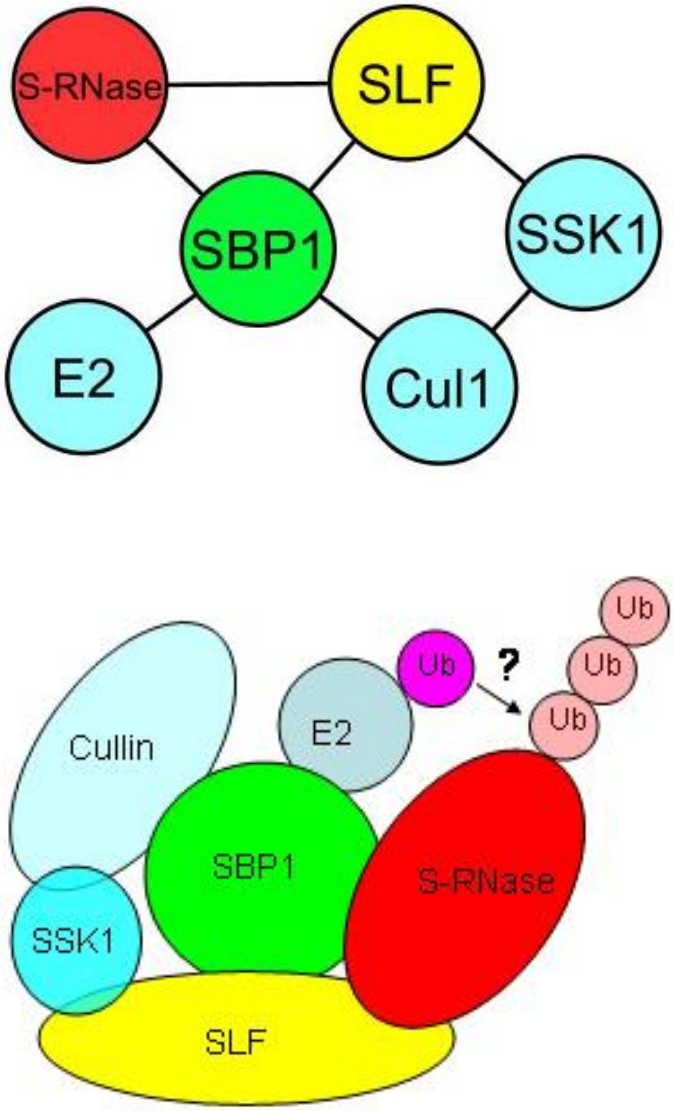


Figure 5: Proposed SCF^{SLF} E3 ubiquitin ligase model. Upper figure: the lines connecting the circled proteins indicate observed protein interactions. Bottom figure: model of proposed SCF^{SLF} E3 ubiquitin ligase complex thought to recognize and degrade non-self S-RNase.

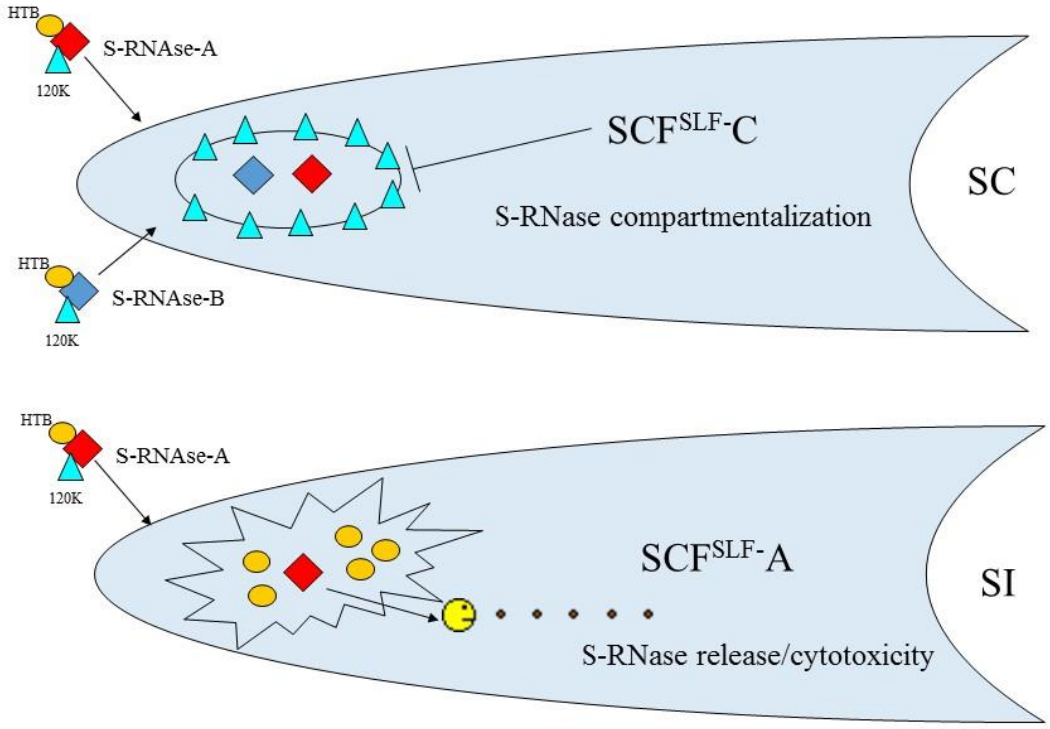


Figure 6: Model of vacuolar sequestration of S-RNase. Upper-half of figure: in compatible pollen tubes, S-RNase is sequestered in a vacuolar compartment bounded by the 120 KDa protein, and HT-B is degraded. Bottom-half of figure: in incompatible pollen tubes, vacuolar compartment breaks down leading to release of cytotoxic S-RNase into cytoplasm. HT-B remains intact in incompatible pollen tubes whereas the 120 KDa protein is no longer present.

Collaborative Recognition of S-RNase Alleles

Prior to the identification of SLF as pollen-S, one of the predictions was that Pollen-S was expected to be similarly polymorphic as S-RNase due to the fact that the entire S-locus was suppressed from recombination (see section "SLF" above). Further studies demonstrated this prediction was partially true. Polymorphism of SLF did exist, however, it was not located in the hypervariable regions as found in S-RNase alleles. Instead, polymorphism of SLF was found distributed across the entire SLF-protein sequence, which was puzzling because based on previous studies the recognition specificities of pollen-S (SLF) and pistil-S (S-RNase) had co-evolved; therefore, a similar format of polymorphism was expected to be present in both proteins located mostly in hypervariable regions (de Nettancourt 1977; Zhang & Xue 2008; Sims & Robbins 2009). A more recent observation (Kubo et al. 2010) also indicated similar mystery. That was SLF proteins from two different S-haplotypes, i.e. *P. hybrida* S_7 and *P. axillaris* S_{19}, were identical, despite the fact that S_7- and S_{19}-RNases shared only 45% identity. Further screening of cDNA libraries, together with characterizing genes previously identified as SLFL (SLF-like) genes, discovered 6 subclasses of SLF genes, designated from SLF1 to SLF6. Immunoprecipitation assays showed that S_7-SLF2 reacted with S_9- and S_{11}-RNases but not with S_5- or S_7-RNases (Kubo et al. 2010). It suggested that in pollen different SLF subtypes (in this case S_7-SLF1 to S_7-SLF6) were expressed as a group, and interacted with different S-RNase alleles to different extents, i.e. instead of single SLF, several subtypes of SLF variants recognize S-RNase as self versus non-self in a collaborative fashion (Fig. 7).

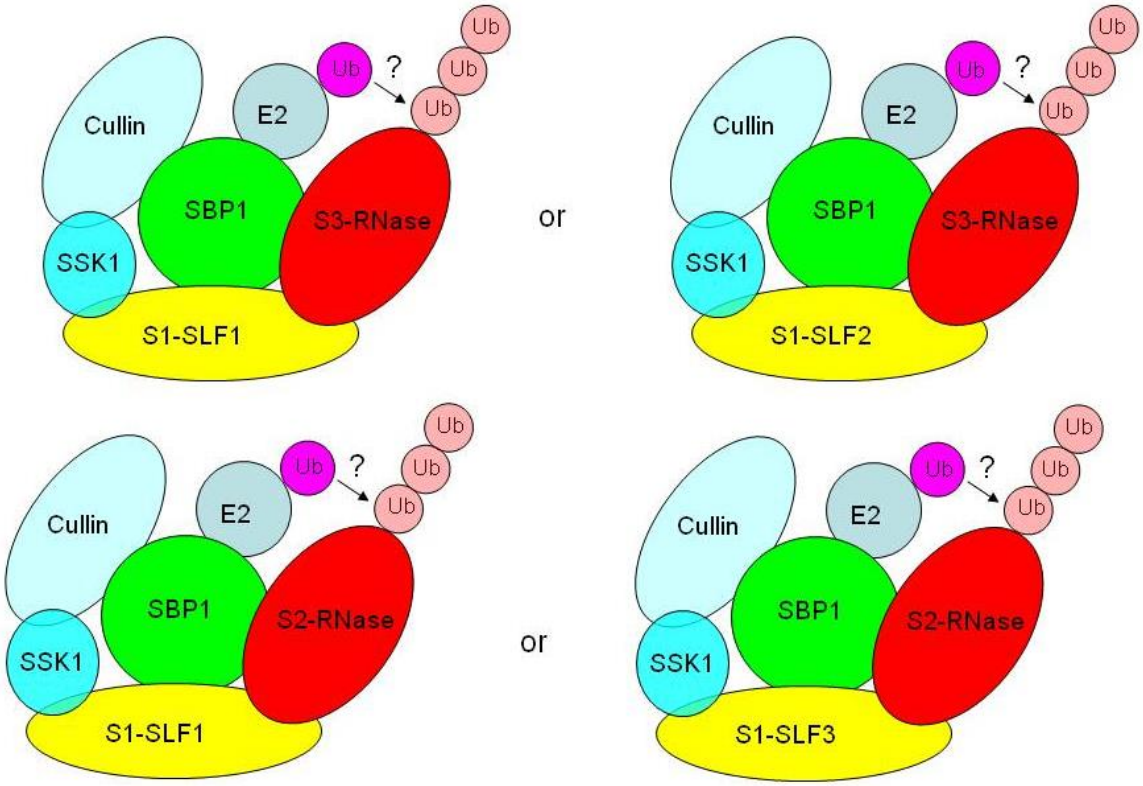


Figure 7: Hypothetical combinatorial recognition of S-RNase by SLF subtypes. Images show one example: S_1-SLF1 and S_1-SLF2 subtypes can recognize S_3-RNase, whereas S_1-SLF1 and S_1-SLF3 subtypes can recognize S_2-RNase, which implies that S_1-SLF2 subtype is not able to recognize S_2-RNase.

The Petunia Genome Project

For the past a few years, my advisor Dr. Sims has been coordinating an international effort to sequence and assemble the genomic DNA sequences for *Petunia axillaris* and *Petunia inflata*. Based on the sequence data, most known SLF variants appear to be present in both genomes, and a large number of those are functional according to the expressed protein sequence. The most striking finding, however, is that S-RNase sequence in the *Petunia axillaris*

line, now designated as S_{ax1}, used for sequencing appears to be 100% identical to the S_1-RNase in the *Petunia hybrida* line that has been used in our lab for years! This finding strongly suggests that the S-locus haplotype is identical in these two lines, which should enable identification and isolation of all potential SLF variants from both *P. hybrida* as well as *P. axillaris*.

Rationale and Preview

Much has been introduced so far about the genetic and molecular mechanisms that control self versus non-self recognition during pollination. Current understanding and models suggest that specific protein interactions mediated by an SCF^{SLF} E3 ubiquitin ligase complex are critical to recognition, acceptance or rejection of pollen grains. Many questions are yet to be answered. However, the emphasis of my research is on the investigation of potential protein interactions, in particular SLF::S-RNase interaction and, SLF::SBP1 interaction. Since it has been interpreted that multiple SLF variants may function collaboratively to govern the S-RNase recognition, it becomes important to identify and isolate the total population of SLF variants in different GSI backgrounds, for the purpose of determining which SLF variants interact with which S-RNase alleles, and which interactions are crucial to GSI recognition. To date, the majority of protein-interaction assays used for the analysis of GSI are either the yeast two-hybrid system or some variation on *in vitro* pull-down assays (his and GST tags) or co-immunoprecipitation from extracts, however I decided to choose Bimolecular Fluorescence Complementation (BiFC) to detect *in vivo* protein-protein interactions. Compared with other two techniques, BiFC presents protein interactions in a natural environment, which provides less-artificial evidence of the existence of interactions between certain proteins. Another reason is

that so far little effort has been made to interpret interactions present among GSI proteins by using BiFC. In addition to investigating protein interactions per se, I wished to develop methodologies that could analyze protein interactions in living cells. Experimental methods described in the next chapter were mainly employed (1) to identify which SLF variants are present in our current stocks, e.g. S_1S_1 *P. hybrida*, (2) to determine the presence of interactions *in vivo* of those variants with S_1- and S_3-RNase proteins, and (3) to demonstrate specific protein domains required for SLF::S-RNase interaction *in vivo*, as well as interaction with other components of the SCF^{SLF} E3 ubiquitin ligase complex. Other approaches, such as semi *in vivo* pollinations localizing S-RNase at the subcellular level at pollen-tube tip, down-regulation of SBP1 studying the role of SBP1 in GSI, western blots monitoring the protein-expression levels *in vivo*, and bioinformatics analyses identifying potential regions at which SLF recognizes S-RNase, were also applied.

Significance

S-RNase-based gametophytic self-incompatibility is a barrier to inbreeding, which affects numerous types of fruit, vegetable, and floriculture crops in the Solanaceae and Rosaceae, including apples, tomatoes, potatoes, almonds, cherries, peaches, pears, and *Petunia*. *Petunia hybrida*, which has been studied in our lab for years, is important both as one of the major economic plants in the US and as a model species for research in plant molecular biology. The work mentioned in this dissertation is directly relevant: (1) to plant genome structure and function by providing information such as number, sequence similarity and function of a family genes encoding SLF proteins that function in GSI; (2) to molecular and cellular genetics of GSI

systems that lead to future plant biotechnology of manipulating breeding barriers for crop improvement; (3) to sustainability of U.S. agricultural and food systems by providing fundamental information of reproductive processes, including GSI, of crops and economic plants.

CHAPTER 2

MATERIALS AND METHODS

Semi *in vivo* Pollination

First, *Petunia hybrida* plants were grouped based on their genotypes, i.e. S-locus genes, and the tentative pollinating combinations were arranged according to the S-locus genes carried by pollen donors. They were either self crosses, i.e. generally incompatible pollinations, or crosses in which S-locus genes carried by the pollen donor did not match those carried by the pollen acceptor, i.e. compatible pollinations. Next, the style of the pollen acceptor was pollinated by contact with isolated anthers from the pollen donor so that pollen grains were deposited onto the surface of the stigma. Temperature and light conditions were recorded while pollinating by hand. Then, pollinated styles were collected after different time intervals, e.g. 6, 12, 18, 24, 36, and 48 hours; fixed in Acetic acid/EtOH (1:3) solution for at least 2 hours at room temperature; sequentially rehydrated in 70%, 50%, 30% EtOH and lastly distilled water; treated with 8 M NaOH at room temperature overnight; rinsed with distilled water; stained with 0.1% (w/v) aniline blue in 0.1 M K_3PO_4 (pH 9) for at least 2 hours under dark conditions at room temperature. Finally, each prepared style was gently squashed on a microscopy slide by a cover slip, and observed under Nikon Eclipse E-600 microscope using the UV-2B filter and epifluorescence. Pollen tubes were visible in between or underneath squashed style tissues, and glowing as bright blue fluorescence (Fig. 2). Images could be saved on a computer connected

with the camera for future use. Based on the scale and the length of pollen tubes measured from captured images, the length that pollen tubes had extended and the distance that pollen-tube tips are away from the base of the style could be accurately determined. For reproducibility, more than 20 replicates were performed for each pollinating combination. Eventually, by pooling all the obtained data, individual pollinating durations would be confirmed, i.e. knowing when after being pollinated the styles should be collected from petunia plants.

In order to allow pollen tubes to grow continuously, styles were harvested after being pollinated for the confirmed pollinating time interval, and incubated on the growing medium [20 mM MES (pH 6); 3 mM $Ca(NO_3)_2$-$4H_2O$; 1 mM KNO_3; 0.8 mM $MgSO_4$-$7H_2O$; 1.6 mM H_3BO_3; 0.5% Agarose] till pollen tubes reached out of the base of the style. Repeated tests indicated how long styles needed to be incubated on growing medium and when pollen tubes were just emerged from the style base for each pollinating pair.

In summary, isolating pollen-tube tips and resizing for the sample-holding "cap" on high-pressure freezing equipment was used to determine the subcellular location of S-RNase at the pollen-tube-tip by performing semi *in vivo* pollinations.

RNA Interference: SBP1 Down-Regulation via Gateway® Cloning

At first, Gateway® PCR primers for SBP1 were designed by adding "attB site" cassettes to each template-specific primer (attB1 site "GGGGACAAGTTTGTACAAAAAAGCAGGCT" to the 5' end of forward primer; attB2 site "GGGGACCACTTTGTACAAGAAAGCTGGGT" to the 3' end of reverse primer). Next, attB-PCR-amplified SBP1 (150 ng) was mixed with 150 ng of plasmid DNA of pDONR vectors (Fig. 8) in TE buffer (pH 8.0). After addition of BP clonase

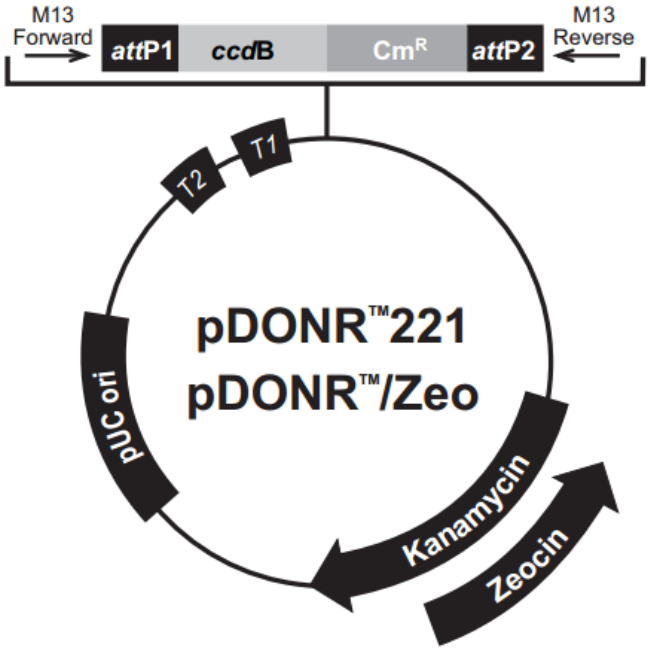


Figure 8: Map of pDONR/pDONR-Zeo vector. Both contain attP sites but carry different antibiotic resistance. The pDONR vector can be selected on Kanamycin medium and pDONR-Zeo is resistant to Zeocin.

and incubation at room temperature for at least one hour, recombination occurred between "attB" sites on PCR-amplified SBP1 and "attP" sites on the pDONR vector, leading to SBP1-pDONR constructs with "attL" sites flanking the inserted SBP1 sequence, known as "BP reactions" (Figs. 9, 10). Each BP reaction was transformed into *E. coli* through electroporation and subjected to plasmid DNA extraction.

Next, 150 ng of plasmid DNA of the SBP1-pDONR construct, i.e. the entry clone, was mixed with 150 ng of plasmid DNA of the destination vector pSTARGATE (Fig. 11) in TE buffer (pH 8.0). After addition of LR clonase and incubation at room temperature for at least one hour, recombination occurred between the "attL" sites of the SBP1-pDONR construct and "attR" sites of pSTARGATE vectors, leading to final SBP1-RNAi constructs with "attB" sites flanking the inserted SBP1 sequence, known as "LR reaction" (Figs. 9, 10). Each LR reaction was transformed into *E. coli* through electroporation and subject to plasmid DNA extraction. Preliminary results indicated that overnight incubation of LR reaction at room temperature often yielded more colonies in transformation. Next, extracted plasmid DNA of SBP-RNAi constructs was transformed into Agrobacterium. One reason for not directly transforming the LR reaction into Agrobacterium was that the LR reaction eventually gave a mixture, rather than single type of DNA, of successfully constructed SBP1 and SBP1-pDONR constructs not recombined with the pSTARGATE vector, which might complicate the following Agrobacterium colony selection. Another reason was that *E. coli* was considered as a better storage host than Agrobacterium. At last, selected SBP1-RNAi-Agro constructs were transformed into plant tissues by the infecting activity of Agrobacterium, which induces RNA interference *in vivo* by forming hpRNA and silencing the genes encoding SBP1 protein.

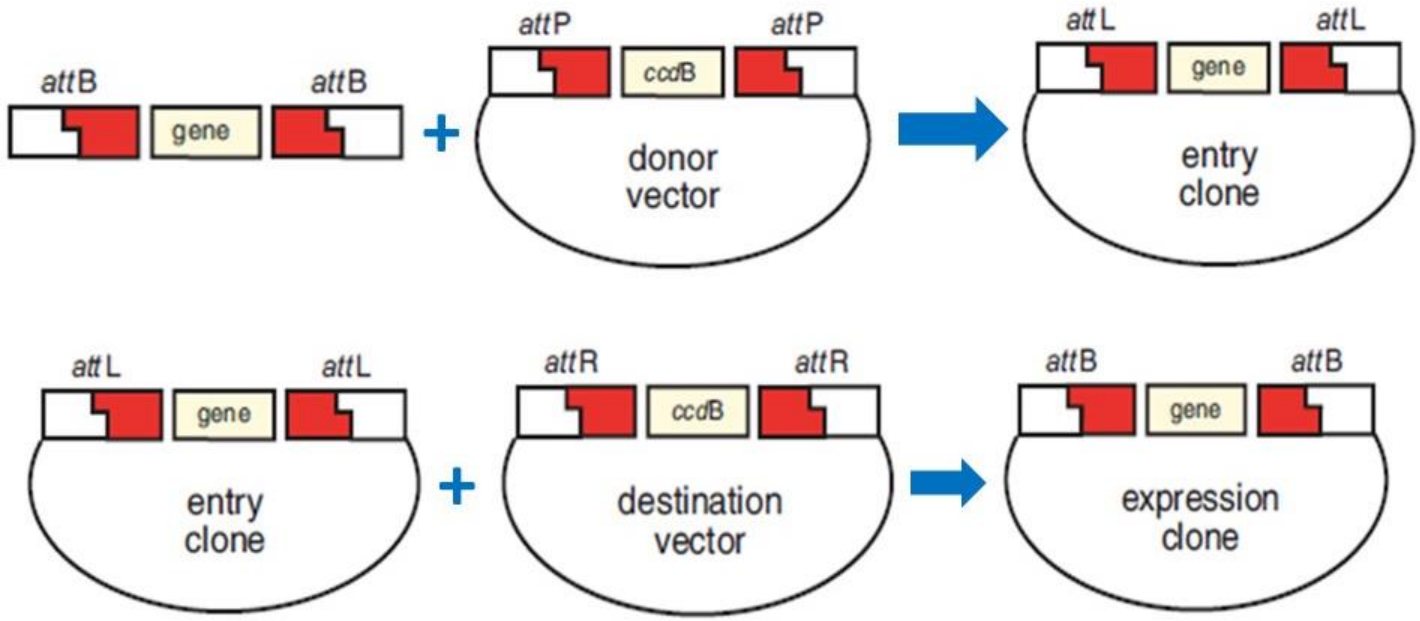


Figure 9: Two-step Gateway® cloning procedure. The upper-half of the figure shows the "BP reaction" in which the target gene amplified by attB-site flanking primers is inserted into pDONR vector, producing an entry clone. The bottom-half of the figure shows the "LR reaction" in which the target gene is inserted into destination vector, in this case pSTARGATE, and produce an expression clone, i.e. an RNAi construct.

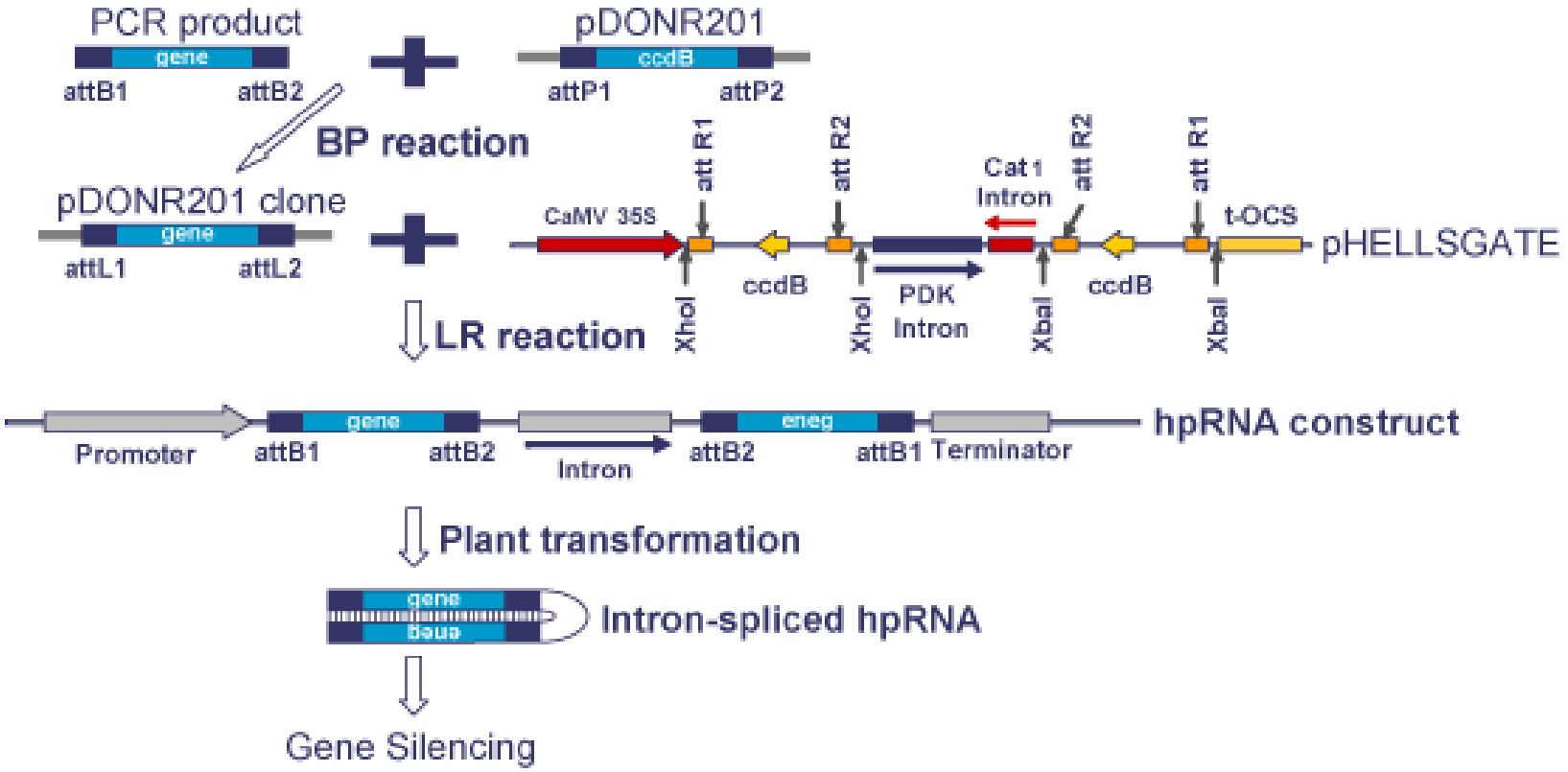


Figure 10: Complete constructing process for RNAi. Preparing RNAi constructs by Gateway cloning, eventually resulting in hairpin RNA and gene silencing of target gene in living plant tissue. pHELLSGATE shown in figure is a similar RNAi vector to pSTARGATE but containing a different promoter (in pSTARGATE it is a 35S promoter).

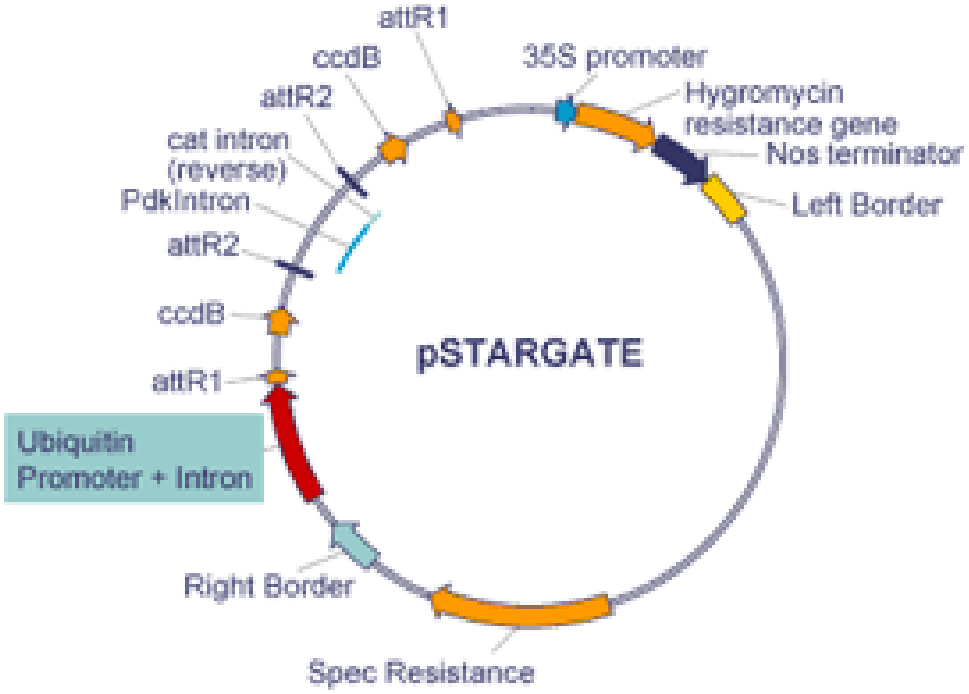


Figure 11: Map of pSTARGATE vector. pSTARGATE vector contains attR sites required in LR reaction, 35S promoter functioning in plant cells, and ubiquitin promoter functioning in pollen and styles.

Plant Transformation, Selection, and Rooting

To inoculate the leaf explants, overnight culture of SBP1-RNAi-Agro construct was prepared and diluted as 1 to 200 with Sigma Murashige and Skoog salts and vitamins medium (4.4 g/L). Next, fully-expanded top leaves from S_1S_1 *Petunia hybrida* 90FS2D3 were harvested, sterilized for 15 minutes in 10% solution of household bleach containing 0.1% Tween, rinsed thoroughly with sterile distilled water, and cut into small squares (1 x 1 cm^2) to produce

wounded edges where Agrobacterium infects. Next, leaf explants were inoculated by diluted overnight culture of SBP1-RNAi-Agro construct in petri dishes for 20 minutes.

After inoculation, explants were sandwiched between two layers of sterile filter paper to remove excess liquid Next, explants were placed with adaxial surface down on petri dishes with Co-cultivation medium (Murashige & Skoog salt pH 5.8; 0.112 g/L Vitamin B5; 30 g/L Sucrose; 2 mg/L 6-BAP; 0.01 mg/L NAA; 8 g/L Agar), and incubated for 2-3 days in an incubator (25 ℃, 14h light/10h dark). Next, explants were transferred to Regeneration and Selection medium (Same recipe as Co-cultivation medium but adding antibiotics such as 250 mg/L cefotaxime to kill untransformed Agrobaterium, and 100 μg/L spectinomycin for pSTARGATE selection), and incubated for 2-3 weeks in incubator till fresh shoots were seen.

Next, shoots were excised and placed upright onto Rooting medium (Murashige & Skoog salt pH 5.8; Vitamin B5; 30 g/L Sucrose; No 6-BAP or NAA; 7 g/L Agar; and antibiotics), and incubated for 4-6 weeks in incubator till roots are formed (Meer 2005). At last, the rooted tissues were cautiously transferred into soil and incubated in the greenhouse in the Department of Biological Sciences till transformants were ready for further analysis.

Multiple Sequence Alignment of S_1-SLF Genes

At first, 40 Genbank entries of SLF variants (SLF1-SLF8), plus the SLF sequences previously sequenced by our laboratory, were downloaded and grouped in text-based files in FASTA format. In bioinformatics, FASTA format is one of the most frequently used formats representing either nucleotide sequences or amino acid sequences. Next, multiple sequence alignment was performed using ClustalX (Thompson et al. 1997) in which nucleotides are

labeled with different colors so that conserved regions, i.e. sequences with the highest similarity, can be easily identified. The results of such analyses provided templates to design PCR primers for amplifying SLF variants from total plasmid DNA of the S_1S_1 cDNA library in our lab. In some cases, primers were designed according to highly conserved internal gene regions, rather to the less conserved 3'-ends, to determine whether a particular variant was present in our plasmid DNA stocks. Eventually, all PCR-amplified SLF variants were used as probes for subsequent plaque hybridizations.

Plaque Hybridization

At first, the phage library containing S_1S_1 *Petunia hybrida* cDNA was plated on NZY amine plates and incubated overnight at 37 ℃. The next day, plaques grown on plates were transferred to nylon membranes via plaque lifting which caused cDNA adhering to the membrane. Next, transferred membranes were denatured in 0.5 N NaOH/ 1.5 M NaCl, neutralized in 0.5 M Tris-HCl, pH 8.0/ 1.5 M NaCl, incubated with 2X SSC (0.3 M NaCl, and 0.03 M Na_3Citrate), and hybridized with PCR-amplified SLF probes, which had already been labeled with ^{32}P-dCTP, at the temperature that was 18 to 20 ℃ below the annealing temperature applied in previous PCR. Hybridization buffer used was: 50% Formamide, 5X SSPE (0.9 M NaCl, 0.05 M NaH_2PO_4 pH 7.4, and 5 mM Na_2EDTA), 1X Denhardt's (0.02% Ficoll, 0.02% PVP, and 0.02% BSA).

Hybridized membranes were washed twice by low-stringency buffer (2X SSC/ 0.2% SDS), twice by high-stringency buffer (1X SSC/ 0.1% SDS), and exposed to X-ray films which produced images of positive plaques. Given the density of plating used, it would be very likely

that any positive signals would come from regions of the petri dish where there were overlapping plaques. Therefore it was necessary to purify positive plaques by re-plating and re-hybridizing. Eventually, a number of single-positive plaques obtained were subject to phagemid excision to extract plasmid DNA containing SLF-gene insertions. One advantage of screening cDNA library is that all library clones have already been in GAL4-AD vectors, so they can be directly used in Yeast two-hybrid (Y2H) assays with current S_1- and S_3-RNase bait constructs, for the purpose of investigating protein interactions between SLF and S-RNase.

TA Cloning and Subcloning

The alternative approach besides plaque hybridization is to use PCR-based methods to clone SLF variants into TA vector pGEM-T (Fig. 12), followed by subcloning into Y2H vectors. Due to the 3'-A overhangs produced by Taq polymerase, PCR-amplified SLF variants were able to be ligated with pGEM-T vector which is a linearized vector with a single, 3'-T overhangs. Ligation reactions were set up by mixing 2X Ligation buffer, 50 ng pGEM-T vector DNA, optimized amount of PCR-amplified SLF genes, 3 units of T4 DNA ligase, and nuclease-free water to a final volume of 10μl. Next, ligation reactions were transformed into E. coli competent cells, and colonies were selected for plasmid DNA extraction. Next, plasmid DNA of SLF-pGEM-T constructs was isolated, sequenced to confirm the presence and orientation of inserted SLF sequences, and directionally subcloned into GAL4-AD and GAL4-BD vectors via digestion by selected restriction enzymes and ligation. On both pAD- (Fig. 13) and pBD-GAL4 vectors (Fig. 14), there are sequences designed as multiple cloning site regions which contain various types of restriction sites and allow directional subcloning by choosing restriction enzymes shared

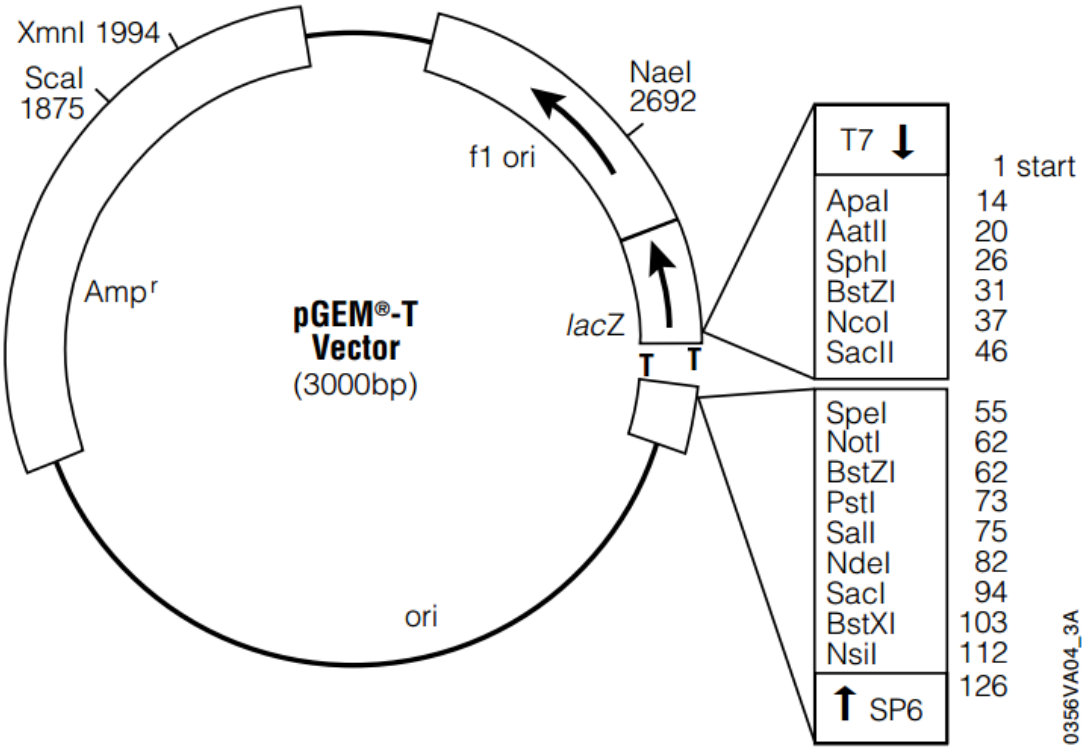
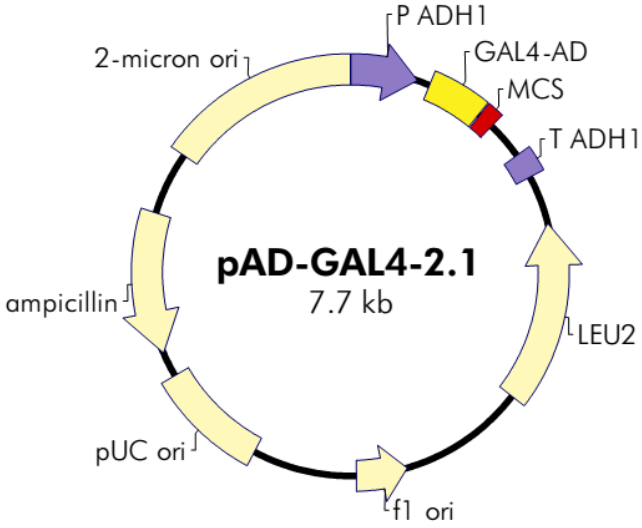


Figure 12: Map of pGEM-T vector. TA cloning occurs between two T-overhangs. Various restriction sites on pGEM-T vector are available for subcloning into yeast two-hybrid vectors following the TA cloning.

yeast *ADH1* promoter 4–408
***GAL4* activation domain** 488–829
multiple cloning site 839–935
yeast *ADH1* terminator 1168–1318
yeast *LEU2* ORF 1615–2709
f1 origin 3483–3789
pUC origin 4427–5094
ampicillin resistance (*bla*) ORF 5245–6102
2μ yeast origin 6489–7653



pAD-GAL4-2.1 Multiple Cloning Site Region
(sequence shown 812–958)

end of GAL4 activation domain

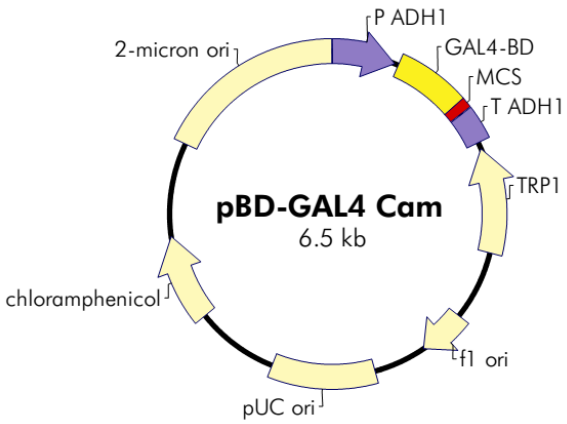
```
                                                BamH I          Nhe I           EcoR I
5´ CCA AAC CCA AAA AAA GAG ATC GAA TTA GGA TCC TCT GCT AGC AGA GAA TTC AAT...

                        Xho I                   Sal I   Xba I
...TCT CTA ATG CTT CTC GAG AGT ATT AGT CGA CTC TAG AGC CCT ATA GTG AGT CGT ATT...
                                                                T7 promoter

    Pst I       Bgl II
...ACT GCA GAG ATC TAT GAA TCG TAG ATA CTG AAA AAC 3´
                  STOP      STOP      STOP
```

Figure 13: pAD-GAL4 vector. Multiple cloning site region (MCS) provides various options of restriction enzymes for subcloning from pGEM-T constructs.

yeast *ADH1* promoter 4–408
***GAL4* DNA binding domain** 434–877
multiple cloning site 878–941
yeast *ADH1* terminator 948–1154
yeast *TRP1* ORF 1197–1871
f1 origin 2322–2628
pUC origin 2970–3637
chloramphenicol resistance ORF 4174–4725
2μ yeast origin 5330–6489



pBD-GAL4 Cam Multiple Cloning Site Region

(sequence shown 854–992)

end of GAL4 binding domain | EcoR I | Srf I | Sal I

```
5´ CAA AGA CAG TTG ACT GTA TCG CCG GAA TTC GCC CGG GCC TCG AGC CCG GGT CGA...

                    T7 promoter
...CTC TAG AGC CCT ATA GTG AGT CGT ATT ACT GCA GCC AAG CTA ATT CCG GGC GAA...

...TTT CTT ATG ATT TAT GAT TTT TAT TAT TAA A 3´
      STOP  STOP          STOP
```

In the MCS of the pBD-GAL4 Cam phagemid vector, there is a non-unique *Xba* I site upstream of the T7 promoter. This *Xba* I site contains the UAG amber suppressor in the same translational reading frame as the GAL4 domain. DNA should therefore be inserted such that the *Xba* I site is not between the GAL4 domain and the DNA insert. The complete sequence and list of restriction sites can be found at *www.stratagene.com*.

Figure 14: pBD-GAL4 vector. Multiple cloning site region (MCS) provides various options of restriction enzymes for subcloning from pGEM-T constructs.

by pGEM-T vectors. The constructs of SLF genes in pAD/pBD vectors obtained via such PCR-based cloning method can also be used to test protein interactions in yeast two-hybrid assays.

Petunia axillaris Genomic Sequences and Gateway® Cloning

Since the S-locus is suppressed for recombination, together with the fact that S-RNase and SLF genes are tightly linked to each other, such identity indicates that the entire S-locus of the *P. axillaris* line and that of the S_1 haplotype of *P. hybrida* are largely identical. Therefore using the sequence data of *Petunia axillaris* to identify and isolate SLF variants of *Petunia hybrida* becomes practical.

To isolate SLF variants from the total sequence data of *P. axillaris*, SLF sequences from various sources that have already been identified were used as probes. Due to the fact that a certain subtype of SLF variants, e.g. S_1-SLF1, S_2-SLF1, S_6-SLF1, etc. (S_x represents the type x S-locus gene; SLF1 indicates the subtype 1 SLF variant), are highly conserved even if they were from different species, these probes were able to search within the sequence data of *Petunia axillaris* and match the SLF genes with high similarity that could also be the SLF variants existed in *Petunia hybrida*.

Next, according to individual subtypes of SLF sequences obtained, Gateway® primers were designed by adding "attB site" cassettes to each template-specific primer (attB1 site "GGGGACAAGTTTGTACAAAAAAGCAGGCT" to the 5' end of forward primer; attB2 site "GGGGACCACTTTGTACAAGAAAGCTGGGT" to the 3' end of reverse primer).. Next, PCR reactions were carried out by using either DNA of *P. hybrida* or *P. axillaris* as template to isolate SLF variants with flanking attB sites. Next, attB-PCR-amplified SLF (150 ng) was mixed with

150 ng of plasmid DNA of pDONR-Zeo vectors (Fig. 8) in TE buffer (pH 8.0). After addition of BP clonase and incubation at room temperature for at least one hour, recombination occurred between "attB" sites on PCR-amplified SLF and "attP" sites on pDONR-Zeo vector, leading to SLF-pDONR constructs with "attL" sites flanking the inserted SLF sequence, known as "BP reaction" (Fig. 9). Each BP reaction was transformed into E. coli through electroporation and subject to plasmid DNA extraction.

Next, 150 ng of plasmid DNA of SLF-pDONR-Zeo construct, i.e. entry clone, was mixed with 150 ng of plasmid DNA of the BiFC vector used in subsequent protein-interaction assays (see section immediately below) in TE buffer (pH 8.0). After addition of LR clonase and incubation at room temperature for at least one hour, recombination occurred between "attL" sites of SLF-pDONR-Zeo construct and "attR" sites of BiFC vectors, leading to final SLF-BiFC constructs with "attB" sites flanking the inserted SLF sequence, known as "LR reaction" (Fig. 9). Each LR reaction was transformed into E. coli through electroporation and subject to plasmid DNA extraction. Preliminary results indicated that overnight incubation of LR reaction at room temperature often yielded more colonies in transformation. Next, Extracted plasmid DNA of SLF-BiFC construct was transformed into Agrobacterium. At last, selected BiFC-Agro constructs were employed in protein-interaction assays as described in below section.

Bimolecular Fluorescence Complementation (BiFC) assays via Agroinfiltration

Agroinfiltration is a technique to transfer genes into plants by injecting Agrobacterium suspension into leaves, and inducing transient expression of desired proteins *in vivo*. At first, selected SLF- and S-RNase-BiFC constructs were incubated at 28 ℃ in liquid $YEP^{Kanamycin}$

culture till the OD_{600} reached 1.0. Next, cultures were centrifuged, and resuspended to OD_{600} of 0.7 with infiltration buffer (10 mM MES pH 5.6; 10 mM $MgCl_2$). Before infiltrating, the resuspensions of different constructs were mixed with equal volume based on what type of protein interactions to be investigated, and incubated at room temperature for 2 hours. In addition, equal-volume resuspension of P19 anti-RNAi Agrobacterium strain was added to each mixture for the purpose of suppressing RNA interference and thus enhancing gene expression. While infiltrating, sterile syringes were used to inject mixed suspension into leaves of *Nicotiana benthamiana*. Young and fully-expanded leaves were always preferred. The tip of the syringe was pressed gently but firmly against the abaxial side of a leaf while applying moderate counter pressure with index finger to the adaxial side. Resuspensions of mixed BiFC constructs would be infiltrated into the subepidermal layers by gently pressing down on the syringe plunger. After infiltration, each leaf was tagged, and plants were placed in greenhouse for 4 days in order to gain optimal protein expression.

Next, infiltrated leaves were collected, cut into small squares (1 x 1 cm^2), and prepared as microscopy slides. Signals could be captured and saved as JPEG images via the confocal microscope connected to a computer owned by the Department of Biological Sciences. Depending on what type of fluorescent protein was reconstituted, a correct mode should be set before scanning samples. That was BP 475-525, BP 505-530 or BP 505-550. Positive control (CNX6::CNX7) was always assayed first for optimizing the scanning conditions, such as excitation value, pin hole value, and detector gain value, which were applied to the rest of the samples. Assuming the interaction between proteins A and B brought the fluorescent fragments (VYNE and VYCE) together (Fig. 15), the reporter protein Venus would reform in its native three-dimensional structure and emit its fluorescent signal that would be observed through

confocal microscope. In addition, the intensity of the fluorescence is proportional to the strength of certain protein interactions. As long as two proteins actually interact with each other, signal can be detected and imaged as the epidermal cells with fluorescent edges.

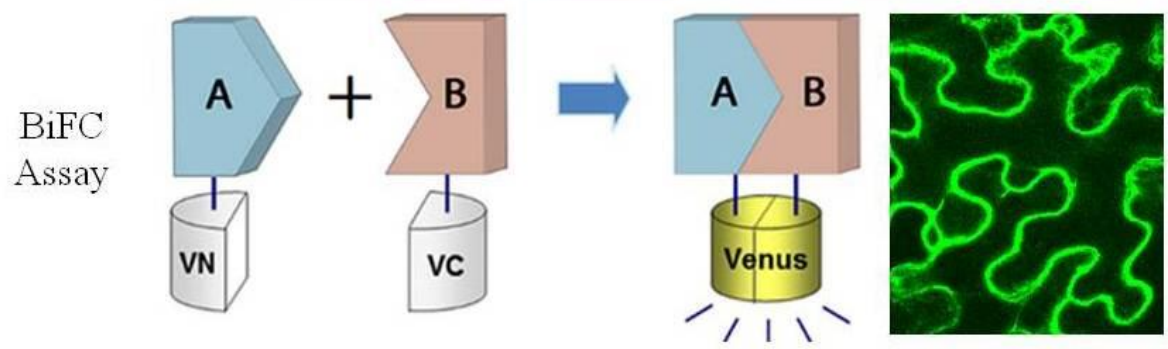


Figure 15: Summary of BiFC assay. Different gene fusions are constructed such as A fused to the N-terminal domain of VYNE, and B fused to the C-terminal domain of VYCE. Interaction of A and B thus brings the two domains of VYNE and VYCE together, which then fluoresce. The right-hand photo shows BiFC fluorescence in the cytoplasm of leaf epidermal cells, as visualized in NIU's confocal microscope.

In addition, co-infiltration can be performed if three-ways BiFC assays are required, i.e. testing the effect of adding a third protein besides SLF and S-RNase. The gene sequences encoding the third protein were cloned into modified BiFC vector whose coding sequence for fluorescent protein had been removed while 35S promoter remained intact. In this case, the third protein could be expressed in plant cell without creating fluorescent protein that might affect the original result. Modified constructs of the third protein were transformed into Agrobacterium,

incubated separately along with other regular BiFC constructs, and mixed with other resuspension as 1:1 ratio before infiltrating leaves. The procedure of Agroinfiltration remained the same.

Another adjustment that can be made for Agroinfiltration is to involve the 26S proteosome inhibitor MG132, for the purpose of testifying the hypothesis of non-self S-RNase being degraded by SCF^{SLF} E3 ubiquitin ligase after interacting with SLF and therefore not being able to show signal under confocal microscope. In this case, 50 μM MG132 will be infiltrated into the same region 12 hours after Agroinfiltration of SLF::S-RNase of interest, which should inhibit the activity of E3 ubiquitin ligase *in vivo*, and allow more accumulation of expected fluorescence, if any, till being harvested for microscopy detection.

Western Blot

Western Blot was employed to monitor the protein-expression levels *in vivo*. At first, numerous Agroinfiltrations were first arranged as: (1) positive control CNX6::CNX7; (2) selected pair of SLF::S-RNase; (3) selected SLF only; (4) selected S-RNase only; and (5) selected pairs whose signals had been observed in previous BiFC tests, as positive controls.

After growing for four days in greenhouse, infiltrated leaves were harvested, grinded in liquid nitrogen, homogenized in the grinding buffer (10 mM Tris pH 9.5, 10 mM EDTA pH 8.0, 100 mM KCl, and 0.1% 2-mercaptoethanol) for protein extraction, and mixed with 5X SDS sample buffer (250 mM Tris, pH 6.8, 10% SDS, 45% glycerol, 5% 2-mercaptoethanol, and 0.1% bromophenol blue). Next, the total protein samples were separated by SDS-PAGE (12% acrylamide in resolving gel; 3.9% acrylamide in stacking gel; Running at 160V for about an hour

until the loading dye was just about to run off the bottom of the resolving gel). After electrophoresis, the resolving gel was sandwiched between PVDF membrane and blotting papers, and the whole cassette was placed in transfer buffer running at 20V for 2.5 hours, in order to transfer bands on gel to the PVDF membranes.

Next, transferred membranes were incubated, in sequence, with the primary antibody that was against the epitope tags on corresponding BiFC vectors, and with the secondary antibody that bound to the primary antibody. Next, blotted membranes were washed thoroughly by TBS-T buffer (2.42 g/L Tris base, 29.24 g NaCl, and 0.1% Tween-20), and treated with chemiluminescent substrate specifically for the secondary antibody. At last, protein that had been expressed in leaves, if any, could be visualized as bands on membranes in the chemiluminescent detector.

Bioinformatic Analysis of SLF Sequences

Multiple sequence alignments were carried out by using a program named MAFFT, one of the fastest multiple-alignment tools that are currently available (Grasso and Lee, 2004). Because of its high performance, MAFFT has become more and more popular in computational analysis of nucleotide or amino acid sequences since it was first released in 2002 (Katoh 2002).

Most SLF variants included in MAFFT alignments were amino acid sequences obtained from *P. axillaris* and *P. inflata* that had been sequenced in Petunia Genome Project, whereas some other SLF variants downloaded from public Genbank were identified in previous researches (Sijacic et al. 2004; Tsukamoto et al. 2005; Kubo et al. 2010; Williams et al. 2014). Three types of alignments were arranged among SLF amino acid sequences as following: (1) All

the SLF variants identified in the genome of S_{ax1} *P. axillaris*; (2) All the SLF variants identified in the genome of S_6 *P. inflata*; and (3) All the subtype-1 SLF variants, i.e. SLF1, with different S haplotypes that were identified in various species of Petunia. Analysis would be investigating different regions of SLF protein sequences, both conserved and variable regions, based on these alignments, and attempting to locate the potential domain(s) that would be responsible for the interactions between SLF variants and S-RNase proteins.

CHAPTER 3

RESULTS

Preliminary Investigation

The majority of the work described in this dissertation used a combination of Bimolecular Fluorescence Complementation (BiFC) assays and bioinformatics analysis of *Petunia* genomic DNA sequences to investigate protein-protein interactions within a proposed SCF^{SLF} E3 ubiquitin ligase complex. Prior to focusing on those experiments, however, I initially pursued some different problems related to gametophytic self-incompatibility in *Petunia hybrida*. Although, due to technical difficulties, those experiments could not be completed, the approaches used and progress made are briefly described in the sections below.

Semi *in vivo* Pollination and Pollen-tube Monitoring

The reason for performing semi *in vivo* pollination was that I intended to find out the subcellular location of S-RNase at pollen-tube tip by High Pressure Freezing/Freeze substitution electron microscopy (HPF/FS). There were some concerns that the fixation and embedding techniques used by Goldraij et al (2006) might result in artifacts, and – in consultation with Dr. Marisa Otegui of the University of Wisconsin-Madison, we were hoping to use HPF/FS as a means to determine the subcellular location of S-RNase and SLF in SC versus SI pollinations. The "caps" used for HPF samples can usually hold only about 2 mm^2 of tissue, so that

sectioning and freezing a whole pollinated style would have been time and cost prohibitive. HPF/FS can freeze samples in liquid nitrogen under high pressure resulting in instant immobilization of the cellular structure of samples, which enables high-quality morphological preservation of biological samples for electron microscopy.

Basically semi *in vivo* pollination allows compatible pollen tubes growing out of the base of a pollinated style after being harvested from Petunia, therefore allowing easy isolation of pollen-tube tips used to find out the subcellular location of S-RNase by high-pressure freezing. To achieve this goal, time intervals during which pollen tubes have been extending but not yet reached the end of the styles have to be confirmed. In addition, pollinated styles are collected and incubated on growing medium in which pollen tubes continue to extend. So it is also necessary to work out the time needed on medium till pollen-tube tips emerge at the end of the styles.

After experimenting repeatedly, I was able to monitor pollen tubes at different status under the microscope, however I could not establish the relationship between status and time. That was significantly uneven growth rate of pollen tubes among different plants or at different hours in the greenhouse. Even the data from the same cross lacked consistency. Many factors could be affecting how fast pollen tubes were extending, such as the vitality of plant itself, the quality of the pollen grains, ambient environment, etc.

Down-Regulating SBP1 via RNA Interference

SBP1 is an important part of the proposed SCF^{SLF} E3 ubiquitin ligase complex because it contains a RING-HC domain characteristic of E3 ubiquitin ligases. SBP1 has also been shown to interact with both S-RNase and SLF in our lab. So down-regulation of SBP1 is predicted to

result in 100% pollen-tube degradation due to inability of inactivating S-RNase, and will provide more evidence of S-RNase's significance in self-incompatibility.

RNA interference is commonly utilized for suppressing the expression of target genes. RNAi is initiated by the enzyme called Dicer, which cleaves the long double-strand RNA into short fragments, i.e. small interfering RNA (siRNA). Each siRNA unwinds into single-strand RNA (ssRNA) and is incorporated into the RNA-induced silencing complex (RISC) which guides the binding to the complementary sequence in the target mRNA, preventing it from producing proteins.

RNAi constructs were prepared via Gateway® Cloning into the pSTARGATE vector which allows the insertion of target gene in both forward and reversed orientation, resulting in a self-complementary double-stranded "hairpin" RNA (hpRNA) in plants after transformation. Besides, pSTARGATE contains the ubiquitin promoter for transgene expression (the more widely used 35S promoter is nonfunctional in pollen and styles). If necessary, Chi2 (style) or LAT52 (pollen) promoters can also be used to drive transgene expression.

An important control that is necessary is to test the effect of SBP1 down-regulation in backgrounds where S-RNase is also down-regulated. Therefore I also needed to build S_1-/S_3-RNase-RNAi constructs. I successfully cloned SBP1-RNAi construct that had been introduced into Petunia leaf discs via Agrobacterium transformation. However, unlike SBP1 genes, I was unable to verify that S_1- or S_3-RNase genes had been successfully cloned into pSTARGATE. Roots were observed to develop from SBP1-RNAi calluses several weeks after being transferred to regeneration and selection medium. However, rooted explants always died after growing in soil for a short time in growing chamber. This may indicate that general down-regulation of SBP1 was destructive to developmental processes.

Plaque Hybridization to Screen S_1S_1 cDNA Library

My preliminary screens of S_1S_1 cDNA library by plaque hybridization, however gave mixed results, with relatively few plaques isolated, and weak hybridization signals. One of the potential (and apparently) real difficulties with plaque-screens for SLF variants is that SLF is expressed at very low levels (at least in mature anthers), so that extremely high numbers of plaques may need to be screened to isolate all SLF variants. Although all library clones were already in GAL4-AD vectors and ready for yeast two-hybrid assays, plaque hybridization was still slower and less efficient than the PCR-based cloning approach, especially after gaining the sequence data from *Petunia axillaris* and knowing that the S-haplotype of *P. axillaris N* and S_1-*P. hybrida* were the same.

Yeast Two-Hybrid (Y2H) Assays

Y2H assays are used to analyze protein-protein interactions by testing for physical interactions between two proteins. The rationale (Fig. 16) behind this test is the activation of a downstream reporter gene by the binding of a transcription factor onto an upstream activating sequence (UAS). In the two-hybrid system, the transcription factor is split into two separate fragments, the binding domain (BD) and the activation domain (AD). The BD is responsible for binding to the UAS and AD is responsible for activating transcription of reporter genes such as *HIS3, ADE2* or LacZ.

To carry out Y2H assays between SLF and S-RNase, Y2H constructs of SLF can be obtained by either TA cloning and subsequent subcloning into the pAD-GAL4 vector (Fig. 13), or screening the S_1S_1 cDNA library where SLF has already been in pAD-GAL4 vector.

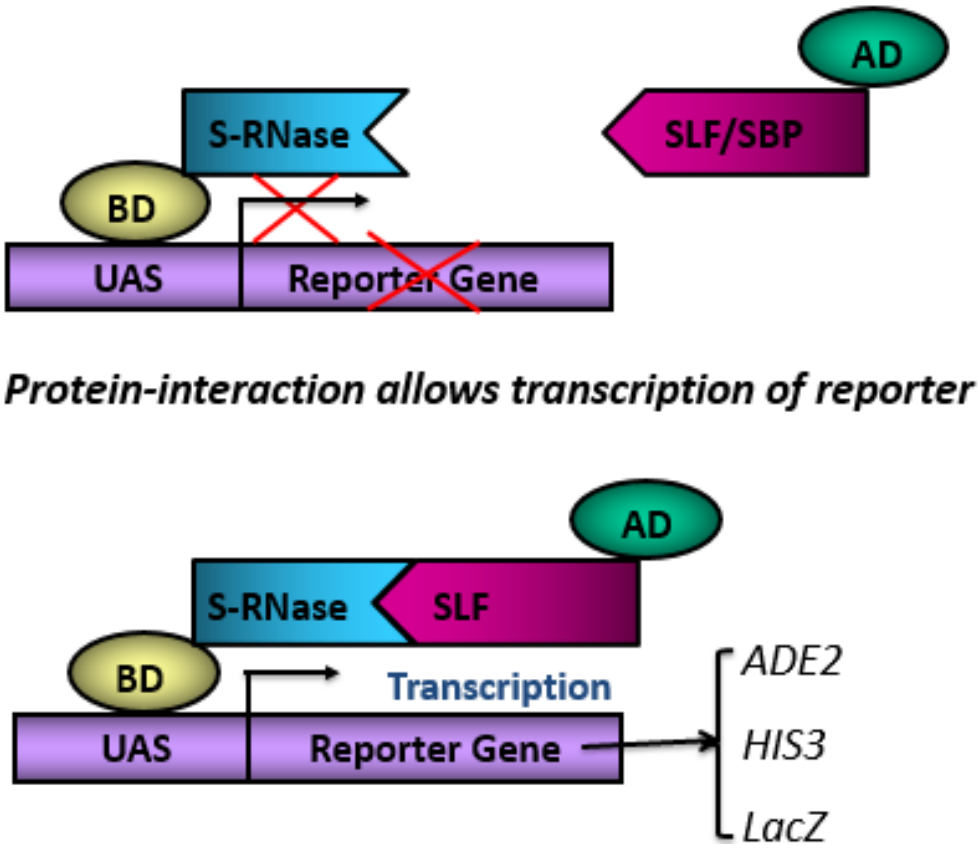


Figure 16: Summary of Y2H assay. SLF variant cloned into the pAD-GAL4 vector is tested against bait constructs, S-RNase cloned in pBD-GAL4 vector. If an interaction occurs between the bait (BD) and prey (AD) proteins, the re-constituted transcription factor activates the reporter genes allowing growth of yeast cells on SD trp$^-$ leu$^-$ his$^-$ ade$^-$ media.

SLF-AD constructs can be tested for the ability to interact with existing bait constructs in pBD-GAL4 vector (Fig. 14), such as S-RNases with different S haplotypes. If an interaction occurs between the bait (BD) and prey (AD) proteins, the re-constituted transcription factor activates the reporter genes allowing growth of yeast cells on SD trp$^-$ leu$^-$ his$^-$ ade$^-$ media. In addition to testing full-length SLF variants, SLF whose F-box is truncated will also be tested, as a previous

study (Qiao et al. 2004) indicated that, in the case of AhSLF-S_2, removal of the F-box was necessary to demonstrate protein interactions by two-hybrid assays.

I had been able to finish cloning *P. hybrida* SLF1-5 into both pAD- and pBD-GAL4 vectors before I obtained sequence data *P. axillaris* from the Petunia Genome Project. Now I need to clone more SLF variants from S_{ax1} *P. axillaris* into Y2H vectors. As soon as BiFC assays detecting *in-vivo*-protein interactions among GSI proteins have been completed, I will be able to test SLF variants from both *P. hybrida* and *P. axillaris* against other proteins, such as S-RNase, and SBP1, to investigate protein interactions in yeast. In addition, we recently obtained Gateway® two-hybrid vectors (Life Technologies), so in the future, all of my current Gateway® BiFC constructs can be easily and rapidly transformed into Y2H constructs.

Main Results

Semi *in vivo* Pollinations and Pollen-tube Monitoring

To obtain the pollen-tube tips that fit the "caps" used on high-pressure freezing equipment, I carried out semi *in vivo* pollination which allowed pollen tubes to extend out the end of the styles, resulting in easy acquisition of only the tip part. *Petunia hybrida* plants with different genotypes are grouped for either compatible crosses or incompatible/self crosses (Table 1). Temperature and light conditions were recorded while pollinating. Since all plants were growing in the greenhouse in our department, conditions such as humidity, temperature, etc. were well maintained within an optimal range.

Table 1: Summary of in-stock *P. hybrida* plants. *P. hybrida* plants with different genotypes involved in semi *in vivo* pollination are listed. Compatible crosses are designed based on the S-haplotype of pollen donor.

	Plant	**Genotype**	**SI behavior**
Self In-Compatible Cross	90FS2D3	S1S1	SI
	90FS2D4	S1S1	SI
	2920-6S	S1S2	SI
	9929-10S	S1S2	High PSC
	9924A-5S	S1S2	Moderate PSC
	99-3-6S	S1S2	High PSC
	FL1FL2-6S	S1S3	High PSC
	FL1GL3-1	S1S3	Moderate PSC
	GL4-3S	S3S3	?
Compatible Cross	90FS2D3 x 9929-10S	S1S1 x S1S2	
	90FS2D3 x 9924A-5S		
	90FS2D4 x 9929-10S		
	90FS2D4 x 9924A-5S		
	9929-10S x FL1FL2-6S	S1S2 x S1S3	
	9929-10S x FL1GL3-1		
	99-3-6S x FL1FL2-6S		
	99-3-6S x FL1GL3-1		
	90FS2D3 x GL4-3S	S1S1 x S3S3	
	90FS2D4 x GL4-3S		
	GL4-3S x 90FS2D3	S3S3 x S1S1	
	GL4-3S x 90FS2D4		

Pollinations lasted for various durations depending on what type of cross it was. At the beginning, styles of all crosses were collected 24 hours after pollinations, and stained with aniline blue. Under microscope, pollen tubes could be found initiated at stigma surface where pollen grains were deposited (Fig. 17), and elongating for certain distance within the styles (Fig. 18). For those styles in which pollen tubes clearly reached or even extended beyond the bottom in 24 hours (Fig. 19), pollinating durations would be reduced to 12 hours in next attempt. If pollen tubes was found barely extended in certain crosses, for example reaching only half style or less (Fig. 20), in the next attempt the pollinations would be prolonged to 36 hours or more. In addition, further adjustments would be made till the time frames were able to be narrowed down to a minimum number of hours.

To optimize the reproducibility, each cross listed in Table 1 was repeated at least 20 times. The final data pool, however, failed to support a reliable pollinating time interval for any crosses, because the growth rate indicated by data was significantly uneven (Fig. 21).

Gateway® Cloning for RNA Interference

To study the role of SBP1 during pollen recognition in GSI, genes encoding SBP1, S_1- and S_3-RNase were PCR-amplified from corresponding genomic DNA or cDNA by Gateway® primers (Table 2; Fig. 22). Purified PCR products were cloned into pDONR vectors (Kanamycin resistant) via BP reaction. pDONR constructs were transformed into *E coli* DH5α, incubated on LB^{Kan} plates and selected by colony PCR (Fig. 23). Plasmid DNA of selected pDONR construct was extracted and subject to LR reaction inserting genes into pSTARGATE vectors (Spectinomycin resistant), i.e. final RNAi constructs. pSTARGATE constructs were transformed

Figure 17: Micrographs of fluorescent pollen tubes. Pollen grains (Blue dots) are deposited on the stigma surface, leading to subsequent extension of pollen tubes (Blue threads developed from pollen grains).

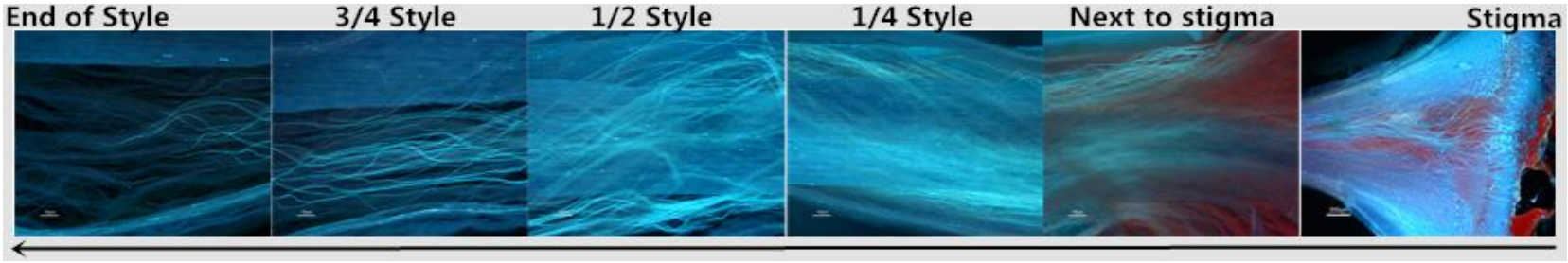


Figure 18: Pollen tubes in an entire style. The entire style of 90FS2D3 self-crossed for 24 hours. Images taken under Nikon Eclipse E-600 with UV illumination. Starting from right-hand image, pollen tubes (Blue threads) were observed extending from the stigma, through the transmitting tract, all the way to the end of the style. The further pollen tubes extended, the few amount of pollen tubes could be found.

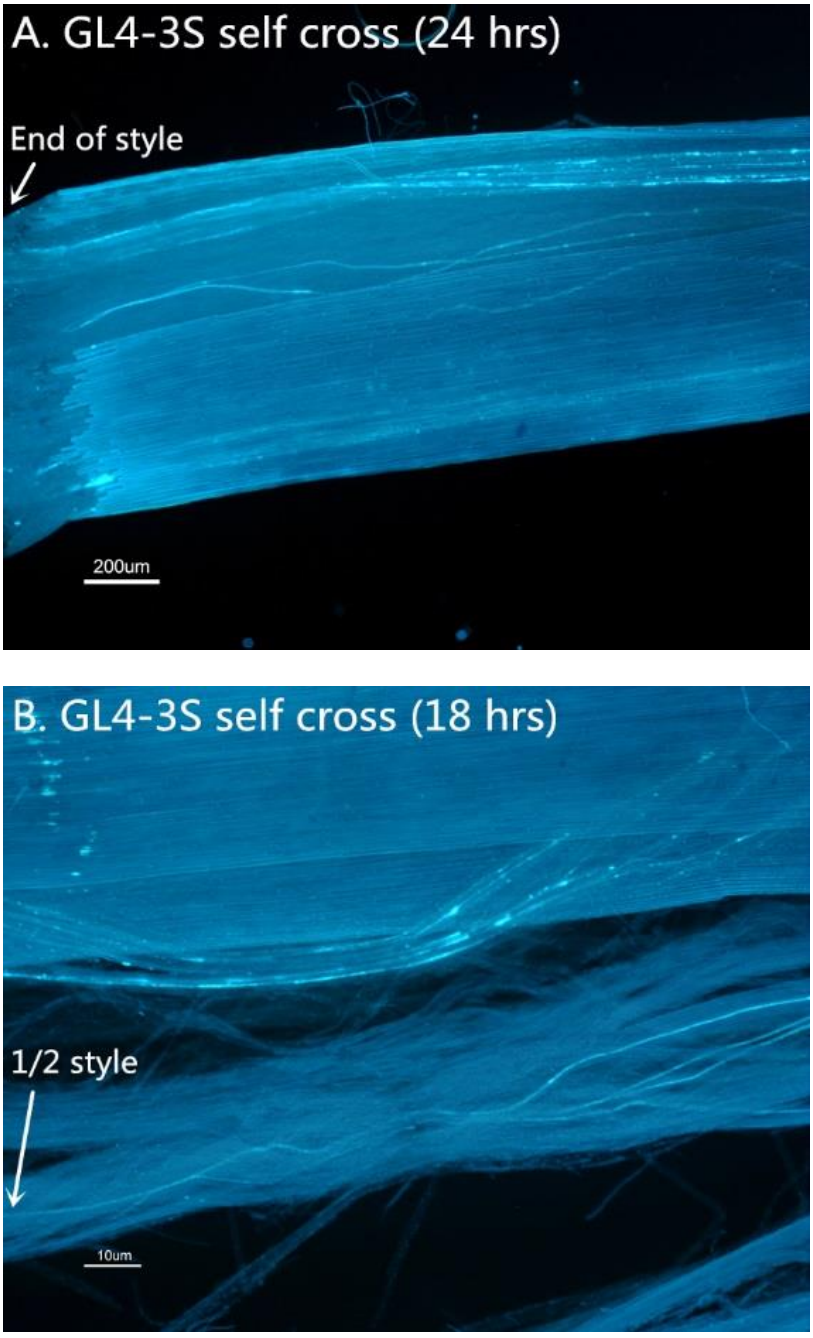


Figure 19: Pollen tubes at different stages #1. (A) GL4-3S self-crossed for 24 hours, pollen tubes almost reaches the bottom of the style; (B) GL4-3S self-crossed for 18 hours, pollen tubes only extended to half style. Together, both images indicate that the style of self-crossed GL4-3S could be harvested 18 hours after being pollinated, cut at 2/3 style spot, and incubated on growing medium for continuous pollen-tube extension, which is the most efficient way to obtain pollen-tube tips in this particular case.

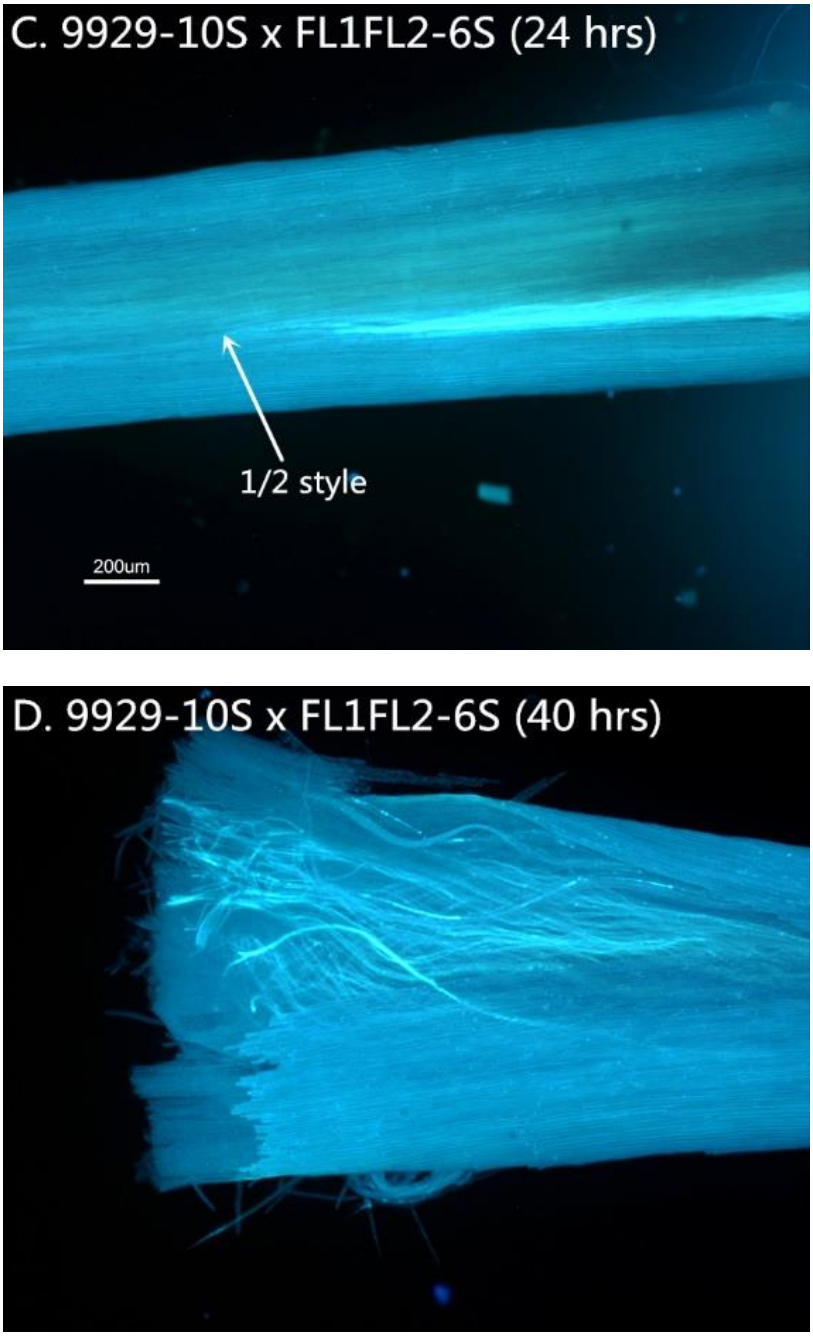


Figure 20: Pollen tubes at different stages #2. (C) 9929-10S x FL1FL2-6S crossed for 24 hours. Pollen tubes were not exposed since the style had not been squashed, however the fluorescence could still be found fading after half-style spot, which meant pollen tubes had stopped growing at the very place; (D) 9929-10S x FL1FL2-6S crossed for 40 hours. It was clear pollen tubes reached beyond the end of the style. Together, both images showed the pollinating duration of 9929-10S x FL1FL2-6S should be ranged from 24 to 40 hours.

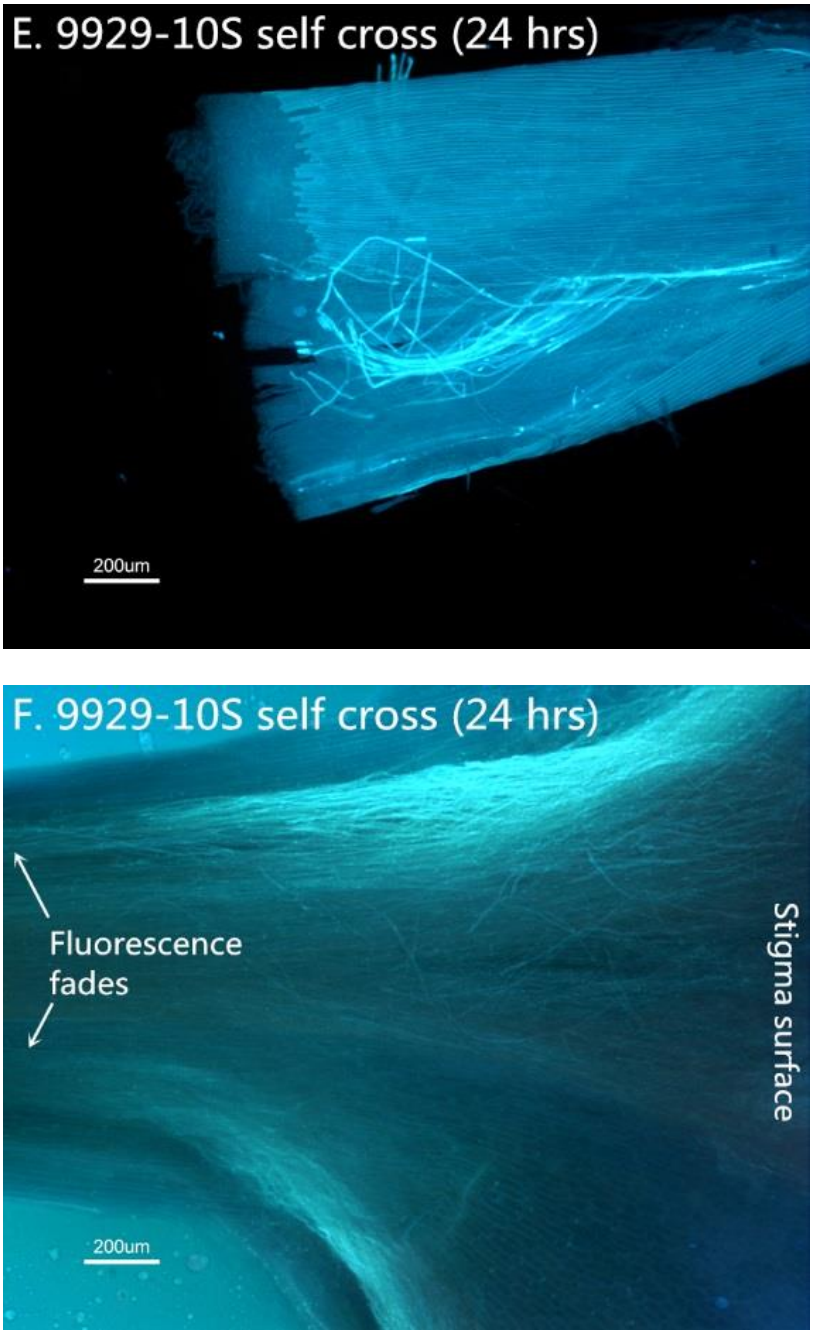


Figure 21: Pollen tubes at different stages #3. E and F indicate one of the examples of uneven growth rate of pollen tubes from the same crossing pattern as well as under the equal greenhouse condition. Pollen tubes reached the end of the style in photo E while gradually stopped extending near stigma in photo F. Examples like this prevent precise time intervals from being worked out.

Table 2: Gateway® primers amplifying SBP1, S_1- and S_3-RNase. Bold sequences are attB sites for Gateway® cloning, and the rest of the primers are gene-specific sequences.

Primer Name	Sequence
S_1_RNAi_2_Fwd	**GGGGACACGTTTGTACAAAAAAGCAGGCT**GCTTGCTCTCCAATTCCTGG
S_1_RNAi_5_Fwd	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TGTCCGAGACCAGTAATTCC
S_1_RNAi_8_Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TCATAGCAACTGTCGTTCGG
S_3_RNAi_1_Fwd	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**CTCTTTCTCCCGTTAGTGCG
S_3_RNAi_7_Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TCGATCAAACCTATCTGCCG
S_3_RNAi_5_Fwd	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**GCCACTGGATTCAAATGAGG
S_3_RNAi_4_Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**CCGAAACAGAATCTTCGTGC
SBP1_RNAi_Fwd1	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**ATCGACTGGCTTGGGTTTGTCTCT
SBP1_RNAi_Rev1	**GGGGACGAGTTTGTACAAGAAAGCTGGGT**AAGATCTGTGGCCCGTCCATTACA
SBP1_RNAi_Fwd2	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TTTGGAACCAAAGAGAAAGA
SBP1_RNAi_Rev2	**GGGGACGAGTTTGTACAAGAAAGCTGGGT**GTTCAAGCTCCATGTTTTTC

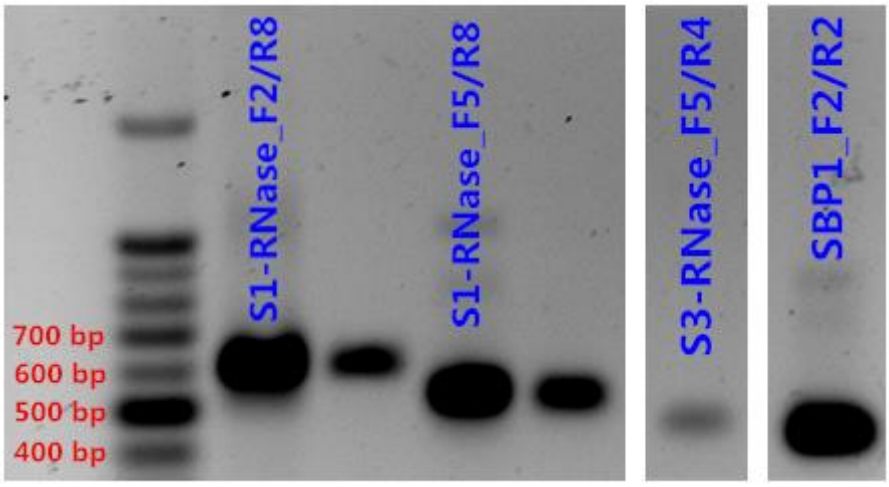


Figure 22: Electrophoresis of PCR of S_1- and S_3-RNase, SBP1.

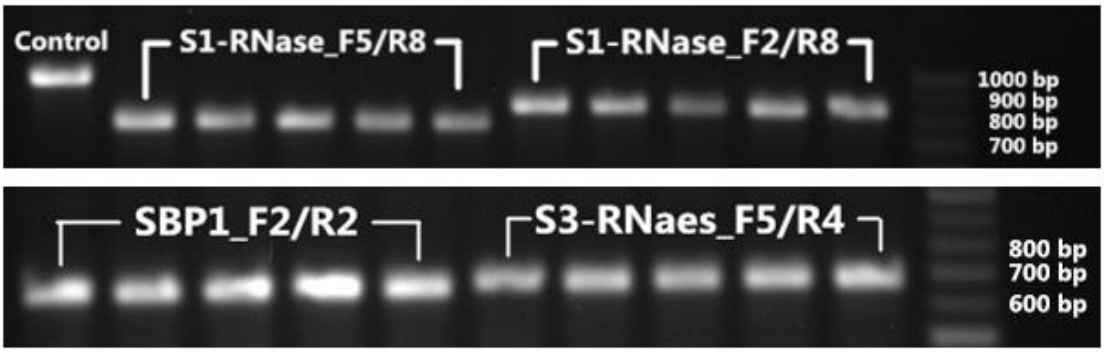
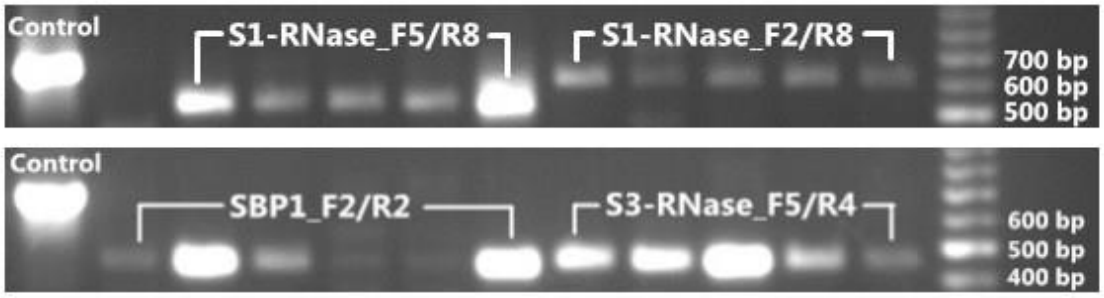


Figure 23: Electrophoresis of colony PCR of S_1- and S_3-RNase, SBP1 in pDONR vectors in *E coli* DH5α.

into *E coli*, incubated on LB^{Spec} plates, and verified by colony PCR (Fig. 24). Plasmid DNA of selected pSTARGATE constructs were extracted, transformed into Agrobacterium, and incubated on YEP^{Spec} plates. Agro-pSTARGATE-constructs confirmed by colony PCR would be employed in leaf inoculation.

However, after transforming the plasmid DNA of selected pSTARGATE constructs into Agrobacterium, only the SBP1-RNAi construct was confirmed (Fig. 25). I was never able to confirm correct insertion of S_1- and S_3-RNase by colony PCR. Electrophoresis of colony PCR showed either no product on the gel or products of smaller size. Thus far, the reason of S_1- and S_3-RNase-pSTARGATE constructs not being normally transformed into Agrobacterium remains unclear.



. Figure 24: Electrophoresis of colony PCR of S_1- and S_3-RNase, SBP1 in pSTARGATE vectors in *E coli* DH5α.

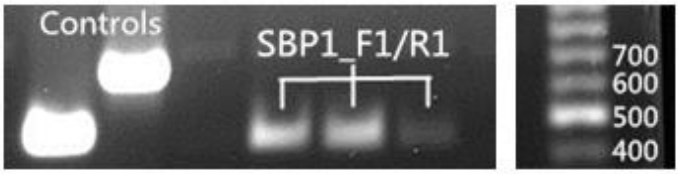


Figure 25: Electrophoresis of colony PCR of SBP1 in pSTARGATE vectors in Agrobacterium.

Multiple Sequence Alignment and PCR Amplification of S_1-SLF genes

Other than S-RNase and SBP1, SLF is equally important as it is directly involved in pollen recognition and also a major component of SCF^{SLF} E3 ubiquitin ligase complex. However, compared with S-RNase, SLF is less conserved in some regions within its sequence which leads to a fact that there are a large family of SLF genes of each S haplotype. So, being able to discover all the SLF variants is crucial to the entire study of protein interactions between SLF and other proteins.

40 SLF variants were grouped in eight different subtypes, i.e. SLF1-8. Within the same subtype, for example SLF1 (Fig. 26), there were SLF1 variants with different types of S haplotypes, such as S_1-SLF1, S_2-SLF1, S_3-SLF1, etc. All the S_x-SLF1 variants were subject to multiple sequence alignment to locate regions that were highly conserved for PCR-primer designing (Table 3).

Two forms of SLF variants were amplified, one with an F-box sequence and the other without. F1 and R2 amplified the entire SLF gene, including the F-box domain. F2 and R1 were internal primers; amplified bands lacked the conserved F-box domain and expected to be more

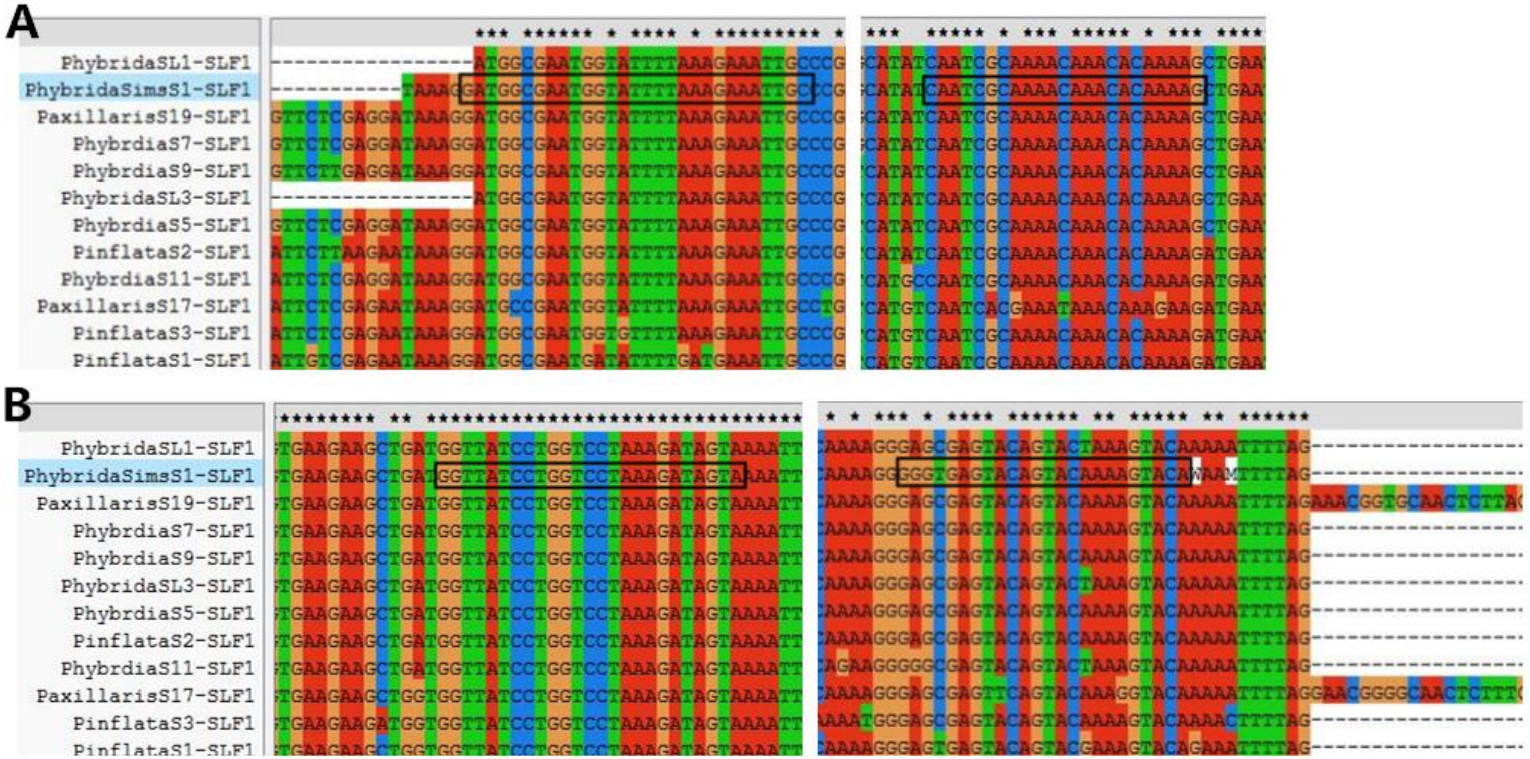


Figure 26: Multiple sequence alignment of S_x-SLF1 variants by ClustalX. "PhybridaSimsS1-SLF1" was the one sequenced in our lab. (A) The left-hand framed sequence was "SLF1-F1" primer and the right-hand framed one was "SLF1-F2" primer. "SLF1-F1" located upstream of F-box coding sequence while "SLF1-F2" was at downstream; (B) The left-hand framed sequence was "SLF1-R1" primer and the right-hand framed one was "SLF1-R2" primer. "SLF1-R1" was internal while "SLF1-R2" was at 3' end.

Table3: PCR primers amplifying SLF variants from S_1S_1 *Petunia hybrida* cDNA library. Genes amplified by "F1+R1" and "F1+R2" included F-box while those amplified by "F2+R1" and "F2+R2" did not.

Primer	Sequence	Comment	Combination	bp of Product
SLF1-F1	GATGCGAATGGTATTTTAAAGAAATTGC	Includes F-box	F1R1	575
SLF1-F2	CAATCGCAAAACAAACACAAAAG	Internal	F1R2	1161
SLF1-R1	TACTATCTTTAGGACCAGGATAACC	Internal	F2R1	425
SLF1-R2	TGTACTTTWGTACTGTACTCGCTC	3' end	F2R2	1011
SLF2-F2	GAACCAGAKCAATTRAAAAGTATHGC	Internal	F2R1	341
SLF2-R1	GGATCCCAATAAACATCTGAAAGC			
SLF3-F1	ATGAAGAAATTGCCCATAGATGTG	Includes F-box	F1R1	712
SLF3-F2	AGATGAATTYATTCTCTTCAAGCGATC	Internal	F1R2	1078
SLF3-R1	GTGGGCATACCAATGAAAGG	Internal	F2R1	542
SLF3-R2	CATCTAATTTGAATTCCTTGACTTCATC	3' end	F2R2	908
SLF4-F1	AATGAAATTATATMGTAAGAATACAAGATGBCG	Includes F-box	F1R1	787
SLF4-F2	CTTCAAAGAAGATGTTGAAAGTTATAAAGGC	Internal	F1R2	1208
SLF4-R1	TTCAGTACTCATGTCAAAACAAAGAATTATC	Internal	F2R1	563
SLF4-R2	TGAAGTTGTGTACTACTTTGGCTTC	3' end	F2R2	983

(Continued on following page)

Table 3 (continued)

Primer	Sequence	Comment	Combination	bp of Product
SLF5-F1	CCAATATTATGAAGATGC CACATGG	Includes F-box	F1R1	491
SLF5-F2	AATCGCACCACAACGGTG	Internal	F1R2	1175
SLF5-R1	ACATTTGATGGATCGGTG GTATC	Internal	F2R1	327
SLF5-R2	CTAAAACTTGTGAACTGG TGTACTG	3’ end	F2R2	1011
SLF7-F1	TTGTTTGGAGATGTTRTG ATTTATATACT	Includes F-box	F1R1	729
SLF7-F2	GATATTCACTCTATTTCTC CAGATCTAGATG	Internal	F1R2	1154
SLF7-R1	ATGTCAAAACAAAGYATA ACCACTGTTT	Internal	F2R1	479
SLF7-R2	TAAAAATTTTGAACTTGA GTACTATSCTCG	3’ end	F2R2	914
SLF8-F1	AGGAATAAGCTTTTGCGT CAAACTCAAAGG	Includes F-box	F1R1	1001
SLF8-F2	ATGCCTTGTCTATTCTTTC TTGTGGAAACG	Internal	F1R2	1550
SLF8-R1	GGAGGAGTATGGTGTATA CGAGTCTTGGAC	Internal	F2R1	686
SLF8-R2	AATTCTTAATTAAGTAAA GGCCACTGGGTTTTGAG	3’ end	F2R2	1235

specific as hybridization probes. PCR was carried out by employing S_1S_1 *Petunia hybrida* cDNA library as DNA template, and electrophoresis gave strong bands of SLF1, SLF2, SLF3 and SLF5, as well as bands of SLF4 and SLF6 with dim signal (Fig. 27).

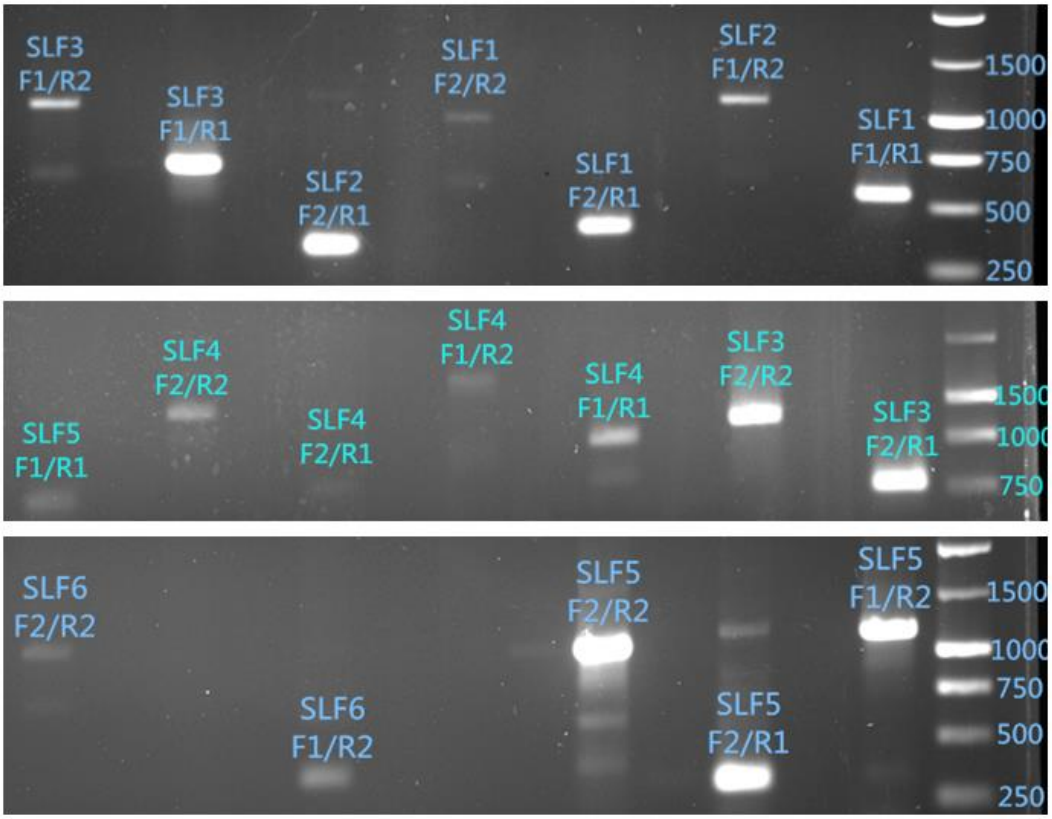


Figure 27: Electrophoresis of PCR amplifying SLF variants from S_1S_1 *Petunia hybrida* cDNA library. F1 & R2 amplified the entire SLF gene, including the F-box domain. F2 and R1 were internal primers; amplified bands lacked the conserved F-box domain and would be more specific as hybridization probes.

Constructing SLF variants for Yeast Two-hybrid assays – Plaque hybridization or TA Cloning?

My original approach was to use plaque hybridization with PCR-amplified SLF variant as hybridizing probes to screen the Y2H cDNA library. The rationale behind this approach was that library clones would already be in GAL4-AD vectors, and could be directly used in Y2H assays with current S_1- and S_3-RNase bait constructs. My preliminary screens, however had given mixed results, with relatively few plaques isolated, and weak hybridization signals. One of the potential (and apparently) real difficulties with plaque screens for SLF variants was that SLF was expressed at very low levels (at least in mature anthers), so that extremely high numbers of plaques may need to be screened to isolate all SLF variants.

The alternative approach was to use PCR-based methods to clone SLF variants into TA vectors (pGEM-T) and then subclone into Y2H vectors (pAD- and pBD-GAL4) by restriction-enzyme digestion and ligation. Because the preliminary PCR amplifications (Fig. 27) demonstrated that SLF variants were present in the cDNA library, this approach should be relatively more straightforward, although it would involve more cloning steps.

According to the electrophoresis results, the preliminary PCR (Fig. 27) showed strong amplification of SLF1, SLF2, SLF3, and SLF5, as well as weak signals from SLF4 and SLF6. Among them, SLF1-F1R1, SLF2-F2R1, SLF3-F1R1, SLF3-F2R2, SLF5-F1R2, and SLF5-F2R2 were selected to be cloned into pGEM-T and transformed into *E coli* DH5α. The presence of inserted genes and their orientations were confirmed by being sequenced in the Core DNA Analysis Lab in our department, for the purpose of subcloning SLF insertions into Y2H vectors in right direction. The inserted SLF genes, however, were found correct in sequences but

improperly oriented in pGEM-T vectors in some cases. Thus, instead of carrying out TA cloning again, I decided to design new primers and amplify from S_1S_1 cDNA library directly and directionally, which eventually turned out more straightforward and efficient.

New primers for PCR followed with restriction-enzyme digestion and ligation were designed. Sequences of either EcoR I or Sal I restriction sites were added to the origin of each primer, since both sites were at multiple cloning regions on pAD- or pBD-GAL4 vectors and could be taken advantage for cloning. Then, PCR (Fig. 28) was performed and products were purified. Both PCR products and Y2H-vector DNA needed to be digested by EcoR I and Sal I restriction enzymes, so they could be ligated based on complimentary overhangs. The ligation reactions were transformed into *E coli* DH5α, and colony PCR (Fig. 29) was applied to confirm that SLF genes were correctly inserted into pAD- or pBD-GAL4 vectors. At last, I was able finish building SLF-Y2H constructs by cloning all combinations (Table 4) into both pAD- and pBD-GAL4 vectors.

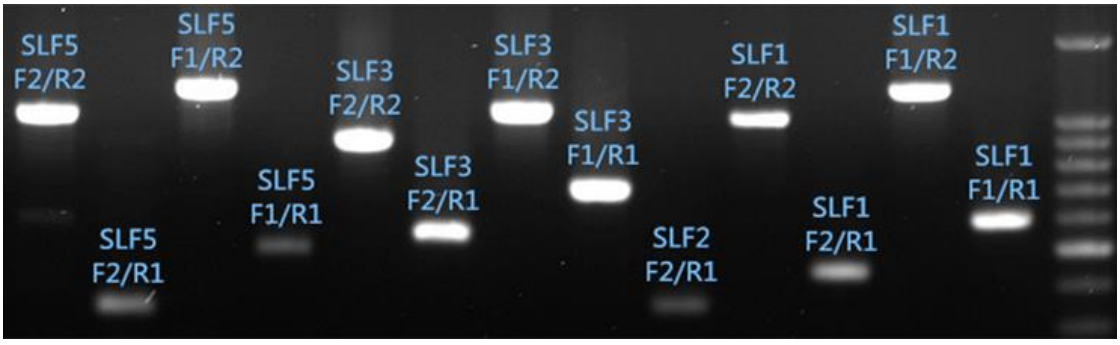


Figure 28: Electrophoresis of PCR amplifying restriction-site-flanking SLF variants from S_1S_1 cDNA library.

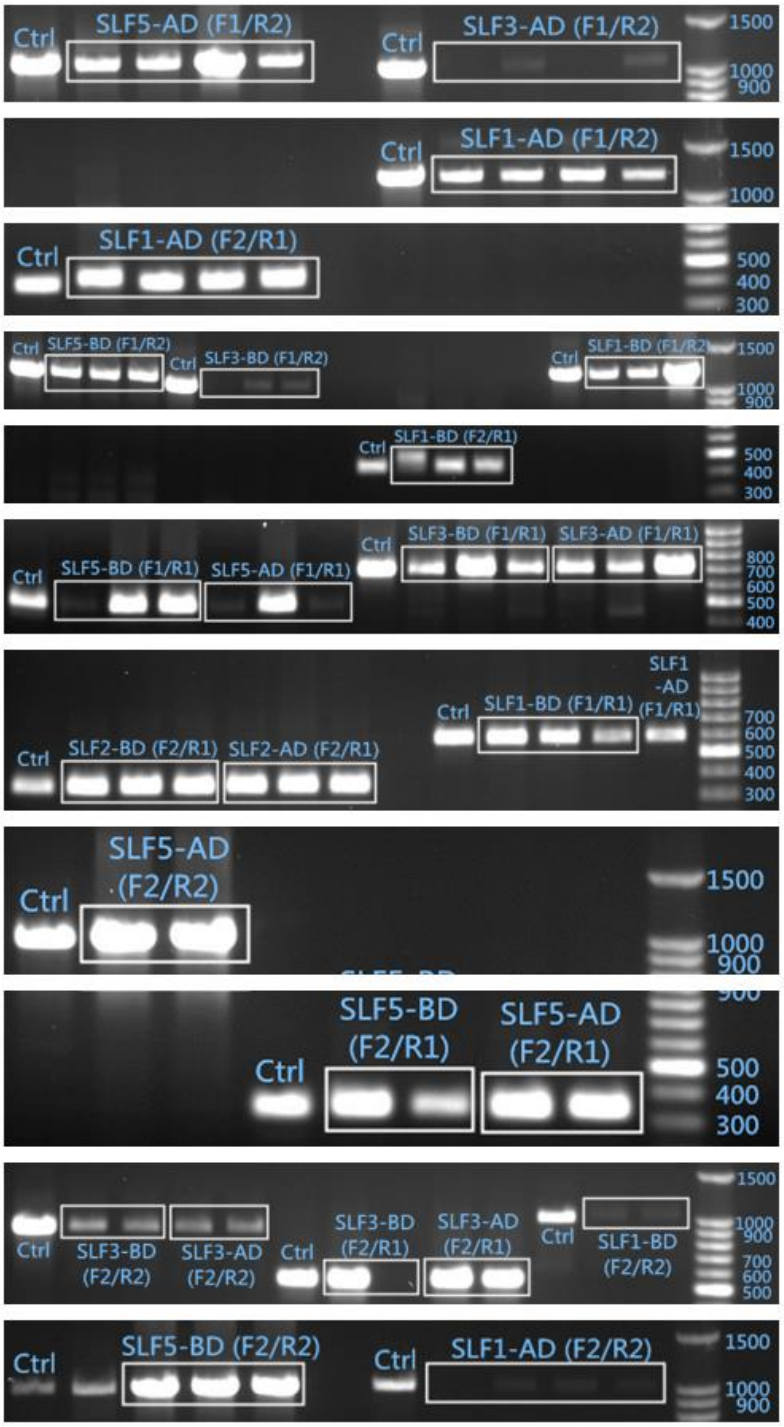


Figure 29: Electrophoresis of colony PCR confirming correct insertion of SLF genes into Y2H vectors.

Table 4: PCR Primers with flanking restriction sites. These primers were used to amplify S_1-SLF variants for subsequent restriction-enzyme digestion and ligation reactions. Sequences (bold) of EcoR I or Sal I restriction sites were added in the interest of flanking amplified genes with both sites in right order.

Oligo	Sequence	Combination	Comment
Y2HSLF1-F1	**CTGTGAATTC**GATGCGAATGGTATTTTAAAGAAATTGC	F1R1	Includes F-Box
Y2HSLF1-F2	**CTGTGAATTC**CAATCGCAAAACAAACACAAAAG	F1R2	Internal
Y2HSLF1-R1	**AGATGTCGAC**TACTATCTTTAGGACCAGGATAACC	F2R1	Internal
Y2HSLF1-R2	**AGATGTCGAC**TGTACTTTWGTACTGTACTCGCTC	F2R2	3’ end
Y2HSLF2-F2	**CTGTGAATTC**GAACCAGAKCAATTRAAAAGTATHGC	F2R1	Internal
Y2HSLF2-R1	**AGATGTCGAC**GGATCCCAATAAACATCTGAAAGC		
Y2HSLF3-F1	**CTGTGAATTC**ATGAAGAAATTGCCCATAGATGTG	F1R1	Includes F-Box
Y2HSLF3-F2	**CTGTGAATTC**AGATGAATTYATTCTCTTCAAGCGATC	F1R2	Internal
Y2HSLF3-R1	**AGATGTCGAC**GTGGGCATACCAATGAAAGG	F2R1	Internal
Y2HSLF3-R2	**AGATGTCGAC**CATCTAATTTGAATTCCTTGACTTCATC	F2R2	3’ end
Y2HSLF5-F1	**CTGTGAATTC**CCAATATTATGAAGATGCCACATGG	F1R1	Includes F-Box
Y2HSLF5-F2	**CTGTGAATTC**AATCGCACCACAACGGTG	F1R2	Internal
Y2HSLF5-R1	**AGATGTCGAC**ACATTTGATGGATCGGTGGTATC	F2R1	Internal
Y2HSLF5-R2	**AGATGTCGAC**CTAAAACTTGTGAACTGGTGTACTG	F2R2	3’ end

Gateway® Cloning SLF Variants from S_1 *Petunia hybrida* and S_{ax1} *Petunia axillaris* for BiFC assays

Gateway® Cloning SLF Variants from S_1 *Petunia hybrida*

To amplify SLF variants and clone them into Gateway® BiFC vectors, Gateway® primers (Table 5) were designed based on the preliminary PCR and electrophoresis results. These new primers gave mixed results (Fig. 30), most likely because primers were designed to amplify the entire SLF coding region (+/- the F-box), and therefore in some cases targeted less-conserved gene regions. For example, PCR amplifications gave decent bands for SLF1 (±F-box) whereas no amplification was observed for SLF2. When using S_1S_1 library plasmid DNA, SLF3_F1-R (attB1Fwd + attB2Rev) gave a good band which appeared to be the correct size. SLF3_ΔF-R (ΔFattB1Fwd + attB2Rev) yielded two closely-spaced bands when using S_1S_1 genomic DNA (SLF genes lack introns) as template. With total S_1S_1 library plasmid DNA as PCR template, SLF4_F1-R (attB1Fwd1 + attB2Rev) did not give any band, nor did SLF4_ΔF3-R (ΔFattB1Fwd3 and attB2Rev). SLF4_ΔF1-R (ΔFattB1Fwd1 + attB2Rev) gave a single weak band whose size was far too large as a correct PCR product. Switching to S_1S_1 genomic DNA (SLF genes lack introns), SLF4dF1_ΔF1-R (ΔFattB1Fwd1 + attB2Rev) gave multiple bands with one prominent band that appeared to be slightly smaller than expected. SLF5_F1-R (attB1Fwd1 + attB2Rev) yielded single band of correct size from the plasmid DNA library. SLF5_ΔF-R (ΔFattB1Fwd + attB2Rev) produced two bands. The upper one appeared to be the correct size as the ΔF-box product but the other one, at 250 bp, was too small.

Table 5: Gateway® primers amplifying *Petunia hybrida* S_1-SLF variants. Bold sequences are attB sites.

Primers	Sequence
S1-SLF1attB1Fwd2	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TA AAGGATGGCGAATGGTATTTTAAAGAAATTGC
S1-SLF1dFBattB1Fwd1A	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**AA CAATGCGCAAAACAAACACAAAAGCTG
S1-SLF1attB2rev2	**GGGGACCACTTTGTACAAGAAAGCTGGGT**CA AATTTTTGTACTTTTGTACTGTACTCGCTC
S1-SLF2dFBattB1Fwd3	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**AA CATGCGYATYAYAASCACARARGATGAAYTMAT YC
S1-SLF2attB2Rev2	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TRA ATTGYTSAACTWGTGTWCCATKWTC
S1-SLF3attB1Fwd1	**GGGGACACGTTTGTACAAAAAAGCAGGCT**RRC YATGAAGAAATTGCCMRWAGATRTG
S1-SLF3dFBattB1Fwd	**GGGGACACGTTTGTACAAAAAAGCAGGCT**AA CAATGGCMACTAACCRYCAMAGATAATTC
S1SLF3attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TA AAWYKYTSAACTTGTGTACTACC
S1-SLF4attB1Fwd1	**GGGGACACGTTTGTACAAAAAAGCAGGCT**AA TGAAAKTAWATHARTAAARAAGACAAGATGBC G
S1-SLF4dFBattB1Fwd1	**GGGGACACGTTTGTACAAAAAAGCAGGCT**AA CATGCATCTWBACCGCACCACAWCWYCGG
S1-SLF4dFBattB1Fwd3	**GGGGACACGTTTGTACAAAAAAGCAGGCT**AA CAATGACCACAACTTCGGAAGATG
S1-SLF4attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TW AMAMWWCDWCAAMWSATGAAACCGAAGG
S1-SLF5attB1Fwd1	**GGGGACACGTTTGTACAAAAAAGCAGGCT**AA CAATGAAGATGCCACATGGAAYTATGAAG
S1-SLF5dFBattB1Fwd	**GGGGACACGTTTGTACAAAAAAGCAGGCT**AA CAATGCACCTCAATCGACCACAACG
S1-SLF5attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TA AACTTGTGAACTGGTGTACTGC

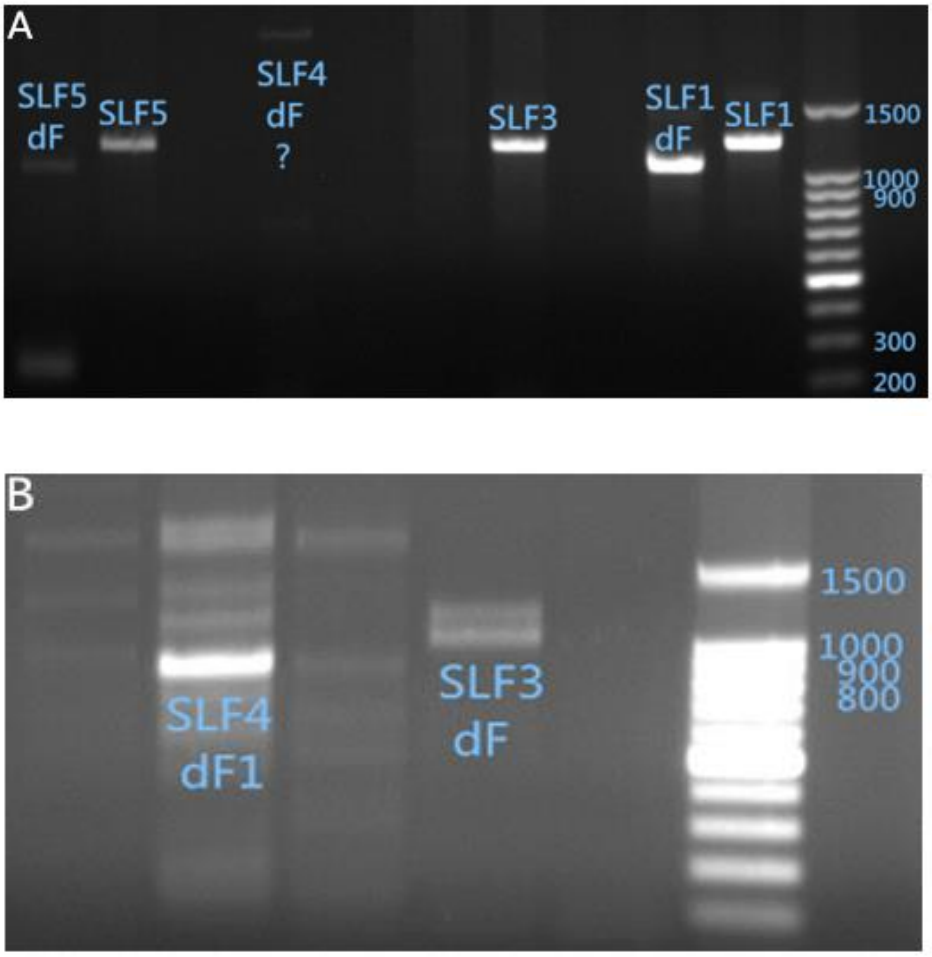


Figure 30: PCR amplification of *P. hybrida* S_1-SLF variants using Gateway® primers. (A) Using S_1S_1 pollen cDNA library as DNA template. (B) Using S_1S_1 genomic DNA as DNA template. F & R should amplify the entire SLF gene, including the F-box domain. Bands amplified by dF and R would lack the conserved F-box domain

.

Selected PCR products of SLF were purified and inserted into pDONR-Zeo vector (Fig. 8) via BP reaction (Fig. 9) which created entry clones in *E coli* DH5α. Plasmid DNA of pDONR-Zeo constructs were extracted, and SLF genes were then inserted into BiFC vectors, i.e. pDEST-SCYNE/SCYCE/VYNE/VYCE, via LR reaction (Fig. 9) which created destination constructs in Agrobacterium that would be verified by colony PCR (Fig. 31) and used in Agroinfiltration into leaves. At last, I had following S_1-SLF-BiFC constructs in stock and ready for BiFC assays: SLF1-SCYNE/SCYCE; SLF1dF-VYNE/SCYCE; SLF3- and SLF3dF-SCYNE/SCYCE/VYNE/VYCE; SLF4dF-SCYNE/SCYCE; SLF5-SCYNE/SCYCE; and SLF5dF-VYNE/SCYCE.

Gateway® Cloning SLF Variants from S_{ax1} *Petunia axillaris*

The sequence data from Petunia Sequencing Project indicated S-RNase sequence of *Petunia axillaris* "N" (designated as S_{ax1}-RNase) is 100% identical to S_1-RNase (our lab) from *Petunia hybrida*. Since the entire S-locus is suppressed for recombination as S-RNase and SLF genes are tightly linked, this finding indicates that the whole S-locus of the *P. axillaris* line and that of the S_1 haplotype of *P. hybrida* should be nearly identical. It can be further deduced that the SLF variants in the sequenced *P. axillaris* line are largely equivalent to those in S_1 *P. hybrida* in our lab. Therefore I was able to utilize the sequence data to design Gateway® primers (Table 6) and isolate more SLF variants for protein-interaction assays. Amplified SLF variants (Fig. 32) were subject to the same procedures of Gateway® cloning, eventually cloned into BiFC vectors and transformed into Agrobacterium as final BiFC constructs (Figs. 33, 34).

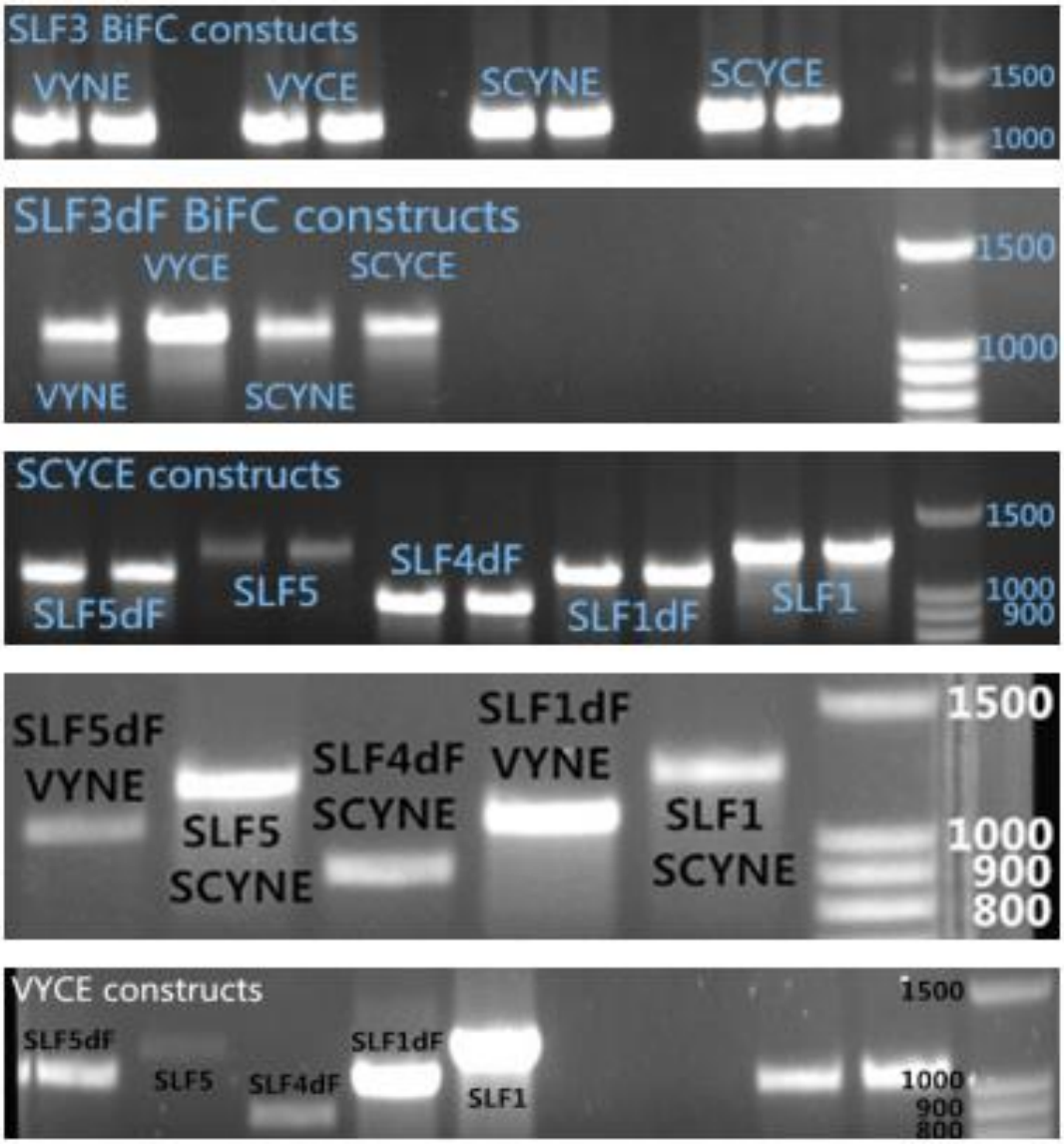


Figure 31: Electrophoresis of colony PCR verifying correct insertion of SLF genes into BiFC vectors in Agrobacterium.

Table 6: Gateway® primers amplifying S_{ax1} *Petunia axillaris* SLF variants. Primers were designed based on the sequencing data of *P. axillaris* and used to clone SLF variants into BiFC vectors via Gateway® cloning technique. Bold sequences are attB sites.

Primer	Sequence
S1-SLF4attB1Fwd1	**GGGGACACGTTTGTACAAAAAAGCAGGCT**AATG AATTTATATCGTAAAGAATACAAGATGGC
S1-SLF4dFattb1Fwd2	**GGGGACACGTTTGTACAAAAAAGCAGGCT**AACA TGAATATTCATCTACACCGCACC
S1-SLF4attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TAAT GTTTTGAACTTGTGTACTACTTTGGC
S1-SLF5dFBattB1Fwd	**GGGGACACGTTTGTACAAAAAAGCAGGCT**AACA ATGCATCTCAATCGCACAACAACGGTGAAG
S1-SLF5attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TAAA TTTGTGAACTGGTGTACTGTTTTCGC
S1-SLF2attB1Fwd1	**GGGGACACGTTTGTACAAAAAAGCAGGCT**ATGG CAAATAGAATTAAAAAACTGCCTGAAGATG
S1-SLF2dFattb1Fwd2	**GGGGACACGTTTGTACAAAAAAGCAGGCT**AACA TGCATCTCAACCGCATCACAACAACAAAG
S1-SLF2attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TAAA TTGAACTTGTGTACCATGTTCGCTTATTTTTTG
S1-SLF6attB1Fwd1	**GGGGACACGTTTGTACAAAAAAGCAGGCT**ATGG CGGATGGAATTATCAAAAAGTTGTC
S1-SLF6dFattb1Fwd2	**GGGGACACGTTTGTACAAAAAAGCAGGCT**AACA TGTATCTCTACAACACAACAACTTCTAGAGATGAA TATATTC
S1-SLF6attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TAAA TTTATAAACTTGTTTTGTATGCTCACTTTCTCTTGG
S1-SLF8attB1Fwd1	**GGGGACACGTTTGTACAAAAAAGCAGGCT**ATGA CGTTGGATGGAATTATGAAACATTTGC
S1-SLF8dFattb1Fwd2	**GGGGACACGTTTGTACAAAAAAGCAGGCT**AACA TGCATCTTAATCGTGCTACAACAACCACAG
S1-SLF8attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TAAT TTTTGTGCCATGCTTGCATCC

(Continued in following page)

Table 6 (continued)

Primer	Sequence
S1-SLF10attB1Fwd1	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCAT GAAGGAGTTGCCCCAAGATGTAG
S1-SLF10dFattB1Fwd2	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCAT GCATACGACCAACTTCAATGATGAATTGG
S1-SLF10attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**GAGT TACATCTAAAATTTTGAAGTTCTATGC
S1-SLF11attB1Fwd1	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCAT GAAGAAGTTTCACGAAGATGTGG
S1-SLF11dFattB1Fwd2	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCAT GCGTACTACAACCTATAAAGATGAATTAC
S1-SLF11attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**GCAA AAATTTTGAACTTTTGTGCCATCCTC
S1-SLF12attB1Fwd1	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCAT GCCGGACGGAATTATTATGAAATTGC
S1-SLF12dFattB1Fwd2	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCAT GTTCAAGCGCTCCTTCAAAGAAGATGTTG
S1-SLF12attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**GAGA AGTTGTGAACTTGTGTACTAC
S1-SLF13attB1Fwd1	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCAT GGATGGAACTATGAAGAAATTGCC
S1-SLF13dFattB1Fwd2	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCAT GCGAAAAACTACCACCAAAGATGAATTC
S1-SLF13attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**GAAA ATTTTTCAACTTGTGTATTGCC
S1-SLF14attB1Fwd1	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCAT GAAGATCGCATTGGAAGAAATCC
S1-SLF14dFattB1Fwd2	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCAT GTGGGCATTCATCATTCTTCAGC
S1-SLF14attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**GTTG AGCTGTATTAAACTCACTTTC

(Continued on following page)

Table 6 (continued)

Primer	Sequence
S1-SLF15attB1Fwd1	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGGGAGATGAAATTGTGGAAAAATTGC
S1-SLF15dFattB1Fwd2	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGCGTACCACCACCACCACC
S1-SLF15attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**GTAACTCTCTAAAATTTTTGGGCATTTGG
S1-SLF16attB1Fwd1	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGGCAGATGAAATTGTGATAAAGTTGC
S1-SLF16dFattB1Fwd2	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGCTCAATCGTAACATCACCACCAACG
S1-SLF16attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**GTACTCCTTGCATTGATCTTTTGGAATTAAAGC
S1-SLF18attB1Fwd1	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGTTGGATGGGACCATGAAGGAATTG
S1-SLF18dFattB1Fwd2	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGCATACAACCAACTTCAAGGATGAAC
S1-SLF18attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**GTAAAATTGTTGAACTTCTGCACCATTG
S1-SLF19attB1Fwd1	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGCAAAAGATGTTGGATGGGACCATAAAAG
S1-SLF19dFattB1Fwd2	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGCGTACAACCGAAGTCAAGGATGAATTG
S1-SLF19attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**GTAAAATTGTTGAACTTCTGCACCATCG
S1-SLF20attB1Fwd1	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGCAAAAGATGTTGGATGGGACCATAAAAG
S1-SLF20dFattB1Fwd2	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TCATGCGTACAACCGAAGTCAAGGATGAATTG
S1-SLF20attB2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**GTAANTTTGACCGTACTCTTTCATTATCC

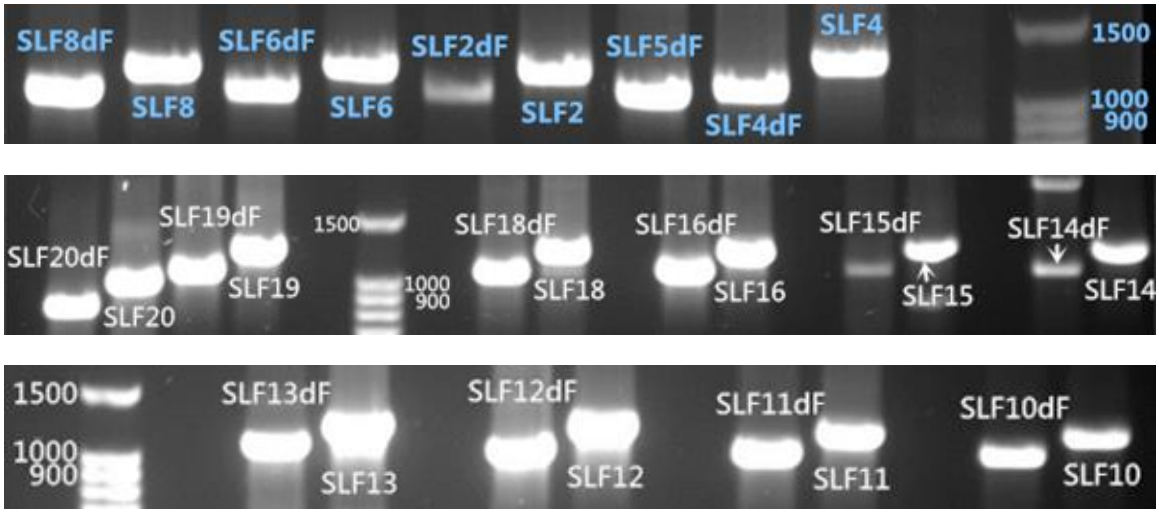


Figure 32: Electrophoresis of PCR of SLF4-20 variants from the *P axillaris* genomic DNA.

Figure 33: Electrophoresis of colony PCR verifying correct insertion of SLF2-8 genes into pDEST-SCYCE vector in Agrobacterium.

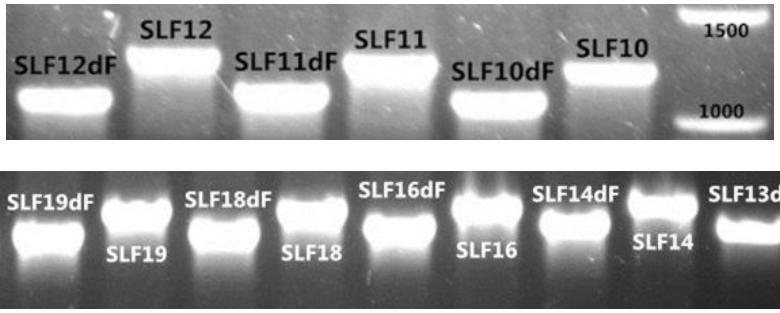


Figure 34: Electrophoresis of colony PCR verifying correct insertion of SLF10-20 genes into pDEST-VYCE vector in Agrobacterium.

Bimolecular Fluorescence Complementation (BiFC) Assays

Over the past several years, the use of fluorescent proteins as molecular tags has become increasingly prevalent. The advantage of fluorescent proteins is that they function in living tissue, and normally these proteins are small and stable enough not to disrupt the function of the tagged protein. BiFC takes this approach one step further. In BiFC, two halves of a fluorescent protein (which cannot independently fluoresce) are separately cloned as fusion-constructs to two proteins thought to interact (e.g. SBP1 and S-RNase). When the two proteins synthesize and interact in the same cell, the protein interaction also brings the two halves of the fluorescent protein together, reconstituting the fluorescence protein, whose fluorescence can then be observed by confocal microscopy (Gehl et al. 2009). BiFC is in current use in my lab, and fluorescence resulting from protein interactions in transient assays in leaf protoplasts has been observed (Diwa Malla, M.S. 2012 and personal communication).

The vectors (Fig. 35) chosen for preparing BiFC constructs are multicolor vectors (Gehl et al. 2009), i.e. pDEST-SCYNE, pDEST-SCYCE, pDEST-VYNE and pDEST-VYCE, which bring fluorescence at different wavelengths (Fig. 36) while reconstituting in different patterns (Venus-Venus 525 nm; SCP3A-SCP3A 480/495 nm; Venus-SCP3A 515 nm). Genes encoding certain target proteins, e.g. SLF, S-RNase, etc., are inserted via Gateway® cloning to replace ccdB genes, and fused with either N-terminal or C-terminal domain of the fluorescent protein. In one BiFC test, it is always necessary to pair one target protein fused with N-terminal domain, i.e. SCYNE or VYNE, with the other one fused with C-terminal domain, i.e. SCYCE or VYCE, resulting in a complementary reconstitution of fluorescent protein. The combination of two N-terminal constructs or two C-terminal constructs will fail to produce any fluorescence.

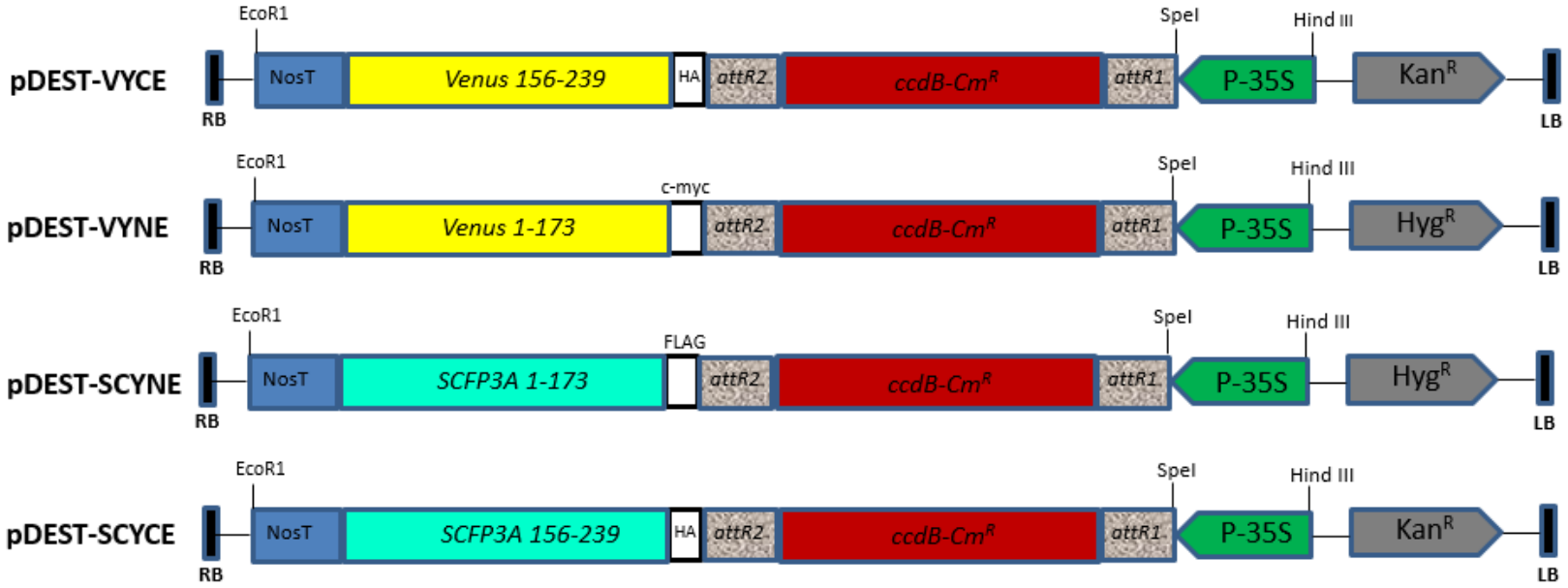


Figure 35: BiFC vectors. Each has a either N-terminal or C-terminal fragment of fluorescent protein. Target genes, e.g. genes encoding SLF, S-RNase, etc., are inserted between two attR sites to replace ccdB gene.

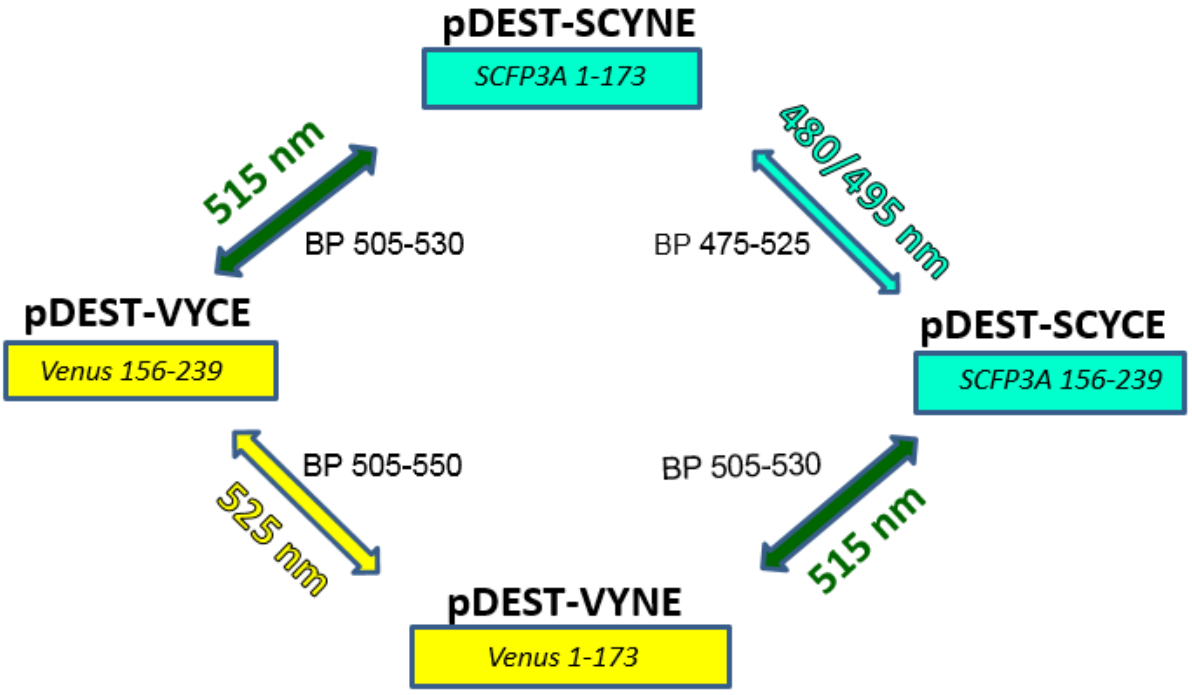


Figure 36: Wavelengths of multicolor BiFC vectors. Different combinations of reconstituting a fluorescent protein leading to emission of fluorescence under different wavelength. Corresponding detecting mode, i.e. BP 475-525, 505-530, or 505-550, is selected while using confocal microscopy.

BiFC assays – SLF1, 3, 4, 5 vs. Full-length S_1-RNase (self)

First of all, BiFC assays (Fig. 37) were performed between self SLF and S-RNase, i.e. both were carrying the same S-locus gene "S_1". Image A is the positive control CNX6::CNX7 ("::" mean both proteins were tested as a pair) which always gave strong signals. Image B is SLF1::CNX6 that has been proven to be qualified as negative control in previous BiFC assays (Malla 2012), which is usually completely dark, whereas sometimes with extremely dim fluorescence (*For all the following BiFC assays, same controls were used. To avoid redundancy, they are only mentioned here once*). Images C and D are SLF1/1dF:: S_1-RNase ("dF" represents removal of F-box sequence in the SLF variant); Images E and F are SLF3/3dF:: S_1-RNase; Image G is SLF4dF:: S_1-RNase; and finally images H and I are SLF5/5dF:: S_1-RNase. Images C to I show much weaker signal than the positive control, but are significantly more visible than the negative control. These finding are consistent with the mechanism of SCF^{SLF} E3 ubiquitination ligase model in which self S-RNase will be not degraded. That is less or no interaction with SLF leading to weak or no fluorescence in BiFC assays.

BiFC assays – SLF1, 3, 4, 5 vs. S_1-RNase Mature (self)

S_1-RNase mature was constructed by cleaving signal peptide from a complete S_1-RNase protein, in order to ask whether the signal-peptide domain was required for recognition of self S-RNase. Images A and B (Fig. 38) are controls; C and D are SLF1/1dF:: S_1-RNase mature; E and F are SLF3/3dF:: S_1-RNase mature; G is SLF4dF:: S_1-RNase mature; and H is SLF5:: S_1-RNase mature. Image of SLF5dF:: S_1-RNase mature was not saved because it was as dark as the negative control (Same reason for images not shown in text below). Since these self SLF::S-

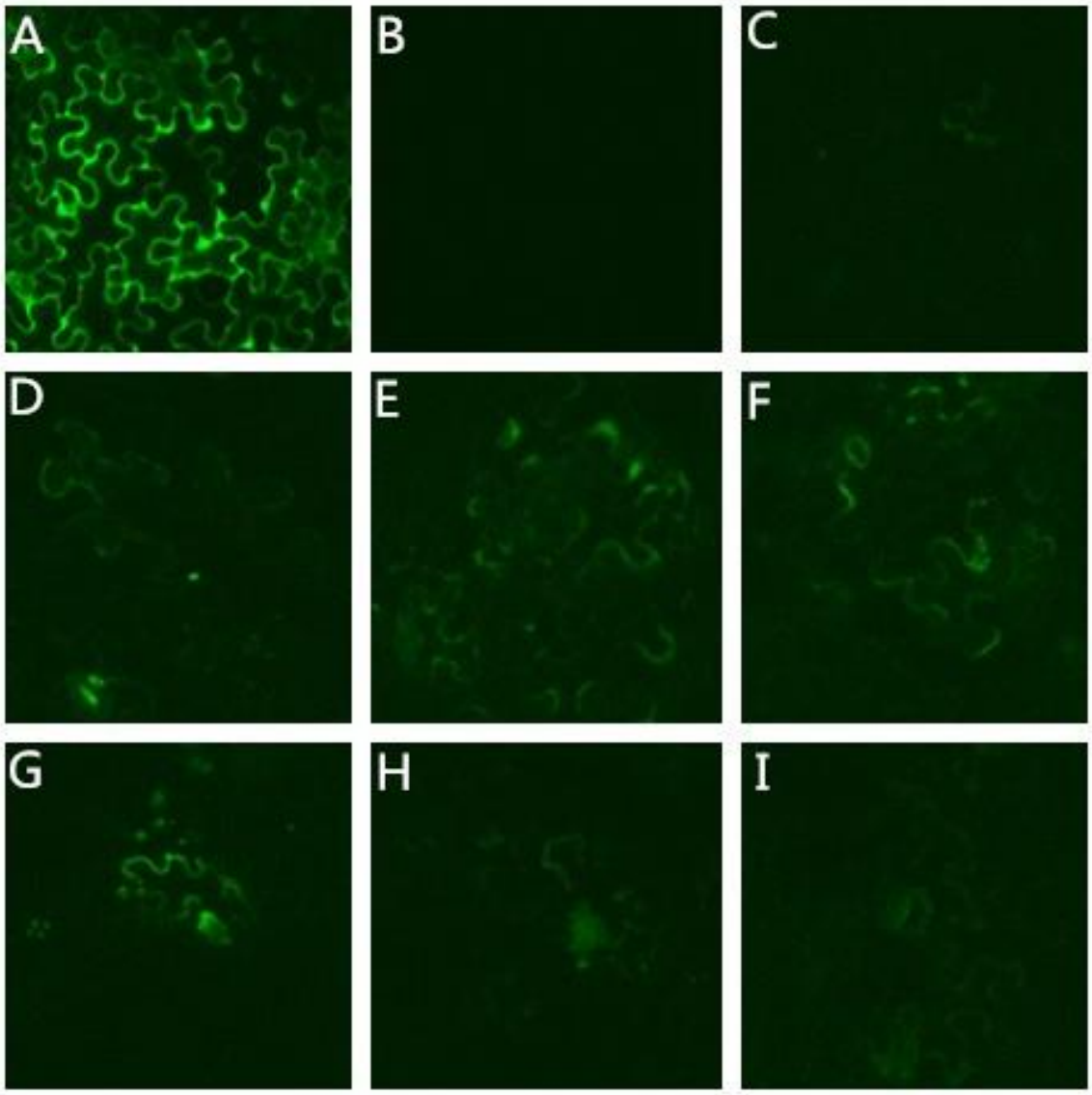


Figure 37: BiFC assays - SLF1, 3, 4, 5 vs. full-length S_1-RNase (self).

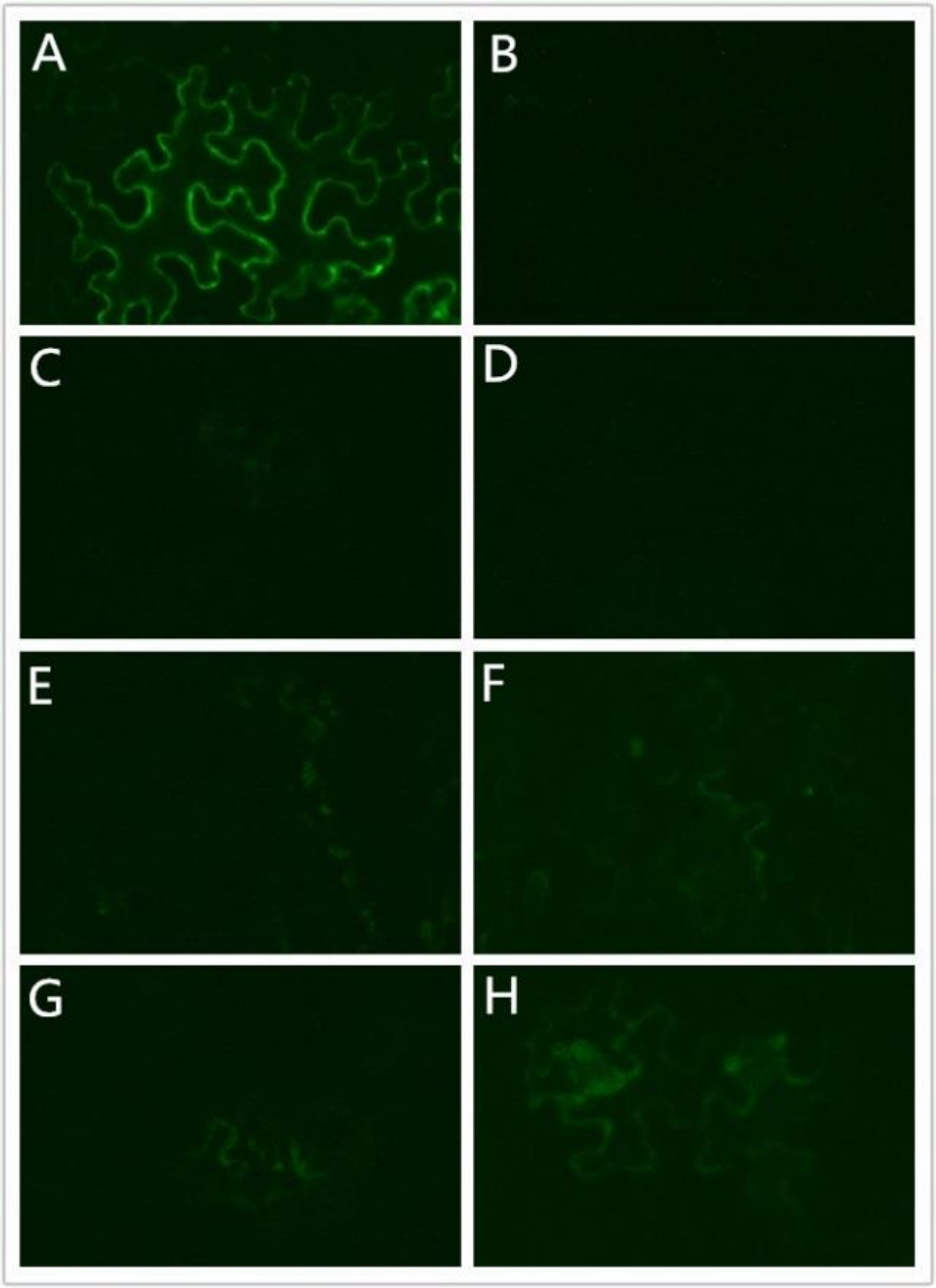


Figure 38: BiFC assays – SLF1, 3, 4, 5 vs. S_1-RNase mature (self).

RNase pairs gave either weak or none fluorescence under confocal microscopy, results of SLF::S_1-RNase mature BiFC assays were consistent with those from SLF::full-length S_1-RNase BiFC assays.

BiFC assays – SLF1, 3, 4, 5 vs. S_3-RNase NT (non-self)

S_3-RNase NT was constructed by removing domains including C4, glycosylation site, and C5 (Fig. 3), while maintaining only the rest of the N-terminal part of S_3-RNase, for the purpose of determining whether the specific C-terminal regions were required for recognition of non-self S-RNase. (In previous two-hybrid experiments, Sims and Ordanic 2001 had shown that the N-terminal portion of the S-RNase was sufficient to interact with SBP1, whereas full-length constructs did not interact, possibly because of misfolding of the larger protein.) Images A and B (Fig. 39) were controls; C and D were SLF1/1dF:: S_3-RNase NT; E and F were SLF3/3dF:: S_3-RNase NT; G was SLF4dF:: S_3-RNase NT; and H was SLF5:: S_3-RNase NT. Unexpectedly, such non-self SLF::S-RNase combinations did not produce stronger signal than self pairs. Although the positive control was not as strong as it was in two "self" tests above, the signal from positive control was still very widely distributed on the edge of the epidermal cells indicating high-level protein expression. Fluorescence from other SLF:: S_3-RNase NT pairs, however, could only be found on limited numbers of cells.

BiFC assays – SLF1, 3, 4, 5 vs. S_3-RNase Mature (non-self)

S_3-RNase mature was constructed by cleaving signal peptide from a complete S_3-RNase protein, in order to ask whether the signal-peptide domain was required for recognition of non-

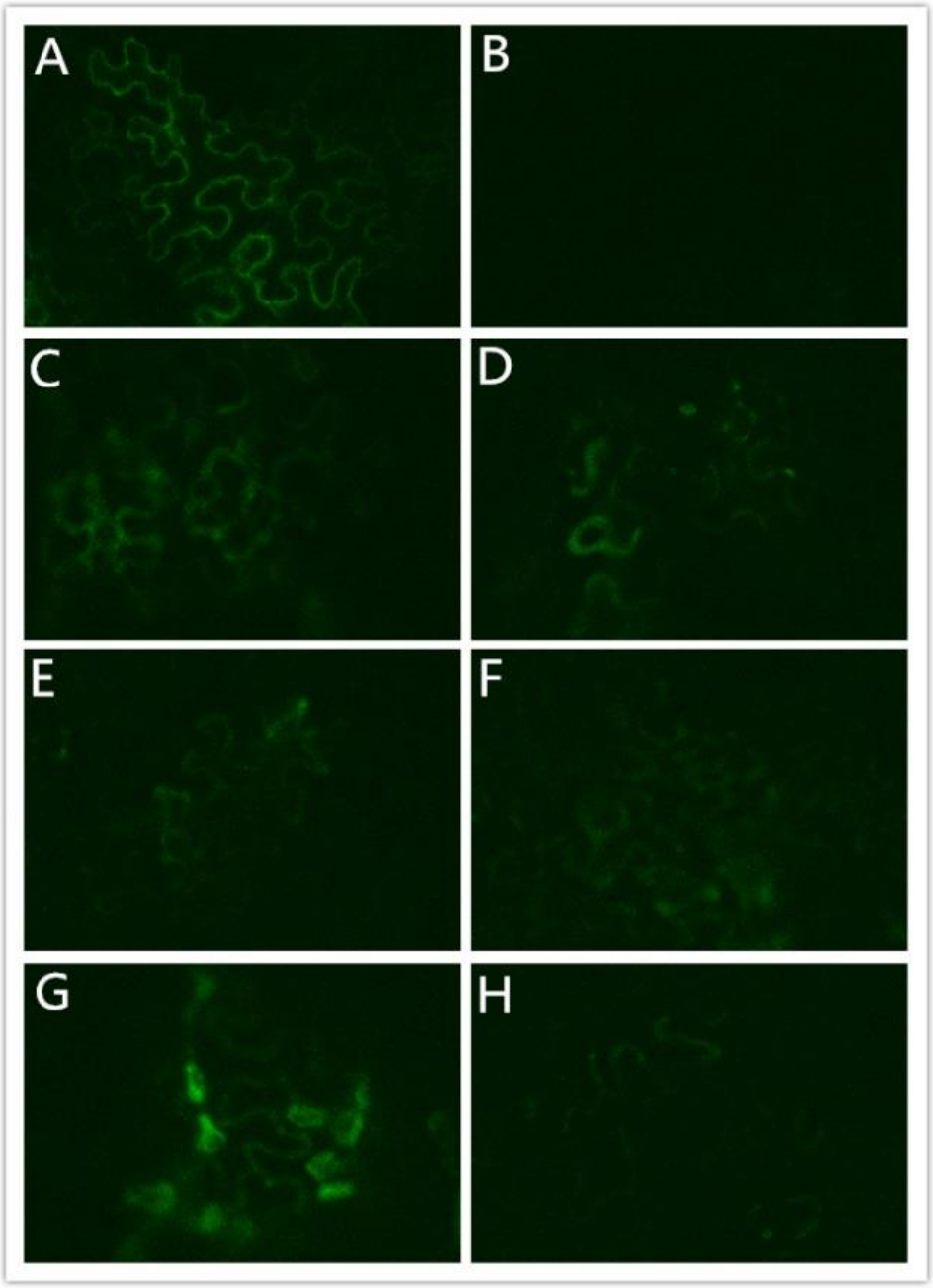


Figure 39: BiFC assays – SLF1, 3, 4, 5 vs. S_3-RNase NT (non-self).

self S-RNase. Combinations of SLF:: S_3-RNase mature did not provide stronger signal as expected either (Fig. 40). Images A and B were controls. The only sample giving visible signal was SLF1::S_3-RNase mature shown in image C.

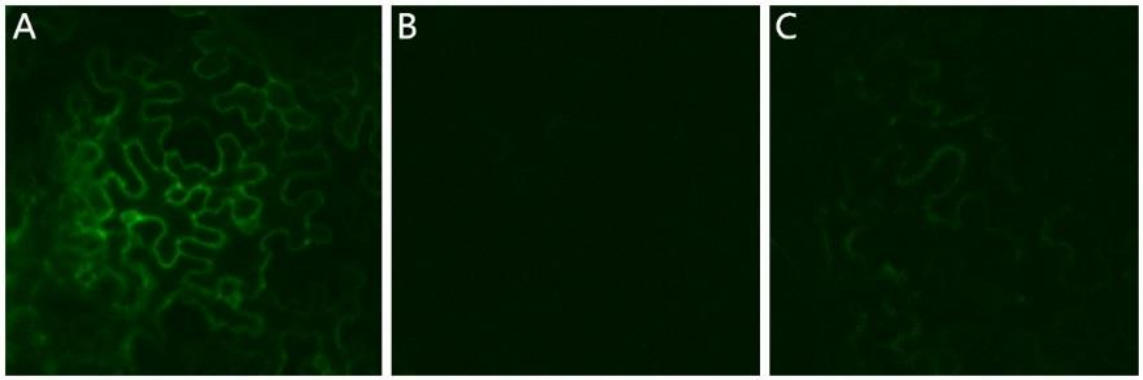


Figure 40: BiFC assays – SLF1, 3, 4, 5 vs. S_3-RNase mature (non-self).

<u>BiFC assays – SLF10, 11, 12, 13, 14, 16, 18, 19, 20 vs. Non-self S_3-RNase (Full-length, Mature, and NT)</u>

SLF10-20 variants were isolated from genomic DNA of *Petunia axillaris* based on the sequence data from the Petunia Genome Project, and were tested with each non-self S-RNase construct in three consecutive BiFC assays. Considering the efficiency and the availability of leaves, the preferable number of samples, including positive and negative controls, processed all together was normally around ten in one BiFC test.

It was observed that SLF10dF, 16, 19 and 20 could interact with full-length S_3-RNase, and gave weak signal around the edge of the epidermal cells (Figs. 41, 42, 43). In BiFC assays between SLF10-20::S_3-RNase mature (Figs. 44, 45, 46), it was found only SLF18, 18dF, 19, 19dF, 20 and 20dF could interact with S_3-RNase mature and gave weak signal. Among the pairs of SLF10-20::S_3-RNase NT (Figs. 47, 48, 49), no signal was found except SLF10 and 10dF showing relatively strong fluorescence which indicated the existence of close interaction between SLF10 and N-terminal domain S_3-RNase.

BiFC assays – SLF1, 3, 4, 5 vs. SBP1/ RBX1 or SSK1

I also carried out BiFC assays by pairing S_1-SLF variants with other proteins involved in SCF^{SLF} E3 ubiquitin ligase model during pollen recognition, such as SBP1/RBX1, and SSK1, for the purpose of investigating the role of these proteins, and looking for potential protein interactions between them and the SLF variants.

The interactions between SLF::SBP1 were found significantly more visible than those of SLF::S-RNase, in terms of the strength of signal itself, as well as the wideness of fluorescent distribution on edge of epidermal cells. Based on the signal shown in the images (Fig. 50) taken under confocal microscopy, it could be observed that SLF3/3dF, SLF4dF and SLF5/5dF interacted with SBP1 powerfully and extensively. SLF1 and 1dF, however, gave very weak signal and unspecific fluorescent noise in the background.

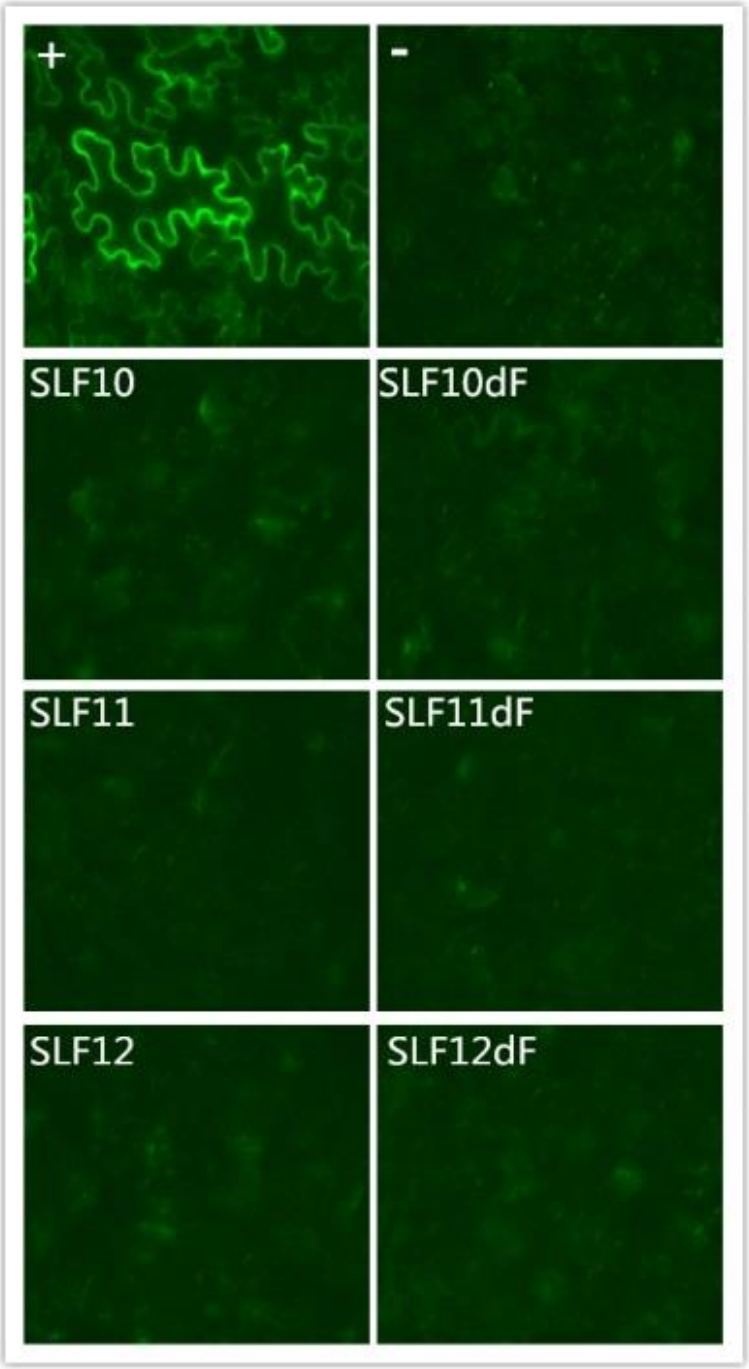


Figure 41: BiFC assays – SLF10, 11, 12 vs. full-length S_3-RNase (non-self).

Figure 42: BiFC assays – SLF13, 14, 16 vs. full-length S_3-RNase (non-self).

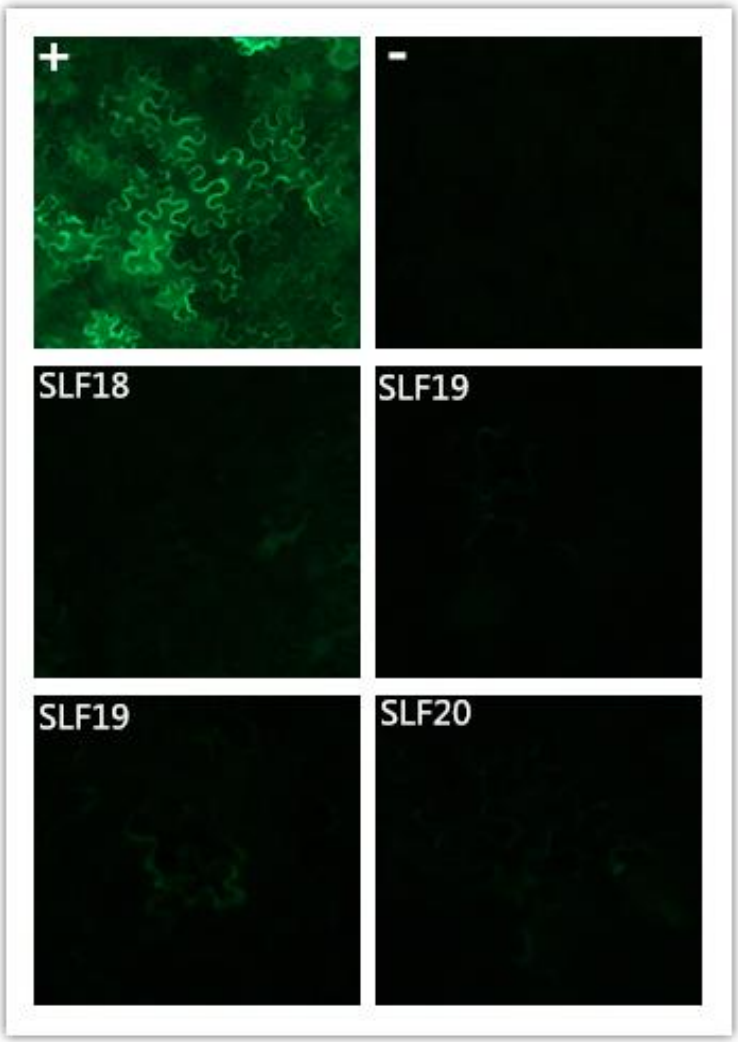


Figure 43: BiFC assays – SLF18, 19, 20 vs. full-length S_3-RNase (non-self).

Figure 44: BiFC assays – SLF10, 11, 12 vs. S_3-RNase mature (non-self).

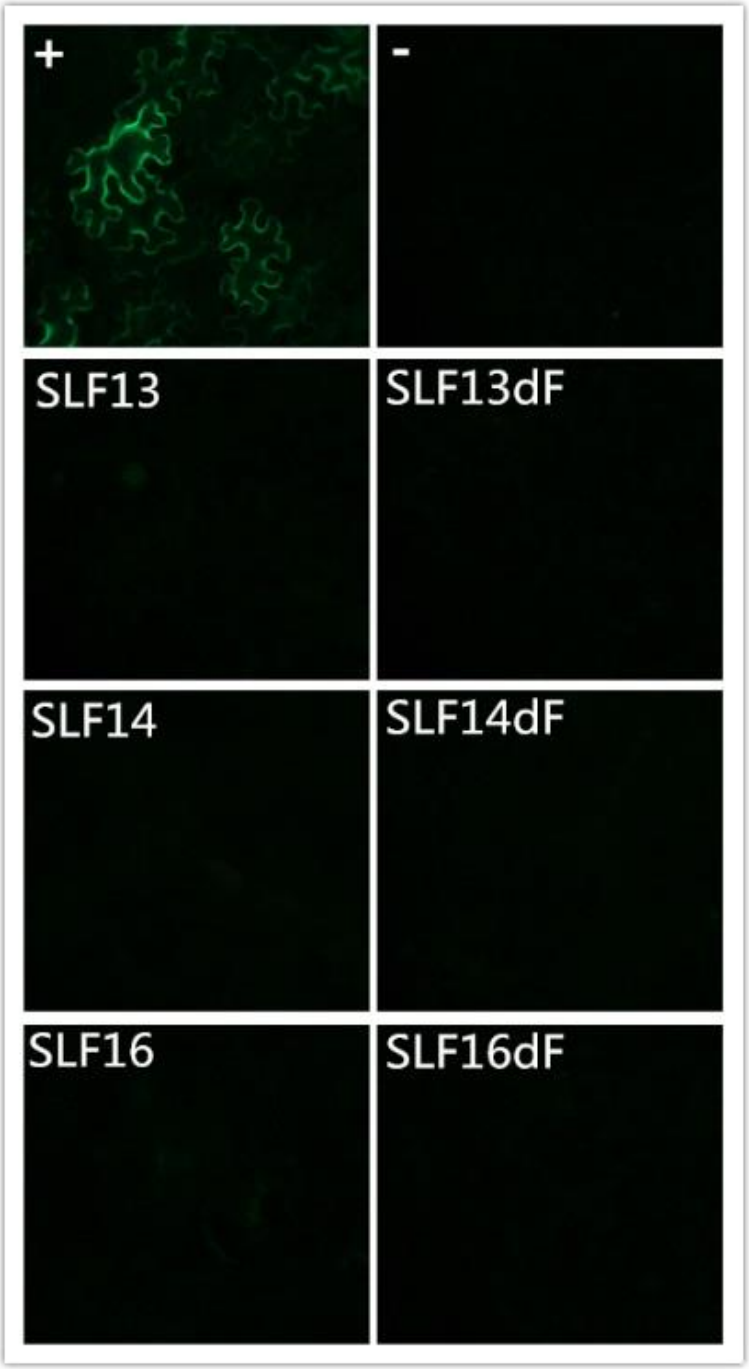


Figure 45: BiFC assays – SLF13, 14, 16 vs. S_3-RNase mature (non-self).

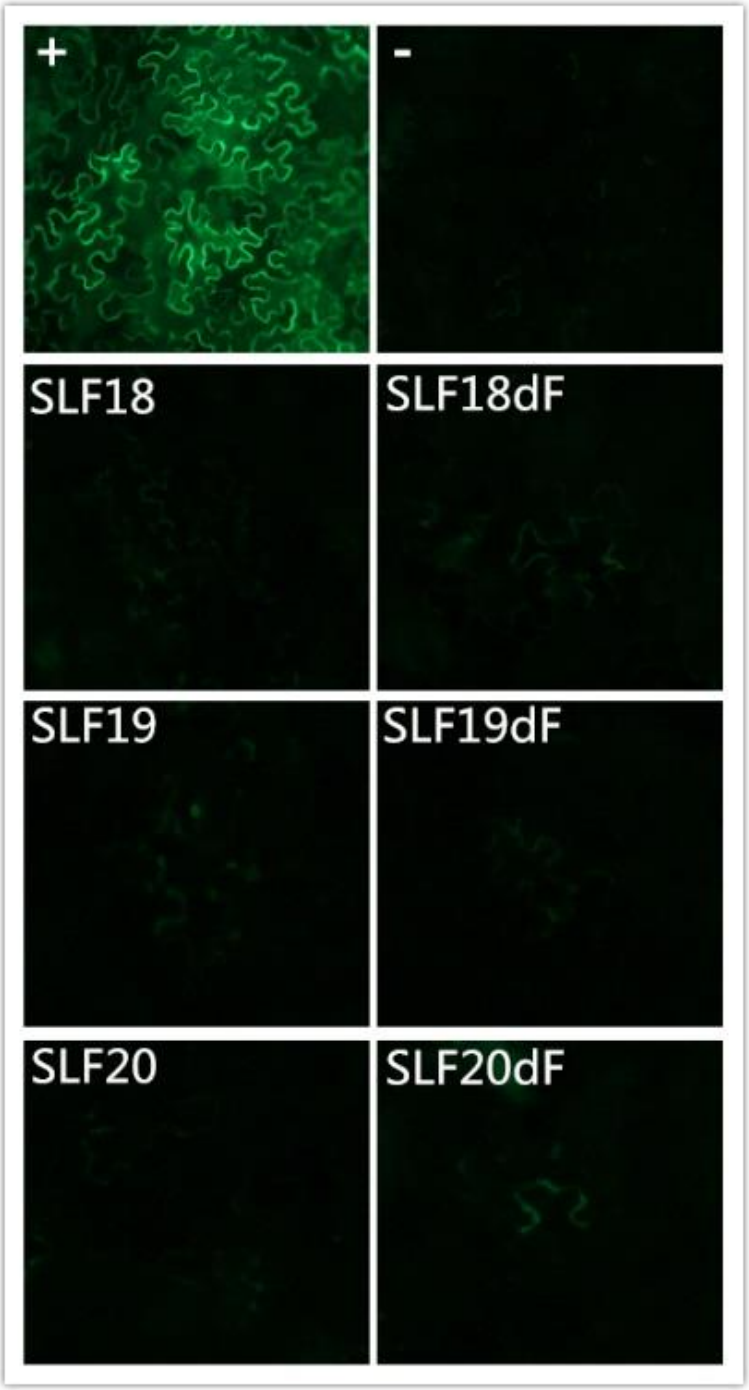


Figure 46: BiFC assays – SLF18, 19, 20 vs. S_3-RNase mature (non-self).

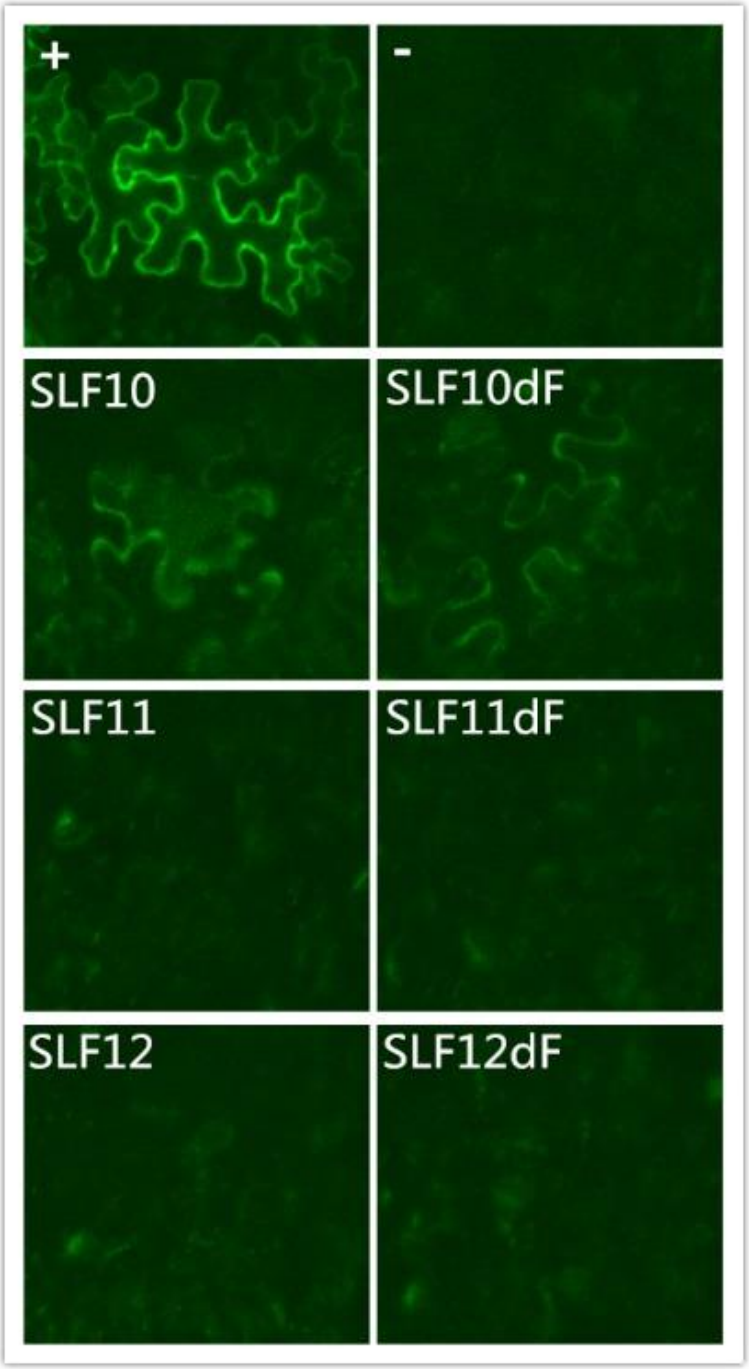


Figure 47: BiFC assays – SLF10, 11, 12 vs. S_3-RNase NT (non-self).

Figure 48: BiFC assays – SLF13, 14, 16 vs. S_3-RNase NT (non-self).

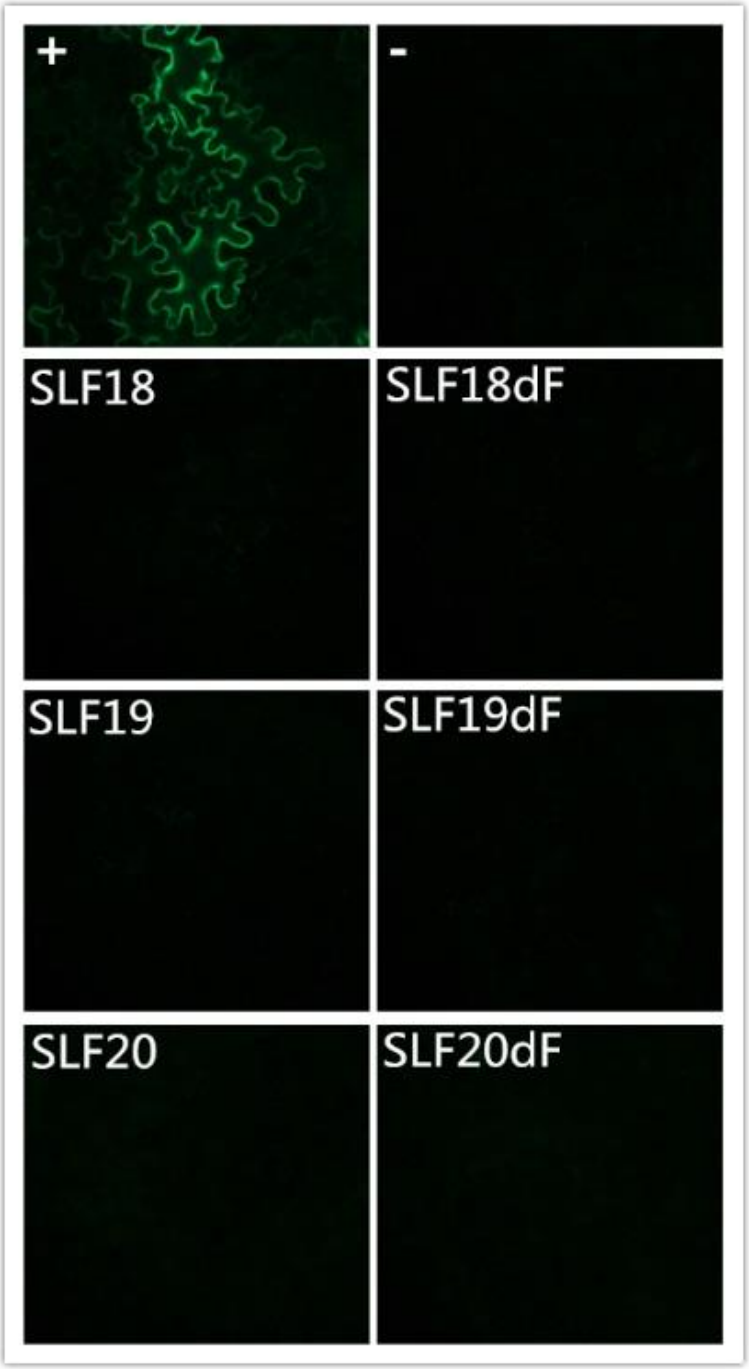


Figure 49: BiFC assays – SLF18, 19, 20 vs. S_3-RNase NT (non-self).

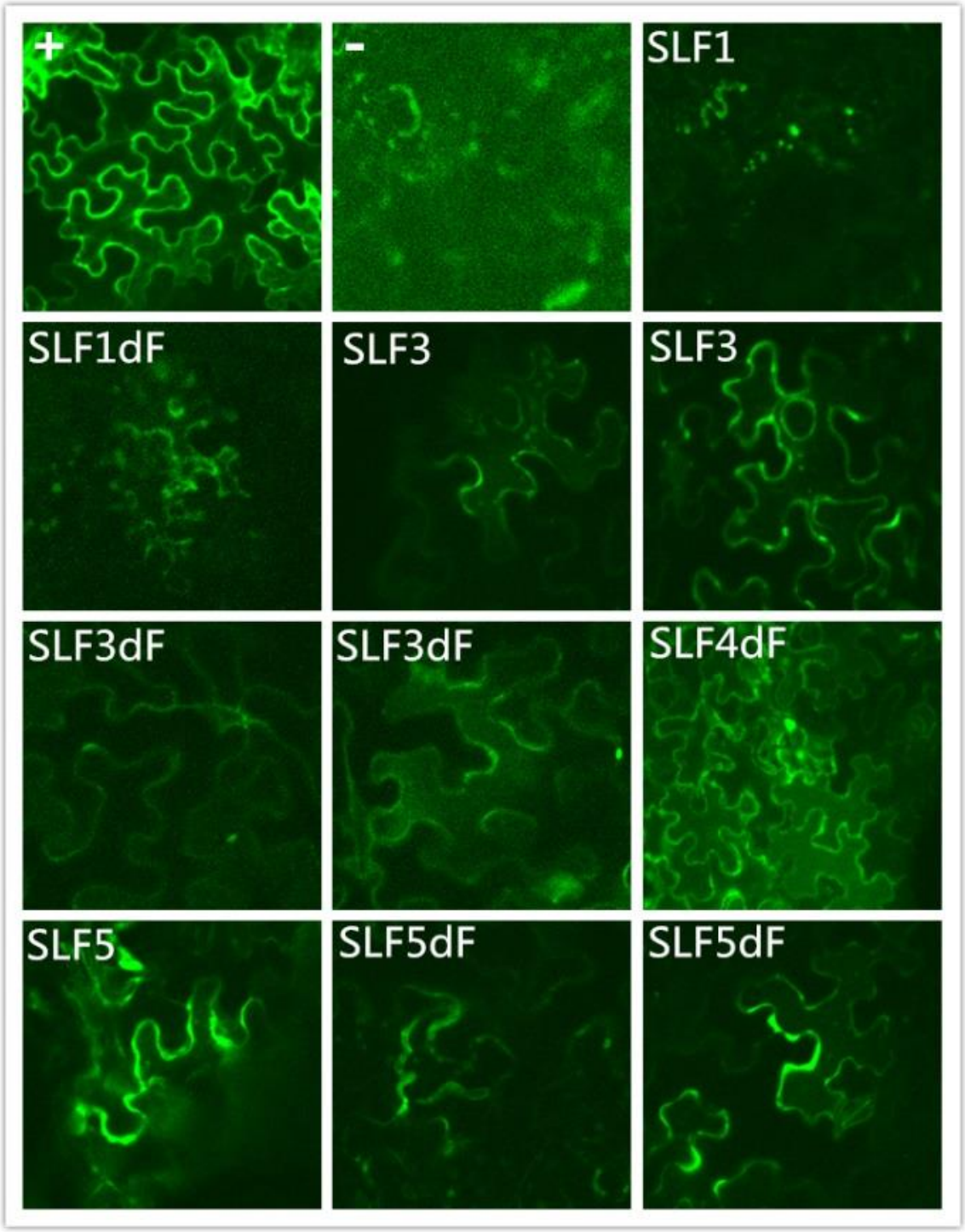


Figure 50: BiFC assays – SLF1, 3, 4, 5 vs. SBP1.

The results from BiFC assays of SLF1-5::RBX1 (Fig. 51) showed similar protein-interaction pattern, except the signals from SLF1/1dF::RBX1 appeared to be more detectable than those from SLF1/1dF::SBP1, indicating stronger interactions existed between SLF1/1dF and RBX1.

The interactions between SLF and SSK1 was nearly undetectable, except SLF4dF::SSK1 yielded fluorescence (Fig. 52). The images of other SLF variants with SSK1 under microscopy was as dark as the negative control, and therefore not presented.

BiFC Assays Involving Hypothesized "Bridge" Proteins

Given the BiFC results presented above, both self and non-self SLF::S-RNase combinations gave relatively dim signals indicating weak interactions in between, which was contrary to the evidence provided by previous co-immunoprecipitation assays and transgenic tests which indicated strong interactions between SLF and non-self S-RNase (Hua and Kao 2006). So I decided to involve more extra protein and tested the hypothesis that the interactions between SLF and non-self S-RNase could be stabilized by "bridge" protein(s) (Fig. 53). Candidates of "bridge" proteins were SBP1/RBX1, SSK1 and Cullin-1 whose participation in pollen recognition had been demonstrated, especially SBP1 and SSK1 had been found interact with SLF in previous BiFC assays (Figs. 50, 52).

Coding sequences of "bridge" proteins were inserted into modified BiFC vectors in which the sequences encoding fluorescent proteins, i.e. Venus or SCFP3A (Fig. 35), had been truncated. Bridge proteins were also prepared as Agrobacterium constructs (Fig. 54), so that they could normally express in leaves without producing fluorescent protein to affect results.

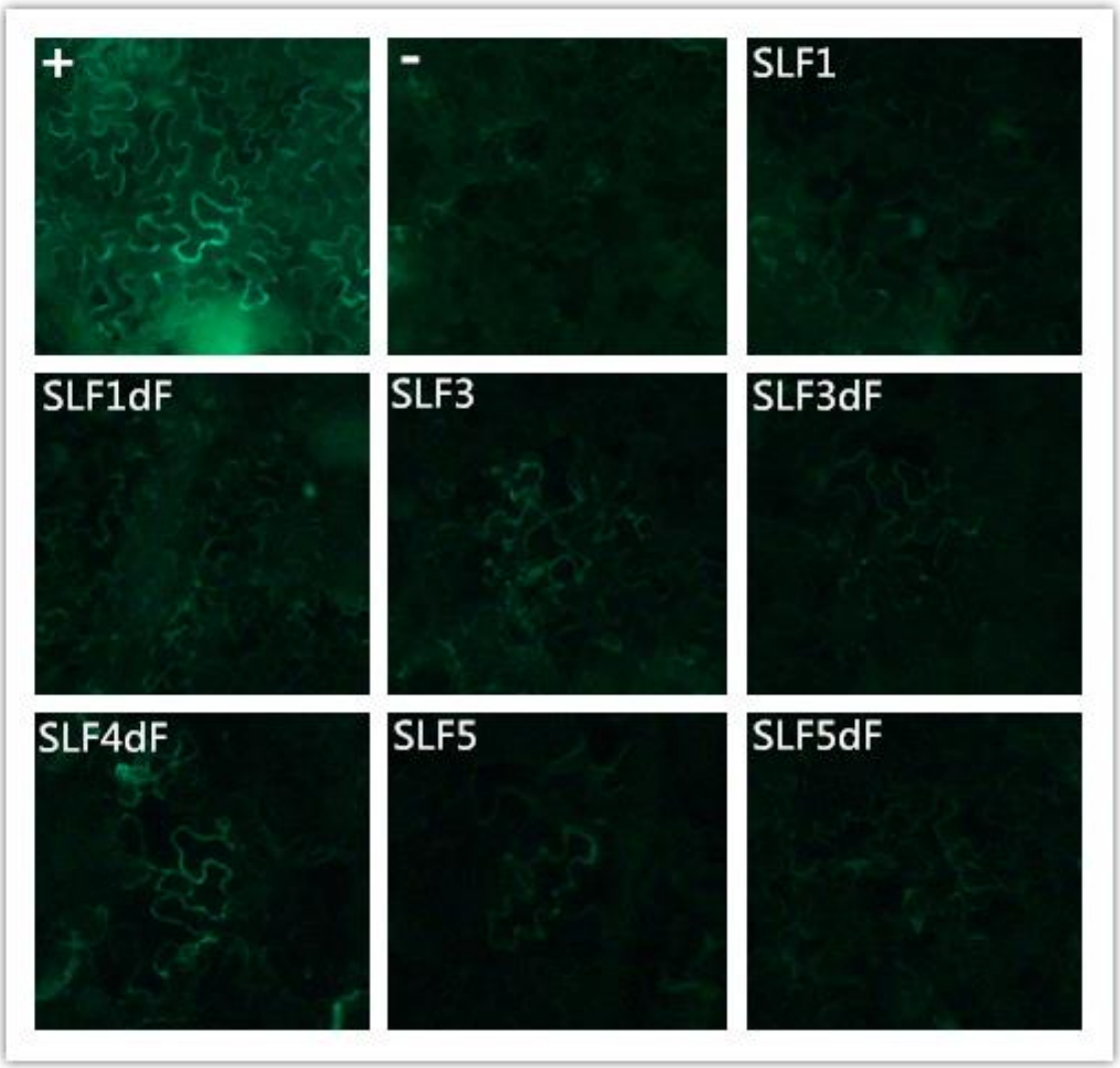


Figure 51: BiFC assays – SLF1, 3, 4, 5 vs. RBX1.

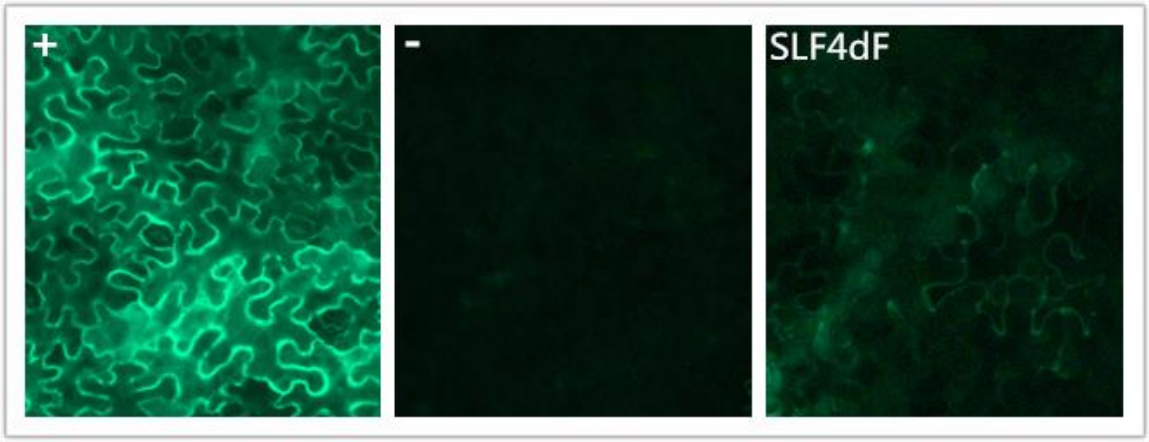


Figure 52: BiFC assays – SLF1, 3, 4, 5 vs. SSK1.

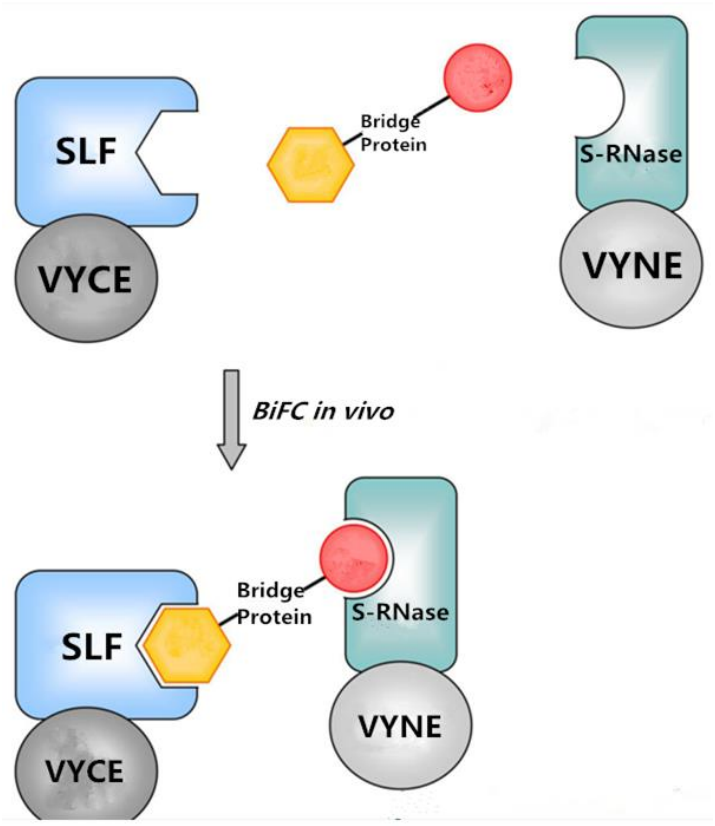


Figure 53: Hypothetical bridge protein that stabilizes the SLF::S-RNase protein interactions in BiFC assays *in vivo*.

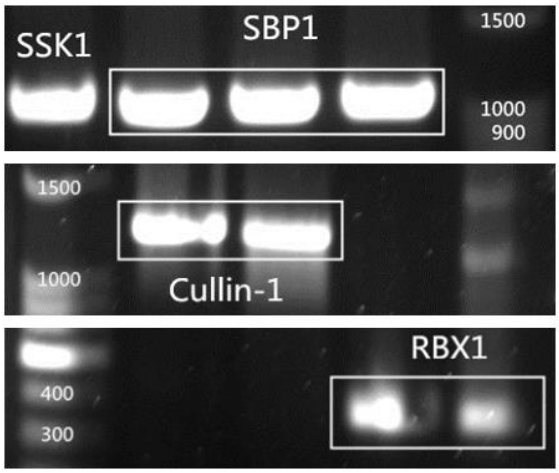


Figure 54: Electrophoresis of colony PCR verifying correct insertion of SBP1, RBX1, SSK1 and Cullin-1 genes into modified BiFC vector.

Selected "bridge" protein(s) was/were co-infiltrated with the pairs of SLF::S-RNase of interest, i.e. 3-way, 4-way or even 5-way BiFC assays. The goal was to discover whether hypothetical bridge protein(s) could strengthen the weak protein interactions found in previous non-self SLF::S-RNase BiFC tests.

In addition, the Zeiss confocal microscopy in our department broke down twice in middle of several BiFC tests, and I had to switch to Nikon Eclipse E-600 which presented signal in a slightly different mode, for example the color of fluorescence. For precise comparisons of signals before or after adding "bridge" protein(s), I carried out SLF1-5::S_3-RNase mature BiFC assays again and took pictures (Fig. 55) under Nikon Eclipse E-600. It was found only SLF1 showing visible fluorescence while others were as dark as the negative control, which was consistent with the results obtained under confocal microscope (Fig. 40). This test was to provide

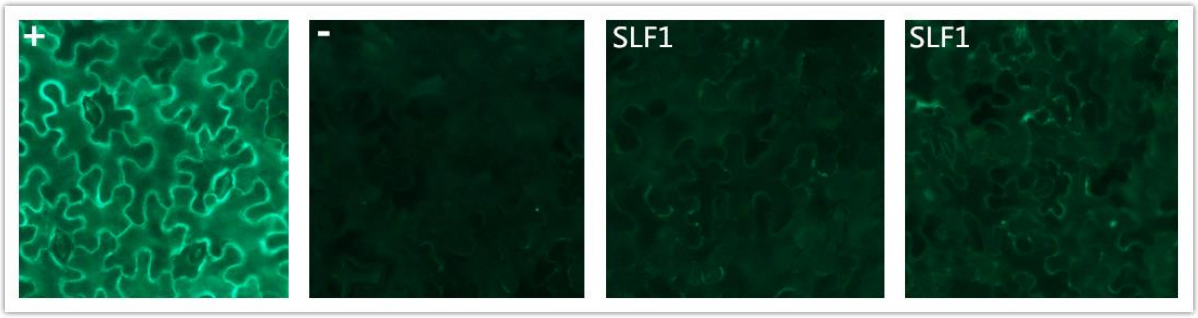


Figure 55: BiFC assays – SLF1-5 vs. S_3-RNase mature. Fluorescence was observed under Nikon Eclipse E-600 microscopy instead of Zeiss confocal microscope.

a general concept of how signals of the same combination of S_1-SLF::S_3-RNase mature were presented under the Nikon Eclipse E-600 microscopy.

Three-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase Mature + "Bridge" SBP1. Based on the images of SLF::S_3-RNase mature + bridge SBP1 (Fig. 56), and meanwhile considering the fluorescent strength of the positive control, it appeared that bridge SBP1 did improve the strength of the signal from SLF1::S_3-RNase mature. Furthermore, the addition of bridge SBP1 brought SLF3:: and SLF5::S_3-RNase mature to be significantly more detectable.

Three-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase Mature + "Bridge" RBX1. The results of BiFC tests including "bridge" RBX1 were mixed (Fig. 57). That was SLF1::S_3-RNase mature gave stronger fluorescence without adding bridge RBX1, while SLF3dF::S-RNase yielded brighter signal after co-filtrating with bridge RBX1.

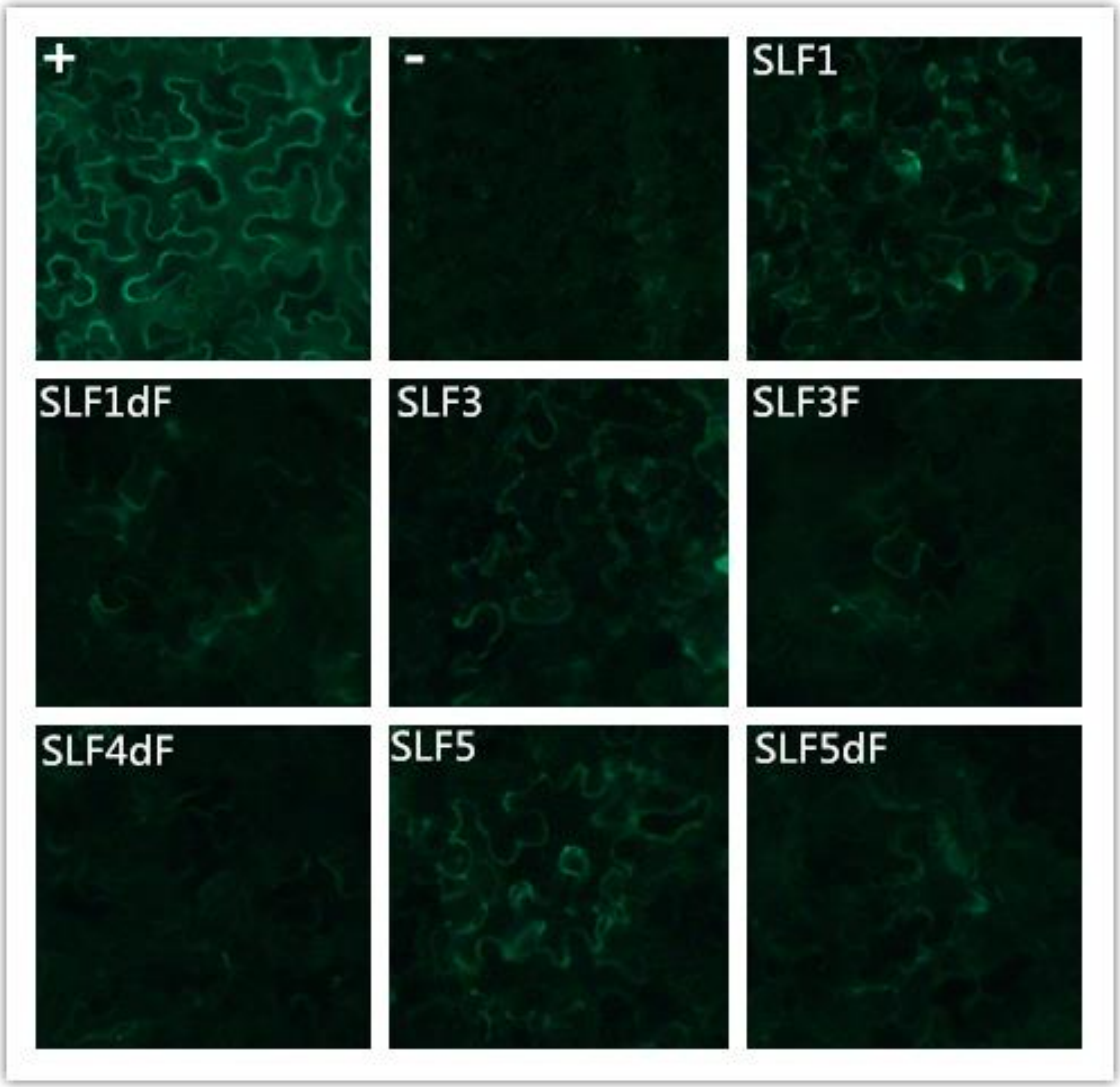


Figure 56: Three-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase mature + "Bridge" SBP1.

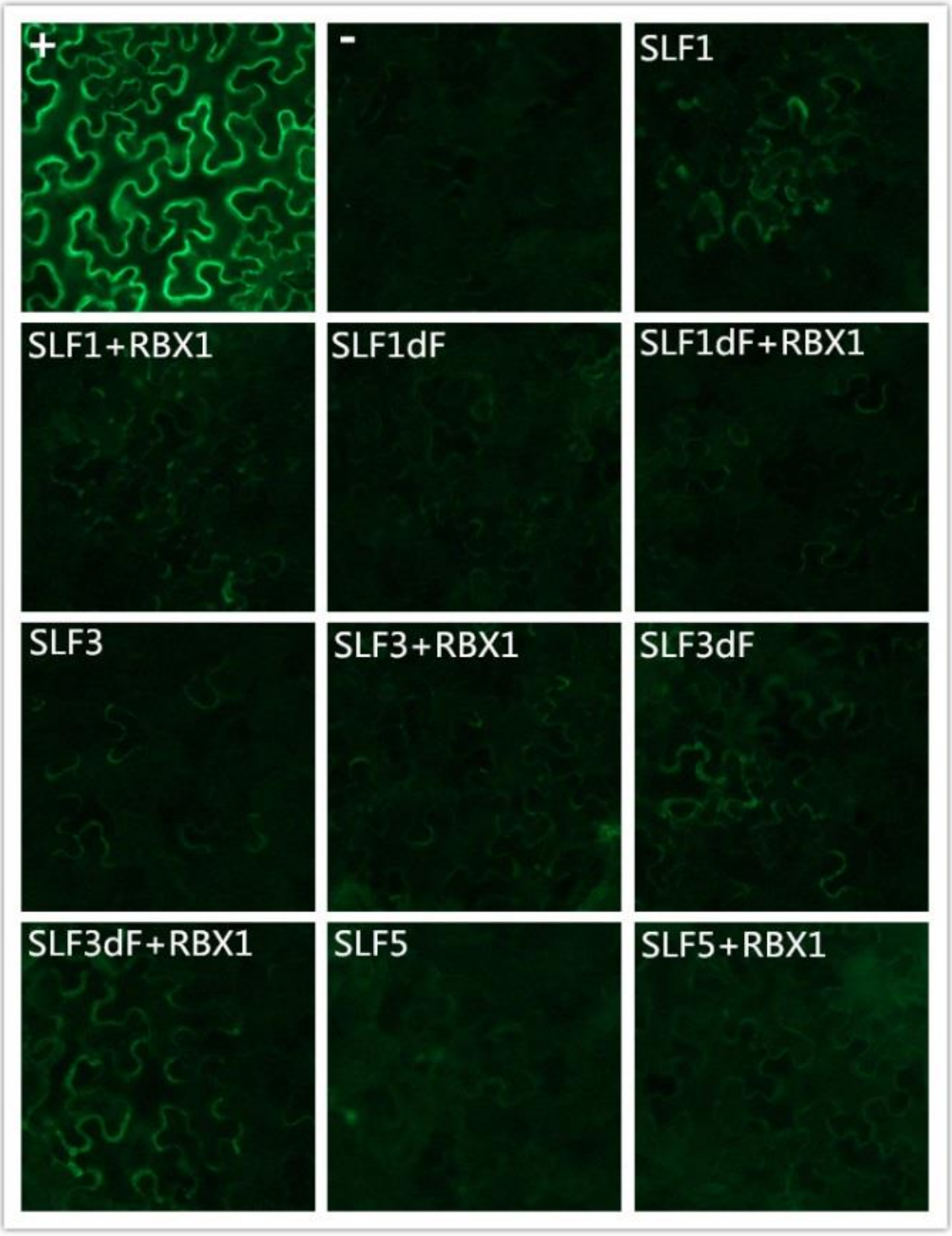


Figure 57: Three-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase mature + "Bridge" RBX1.

Three-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase Mature + "Bridge" SSK1. Co-infiltrating SSK1 with different pairs of SLF::S_3-RNase mature failed to show any effect of strengthening fluorescent signals (Fig. 58).

Three-way BiFC – Reverse SLF1, 2, 3, 4, 5, 6, 8, S3b vs. S_3-RNase Mature +/- "Bridge" SSK1. One more question that could be asked was whether the orientation of BiFC constructs affected the strength of fluorescent signals or not. To answer, selected reverse S_1-SLF variants (Gateway® PCR Primers are listed in Table 7) were cloned into the pDEST-VYCE(R) vectors (Figs. 59, 60) in which fluorophore coding sequence is located upstream of the SLF gene. BiFC tests between S_1-SLF-VYCE(R) and S_3-RNase-VYNE, with or without bridge SSK1, were carried out to find out if the results were consistent with the results between S_1-SLF-VYCE and S_3-RNase-VYNE, +/- bridge SSK1. Results (Fig. 61) indicated that bridge SSK1 could only strengthen the protein interactions existed in SLF2::S_3-RNase mature pair.

Four-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase Mature + "Bridges" SBP1 and SSK1. Signals were barely detectable while co-infiltrating with two hypothetical "bridge" proteins SBP1 and SSK1 at the same time (Fig. 62). No effect of signal improvement was found by adding bridges SBP1 and SSK1.

Four-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase Mature + "Bridges" SBP1 and Cullin-1. Signals were weak under microscopy. Although SLF5dF::S_3-RNase mature pair, with bridges SBP1 and Cullin-1, appeared to be fairly bright, it was mostly unspecific fluorescence in the background, instead of locating on the edge of the epidermal cells like the positive control (Fig. 63). No sign of signal strengthening was verified by adding bridges SBP1 and Cullin-1 at the same time.

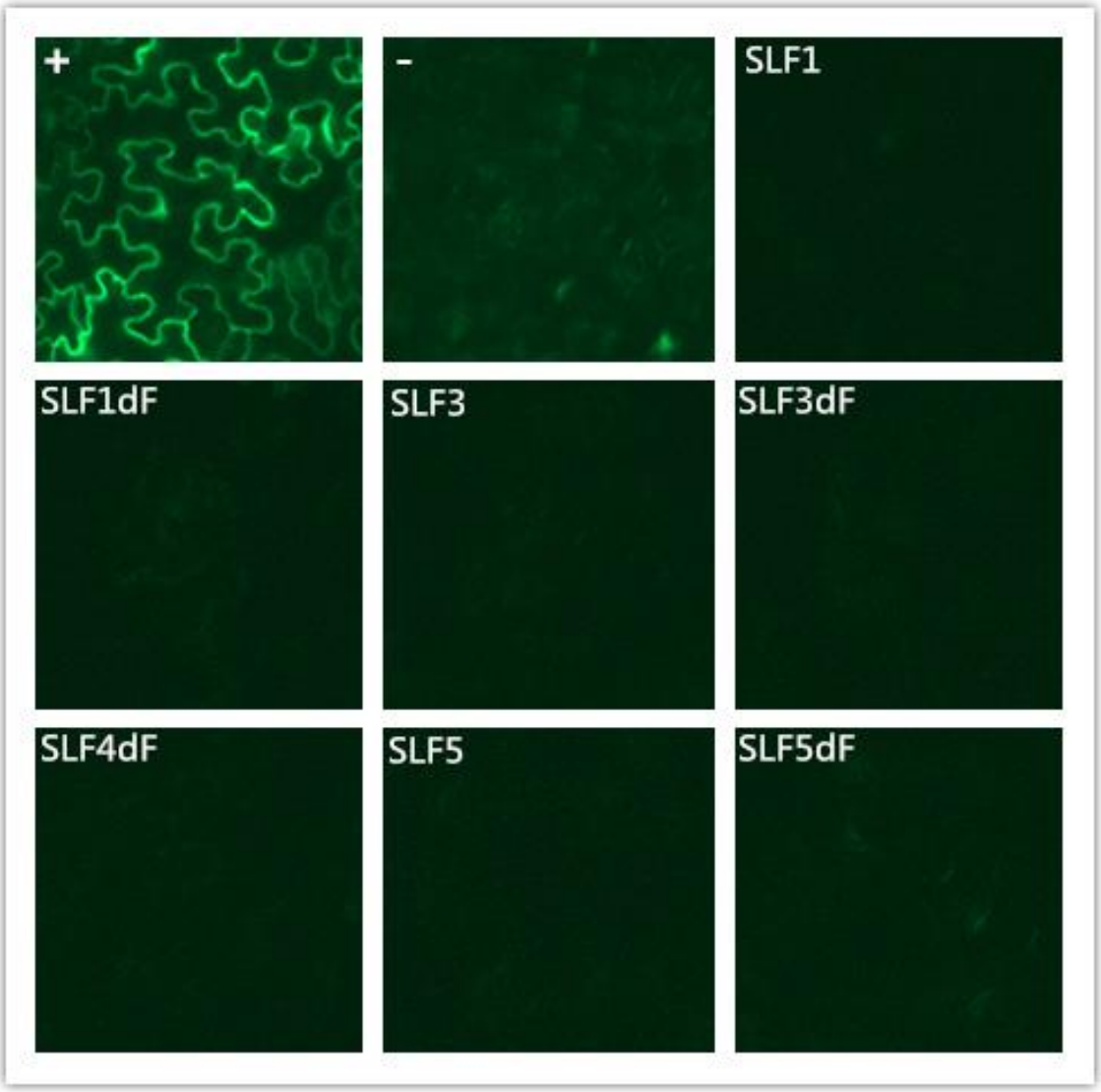


Figure 58: Three-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase mature + "Bridge" SSK1.

Table 7: Gateway® primers amplifying reverse S_{ax1} *Petunia axillaris* SLF variants. Primers were designed based on the sequencing data of *P. axillaris* and used to clone SLF variants into reverse BiFC vectors via Gateway® cloning technique. Bold sequences are attB sites.

Oligo	Sequence
S1-SLF1attB1GW2Fwd	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TAATGGCGAATGGTATTTTAAAGAAATTGCCCG
S1-SLF1attB2GW2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**CCTAAAATTTTTGTACTTTTGTACTGTACTCGCTCGC
S1-SLF2attB1GW2Fwd	**GGGGACAAGTTTGTACAAAAAAGCAGGCT**TAATGGCAAATAGAATTAAAAAACTGCCTGAAGATG
S1-SLF2attB2GW2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TCTAAAATTGTTGAACTTGTGTACCATGTTCGC
S1-SLF3attB1GW2Fwd	**GGGGACACGTTTGTACAAAAAAGCAGGCT**TAATGACGGCCATGAAGAAATTGCCC
S1-SLF3attB2GW2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TCTAAAAATTTTGAACTTGTGTACTACCCTTAGGAATTGG
S1-SLF4attB1GW2Fwd	**GGGGACACGTTTGTACAAAAAAGCAGGCT**TAATGAATTTATATCGTAAAGAATACAAGATGGCGGACAG
S1-SLF4attB2GW2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TCTAAATGTTTTGAACTTGTGTACTACTTTGGCTTCC
S1-SLF5attB1GW2Fwd	**GGGGACACGTTTGTACAAAAAAGCAGGCT**TAATGAAGATGCCACATGGAATTATGAAGAAATTGC
S1-SLF5attB2GW2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TCTAAAATTTGTGAACTGGTGTACTGTTTTCGC

(Continued on following page)

Table 7 (continued)

Oligo	Sequence
S1-SLF6attB1GW2Fwd	**GGGGACACGTTTGTACAAAAAAGCAGGCT**TAATGGCGGATGGAATTATCAAAAAGTTGTCC
S1-SLF6attB2GW2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TCTAAAATTTATAAACTTGTTTTGTATGCTCACTTTCTCTTGG
S1-SLF8attB1GW2Fwd	**GGGGACACGTTTGTACAAAAAAGCAGGCT**TAATGACGTTGGATGGAATTATGAAACATTTGCC
S1-SLF8attB2GW2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TTTAAATTTTTGTGCCATGCTTGCATCCC
S1-SLFS3battB1GW2Fwd	**GGGGACACGTTTGTACAAAAAAGCAGGCT**TAATGAAGGAGTTGCCCCAAGATGTAGTG
S1-SLFS3battB2GW2Rev	**GGGGACCACTTTGTACAAGAAAGCTGGGT**TTTAGTTACATCTAAAATTTTGAAGTTCTATGCAATCATTATTTCTTGG

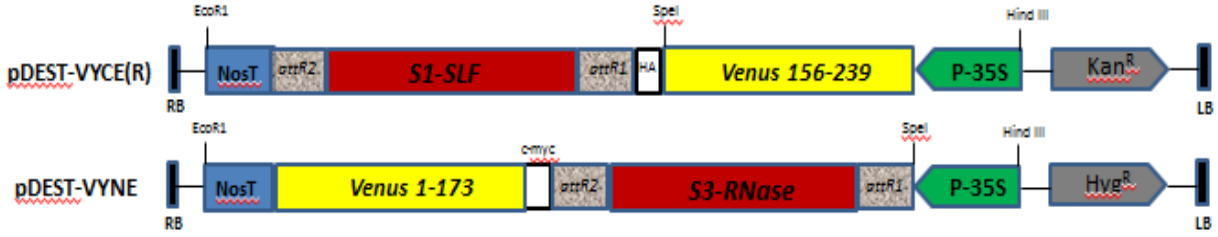


Figure 59: Reverse versus regular BiFC vector. In reverse BiFC vector – pDEST-VYCE(R), the fluorophore coding sequence is located upstream of the target gene, in this case SLF gene. Genes encoding S-RNase, however, in inserted in regular N-terminal BiFC vector, in order to asked is whether the construct orientation affects the strength of fluorescent signals or not.

Figure 60: Electrophoresis of colony PCR verifying correct insertion of SLF variants into the reverse BiFC vectors in Agrobacterium.

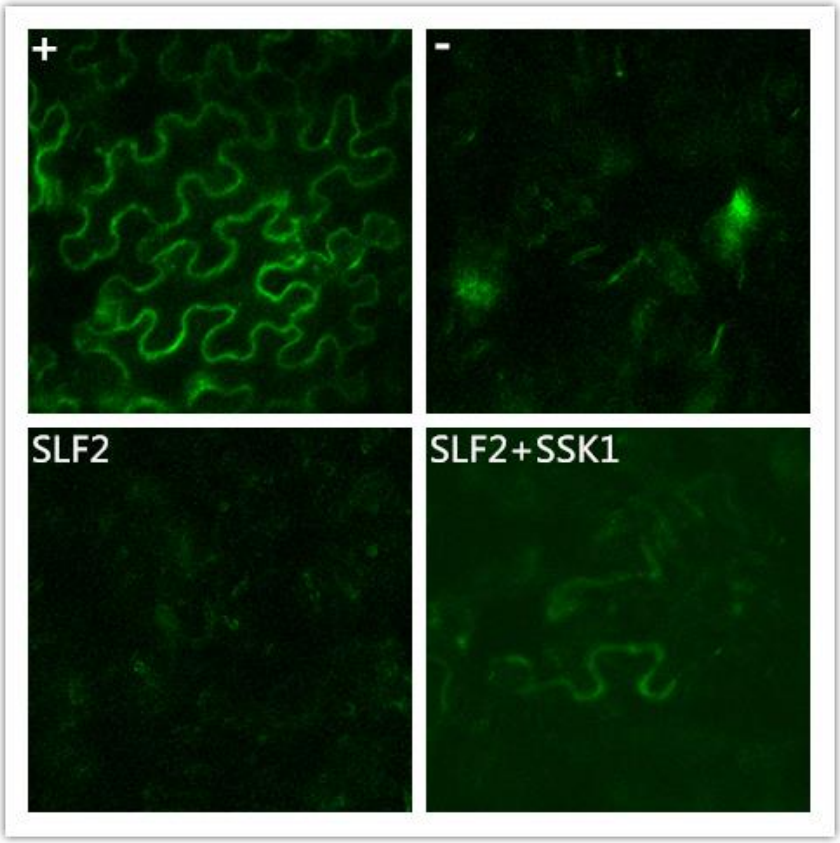


Figure 61: Three-way BiFC – Reverse SLF1, 2, 3, 4, 5, 6, 8, S3b vs. S_3-RNase mature + "Bridge" SSK1.

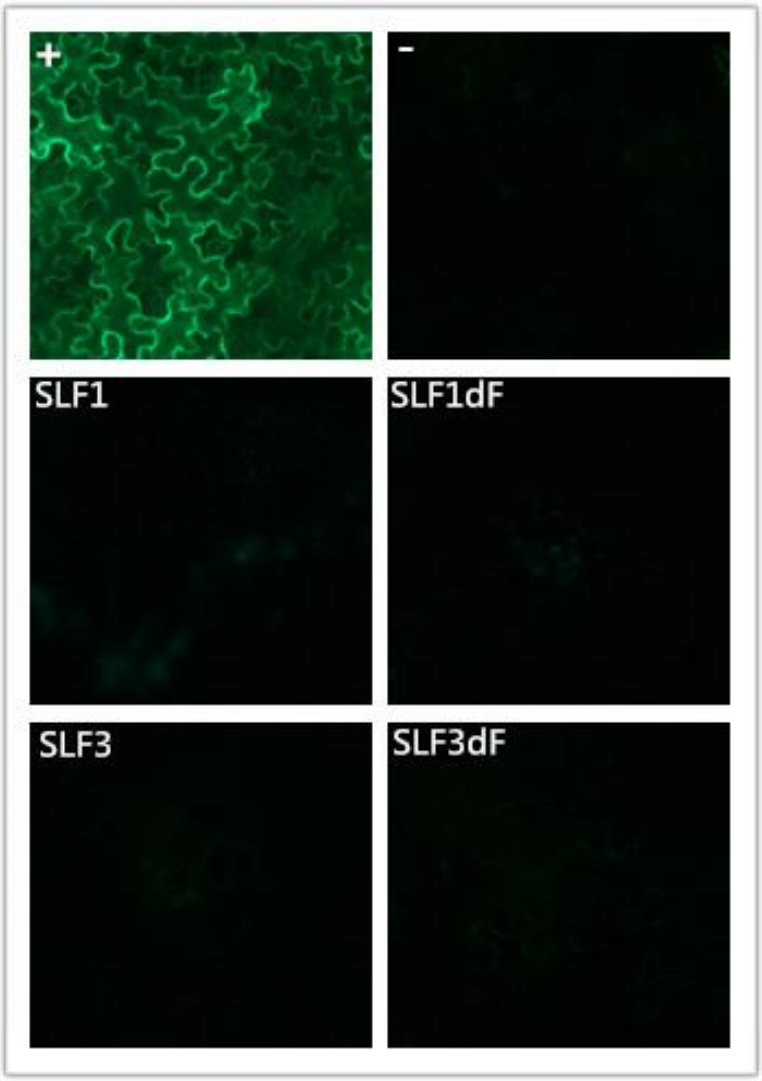


Figure 62: Four-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase mature + "Bridges" SBP1 and SSK1.

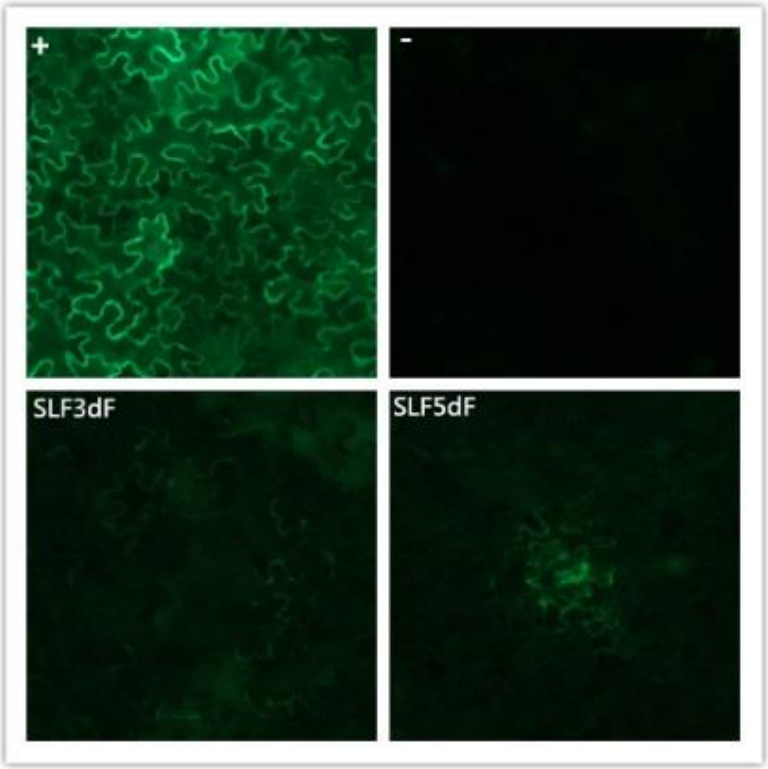


Figure 63: Four-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase mature + “Bridges” SBP1 and Cullin-1.

Four-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase Mature + "Bridges" SSK1 and Cullin-1. BiFC assays were carried out by co-infiltrating SSK1 and Cullin-1 together, however no images were presented due to no signal observed except from positive control.

Five-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase Mature + "Bridges" SBP1, SSK1, and Cullin-1. Co-infiltration of SBP1, SSK1, and Cullin-1 at a time was found greatly improve the fluorescence emitted from SLF3dF::S_3-RNase mature. In addition, SLF3::S_3-RNase was also able to give weakly visible signal, but failed to demonstrate the enhancing effect of co-filtrating all three bridges SBP1, SSK1, and Cullin-1 (Fig. 64).

Five-way BiFC – SLF1, 3, 4, 5 vs. S_1-/ S_3-RNase + "Bridges" SBP1, SSK1 and Cullin-1 (Control assay). A control test was performed between SLF and S_1-/S_3-RNase while co-infiltrating three hypothetical "bridge" proteins at the same time. Results (Fig. 65) indicated the S haplotype of the S-RNase was not related to the signal strength in such 5-way BiFC assays.

Four-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase Mature + "Bridges" RBX1 and SSK1 & Four-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase Mature + "Bridges" RBX1 and Cullin-1 & Four-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase Mature + "Bridges" RBX1, SSK1, and Cullin-1. BiFC tests were also arranged by replacing SBP1 with RBX1, while SSK1 and Cullin-1 remained the same. However, the only enhanced fluorescence was from the pair of SLF3dF::S_3-RNase mature while co-infiltrating RBX1 and Cullin-1 as assumptive "bridge" proteins (Fig. 66).

BiFC assays – SLF1, 3, 4, 5 vs. SLF1, 3, 4, 5 & BiFC assays – SSK1 vs. RBX1. There were a few other BiFC assays carried out but without saving any images due to a complete lack of signal, such as the tests investigating the potential interactions between S_1-SLF variants, i.e. SLF::SLF, and the BiFC assay of between SSK1 and RBX1.

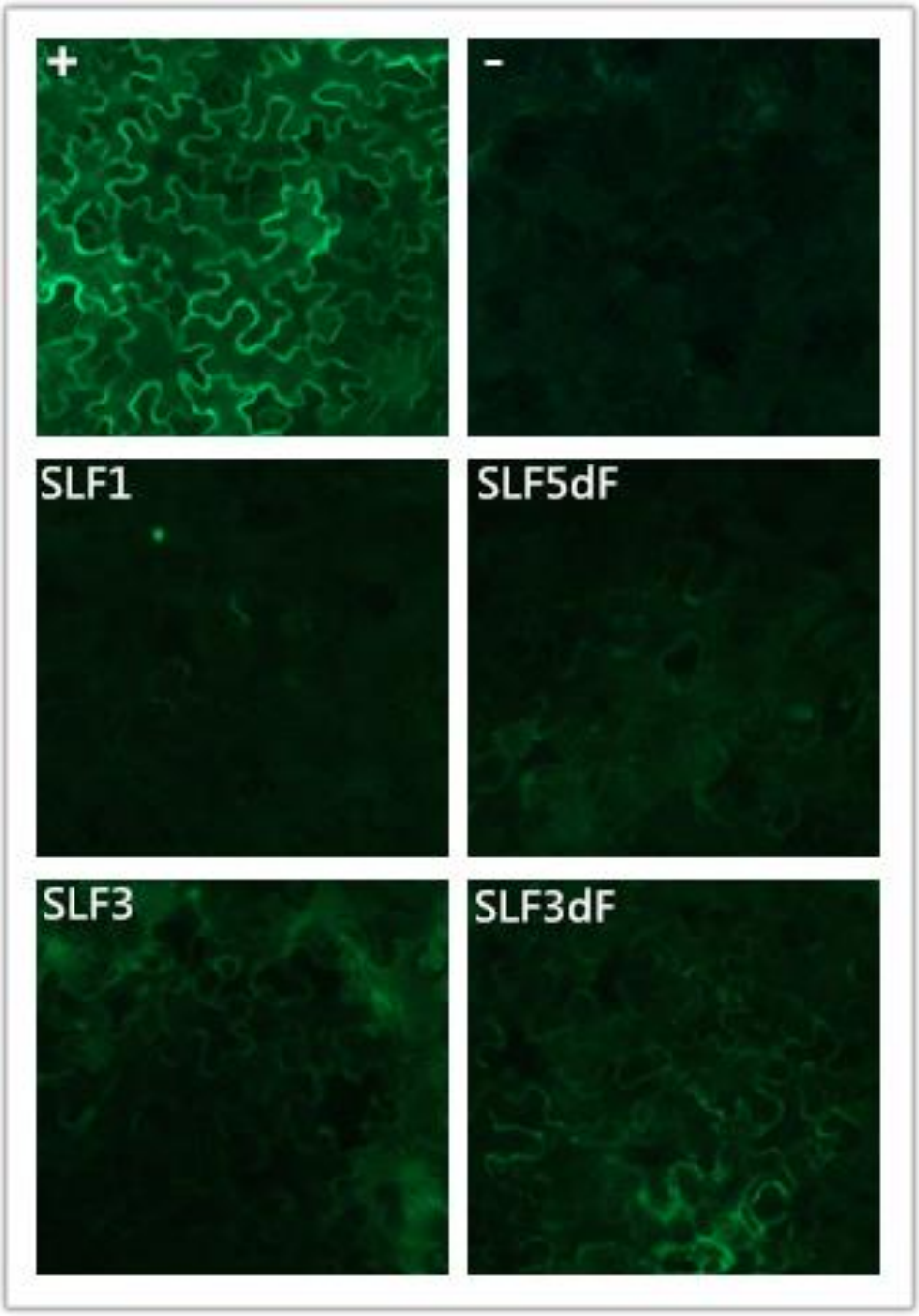


Figure 64: Five-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase mature + "Bridges" SBP1, SSK1, and Cullin-1.

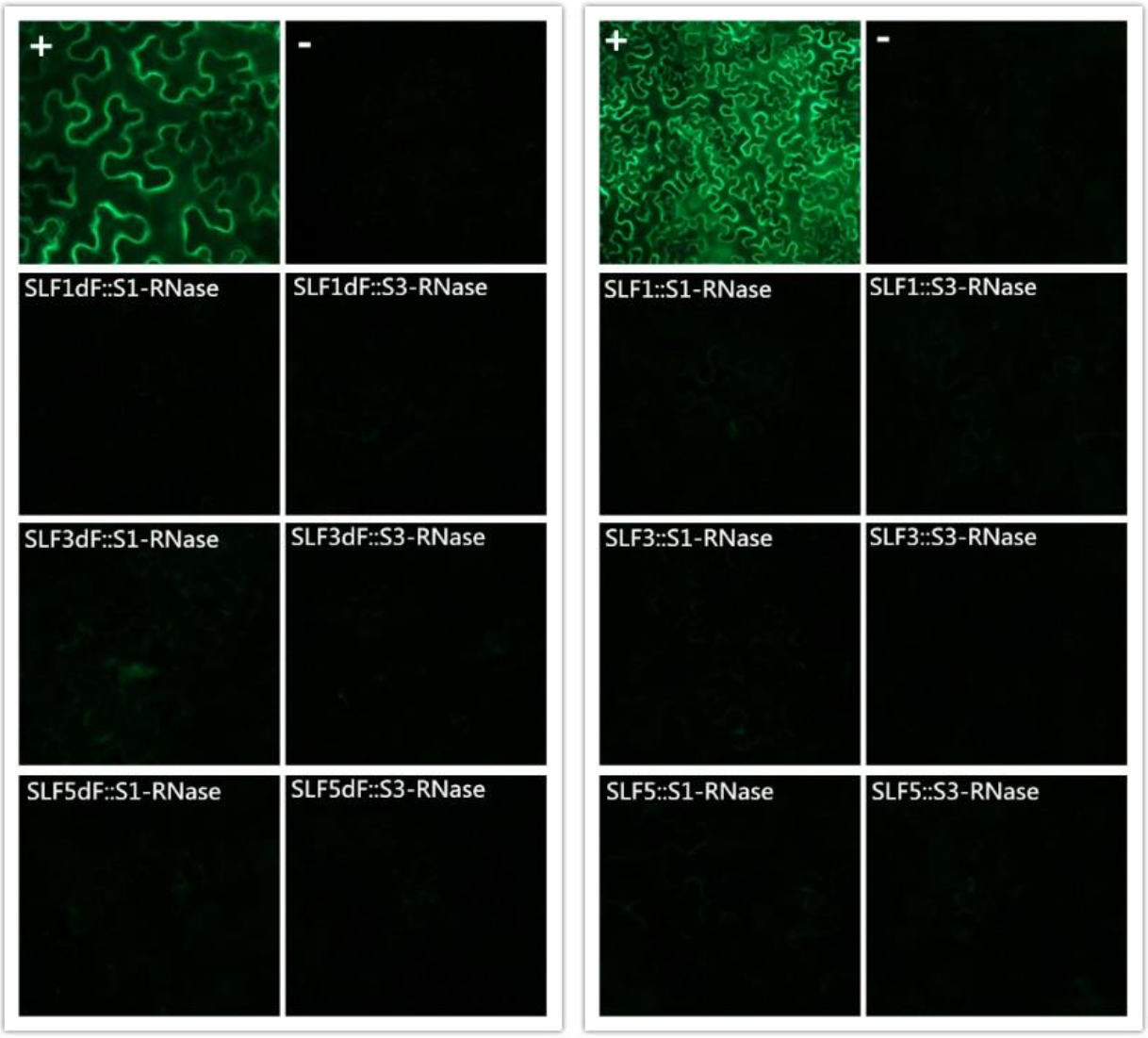


Figure 65: Control tests of Five-way BiFC – SLF1, 3, 4, 5 vs. S_1-/ S_3-RNase + "Bridges" SBP1, SSK1 and Cullin-1.

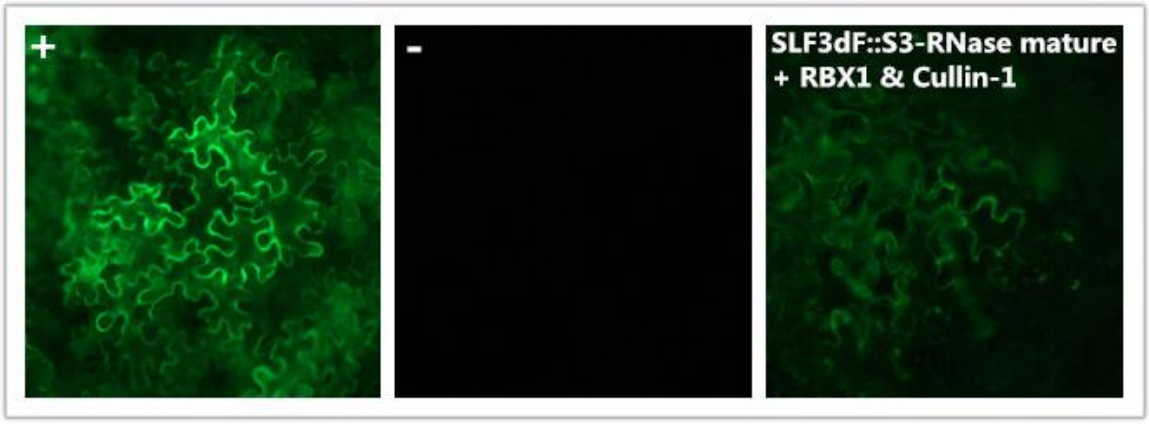


Figure 66: Four-way BiFC – SLF1, 3, 4, 5 vs. S_3-RNase mature + "Bridges" RBX1 and Cullin-1.

Western Blot Monitoring Protein Expression

Besides the signal enhancement created by the hypothetical bridge proteins, another possible reason of fluorescence not being observed is either S-RNases have been poorly expressed *in vivo* or the non-self SLF::S-RNase interactions have led to ubiquitination and degradation of the S-RNase.

Because all of the BiFC vectors in use have different epitope tags (Fig. 35) incorporated into the fusion gene, i.e., VYNE (HA), VYCE (c-myc), SCYNE (FLAG) and SCYCE (HA), protein-expression levels can be determined by Western Blot analyses using commercially available antibodies against the epitopes. For example, the expression levels of S-RNase protein in S-RNase::SBP1 BiFC assays (giving strong fluorescent signals) versus S-RNase::SLF BiFC assays (showing weak fluorescent signals) are experimented for comparison. If Western blotting

shows that even though no BiFC interaction is observed in S-RNase::SLF BiFC assays, the S-RNase protein levels are the same as when interaction is observed in S-RNase::SBP1 BiFC assays, then the possibility of poorly-expressed S-RNase proteins leading to weak interactions could be excluded. Conversely, if a lower level of S-RNase protein is found in S-RNase::SLF BiFC assays which gives weak interaction (but meanwhile SLF is normally expressed), it might imply that S-RNase degradation is responsible for the weak BiFC signal.

The pair of S_1-SLF1::S_3-RNase Mature was selected for Western Blots, along with three positive controls: CNX6::CNX7 used as positive control in BiFC assays; SLF3/ SLF4dF::SBP1 in which signal was observe in previous BiFC tests. However, the results (Fig. 67) of Western Blot analyses showed that only the expression of CNX6::CNX7 was detected in leaves, and the rest were negative indicating no expression or at least expression that was unable to be detected by Western Blot.

Multiple Sequence Alignment of Various SLF Proteins

Three multiple sequence alignments were performed via MAFFT among the SLF proteins sequences acquired from either the Petunia Genome Project or previous researches. The first one (Fig. 68) was the alignment of SLF variants (Subtype 1-20) identifies from the genome of S_{ax1} *P. axillaris*. Generally, SLF of different subtypes were highly variable, despite the presence of several scattered short fragments of amino acids that were relatively conserved. The second one (Fig. 69) was the alignment of SLF variants (Subtype 1-16) identifies from the genome of S_6 *P. inflata*, which was similar to the alignment of SLF variants from S_{ax1} *P. axillaris*.

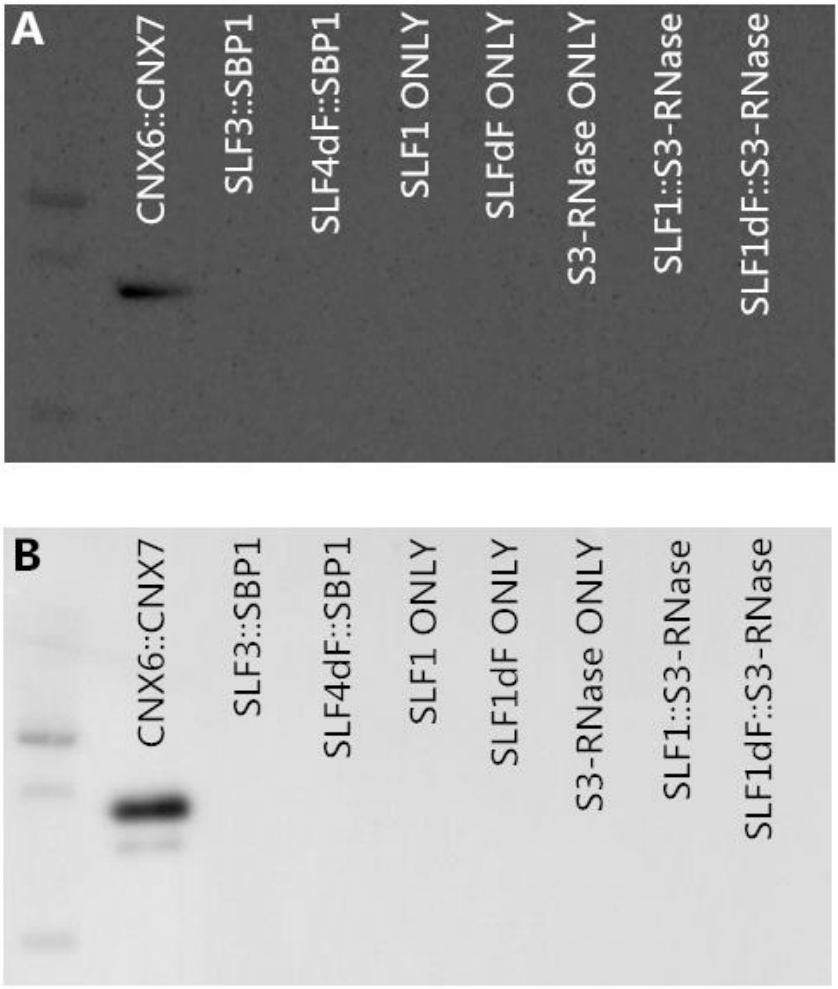


Figure 67: Western blot membranes visualized by chemiluminescent detection. (A) Blotted with antibody against FLAG tag on pDEST-SCYNE. (B) Blotted with antibody against HA tag on pDEST-SCYCE/VYCE. The band in image A and the top band in image B indicated the same protein expressed by CNX6::CNX7. The smaller band from image B is the protein expressed by CNX6-VYCE only.

```
SLF1_1          ---------MANGILKKLPEDLVFLILLTFPVKSLMRFKCISKAWSTLIQSTTFINRHIN
SLF1_2          ---------MANGILKKLPEDLVFLILLTFPVKSLLRFKCISKAWSILIQSTTFINRHIN
SLF2            ---------MANR-IKKLPEDVVIYILLRLPVKSLLRFKCVSKYWYTLILTNTFVKLHLN
SLF11           --------------MKKFHEDVVIYILLRLPVKSLMRLKCISKTWYSLMQSSTFINLHLN
SLF8            M--------TLDGIMKHLPEDIAMHILLRFPVKSLLRFKFISKSWSTLIESSTFINIHLN
SLF3            -----------MTAMKKLPIDVVINILFRLPVKSLTRFKCVAKSWYSLIQSVDFINRHLN
SLF13           ----------MDGTMKKLPEDMRIYILLRLPVKSLTRFKCVTKSWHTLIQSFNFINFHLN
SLF14           MKIALEEIHDGNGVVKKLPKDVVNNITLKLPVKSLLRFKCVSQFWYAYIQSWAFIILQRN
SLF16           ---------MADEIVIKLPKDVVMYILLKFPVKSLLRFKRVSRNLYTLIQSSIFINLHLN
SLF15           ---------MGDEIVEKLPKDIVIYIFLMVPVKSLVRFKCVSKDWYTLIQSSTFISLHFN
SLF4            MNLYRKEYKMADRILLKLPQDVFIYILLRLPVKLLLRFRCVSKSCFTLIQSSTFMNIHLH
SLF12           ---------MPDGIIMKLHQDIIIYMLLRLPVKFLLRFKCISKYCHTLTKSSTFINIHLN
SLF5            M-------KMPHGIMKKLPEDVILCIFLRIPVKSLLRFKCVSKNYYTLLESTTFINLHLN
SLF10           --------------MKELPQDVVIYIFVMLPVKSLLRFKCTDKTFCHIIKSSTFINLHLN
SLF18           ---------MLDGTMKELPQDVVSYILVMLPVKSLLRFKCSCKTFCNIIKSATFINLHLN
SLF19           MQ------KMLDGTIKELPSDVVIYILLMLPAKSLLRFKCICKILCKFINSSTFINLHLN
SLF19           MQ------KMLDGTIKELPSDVVIYILLMLPAKSLLRFKCICKILCKFINSSTFINLHLN
SLF20           MQ------KMLDGTIKELPSDVVIYILLMLPAKSLLRFKCICKILCKFINSSTFINLHLN
SLF6            ---------MADGIIKKLSQDVVIFIFIRLPVKSLMRFKFVSKTFFTLIRSSTFINLYLY
                             : .:  *:   : . .*.* * *::   :       :  *:

SLF1_1          R--KTNTKAEFILFKRSIKD-EEEEFINILSFFSG---HDDVFNPLFPDIDVSYMTSK-C
SLF1_2          R--KTNTKAEFILFKRSIKD-EEEEFINILSFFSG---NDDVLNPLFPDIDVSYMTSK-C
SLF2            R--ITTTKDEFILFIRTFRE-EPDQLRNITTFFSG--DDNNDLSPLFPDLDVSDLTSS-P
SLF11           R--TTTYKDELLFFKRSIKL-EPDLFKNILSFLSR--DNEDDLYPVSPDIDVPYLTSD-Y
SLF8            R--ATTTTDEFLLFSRSYRE-ETEGFKNVLSILSS--GNNDNLIPVVSDLELPYLTFT-E
SLF3            R--ATTIKDEFILFKRSFK--EPEGFRNVMSFLLGG-VGEDDLDPISPDVDVPYLSTT-Y
SLF13           R--KTTTKDEFILFRRSTK--HPDGFSHVLSFLVDH-EGRDDLDPICPDIDMPYLTTGFA
SLF14           C--ASSVNDEIILFKRSFKE-EHDHFKSIMSFLSSG-HDSDDFHHVSPDLEVPYLTNT-T
SLF16           R--NITTNDDLILFKRSLKE-EPNLFKSIMSFLSSG-HDDYDLHYVSPDLDVPYLTNT-G
SLF15           RTTTTTTKDEYMLVKRSFKE-ESNRFRSVMSFLSGGLDDDDDLYPVSPDLDVPFLTTT-N
SLF4            R--TTTSEDEYILFKRSFKE-DVESYKGIFSFYSSH-NDDGDLNSIFSDFDVPNMTSL-Y
SLF12           R--ATTSEDEYILFKRSFKE-DVESYKGIFSFLSS--NNGDDLNCIFPDLDVPNMTSL-Y
SLF5            R--TTTVKDEFILLKRSFKE-DINQYKTIFSFLSGDGHDHDYLNPIFPDFDVPNTTDT-Q
SLF10           H--TTNFNDELVLLKRSFETDEYNFYKSILSFLFA--KEDYDFKPISPDVEIPHLTTT-A
SLF18           H--TTNFKDELVLLKRSFKTDEYNFYKSTISFLFS--KEDYDFKPFSPDVEIPHLTTT-S
SLF19           R--TTEVKDELIIFKRSFKQDEHNLYKSVLSFHIN--EDYFNLKPIAPDFEIPYLTNT-C
SLF19           R--TTEVKDELIIFKRSFKQDEHNLYKSVLSFHIN--EDYFNLKPIAPDFEIPYLTNT-C
SLF20           R--TTEVKDELIIFKRSFKQDEHNLYKSVLSFHIN--EDYFNLKPIAPDFEIPYLTNT-C
SLF6            N--TTTSRDEYILLKRCFIQ-ENNQYKTILSFLAG---DDDYLNPIFQDLDVTHLTST-R
                         : ::. *     . :      ::          :  .  *.::.  :
```

Figure 68. Multiple protein-sequence alignment of S_{ax1}-SLF variants from *P. axillaris*. All the protein sequences above are translated from the S_{ax1}-SLF genes of *P. axillaris* sequenced in Petunia Genome Project. Continued on following page (1/4).

```
SLF1_1          DCTFTPLIGPCDGLIALTDTIITIVLNPATRNFRVLPPSPFGCPKGYHRSVE-GVGFGFD
SLF1_2          DCTFTPLIGPCDGLIALTDTIITIVLNPATRNFRVLPPSPFGCPKGYHRSVE-GVGFGFD
SLF2            CTIFNQIIGPCHGLIALTDSFIIIILNPATRKYIMLPPSPFGCPKGYHRSVE-GIGFGYD
SLF11           CSRFHQLIGPCRGLIALTDFTVIVLLNPATRKYRLLPGSPFVCPKGFTFVTR-GVGFGYS
SLF8            YYLFNKLVGPCNGLIVLTDFEIIVLFNPATKNYMLIPPSPFVCPKGFHRSFRGGVGFGFD
SLF3            SCICHQLTGPCHGLILLTDSTNLVLLNPATRNYRLLPPSPFGIQRGFYRSVA-GVGFGYD
SLF13           SSTSHQFTGPSNGLILLTDSLNFLLLNPATRSYRLLPPNPFCCPRGFLRLIY-GVGFGYD
SLF14           SCTFHRFIGPCHGLIVLTDKVTTVLFNPATRNYRLLKPSPFGSPLGFHRSIN-GIAFGFD
SLF16           GCTFHRFMGPCHGLIVLTDCEETVLFNPSTRNYRLLQPSPYDSPLGFHRSIN-GIAFGFD
SLF15           SCTFPRIMGPCNGLIVLTDKITTVLFNPATRSYRLLQPGRFGCPVGFHRSIN-GVGFGFD
SLF4            SIDYDKLIGPCHGLIAVMDSRSTILFNPSTRKYRLLPSSPFGIPKGYYRSIE-SGGFGFD
SLF12           SITQDKLIGPCHGLVAVMNVSSTILLNPATRKYRLLPSSPFGVPKGFYRDIE-NGGFGFD
SLF5            SIIFDQLIGPCHGLIALMDDLTTIIFNPSTRNFRLLPPSPFDRPKGYHRSIK-CLGFGFD
SLF10           ACICHRLIGPCNGLIVLTDSLTTIVFNPATLKYRLIPPCPFGIPRGFRRSIS-GIGFGFD
SLF18           ACVFHQLIGPCNGLIALTDSLTTILFNPTTRYYRLIPPCPFGIPRGFRRSIS-GFGFGFD
SLF19           ACVCHQLIGPCNGLIVLTDSQTFILFNPTTRHYKLIPPCPFGVPRGFRRSIS-GVGFGFD
SLF19           ACVCHQLIGPCNGLIVLTDSQTFILFNPTTRHYKLIPPCPFGVPRGFRRSIS-GVGFGFD
SLF20           ACVCHQLIGPCNGLIVLTDSQTFILFNPTTRHYKLIPPCPFGVPRGFRRSIS-GVGFGFD
SLF6            NCDHDQLIGPCHGLMALMDTQTTILFNPSTRNYRPLGPSPFGCPQGFHRCIQ-AVGFGFD
                      : **. **: : :    :::**:*  :  :    :    *:        .**:.

SLF1_1          TISNYYKVVRISEVYC-EEADGYPGPKDSKIDVCDLSTDSWRELDHV--QLPSIYWVPCA
SLF1_2          TISYYYKVVRISEVYC-EEADGYPGPKDSKIDVCDLSTDSWRELDDV--QLPSIYWVPCA
SLF2            SIVNDYKVVRLSDVYW-DPPTDYPGPREPKVDIYDLSIDSWRKLD---LEFPSIYYLPCS
SLF11           TAENYYKLVRIFEVYT-DPYDRDLDARHSKVEVYDSCTDCWRDLDLTVKLLPKVRRFACS
SLF8            SIVKDYKFVTISEVFM-DSEW-VPDEKEQKVEVYDLRLDSWRDLNHVDQQLPTVYYYPCF
SLF3            SVHKTYKVVRISEVYG-EPPFNCPSVMEWKGEVYNSSTDSWRELDCVDQELPWPYNFAYS
SLF13           SIQKNYKVIRVSRVYG-DPPYNDRSEMSWESEIYDSGTDSWRQLTNVDQELPGPYMHPYS
SLF14           SIANEYKIVRLAKIRG-EPPFYCYTVREWRVEVYELSINSWREVENVDQQLPYVHWYPCA
SLF16           SIGNEYKIARLAELRG-EPPFNCFTMKEWRVEVYELSIDSWREIENVDQQLPYVHWYPCG
SLF15           SVANSYKIVRIAEVNG-EPPFYCYTMREWKVEIYEFSVDAWREQDQVYRQLPNVFWYPCF
SLF4            SVVNDYKVFRISDVYT-EDRFGYPEEGERKVEVYEVGIDIWRELDHVDQELPRLFWL-TS
SLF12           SVVNDYKVFIISEVYT-EDRYGYPEEGERKVEVYELGIDVWRELDHVDQQLPKLFWM-TS
SLF5            SVVNDYKVVRICEFLK-DDCYGYVQVEEENVEIYELGIDCWRELDHINQQFPTIFWVPCS
SLF10           SDANDYKVVRLSEVYK-E-----PCDKEMKVDIYDFSVDSWREL--LGQDVPFVFWFPCA
SLF18           SNANDYKVVRISEVYK-H-----HYDKDMKVDIYDFSVDTWRVLNLLGQKLPIVFWMPCS
SLF19           SNANDYKVVRISEVYK-D-----FCDKDMKVDIFDFSVDSWRELNLLGQKLPIVIWFPCS
SLF19           SNANDYKVVRISEVYK-D-----FCDKDMKVDIFDFSVDSWRELNLLGQKLPIVIWFPCS
SLF20           SNANDYKVVRISEVYK-D-----FCDKDMKVDIFDFSVDSWRELNLLGQKLPIVIWFPCS
SLF6            RVSNDYKVVRISIIYKVDYDDEYPEERDRKFEVYDLGIDYWRELDNLSQELTTFCVTHCS
                     **.  :  .   .           . :: :   : **        ..
```

Figure 68. Continued (2/4).

```
SLF1_1          GMLYKEMVHWFA---TTDT-SMVILCFDMSTEMFHDMKMPDTCSRITHELYYGLVVLCES
SLF1_2          GILYKEMVHWFA---TTDT-SMVILCFDMSTEMFHDMKMPDTCRRITHELYYGLVILCES
SLF2            EMYYKEAVHWFI---ITDT--VVIFCFDISTEIFRTMEMPGTCT-FFDGPRYGLVVLKDC
SLF11           EIFFKETFHWCA---HDDT--VMILCFDISLETFHYMKLPDHCH-FWDNKGYGLTVLNND
SLF8            EMLYNGVFHWYA---INDRLDHVILSFDISTEIFNSIKMPATGK-SSGGKKYGLIVLNES
SLF3            EIFYEGAFHWYA---HKNV--VLILCFDINTETFRTMEVPEPCA-SYDEKCHSLLVLDEF
SLF13           ELFYKGTFHWYA---QGHM--RLLLCFDINTENFRTMQVPKTCA-VRDEKCHSLVVFDES
SLF14           ELFYKGTSHWFG---NTNT--VVILGFDMSTETFRNIKMPNTCH-FKDRKCYGLVVLNES
SLF16           ELFYKGASHWFG---HANR-ARVILCFDMTTETFRDIKMPNTCH-YKDRKCYGLVVLNGC
SLF15           EMFYKGASHWFA---HANT--IVILCFDIITETFRSIKFPNTCH-FQDENCYSLVILNDS
SLF4            SMYYNGVYHWITTLNHEDK--LIILCFDMSTEIFRNINTPDTRQ-YSSGTCHSLVLLDEC
SLF12           SMPYNGTYHWLITLSYEHR--LILLCFDMSTEIFRYIKTPNTRY-FSSGTRHSLVLLNDC
SLF5            QIFYMGTFHWIA--------QRVILCFNMSTEIFHHIRMPDPYH---NIRNHGLVILNNS
SLF10           EILYKRNFHWFA---FADV--VVILCFEMNTEKFHNMGMPDACH-FADGKCYGLVILFKC
SLF18           EILYKRNFHWFA---TADE--AVILRFDLSTEKFYNIEMPDTCH-YLDGMYYSLVILYKC
SLF19           EILYKRNYHCFA---FAND--VIILCFDFSTELFNNIGMPNT-H-DLDGMSYGLAILYKF
SLF19           EILYKRNYHCFA---FAND--VIILCFDFSTELFNNIGMPNT-H-DLDGMSYGLAILYKF
SLF20           EILYKRNYHCFA---FAND--VIILCFDFSTELFNNIGMPNT-H-DLDGMSYGLAILYKF
SLF6            QMFYKGACHWIA---SLDIDAYIILCFDMSSETFRSLKIPESCH-IINGPTCRLALVHDT
                 : :    *             ::: *::  * *  :  *             * :.

SLF1_1          FTLIGYSNPISSIDPVEDKMHIWVMMEYGVSESWIMKYTIK--PLSI-ESPLAVWKNHIL
SLF1_2          FTLIGYSNPISSIDPVEDKMHIWVMMEYGVSESWIMKYTIR--PLSI-ESPLAVWKNHIL
SLF2            LTLICYPDPMCSVDPTEDLIDIWMMEEYGASESWIKIYTIRPVPIPI-ECPLAIWKDHLL
SLF11           LTFITYPNPRCALDPGQEFTDIWIMEKYGVNGTWIKKYTIR--PLPI-ESSLAISKDHLL
SLF8            LTLISYPNPDSEMDPSKDSMDIWIMMEYGVYESWTKKYIIK--PLPI-ESPLTIWRDHLL
SLF3            LTLFCYPDPRRESSPIQETIEIWTMQEYRVNESWIKKHTIK--SPLI-ESPLAIWKDRLL
SLF13           LTFICYPDPRRESSPVQETIEIWIMQEYSVNESWIKKYTIR--PPPI-ESPLAIWKDRLL
SLF14           ITLICYPYPGCEIDPAIDFMEIWIMKEYGVNDSWNKKYTIV--PLAI-ESPLAIWKNHLL
SLF16           LTLICYPYPGCEIDPAIDFMEIWIMKEYGINESWSMKYKIT--PLAI-ESPLAIWKDHLL
SLF15           LTLICYPYPEKVVEYEKDFMEIWIMMEYGVDESWIKKYSIT--PLSI-ETPLAVWKDHLL
SLF4            LSFMCHPYLGPEIGPTTDLIDIWMMKDYNVYESWTKKYTIR--VLPIDESPLAVWKDSLL
SLF12           LSFMCHPFPGPEIDPTKDFIDIWMMKDYNVYESWINIYTIR--ILPIHEFPLAIWNDSLL
SLF5            LTLICYRSVAPTSDPIEDLMEIWILTDYDVSESWVKNFTIR--SLPI-KIPLAIWKDNLL
SLF10           MTLICYPDPM-PSSPTEKLTDIWIMKEYGEKESWIKRCSIR--LLP--ESPLAVWKDEIL
SLF18           LTLICHP-----ASPIEDLVEIWLMQEYGQKESWIKKRTIK--LLHI-QSPLAVWKDDIL
SLF19           LTLICYHYSM-FTEPTEDLVDIWIMKEYGQKESWIKRFSIK--VLSI-ESPLAVWKDELL
SLF19           LTLICYHYSM-FTEPTEDLVDIWIMKEYGQKESWIKRFSIK--VLSI-ESPLAVWKDELL
SLF20           LTLICYHYSM-FTEPTEDLVDIWIMKEYGQX----------------------------
SLF6            LTLIYYPYPETEIPVEKDLINIWFMKEYNVYESWIRKYTIR--GLLI-DSPLTVWKGYLL
                :::: :           .  .** : .*
```

Figure 68. Continued (3/4).

```
SLF1_1          LLQSRSGLLISYDLNSGEAKELNLHGFPDTLSVKVYKECLTSIPKGS-EYSTKVQKF---
SLF1_2          LLQSRSGLLISYDLNSGEAKELNLHGFPDTLSVKVYKECLTSIPKGS-EYSTKVQKF---
SLF2            LLQTKSGFLISYDINSDEVKEFNLNGDLESLRVLVYTESLTTIQKIS-EHGTQVQQF---
SLF11           LLQSISGTLTSYNLNSDELKEFSFQGFTSTLRLVVYKESLTIIPRES-EDGTKVQNF---
SLF8            LLQSKSGLLVSYDLSSNEVKEFDLHGYPKSLRVLVYKESLISIPKRGCKHGTKI------
SLF3            LFQDKSGILISYDLNSDEVKEFKLDGYPATLRIIIYKESLTPIPKGS----TQVQNF---
SLF13           LLQDKSGDLIAYDLNLDEVKEFNLHGHPESLRVIVYKESLAPIPIGN----TQVEKF---
SLF14           LLQSITGHLISYNLNSDEIKEFNLHGWPKSLRVKIYKESLTFVPKES-EFNTAQ------
SLF16           LLQSISGYLISYDLNSDEIKEFELNGWPESLRVNIYKESLALIPKDQCKE----------
SLF15           LLESRSGSLISYDLNSGEVKELNLHCWPPSFRIAVYQESLTLIPEER-EHSTKCPKILES
SLF4            FFQGKSGYLMSYDFKSKEVKERNLHGCQKSMRAIVYKECLVPIPRGS-QSSTQVQNI---
SLF12           FFQGKTGYLMSYNLNTDEVKELSLNGCKRSMRAIVYKESLAPIPEGS-ESSTQVHNF---
SLF5            LFQNRSGYLMVYDLRTDNVNELNIHGCPESMRVTVYKENLTIIPSGS-ENSTPVHKF---
SLF10           LLHSKTGHLIAYDFNSNEVQELDLHGYPESLRIIIYRESLTAIPRNN--DCIELQNFRCN
SLF18           ILETKSGQLITYDLNSDEVKELNLLGYPTSLRVIVYKESLTPVPRND--NGAEVQQF---
SLF19           LLQTKNGQLIAYDLNSDEVQDLSLHGCPESLRVIVYKESLTLIPRKD--DGAEVQQF---
SLF19           LLQTKNGQLIAYDLNSDEVQDLSLHGCPESLRVIVYKESLTLIPRKD--DGAEVQQF---
SLF20           ------------------------------------------------------------
SLF6            LYQSRSGCLMSYNLNSNDVREFNFHGYPKSLRAIVYKDSLTSIPRES-EHTKQVYKF---
```

Figure 68. Continued (4/4).

```
SLF1_1          ---------MANGILKKLPDDLVFLILLTFPVKSLLRFKCISKAWSILIQSTTFINLHIN
SLF1_2          --------MMPNGILKKLHEDLIFLILLTFPVKSLLRFKCISKAWSILIQSTTFINLHIN
SLF2_1          --------------MKKFHEDMMIYILLRFPVKSLLRFKCISKVYYTLILSNTFVKLHLN
SLF2_2          --------------MKKLPKDVLSYILLRFPVKSLFRFKCICKAWYTLVLTNTFVKLHLN
SLF7_1          ---------MAEGILKRLFGDVVIYIFLRLPLKTLLRFKCISKTLYAIIQSSTFINLHLN
SLF7_2          ---------MAEGILKRLFGDVMIYILLRLPLKTLLLFKCISKTFYNIIQSSTFINLHLN
SLF7_3          ---------MAEGILKRLLGDVVIYILLRILLKSLLRFKCISKTLYAIIQSSTFIILHLN
SLF14_1         MKIALEEIHDGNGVVKKLPKDVVNNITLKLPVKSLLRFKCVSQFWYAYIQSWAFIILQRN
SLF14_2         MKIALEEIHDGNGVVKKLPKDVVNNITLKLPVKSLLRFKCVSQFWYAYIQSWAFIILQRN
SLF16_1         ---------MADEIVIKLPKDVVMYILLKFPVKSLLRFKRVSRNLYTLIQSSIFINLHLN
SLF16_2         ---------MADGIVIKLPKDVVTYIFLTFPVKSLLRLKCVSRNLHTFIQSSAFINLHLN
SLF17           ---------MADGIVIKLPKDVVTYIFLTFPVKSLLRLKCVSRNLHTFIQSSAFINLHLN
SLF16_3         ---------MADGIMVKLPKDVVTYILLTLPVKSLLRFKCVSRKYYTLMKSSKFINLHFN
SLF15_1         ---------MGDEIVEKLPKDIVIYIFLMVPVKSLIRFKCVSKVWYILIQSSTFINLHFN
SLF15_2         ------------------------------------------------------------
SLF11_1         ---------MVDGIMKKIHEDVVICILLGLPVKSLMRLKCISKTLYTLVQSSTFINLHLN
SLF11_2         ---------MVDGIMKKFHKDVVIYILLRMPVKSLMRLKCISKIWYTLMQSSTFINLHLN
SLF9_1          --------------MKELPQDVVIYILVMLPVKSLLRFKCNSKTFCIIIKSSIFINLHLN
SLF10_2         --------------MKELPQDVVIYILVMLPVKSLLRFKCNSKTFCIIIKSSIFINLHLN
SLF9_2          --------------MKELPQDVVIYILVMLPVKSLLRFKCSCKTFYNIIKSSTFIDLHLN
SLF9_3          --------------MKELPQDVVIYIFVMLPVKSLLRFKCTCKTFCHIIKSSTFINLHLN
SLF10_1         --------------MKELPQDVVIYIFVMLPVKSLLRFKCTCKTFCHIIKSSTFINLHLN
SLF3            ---------MT--AMKKLPIDVVINILFRLPVKSLARFKCVTKSWYALIQSADFINRHLN
SLF13_1         ---------MMYGTMKKLPEDMRIYILLRLPVKSLMRFKCVTKSWHTLIQSFNFINFHLN
SLF13_2         ----------MDGTMKKLPEDMRIYILLRLPVKSLTRFKCVTKSWHTLIQSFNFINFHLN
SLF4_1          MKLYSKEYKMSDRNIMKLPEDVFIYILLRLPVKLLLRFRCVSKSCYTLIQSSTFINIHLH
SLF4_2          MKLYSKEYKMSDRNIMKLPEDVFIYILLRLPVKLLLRFRCVSKSCYTLIQSSTFINIHLH
SLF5_1          MKLYSKEYKMSDRNIMKLPEDVFIYILLRLPVKLLLRFRCVSKSCYTLIQSSTFINIHLH
SLF12_2         MKLYSKEYKMSDRNIMKLPEDVFIYILLRLPVKLLLRFRCVSKSCYTLIQSSTFINIHLH
SLF12_1         ---------MPDGIIMKLHQDIIIYMLLRLPVKFLLRFKCISKYCYTLTKSSTFINIHHN
SLF5_2          -------MKMPHGIMKKLPEDVILCIFLKVPVKSLLRFKCVSKNYYTLLESTTFINLHLN
SLF8_1          --------MMLDGIMKHLPADIAMHILLRFPVKSLLRFKFISKSWSTLIESSTFINIHLN
SLF8_2          --------MMLDGIMKHLPEDVGMYILLRFPVKSLLRFKFISKSWYTLIESSTFIKIHLN
SLF6_1          ---------MADGIIKKLSEDVVIFIFFRLPVKSLMRFKFVSKSFFTLIESSTFINIYLY
SLF6_2          ---------MADGIIKKLSEDVVIFIFFRLPVKSLMRFKFVSKSFFTLIESSTFINIYLY
```

Figure 69. Multiple protein-sequence alignment of S_6-SLF variants from *P. inflata*. All the protein sequences above are translated from the S_6-SLF genes of *P. inflata* sequenced in Petunia Genome Project. Continued on following page (1/8).

```
SLF1_1          RKT-NTNAEFILFKRSIK-DEEEEFINILSFFSG---HDDVLNPLFPDIDVSYMTSK-CD
SLF1_2          RKT-NTKAEFILFKRSIK-DEEEEFINILSFFSG---NDNVLNPLFPDIDVSYMTSK-CD
SLF2_1          RIR-TTKDEFILFIRTFR-EEPEQLKSIASFFSC--DDNNDLNTLSPDVDVTDLTST-SC
SLF2_2          RIT-TTKDEFILFIRTFR-EEPDQLRSVASFISG--DENNDLNTLFPDVDVSDLTST-CC
SLF7_1          RTT-TTNDEFILFNRSIK-EAHNEFKSVMSFYAC-SHDNCDIHSISPDLDVPNMKPS-IS
SLF7_2          RTT-TTNDEFVLFNRSIK-EAHNEFKSVMSFYAC-SHDNYDIHSISPYLDVTNMKPS-IS
SLF7_3          RTT-TTNDEFILFNRSIK-EAPDEFRSVLSSYAS-NNECYEIHSISPDLNVPYLKPK-SS
SLF14_1         CAS-SVNDEIILFKRSFK-EEHDHFKSIMSFLSS-GHDSDDFHHVSPDLEVPYLTNT-TS
SLF14_2         CAS-SVNDEIILFKRSFK-EEHDHFKSIMSFLSS-GHDSDDFHHVSPDLEVPYLTNT-TS
SLF16_1         RDI-TTNDDLILFKRSLK-EEPNLFRNIMSFLSS-GHDDYDLHYVSPDLDVPYLTNT-GG
SLF16_2         RTS-IINEEFILFKRSLK-EEPDRFRNIMSFLSS-GHDNYDLHHVSPDLDVPYLTTT-GA
SLF17           RTS-IINEEFILFKRSLK-EEPDRFRNIMSFLSS-GHDNYDLHHVSPDLDVPYLTTT-GA
SLF16_3         GT--ITNDDFILFKRSFK-EEADQFRSIMSFLSS-IDDNYDLLHVSPDLDVPYLTTT-GA
SLF15_1         RTTATTKDEYMLIKRSFK-EESNRFRSVMSFLSG-GLDDDDLYPVSPDLDVPYLTTT-NS
SLF15_2         ------------------------------------------------------------
SLF11_1         RTT-TYNDELIFFKRSIK-LEPDLFKNILSFLSS--DNKDDLNPVSPDIDVPYLTSD-YC
SLF11_2         RTT-SYNDELIFFKRSIK-LEPDLYKNILSFLSS--NNEDDLTPVYPDVDVPYLTSD-YC
SLF9_1          HMT-NVKDELVLLKRSFKTDEYNFYKSILSFLSS--KEDYDFKPISPDVEIPHLTTT-SA
SLF10_2         HMT-NVKDELVLLKRSFKTDEYNFYKSILSFLSS--KEDYDFKPISPDVEIPHLTTT-SA
SLF9_2          HTT-NGNDELVLLKRSFKTDEYNFYKSMLSFLSR--KEDYDFKPISPDVEIPHLTTT-SA
SLF9_3          HTT-NFNDELVLLKRSFETDEYNFYKSILSFLFA--KEDYDFKPISPDVEIPHLTTT-AA
SLF10_1         HTT-NFNDELVLLKRSFETDEYKFYKSILSFLFA--KEDYDFKPISPDVEIPHLTTT-AA
SLF3            RAT-TIKDEFILFKRSFK--EPEGFKNVMSFLLG-GVGDDNLDPISPDVDVPYLSTS-YS
SLF13_1         RKS-TTKDEFILFRRSIK--HPDGFSHVLSFLVD-HEGKDDLYPICPDIDMPYLTTGFAS
SLF13_2         RKS-TTKDEFIVFRRSIK--HPDGFSHVLSFLVD-HEGKDDLDPICPDIDMPYLTTGFAS
SLF4_1          RTT-TSEDEYILFKRSFK-EDVESYKGIFSFYSS-HNDDGDLNSIFPDLDVPNMTSL-YS
SLF4_2          RTT-TSEDEYILFKRSFK-EDVESYKGIFSFYSS-HNDDGDLNSIFPDLDVPNMTSL-YS
SLF5_1          RTT-TSEDEYILFKRSFK-EDVESYKGIFSFYSS-HNDDGDLNSIFPDLDVPNMTSL-YS
SLF12_2         RTT-TSEDEYILFKRSFK-EDVESYKGIFSFYSS-HNDDGDLNSIFPDLDVPNMTSL-YS
SLF12_1         RAT-TSDDEYILFKRSFK-EDVERYKGIFSFLSG--NNGDDLNCTFPDLDVPNMTSL-YS
SLF5_2          RTT-TVKDEFILLKRSFK-EDINQYKTIFSFLSGDGHDHDYLNPIFPDFDVPNMTDT-QS
SLF8_1          RAT-TTKTEFLLFSRSYR-EETEGFKNVLSILSS--GSNDDFIPVVSHLELPYLTFT-EY
SLF8_2          RAT-TTKNEFLLFSRSYR-EETEGFKNVLSIFSS--GNDDDLIPVISDLCLPYLNFT-QY
SLF6_1          NTT-TSRDEYILLKRCFI-QENSQYKTILSFLAG--DDDDYLNPIFQDLDVTHLTST-RN
SLF6_2          NTT-TSRDEYILLKRCFI-QENSQYKTILSFLAG--DDDDYLNPIFQDLDVTHLTST-RN
```

Figure 69. Continued (2/8).

```
SLF1_1          CTFTPLIGPCDGLVALTD-TIITIVLNPATRNFRVLPPSPFGCPKGYHRSVE-GVGFGFD
SLF1_2          CTFNPLIGPCYGLIALTD-TIITIILNPATRNFRVLPPSPFGCPKGYHRSVE-GVGFGFD
SLF2_1          TIFNQLIGPCHGLIALTD-SFIIIVLNPGTRKYIVLPPSPFGCPKGYHRSIE-GIGFGFD
SLF2_2          TIFNQLIGPCNGLIALTD-SFIIIVLNPATRKYIVLPPSPFGCPKGYHRSVE-GIGFGFD
SLF7_1          SVSHRLIGPCHGLIVLTD-TVETILLNPATRNYRILRPSPFDCPLGFCRSIV-GVGFGFD
SLF7_2          SVSHRLIGPCHGLIVLTD-TVETILLNPATRNYRILRPSPFDCPMGFCRSIV-GVRFGFD
SLF7_3          SVPHRLLGPCHGLFVLTD-MVETILLNPATRNYRLLRSSPFVCPLGFCRSTR-GVGFGFD
SLF14_1         CTFHRFIGPCHGLIVLTD-KVTTVLFNPATRNYSLLKPSPFGSPLGFHRSIN-GIAFGFD
SLF14_2         CTFHRFIGPCHGLIVLTD-KVTTVLFNPATRNYSLLKPSPFGSPLGFHRSIN-GIAFGFD
SLF16_1         CTFHRFMGPCHGLIVLTD-CEETVLFNPSTRNYRLLQPSPYDSPLGFHRSIN-GIAFGFD
SLF16_2         CTSHRFMGPCHGLIVFTDGEETEVLFNPSTRNYRLLTPSPFDSPLGFHRSID-GIAFGFD
SLF17           CTSHRFMGPCHGLIVFTDGEETEVLFNPSTRNYRLLTPSPFDSPLGFHRSID-GIAFGFD
SLF16_3         CVSHTFMGPCQGLIALTD-IETTVLLNPATRNFRLLQPSPFGDTLGFHRTIM-GNAFGFD
SLF15_1         CTFHKIMGPCNGLIVLTD-KITTVLFNPATRSYRLLQPGRFGCPVGFHRSIN-GVGFGFD
SLF15_2         ----------------MD--------------------------------------------
SLF11_1         SRFHQLIGPCRGLIALTD-FTTIVLLNPATRNYRLLPGSSFVCPKGFTFVTR-GVGFGHS
SLF11_2         SRFHQLIGPCRGLIALTD-FTVIVLLNPATRKYRLLPGSPFVCPKGFTFVTR-GVGFGYS
SLF9_1          CVFHQLIGPCNGLIALTD-SLTTIVFNPATRKYRLIPPCPFGIPRGFRRSIS-GIGFGFD
SLF10_2         CVFHQLIGPCNGLIALTD-SLTTIVFNPATRKYRLIPPCPFGIPRGFRRSIS-GIGFGFD
SLF9_2          CVFHQLIGPCNGLIALTD-SLTTIVFNPATRKYRLLPPCPFGIPRGFRRSIS-GIGFGFD
SLF9_3          CVCHRLIGPCNGLIVLTD-SLTTIVFNPATLKYRLIPPCPFGIPRGFRRSIS-GIGFGFD
SLF10_1         CVCHRLIGPCNGLIVLTD-SLTTIVFNPATLKYRLIPPCPFGIPRGFRRSIS-GIGFGFD
SLF3            CICHQLTGPCHGLILLTD-STNLVLLNPAIRNYRLLPPSPFGIQRGFYRSVA-GVGFGYD
SLF13_1         STSHQFTGPSNGLILLTD-SLNFLLLNPATRSYRLLPLNPFCCPRGFLRLIY-GVGFGYD
SLF13_2         STSHQFTGPSNGLILLTD-SLNFLLLNPATRSYRLLPPNPFCCPRGFLRLIY-GVGFGYD
SLF4_1          IDYDKIIGPCHGLIAVMD-SRSTILFNPSTRKCRLLPSSPFGIPKGYYRSID-SGGFGFD
SLF4_2          IDYDKIIGPCHGLIAVMD-SRSTILFNPSTRKCRLLPSSPFGIPKGYYRSID-SGGFGFD
SLF5_1          IDYDKIIGPCHGLIAVMD-SRSTILFNPSTRKCRLLPSSPFGIPKGYYRSID-SGGFGFD
SLF12_2         IDYDKIIGPCHGLIAVMD-SRSTILFNPSTRKCRLLPSSPFGIPKGYYRSID-SGGFGFD
SLF12_1         ITQDKLIGPCHGLVAVMN-VSSTILLNPATRKYRLLPSSPFGVPKGFYRNIE-NGGFGFD
SLF5_2          IIFDQLIGPCHGLIALMD-DLTTIIFNPSTRNFRLLPPSPFDRPKVYHRSIK-CLGFGFD
SLF8_1          YLFNKLVGPCNGLIVLTD-FEIIVLFNPATKNYMLIPPSPFVCPKGFHRSFRGGVGFGFD
SLF8_2          YLFNNLVGPCNGLIVLTD-YEIIVLFNPATKNYMLIPPSPFVCPKGFHRSFRGGIGFGFD
SLF6_1          CDHDQLIGPCNGLMALMD-TQTTILFNPSTRNYRPLRPSPFGCPQGFYRCIQ-AVGFGFD
SLF6_2          CDHDQLIGPCNGLMALMD-TQTTILFNPSTRNYRPLRPSPFGCPQGFHRCIQ-AVGFGFD
                                   :
```

Figure 69. Continued (3/8).

```
SLF1_1          TISNYYKVVRISEVYC-EEADGYPGPKDSKIDVCDLSTDSWRELDHV--QLPSIYWVPCA
SLF1_2          TISNYYKVVRISEVYC-EEADGYPGPKDSKIDVCDLGTDSWRELDHV--QLPSIYWVPCA
SLF2_1          SIVNEYKVVRLSDVYW-DPPTDYPGPREPKVDIYDLGIDSWREID---IDFPPIYYLPCS
SLF2_2          SIVDDYKVVRLSDVYW-DPPTDYFGPREPKVDIYDLSIDSWRELD---LEFPSIYYLPCS
SLF7_1          SIASDYKIVRVLEDYG-DPPFYDFALRKWKIDVHELTIDSWRELDYMELQLPHIHRYPCS
SLF7_2          STANDYKIVWVLEDYG-DPPFYCYGLSKWRIDVYELTIDSWRELDYIDLEWPFDYRYPYS
SLF7_3          SIANDYKIVRILNDYG-DPPFYDFAMREWKVDVYELRTDSWRKLDHVYLRLPFMHRYPCS
SLF14_1         SIANEYKIVRLAEIRG-EPPFYCYTVREWRVEVYELSIDSWREVENVDQQLPYVHWYPCA
SLF14_2         SIANEYKIVRLAEIRG-EPPFYCYTVREWRVEVYELSIDSWREVENVDQQLPYVHWYPCA
SLF16_1         SIGNEYKIARLAELRG-EPPFNCFTMKEWRVEVYELSIDSWREIENVDQQLPYVHWYPCG
SLF16_2         SIGNDYKIVRIAELHG-EPPFNCFSTREWRVEVFEMSIDSWREVENVDQQLRYVHWYPSA
SLF17           SIGNDYKIVRIAELHG-EPPFNCFSTREWRVEVFEMSIDSWREVENVDQQLRYVHWYPSA
SLF16_3         SIANEYKIVRIAELRG-EPPFNCFTMVEWRVEVYELSTDSWRELENVDQQLPYVHWYPCA
SLF15_1         FIANSYKIVRIAEVNG-EPPFYCYTMREWKVEIYESSVDAWREQDQVYRQLPNVFWYPCF
SLF15_2         ----------LALMHG-----------ENKIKCIDNC---------------PMYFGILV
SLF11_1         TAENYYKLVRIFEVYT-DPYDRDLDARHSKVEVYDSCTDCWRDLDLTVKLLPKVRRFASS
SLF11_2         TAENYYKLVRIFEVYT-DPYDRDLDARHSKVEIYDSCTDCWRDLDLTVKLLPKVRRFACS
SLF9_1          SDANNYKVVRLSEVYK-EP-----CDKEMKVDIYDFSVDSWREL--LGQEVPIVYWLPCA
SLF10_2         SDANNYKVVRLSEVYK-EP-----CDKEMKVDIYDFSVDSWREL--LGQEVPIVYWLPCA
SLF9_2          SDANDYKVVRLSEVYK-EP-----CDKEMKVDIYDFSVDSWREL--LGQEVPIVYWLPCA
SLF9_3          SDANDYKVVRLSEVYK-EP-----CDKEMKVDIYDFSVDSWREL--LGQDVPFVFWFPCA
SLF10_1         SDANDYKVVRLSEVYK-EP-----CDKEMKVDIYDFSVDSWREL--LGQDVPFVFWFPCA
SLF3            SVHKTYKVVRISEVYG-EPPFNCPSVMEWKGEVYNSSTDSWRELDCVDQELPWPYNFAYS
SLF13_1         SIQKNYKVIRVSRVYG-DPPYNDRGEMSWESEVYDSSTDSWRQLANVDQELPGPYMHPYS
SLF13_2         SIQKNYKVIRVSRVYG-DPPYNDRSEMSWESEVYDSSTDSWRQLTNVDQELPGPYMHPYS
SLF4_1          SVVNDYKVFRISDVYT-EDRYGYPEEGERKVEVYEVGIDIWRELDHVDQDLPRLFWL-TS
SLF4_2          SVVNDYKVFRISDVYT-EDRYGYPEEGERKVEVYEVGIDIWRELDHVDQDLPRLFWL-TS
SLF5_1          SVVNDYKVFRISDVYT-EDRYGYPEEGERKVEVYEVGIDIWRELDHVDQDLPRLFWL-TS
SLF12_2         SVVNDYKVFRISDVYT-EDRYGYPEEGERKVEVYEVGIDIWRELDHVDQDLPRLFWL-TS
SLF12_1         SVVNDYKVFRISEVYT-EDSFGYPEEGERKVEVYELGIDVWRELDHVDQQLPKLFWM-TS
SLF5_2          SVVNDYKVVRISEFLK-DDCYGYVQVEEENVEIYELGIDCWRELDHINQQFPTIFWVPCS
SLF8_1          SIVKDYKFVTISEVFM-DSEWV-PDEKEQKVEVYDLCFDSWRDLNHVDQQLPTVYYYPCF
SLF8_2          SIVKDYKFVTISEVFR-NSEWG-PEETEQKVEVYDLRIDSWRDLNHVDQQLPTVYYYPCF
SLF6_1          TVSNDYKVVRISIIYKVDYDDEYPEERDRKFEVYDLGIDYWREIDNLSQELTTYSVTHCS
SLF6_2          TVSNDYKVVRISIIYKVDYDDEYPEERDRKFEVYDLGIDYWRELDNLSQELTTFCVTHCS
                          :                  . .  :
```

Figure 69. Continued (4/8).

```
SLF1_1          GMLYKEMVHWFA---TTDI-M--VILCFDMSTEMFHDMKMPDTCSRITHELYYGLVIFCE
SLF1_2          GTLYKEMVHWFA---TTDTSM--VILCFDMSTEMFHDMKMPDTCSRITHELYYGLVIFCE
SLF2_1          EMYYKEAVHWFI---VTDT-V--VIFCFDISTEIFRTMKMPDSC-TFFDGPRYGLTVLNG
SLF2_2          EMYFKEAVHWFI---ITDT-V--VILCFDISTEIFRTMKMPGNC-TFLDGPRYGLTISND
SLF7_1          EMFYNGATHWFG---RTET-V--VILCFDMSTETFRNMKMPDAC-HFKDRKSYGLVVLND
SLF7_2          DMFYNGATHWFG---GRET-V--VILCFDISTETFRNMKMPDAC-HFKDRKSYGLVVLND
SLF7_3          EMFYNGATHWFG---STKT-V--LILCFDMSTETFRSMKMPDAC-HFKDRKSYGLVVLND
SLF14_1         ELFYKGTSHWFG---NTNT-V--VILGFDMSTETFRNIKMPNTC-HFKDRKCYGLVVLNE
SLF14_2         ELFYKGTSHWFG---NTNT-V--VILGFDMSTETFRNIKMPNTC-HFKDRKCYGLVVLNE
SLF16_1         ELFYKGASHWFG---HANR-AR-VILCFDMTTETFRDIKMPNTC-HYKDRKCYGLVVLNG
SLF16_2         DLFYKGASHWFG---NENR-VH-VIVCFDMCTEIFRTFKMPSTC-HYKDKNFYCLVVLNK
SLF17           DLFYKGASHWFG---NENR-VH-VIVCFDMCTEIFRTFKMPSTC-HYKDKNFYCLVVLNK
SLF16_3         ELFYKGASHWFA---HANG-GR-VILCFDMSTQTFRNIKMPNTC-HYNDRKCYGLLVLNE
SLF15_1         EMFYKGASHWFA---HANT-M--VILCFDMITETFRRMKFPNTC-HFQDENCYSLVILND
SLF15_2         LIFYKGASHWFA---HTNT-M--VILCFDMITETFRRMKFPNTC-HFQDQNCYSLVILND
SLF11_1         EIFYKETFHWCA---HDDT-V--MILCFDISLETFHYMKLPDHC-HFWDNKGYGLTVLSN
SLF11_2         EIFYKETFHWCA---HDDI-V--MILCFDISLETFHYMKLPDHC-HFWDNKGYGLTVLSN
SLF9_1          EILYKRNFHWFA---FADD-V--VILCFDMNTEKFHHMGMPDAC-HFDDGKCYGLVILCK
SLF10_2         EILYKRNFHWFA---FADD-V--VILCFDMNTEKFHHMGMPDAC-HFDDGKCYGLVILCK
SLF9_2          EILYKRNFHWFA---FADD-V--VILXFDMNTEKFHHMGMPDAC-HFDDGKCYGLVILCK
SLF9_3          EILYKRNFHWFA---FADV-V--VILCFDMNTEKFHNMGMPDAC-HFDDGKCYGLVILFK
SLF10_1         EILYKRNFHWFA---FADV-V--VILCFDMNTEKFHNMGMPDAC-HFDDGKCYGLVILFK
SLF3            EIFYEGAFHWYA---HKNV-V--LILCFDINTETFRTMEVPEPC-ASYDEKCHSLLVLDE
SLF13_1         ELFYKGTFHWYA---QGHM-R--LLLCFDINTENFRTMQVPKTC-AVRDEKCHSLVVFDE
SLF13_2         ELFYKGTFHWYA---QGKM-R--LLLCFDINTEIFWTMQVPKTC-ASRDEKCHSLVVFDE
SLF4_1          SMYYNGAYHWITTLNHEDK-L--IILSFDMSTEIFRNINTPDTR-QFSSGTCHSLVLLDG
SLF4_2          SMYYNGAYHWITTLNHEDK-L--IILSFDMSTEIFRNINTPDTR-QFSSGTCHSLVLLDG
SLF5_1          SMYYNGAYHWITTLNHEDK-L--IILSFDMSTEIFRNINTPDTR-QFSSGTCHSLVLLDG
SLF12_2         SMYYNGAYHWITTLNHEDK-L--IILSFDMSTEIFRNINTPDTR-QFSSGTCHSLVLLDG
SLF12_1         SMPYNGTYHWLITLSYEHK-L--ILLCFDMSTEIFRYITTPNTR-YFSSGTRHSLVLLND
SLF5_2          QIIYMGTFHWIA---Q----R--VILCFNMNTEIFHHIRMPDPC-H--NIRNHGLVILNK
SLF8_1          EMLYNGAFHWYA---INDR-LDHVILSFDISTEIFHSIKMPATG-KSSGGKKYGLIVLNE
SLF8_2          EILYNGAFHWYA---IEDR-FD-VILSFDISTEIFCSIILPVTR-KSSGGKNYGLVVLNE
SLF6_1          QMFYKGACHWIASL-DIDA-Y--IILCFDMSSETFRSLKIPESC-HIIYGPTCKLALVHD
SLF6_2          QMFYKGACHWIASL-DIDA-Y--IILCFDMSSETFRSLKIPESC-HIINGPTCRLALVHD
                   :    **            ::. *::  : *  :  *           * :
```

Figure 69. Continued (5/8).

```
SLF1_1          SFTLIGYSNPISSIDPVEDKMHIWVMMEYGVSESWIMKYTIK--PLSI-ESPLAVWKNH-
SLF1_2          SFTLIGYSNPISSIDPVEDKMHIWVMMEYGVSQSWIMKYTIK--PLSI-ESPLAVWKNH-
SLF2_1          HLTLICYPDPMSSIHPTEDLIDIWMMEEYGASESWIKIYTIRPVPIPI-ESPLAIWKNQ-
SLF2_2          HLTLICYPDPMSSIDPTEDLIELWTMEEYGASESWIKKYTIRPVPIPI-ESPLAIWKDH-
SLF7_1          SLTLICYRHPGCIIDPTKDFMEIWTMKEYGVGESWIKKYTIT--PLSI-QSPLAVWKNH-
SLF7_2          SLALICYRHPRCVIDPAKDFMEIWTMKEYGVGESWIKKYTIT--PLSI-ESPLAVWKNH-
SLF7_3          SLTLICYRHPECIIDPAKDFMEIWTMKEYGVGESWIRKYTIT--PLSI-ESPLAVWKNH-
SLF14_1         SLTLICYPYPGCEIDPSIDFMEIWIMEEYGVNDSWSKKYTIV--PLAI-ESPLAIWKNH-
SLF14_2         SLTLICYPYPGCEIDPAVDFMEIWIMEEYGVNDSWSKKYTIV--PLAI-ESPLAIWKNH-
SLF16_1         CLTLICYPYPGCEIDPAIDFMEIWIMKEYGINESWSMKYKIT--PLAI-ESPLAIWKDH-
SLF16_2         CLTLICYPYLGYEIDPAIDFMEIWIMKEYGIYESWSKTYRIR--PLAI-ESPLAIWKDH-
SLF17           CLTLICYPYLGYEIDPAIDFMEIWIMKEYGIYESWSKTYRIR--PLAI-ESPLAIWKDH-
SLF16_3         CLTLICYPYPGCKIDTAIDFMEIWVLKEYGVNDSWSKKYTVS--PLAI-ESPLAIWKDH-
SLF15_1         SLTLICYPYPKKVVEHEKDFMEIWIMMEYGVDESWIKKYSIT--PLSI-ETPLAVWKDH-
SLF15_2         SLTLICYPYPEKVVEHQKDFMEIWIMMEYGVDESWIKKYSIT--PLSI-ETPLAVRKDH-
SLF11_1         YLTFITYPNPRCALDPGQEFTDIWIMEEYGVNGTWIKKYTIK--PLPI-ESSLSIWKDH-
SLF11_2         YLTFITYPNPRCALDPGQELTDIWIMEEYGINGTWIKRYTIR--PLPI-ESSLSFWKDH-
SLF9_1          CMTLICYPDPMPS-SPTEKLTDIWIMKEYGEKESWIKRCSIR--LLP--ESPLAVWKDE-
SLF10_2         CMTLICYPDPMPS-SPTEKLTDIWIMKEYGEKESWIKRCSIR--LLP--ESPLAVWKDE-
SLF9_2          CMTLICYPDPMPS-SPTEKLTDIWIMKEYGEKESWIKRCSIR--LLP--ESPLAVWKDE-
SLF9_3          CMTLICYPDPKPS-SPTEKLTDIWIMKEYGEKESWIKRCSIR--LLP--ESPLAVWKDE-
SLF10_1         CMTLICYPDPKPS-SPTEKLTDIWIMKEYGEKESWMKRCSIR--LLP--ESPLAVWKDE-
SLF3            FLTLFCYPDPRRESSPIQETIEIWTMQEYRVNESWIKKHTIK--SPPI-ESPLAIWKDRL
SLF13_1         SLTFICYPDPRRESSPVQETIEIWIMQEYSVNESWIKKYTIK--PPPI-ESPLAIWKDR-
SLF13_2         CLTFICYPDPRRESSPIQETIEIWIMQEYSVNESWIKKYTIR--CPPI-ESPLAIWKDR-
SLF4_1          CLSFMCHPYLGPEIDPTTDLIDIWMMKDYKVYESWAKKYTIR--MLPIDESPLAVWKDS-
SLF4_2          CLSFMCHPYLGPEIDPTTDLIDIWMMKDYKVYESWAKKYTIR--MLPIDESPLAVWKDS-
SLF5_1          CLSFMCHPYLGPEIDPTTDLIDIWMMKDYKVYESWAKKYTIR--MLPIDESPLAVWKDS-
SLF12_2         CLSFMCHPYLGPEIDPTTDLIDIWMMKDYKVYESWAKKYTIR--MLPIDESPLAVWKDS-
SLF12_1         CLSFICHPFLGPEIDPTKDFIDIWMMKDYNVYESWINIYTIR--VLPIHEFPLAIWKDS-
SLF5_2          SLTLICYRSVAPTSDPIEDLMEIWILKDYDVSESWVKKFTIR--SLPI-KIPLAIWKDN-
SLF8_1          SLTLICYPNPDCEMDPSKDSMDIWIMMEYGIYESWTKKYIIK--PLPI-ESPLTIWRDH-
SLF8_2          SLTLICYHDPYSEIHPTKDSMDIWVMMEYGVHESWTKKYIIK--PLPI-GSPLTIWRDH-
SLF6_1          TLTLIYYPYPEPEIPVEKDLINIWFMKEYNVYESWIRKYTIR--GLLI-DSPLTVWKGY-
SLF6_2          TLTLIYYPYPETEIPVEKDLINIWFMKEYNVYESWIRKYTIR--GLLI-DSPLTVWKGY-
                 :::: :           .  .:* : :*    :*     :          .*:. :.
```

Figure 69. Continued (6/8).

```
SLF1_1          ------ILLLQ-SRSGVLLSYDLNTGEAKELNLHGFPDSLSVK--VYKECLTSIPK-GSE
SLF1_2          ------ILLLQ-SRSGVLLSYDLNTGEAKELNLHGFPDSLSVK--VYKECLTSIPK-GSE
SLF2_1          ------LLLLQ-TKSGFFISYDLTSDEVKEFNLNGHLESLRVI--VYTESLTTISR-ISD
SLF2_2          ------LLLLQ-TKSGFLISCDTNSDEVKEFNLNGHLESLRVI--VYTESLTTIPR-INE
SLF7_1          ------FLLFEYRPSGVLFSYDLNSDDVKELNLHGWPQSLRVT--IYKESLTLIPK-GSE
SLF7_2          ------FLLLEYHRSGVLFSYDLNSDEVKELNLHGWPQSLRVS--IYKESLTLIPK-GNE
SLF7_3          ------FLLLEYRPSGVLFCYDLNSDEFKELNLHGWRESLRVT--TYRESLTLIPK-GSE
SLF14_1         ------LLLLQ-SITGHLISYNLNSDEIKEFNLHGWPKSLRVK--IYKESLTLIPK-ESE
SLF14_2         ------LLLLQ-SITGHLISYNLNSDEIKEFNLHGWPKSLRVK--IYKESLTLIPK-ERE
SLF16_1         ------LLLLQ-SISGYLISYDLNSDEIKEFELNGWPESLRVN--IYKESLALIPK-DQC
SLF16_2         ------LLLLQ-SISGYLISYDLNSGEVKEFELNGWPDSLRVT--VYKESLALIPN-SKR
SLF17           ------LLLLQ-SISGYLISYDLNSGEVKEFELNGWPDSLRVT--VYKESLALIPN-SKR
SLF16_3         ------LLLLQ-SISGYLITYDLNSDEIKELNLSGWPESLRVV--VYKESLTLIPK-GHE
SLF15_1         ------LLLLE-SRSGSLISYDLNSGEVKQLNLHCWPXSSNITYIVGQQVLEL-------
SLF15_2         ------LLLLE-SRSGSLISYDLNSGEVKQLNLHCWPTSFRIA--VYKESLTLIPE-ERE
SLF11_1         ------LLLLQ-TTRGTLSSYNLSSDELKEFNFQGFTSTLRLV--VYKESLTIIPR-ESE
SLF11_2         ------VLLLQ-TTSGTLSSYNLSSDELKEFNFQGFTSTLRLV--VYKESLTIIPR-ESQ
SLF9_1          ------ILLLQ-SKIGHLITYDHNSDEVEELDLHGLPTSLRVI--IYRESLTPIPR-SKD
SLF10_2         ------ILLLQ-SKIGHLITYDHNSDEVEELDLHGLPTSLRVI--IYRESLTPIPR-SKD
SLF9_2          ------ILLLQ-SKIGHLIAYDHNSDEVKELDLHGLPTSLRVI--IYRESLTLIPR-SKD
SLF9_3          ------ILLLH-SKMGHLMAYDLNSNEVQELDLHGYPESLRII--IYRESLTAIPR-NND
SLF10_1         ------ILLLH-SKMGHLMAYDLNSNEVQELDLHGYPESLRII--IYRESLTAIPR-NND
SLF3            LLFQDXLLLFQ-DKSGILISYDLNSDEVKEFKLDGYPATLRVI--IYKESLTPIPK-GS-
SLF13_1         ------LLLLQ-DKSGVLIAYDLNLDEVKEFKLHGHPESLRVI--VYKESLTPIPT-GS-
SLF13_2         ------LLLLQ-DKSGVLISYDLNSDEVKEFKFHGHPESLRVI--VYKESLTPIPI-GS-
SLF4_1          ------LLFFQ-GKSGYLMSYNFKSEEVKEWNLHGCQKSMRAI--VYKESLVPIPR-GSQ
SLF4_2          ------LLFFQ-GKSGYLMSYNFKSEEVKEWNLHGCQKSMRAI--VYKESLVPIPR-GSQ
SLF5_1          ------LLFFQ-GKSGYLMSYNFKSEEVKEWNLHGCQKSMRAI--VYKESLVPIPR-GSQ
SLF12_2         ------LLFFQ-GKSGYLMSYNFKSEEVKEWNLHGCQKSMRAI--VYKESLVPIPR-GSQ
SLF12_1         ------LLFFQ-GKTGYLMSYDLNTEEVKELNLNGCKRSMRAI--VYKESLAPIPE-GSE
SLF5_2          ------LLLFQ-NRSGYLMVYDLRTDNVKELNIHGCPESMRAT--VYKENLTIIPS-GSE
SLF8_1          ------LLLLQ-SKNGLLVSYDLSSNEVKEFDLHGYPKSLRVL--VYKESLISIPKRGCK
SLF8_2          ------LLLLQ-SKSGLLISYDLSSNEVKEFDLHGYPKSLRVL--VYKESLISIPRRGCE
SLF6_1          ------LLLYQ-NRSGCLMSYNLNSNGVREFSFHGYPKSLRAI--VYKDSLTSIPR-ESE
SLF6_2          ------LLLYQ-NRSGCLMSYNLNSNDVSEFNFHGYPKSLRAI--VYKDSLTSIPR-ESE
                      .*: .    * :   :       : .:     :         : *
```

Figure 69. Continued (7/8).

```
SLF1_1          YSTKVQKF----
SLF1_2          YSTKVQKF----
SLF2_1          NGTQVQQF----
SLF2_2          HGTQIQQF----
SLF7_1          HSTQVQNF----
SLF7_2          DSTQVQNF----
SLF7_3          NSTRVQSF----
SLF14_1         FNTAQ-------
SLF14_2         FNTAQ-------
SLF16_1         KE----------
SLF16_2         PRA---------
SLF17           PRA---------
SLF16_3         HDLRLT------
SLF15_1         ------QFTRKA
SLF15_2         HSTKCPKF-LES
SLF11_1         HGTRVQTF----
SLF11_2         DGTKVQTF----
SLF9_1          -SIELEQF----
SLF10_2         -SIELEQF----
SLF9_2          -SIDLEQF----
SLF9_3          -CIELQNF-RCN
SLF10_1         -CIELQNF-RCN
SLF3            --TQVQNF----
SLF13_1         --TQVEKF----
SLF13_2         --TQVENF----
SLF4_1          SSSQLQNI----
SLF4_2          SSSQLQNI----
SLF5_1          SSSQLQNI----
SLF12_2         SSSQLQNI----
SLF12_1         SSTQVHNF----
SLF5_2          NSTPVHKF----
SLF8_1          HGTKFKNC----
SLF8_2          HGTKKYNC----
SLF6_1          HTKQVYKF----
SLF6_2          HTKQVYKF----
```

Figure 69. Continued (8/8).

The third one (Fig. 70) was the alignment of SLF1 with different S haplotypes from three Petunia species, of which (1) $PinS_1$-SLF1 and $PinS_3$-SLF1 were from Sijacic et al. (2004); (2) $PaxS_{17}$-SLF1 and $PaxS_{19}$-SLF1 were from Tsukamoto et al. (2005); (3) PhS_5-SLF1, PhS_7-SLF1, PhS_9-SLF1 and PhS_{11}-SLF1were from Kubo et al. (2010); (4) $PinS_2$-SLF1 and $PinS_5$-SLF1 were from Williams et al. (2014); and (5) $PaxS_{ax1}$-SLF1 and $PinS_6$-SLF1 were from the sequence data of Petunia Genome Project (2014). The result indicated that the protein sequences of subtype-1 SLF, i.e. SLF1, even though they were not from the same species or carrying the same S haplotype, were significantly more conserved compared with the alignments among different subtypes of SLF variants but with the same S haplotype. Similar alignments were also performed by using other subtypes of SLF protein sequences, e.g. SLF2, SLF3, SLF5, etc. (Data not shown), and all results indicated that SLF variants of the same subtype were mostly conserved.

```
PaxS17-SLF1      -MPNGILKKLPEDLVFLILLTFSVKSLMRFKCISKAFSILIQSTTFINRHVNHEINKEDE
PaxS19-SLF1      -MANGILKKLPEDLVFLILLTFPVKSLLRFKCISKAWSILIQSTTFINRHINRKTNTKAE
PhS7-SLF1        -MANGILKKLPEDLVFLILLTFPVKSLLRFKCISKAWSILIQSTTFINRHINRKTNTKAE
PhSL1-SLF1       -MANGILKKLPEDLVFLILLTFPVKSLLRFKCISKAWSILIQSTTFINRHINRKTNTKAE
PaxSax1SLF1_2    -MANGILKKLPEDLVFLILLTFPVKSLLRFKCISKAWSILIQSTTFINRHINRKTNTKAE
PaxSax1SLF1_1    -MANGILKKLPEDLVFLILLTFPVKSLMRFKCISKAWSTLIQSTTFINRHINRKTNTKAE
PinS5-SLF1       -MANGILKNLPEDLVFLILLTFPVKSLMRFKCISKAWSTLIQSTTFINRHINRKTNTKAE
PhS5-SLF1        -MANGILKKLPEDLVFLILLTFPVKSLLRLKCISKAWSILIQSTTFINRHINRKTNTKAE
PhS9-SLF1        -MANGILKKLPEDLVFLILLTFPVKSLLRFKCISKAWSILIQSTTFIKRHINRKTNTKAE
PhSL3-SLF1       -MANGILKKLPEDLVFLILLTFPVKSLLRFKCISKAWSILIQSTTFIKRHINRKTNTKAE
PinS6-SLF1_1     -MANGILKKLPDDLVFLILLTFPVKSLLRFKCISKAWSILIQSTTFINLHINRKTNTNAE
PinS2-SLF1       -MANGILKKLPEDLVFLILLTFPVKSLMRFKCISKAWSILIQSTTFINRHINRKTNTKDE
PinS6-SLF1_2     MMPNGILKKLHEDLIFLILLTFPVKSLLRFKCISKAWSILIQSTTFINLHINRKTNTKAE
PhS11-SLF1       -MANGILKKLPEDLLFLILLTFPVKSLMRFKCISKAWSILIQSTTFINCHANRKTNTKDE
PinS1-SLF1       -MANDILMKLPEDLVFLVLLTFPVKSLLRFKCISKAWSILIQSTTFINRHVNRKTNTKDE
PinS3-SLF1       -MANGVLKKLPEDLVCLILLTFPVKSLMRFKCISKTWSILIQSTTFINRHVNRKTNTKDE
                  *.*.:* :* :**: *:****.****:*:*****::* ********: * *:: *.: *

PaxS17-SLF1      FILFKRAIKDEEEEFINILSFFSGHDDVLNPLFPDIDVSYMTSKFNCTFNPLIGPCDGLI
PaxS19-SLF1      FILFKRSIKDEEEEFINILSFFSGNDDVLNPLFPDIDVSYMTSKCDCTFTPLIGPCDGLI
PhS7-SLF1        FILFKRSIKDEEEEFINILSFFSGNDDVLNPLFPDIDVSYMTSKCDCTFTPLIGPCDGLI
PhSL1-SLF1       FILFKRSIKDEEEEFINILSFFSGNDDVLNPLFPDIDVSYMTSKCDCTFTPLIGPCDGLI
PaxSax1SLF1_2    FILFKRSIKDEEEEFINILSFFSGNDDVLNPLFPDIDVSYMTSKCDCTFTPLIGPCDGLI
PaxSax1SLF1_1    FILFKRSIKDEEEEFINILSFFSGHDDVFNPLFPDIDVSYMTSKCDCTFTPLIGPCDGLI
PinS5-SLF1       FILFKRSIKDEEEEFINILSFFSDHDDVFNPLFPDIDVSYMTSKCDCTFTPLIGPCDGLI
PhS5-SLF1        FILFKRSIKDEEEEFINILSFFSGHDDVLNPLFPDIDVSYMTSKCDCSFNPLIGPCDGLI
PhS9-SLF1        FILLKRSIKDEEEEFINILSFFSGHDDVLNPLFPDIDVSYMTSKCDCTFTPLIGPCDGLI
PhSL3-SLF1       FILLKRSIKDEEEEFINILSFFSGHDDVLNPLFPDIDVSYMTSKCDCTFTPLIGPCDGLI
PinS6-SLF1_1     FILFKRSIKDEEEEFINILSFFSGHDDVLNPLFPDIDVSYMTSKCDCTFTPLIGPCDGLV
PinS2-SLF1       FILFKRAIKDDEEEFINILSFFSGHVDVLNPLFPDMDVSYMTSKCDCTFNPLIGPCDGLI
PinS6-SLF1_2     FILFKRSIKDEEEEFINILSFFSGNDNVLNPLFPDIDVSYMTSKCDCTFNPLIGPCYGLI
PhS11-SLF1       FILFKRAIKDEEEEFINILSFFSGHNDVLNPLFPDIDVSYMTSKCDCAFNPLIGPCDGLI
PinS1-SLF1       FIIFKRSIKDEQEGFKDILSFFSGHDDVLNPLFPDVEVSYMTSKCNCTFNPLIGPCDGLI
PinS3-SLF1       FILFKRAIKDEQEEFRDILSFLSGHDDVLNPLFADIDVSYMTSKCNCAFNPLIGPCDGLI
                 **::**:***::* * :****:*.: :*:****.*::******* :*:*.****** **:
```

Figure 70. Multiple protein-sequence alignment of S_x-SLF1 variants from different Petunia species. "S_x" indicates various S haplotypes. "Pax" – *Petunia axillaris*; "Ph" – *Petunia hybrida*; "Pin" – *Petunia inflata*. Continued on following page (1/4).

```
PaxS17-SLF1      ALTDSIITIILNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGLDTISNYYKVVRISEVYC
PaxS19-SLF1      ALTDTIITIVLNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGFDTISYYYKVVRISEVYC
PhS7-SLF1        ALTDTIITIVLNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGFDTISYYYKVVRISEVYC
PhSL1-SLF1       ALTDTIITIVLNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGFDTISYYYKVVRISEVYC
PaxSax1SLF1_2    ALTDTIITIVLNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGFDTISYYYKVVRISEVYC
PaxSax1SLF1_1    ALTDTIITIVLNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGFDTISNYYKVVRISEVYC
PinS5-SLF1       ALTDTIITIVLNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGFDTISNYYKVVRISEVYC
PhS5-SLF1        ALTDTIITIVLNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGFDTISNYYKVVRISEVYC
PhS9-SLF1        ALTDTIITIVLNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGFDTISNYYKVVRISEVYC
PhSL3-SLF1       ALTDTIITIVLNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGFDTISNYYKVVRISEVYC
PinS6-SLF1_1     ALTDTIITIVLNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGFDTISNYYKVVRISEVYC
PinS2-SLF1       ALTDTIITIVLNPATRNFRVLPASPFGCPKGYHRSVEGVGFGLDTISNYYKVVRISEVYC
PinS6-SLF1_2     ALTDTIITIILNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGFDTISNYYKVVRISEVYC
PhS11-SLF1       ALTDSIITIILNPATRNFRVLPPSPFGCPKGYHRSVEGVGFGLDTISNYYKVVRISEVYC
PinS1-SLF1       ALTDSIITILLNPATRNFRLLPPSPFGCPKGYHRSVEGVGLGLDTISNYYKVVRISEVYC
PinS3-SLF1       ALTDTIITIILNPATRNFRLLPPSPFGSPKGYHRSVEGVGFGLDTISNYYKVVRISEVYC
                 ****:****:*********:**.****.************:*:**** ************

PaxS17-SLF1      EEAGGYPGPKDSKIDVFDLRTDTWKELDHVQLPLIYWLPCSGMLYKQMVHWFATTDM-MV
PaxS19-SLF1      EEADGYPGPKDSKIDVCDLSTDSWRELDHVQLPSIYWVPCAGMLYKEMVHWFATTDTSMV
PhS7-SLF1        EEADGYPGPKDSKIDVCDLSTDSWRELDHVQLPSIYWVPCAGMLYKEMVHWFATTDTSMV
PhSL1-SLF1       EEADGYPGPKDSKIDVCDLSTDSWRELDHVQLPSIYWVPCAGMLYKEMVHWFATTDTSMV
PaxSax1SLF1_2    EEADGYPGPKDSKIDVCDLSTDSWRELDDVQLPSIYWVPCAGILYKEMVHWFATTDTSMV
PaxSax1SLF1_1    EEADGYPGPKDSKIDVCDLSTDSWRELDHVQLPSIYWVPCAGMLYKEMVHWFATTDTSMV
PinS5-SLF1       EEADGYPGPKDSKIDVCDLSTDSWRELDHVQLPSIYWVPCAGMLYKEMVHWFATTDTSMV
PhS5-SLF1        EEADGYPGPKDSKIDVCDLSTDSWRELDHVQLPSIYWVPCAGMLYKEMVHWFATTDTSMV
PhS9-SLF1        EEADGYPGPKDSKIDVCDLSTDSWRELDHVQLPSIYWVPCAGMLYKEMVHWFATTDTSMV
PhSL3-SLF1       EEADGYPGPKDSKIDVCDLSTDSWRELDHVQLPSIYWVPCAGMLYKEMVHWFATTDTSMV
PinS6-SLF1_1     EEADGYPGPKDSKIDVCDLSTDSWRELDHVQLPSIYWVPCAGMLYKEMVHWFATTDI-MV
PinS2-SLF1       EEADGYPGPKDSKIDVCDLSTDSWRELDHVQLPSIYWVPCAGMLYKEMVHWFATTDMSMV
PinS6-SLF1_2     EEADGYPGPKDSKIDVCDLGTDSWRELDHVQLPSIYWVPCAGTLYKEMVHWFATTDTSMV
PhS11-SLF1       EEADGYPGPKDSKIDVCDLSTDSWRELDHVQLPSIYWVPCSGMLYKEMVHWFATTDI-MV
PinS1-SLF1       EEAGGYPGPKDSKIDVCDLGTDSWRELDHVQLPLIYWVPCSGMLYKEMVHWFATTDESMV
PinS3-SLF1       EEDGGYPGPKDSKIDAFDLSTDSWRELDHVQLPLIYWLPCSGMLYKEMVHWFATTDMSTV
                 ** .************. ** **:*:***.**** ***:**:* ***:*********   *
```

Figure 70. Continued (2/4).

```
PaxS17-SLF1      ILCFDISTEMFRNMKMPDTCCLITHELYYGLVILCESFTLIGYSNPISSIDPARDKMHIW
PaxS19-SLF1      ILCFDMSTEMFHDMKMPDTCSRITHELYYGLVILCESFTLIGYSNPISSIDPVEDKMHIW
PhS7-SLF1        ILCFDMSTEMFHDMKMPDTCSRITHELYYGLVILCESFTLIGYSNPISSIDPVEDKMHIW
PhSL1-SLF1       ILCFDMSTEMFHDMKMPDTCSRITHELYYGLVVLCESFTLIGYSNPISSIDPVEDKMHIW
PaxSax1SLF1_2    ILCFDMSTEMFHDMKMPDTCRRITHELYYGLVILCESFTLIGYSNPISSIDPVEDKMHIW
PaxSax1SLF1_1    ILCFDMSTEMFHDMKMPDTCSRITHELYYGLVVLCESFTLIGYSNPISSIDPVEDKMHIW
PinS5-SLF1       ILCFDMSTEMFHDMKMPDTCSRITHELYYGLVVLCESFTLIGYSNPISSIDPVEDKMHIW
PhS5-SLF1        ILCFDMSTEMFHDMKMPDTCSRITHELYYGLVVLCESFTLIGYSNPISSIDPVEDKMHIW
PhS9-SLF1        ILCFDMITEMFHDLKMPDTCSRITHELYYGLVILCESFTLIGYSNPISSIDPVEDKMHIW
PhSL3-SLF1       ILCFDMITEMFHDLKMPDTCSRITHELYYGLVILCESFTLIGYSNPISSIDPVEDKMHIW
PinS6-SLF1_1     ILCFDMSTEMFHDMKMPDTCSRITHELYYGLVIFCESFTLIGYSNPISSIDPVEDKMHIW
PinS2-SLF1       ILCFDMSTEMFHDMKMPDTCSRITHELYYGLVILCESFTLIGYSNPISSTDPAHDKMHIW
PinS6-SLF1_2     ILCFDMSTEMFHDMKMPDTCSRITHELYYGLVIFCESFTLIGYSNPISSIDPVEDKMHIW
PhS11-SLF1       ILCFDMSTEMFHTMKMPDTCSRITHELYYGLVILCESFTLIGYSNPISSIDPVKDKMHIW
PinS1-SLF1       ILCFDMSTEMFRNMEMPDSCSPITHELYYGLVILCESFTLIGYSNPISSIDPVKDKMHIW
PinS3-SLF1       ILCFDMSTEMFRNMKMPDTCS-VTHKQYYGLVILCESFTLIGYPNPVSPIDPAHDKMHIW
                 *****: ****: ::***:*  :**: *****::*********.**:*. **..******

PaxS17-SLF1      VMMEYGVSESWIMKYTIRPISIKSPLAIWKNNILLLQNRSGILISYDLNSGEAKEFNLHG
PaxS19-SLF1      VMMEYGVSESWIMKYTIRPLSIESPLAVWKNHILLLQSRSGLLISYDLNSGEAKDLNLHG
PhS7-SLF1        VMMEYGVSESWIMKYTIRPLSIESPLAVWKNHILLLQSRSGLLISYDLNSGEAKDLNLHG
PhSL1-SLF1       VMMEYGVSESWIMKYTIKPLSIESPLAVWKNHILLLQSRSGLLISYDLNSGEAKELNLHG
PaxSax1SLF1_2    VMMEYGVSESWIMKYTIRPLSIESPLAVWKNHILLLQSRSGLLISYDLNSGEAKELNLHG
PaxSax1SLF1_1    VMMEYGVSESWIMKYTIKPLSIESPLAVWKNHILLLQSRSGLLISYDLNSGEAKELNLHG
PinS5-SLF1       VMMEYGVSESWIMKYTIKPLSIESPLAVWKNHILLLQSRSGLLISYDLNSGEAKELNLHG
PhS5-SLF1        VMMEYGVSESWIMKYTIRPLSIESPLAVWKNHILLLQSRSGLLISYDLNSGEAKELNLHG
PhS9-SLF1        VMMEYGVSESWIMKYTIKPLSIESPLAVWKNHILLLQSRSGLLISYDLNSGKAKELNLHG
PhSL3-SLF1       VMMEYGVSESWIMKYTIKPLSIESPLAVWKNHILLLQSRSGLLISYDLNSGKAKELNLHG
PinS6-SLF1_1     VMMEYGVSESWIMKYTIKPLSIESPLAVWKNHILLLQSRSGVLLSYDLNTGEAKELNLHG
PinS2-SLF1       VMMEYGVSESWIMKYTIRPLSIESPLAVWKNHILLLQCRSGLLISYDLNSGEAKELNLHG
PinS6-SLF1_2     VMMEYGVSQSWIMKYTIKPLSIESPLAVWKNHILLLQSRSGVLLSYDLNTGEAKELNLHG
PhS11-SLF1       VMIEYGVSESWIMRYTIKPLSIESPLAVWKNHILLLQSRSGLLISYDLHSGDAKELSLHG
PinS1-SLF1       VMMEYGVSESWIMKYTIKPLSIESPLAVWKKNILLLQSRSGRLISYDLNSGEAKELNLHG
PinS3-SLF1       VMMEYGVSESWIMKYTIRPLSIESPLAVWKKNILLLQSRSGLLISYDLNSGQAKELNLHG
                 **:*****:****:***:*:**:****:**::***** *** *:****::*.**::.***
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Figure 70. Continued (3/4).

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PaxS17-SLF1      FPGSLSVIVYKECLTSIPKGSEFSTKVQKF
PaxS19-SLF1      FPDSLSVKVYKECLTSIPKGSEYSTKVQKF
PhS7-SLF1        FPDSLSVKVYKECLTSIPKGSEYSTKVQKF
PhSL1-SLF1       FPDTLSVKVYKECLTSIPKGSEYSTKVQKF
PaxSax1SLF1_2    FPDTLSVKVYKECLTSIPKGSEYSTKVQKF
PaxSax1SLF1_1    FPDTLSVKVYKECLTSIPKGSEYSTKVQKF
PinS5-SLF1       FPDTLSVKVYKECLTSIPKGSEYSTKVQKF
PhS5-SLF1        FPDTLSVKVYKECLTSIPKGSEYSTKVQKF
PhS9-SLF1        FPDTLSVKVYKECLTSIPKGSEYSTKVQKF
PhSL3-SLF1       FPDTLSVKVYKECLTSIPKGSEYSTKVQKF
PinS6-SLF1_1     FPDSLSVKVYKECLTSIPKGSEYSTKVQKF
PinS2-SLF1       FPDSLSVKVYKECLTSIPKGSEYSTKVQKF
PinS6-SLF1_2     FPDSLSVKVYKECLTSIPKGSEYSTKVQKF
PhS11-SLF1       FPDSLSVKVYKECLTSIPEGGEYSTKVQKF
PinS1-SLF1       FPDSLSVIVYKECLTSIPKGSEYSTKVQKF
PinS3-SLF1       FPDSLSVIVYKECLTSIQNGSEYSTKVQNF
                 **.:*** ********* :*.*:*****:*
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Figure 70. Continued (4/4).

CHAPTER 4

DISCUSSION

Semi *in vivo* Pollinations: Rationale and Technical Issues

To determine the subcellular location of S-RNase and SLF post-pollination, I intended to use High-Pressure Freezing/Freezing-Substitution (HPF/FS) immune-electron microscopy. HPF/FS instantly immobilizes cellular structures and enables better morphological conservation of samples for subsequent electron microscopy. One issue for HPF/FS is that the sample holder on the equipment can only contain a size of 2 mm^2. Additionally, the region that I needed to observe, where most S-RNases were found, was the pollen-tube tip which was extremely hard to isolate by conventional style sectioning. So I proposed to apply the technique termed "semi *in vivo"* pollination which allows pollen tubes to grow *in vivo* after pollination, and allows relatively easier isolation of pollen-tube tips as they emerge from the base of a cut style.

To carry out semi *in vivo* pollination, I needed to determine how fast pollen tubes travel *in vivo* and how fast they grow on medium. The purpose of determining the growing speed of pollen tubes *in vivo* was to determine the time required for pollen tubes to extend but not yet to reach the end of the style. Styles collected under this circumstance could be either trimmed shorter or directly transferred to growth medium where pollen tube continued to grow and eventually emerged from the end (or trimmed end) of the style. Trimming may lessen the incubating time on growth medium, and where to trim, however, could only be decided based on

well-tested data. Even though all Petunia plants chosen were cultivated in the greenhouse operated by the Department of Biological Sciences where temperature, humidity and lighting were kept as stable as possible, it seemed pollen tubes were affected by more than the ambient environment, but also by the vitality of plants themselves and by the quality of pollen grains and styles. For example: (1) self-compatible crosses which normally germinated fast sometimes did not germinate at all, (2) the same cross germinated well one day but did not germinate the next day, or (3) the same cross gave different results after being pollinated for the same duration (Fig. 21), often occurred in my attempts. At last, I was not able to make solid conclusion of how fast pollen tubes grown *in vivo*.

Despite the lack of data of pollen-tube growth *in vivo*, as an alternative plan, I could transfer the entire style to growth medium without trimming, only spending longer time on *in vitro* incubation. It was still possible to obtain pollen-tube tips by experimenting in this way. Nevertheless, I encountered the second technical issue that was the pollen-tube growing rate on medium was not consistent either. The original medium [20 mM MES (pH 6); 3 mM $Ca(NO_3)_2$-$4H_2O$; 1 mM KNO_3; 0.8 mM $MgSO_4$-$7H_2O$; 1.6 mM H_3BO_3; 0.5% Agarose] used was from Cheung et al. (1995), termed basal medium. Low-melting agarose was chosen because I could cover the end of the style with molten medium at a relatively low temperature (around 30 ℃) without concern about damaging styles at higher temperature. In addition, basal medium supplemented with casein hydrolysate, 5.0% sucrose, and 15% PEG which had been proven to promote pollen-tube elongation (Lush et al. 1997) was tested. After testing repeatedly with different recipes, I was able to observe pollen tubes extending out of the stylar base (Fig. 71), but I could never confirm a reliable timing when pollen-tube tips just emerged from the end of the style and therefore tips could be isolated.

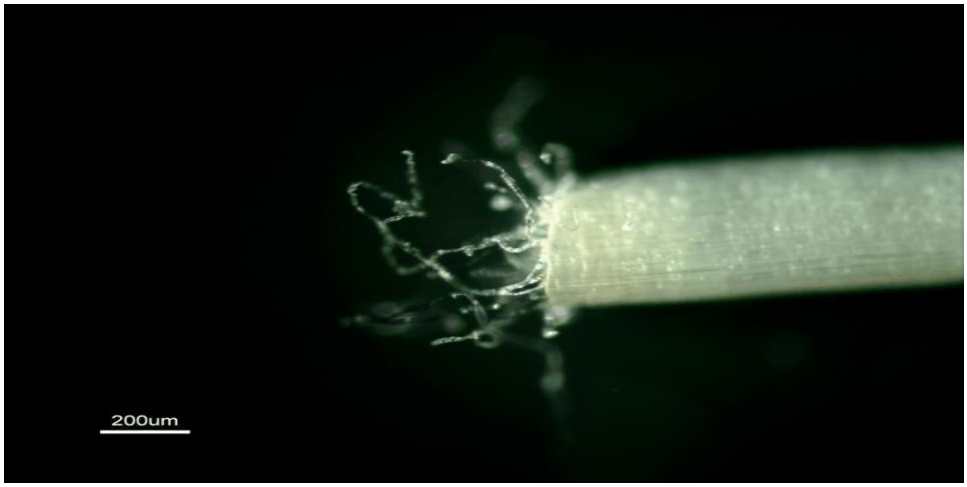

Figure 71: Pollen tubes emerging from the base of a *Petunia hybrida* style after semi *in vivo* pollination.

Together, inducing pollen tubes to extend out the end of the style by semi *in vivo* pollination and incubation on growth medium was feasible, but using a plant incubator or growth chamber may be necessary in the future for controlling the ambient conditions more precisely so that it can minimize the influence from the plant itself.

Down-regulation of SBP1 via RNAi

As a major component of the SCF^{SLF} E3 ubiquitin ligase complex, SBP1 is featured by its C-terminal RING-HC domain, which is correlated with E3 ubiquitin ligase activity. SBP1 has also been shown to interact with both S-RNase and SLF in our lab (Sims et al. 2010; Diwa Malla, M.S. thesis 2010). Therefore, down-regulation of SBP1 was predicted to result in 100%

pollen-tube degradation due to the inability to inactivate the S-RNase, and would provide more evidence of SBP1's role in gametophytic self-incompatibility.

An important control is necessary that was to test the effect of SBP1 down-regulation in backgrounds where S-RNase is also down-regulated. Therefore, the rationale was to make RNAi-SBP1 and RNAi-S_1-RNase S_1S_1 *Petunia hybrida* plants (i.e. where SBP1 or S-RNase is down-regulated in S_1S_1 background) and RNAi-S_3-RNase plants (S_3-RNase down-regulated in S_3S_3-background). RNAi constructs will be transferred into S_1S_1 Petunia leaves. The initial transgenic plants (T1) will be hemizygous for the transgene, therefore 50% of pollen will be SBP1-minus in RNAi-SBP1 plants, leading to only half compatibility (if used in an otherwise fully-compatible cross). To obtain homozygous RNAi-SBP1 plants, two approaches will be adopted: (1) Bud pollination, at a developmental stage prior to the increase in expression of S-RNase, should allow self-pollination. Southern blots and/or qPCR will be used to identify homozygous progeny. (2) RNAi-SBP1 T1 plants (pollen donor) will be crossed to S_3S_3 plants and RNAi-S_3-RNase S_3S_3 down-regulated plants (pollen acceptor). If SBP1 is indeed involved in inhibiting non-self S-RNases, then these two crosses should result in different outcomes. Normally, the cross S_3S_3 x S_1S_1 should be fully compatible. Because SBP1-(RNAi) pollen should not be able to inhibit the S_3-RNase, however, the S_3S_3 x S_1-(RNAi-SPB1) cross should be either half or fully incompatible, depending on whether the pollen parent is heterozygous or homozygous for the transgene. The S_3S_3-(RNAi-S_3-RNase) cross will control for non-specific effects on pollen fertility, since this cross should again be compatible.

Given the strategy described above, I was, however, only able to finish constructing SBP1 for RNAi, but never able to verify correct inserts of S_1- or S_3-RNase genes in the pSTARGATE vector as proper constructs in Agrobacterium. In the future, it may be necessary to

browse the coding sequences of S_1- and S_3-RNase again and design new Gateway® primers for PCR amplification. In addition, as an alternative constructing method, conventional restriction-enzyme digestion and ligation may be applied instead of direct Gateway® cloning into pSTARGATE vector, although it may involve more steps of cloning. Lastly, selecting a different vector for RNAi may also solve the problem.

Plant Transformation, Regeneration, and Rooting

When I was trying to confirm the insertion of S_1- or S_3-RNase genes while having SBP1-RNAi construct confirmed already, I started to use SBP1-RNAi to infect leaves from S_1S_1 *Petunia hybrida* with genotype 90FS2D3 for the purpose of optimizing the conditions for plant transformation, selection and rooting. First, on co-cultivation medium (Meer 2005), calluses were observed to develop from infected leaf squares. Next, after being transferred to the regeneration and selection medium, calluses grew bigger over time (Fig. 72). New shoots, however, were never observed. I attempted to cut off small pieces of callus and transferred them onto the rooting medium, although according to the protocol (Meer 2005) it was new shoot developing from callus that should be transferred. After 3-4 weeks, surprisingly I was still able to identify roots (Fig. 73) from those calluses under microscope. But a short time after being planted into soil and incubated in growing chamber, those rooted explants stopped growing and start to corrupt. Considering that antibiotics (Spectinomycin) was omitted in rooting medium to accelerate the rooting process, the fungus contamination may occur and damage the vulnerable explant. In the future, the proper concentration of antibiotics in rooting medium needs to be

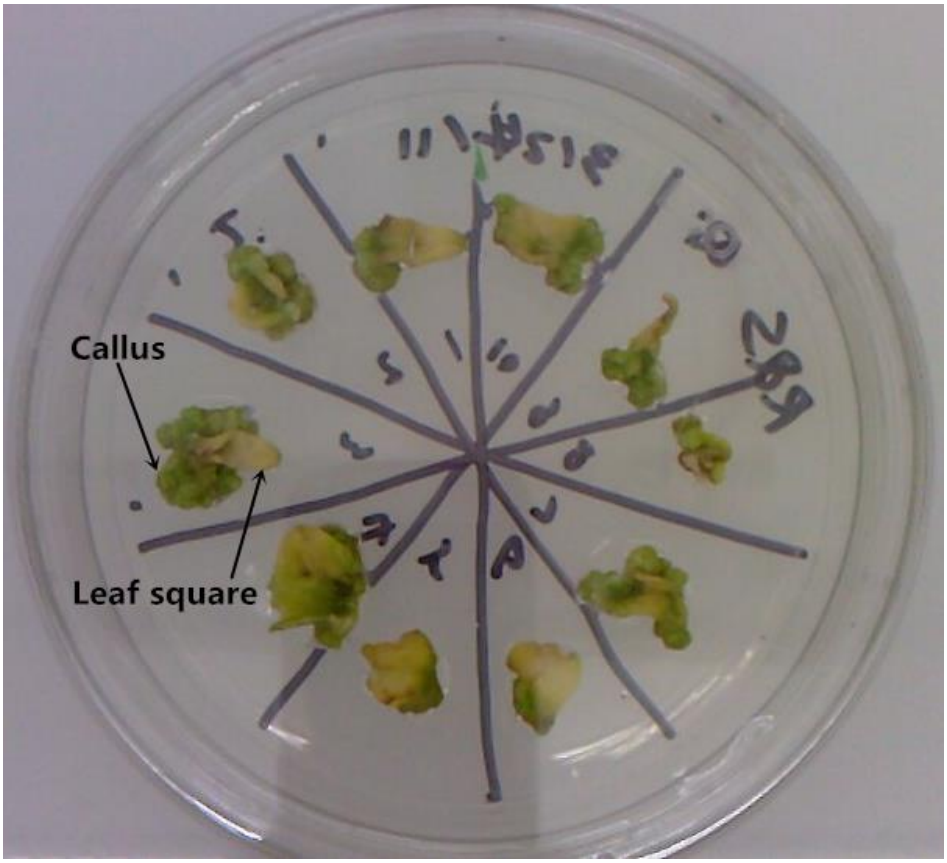

Figure 72: Callus developed from infected leaf squared from S_1S_1 *Petunia hybrida* (Genotype 90FS2D3).

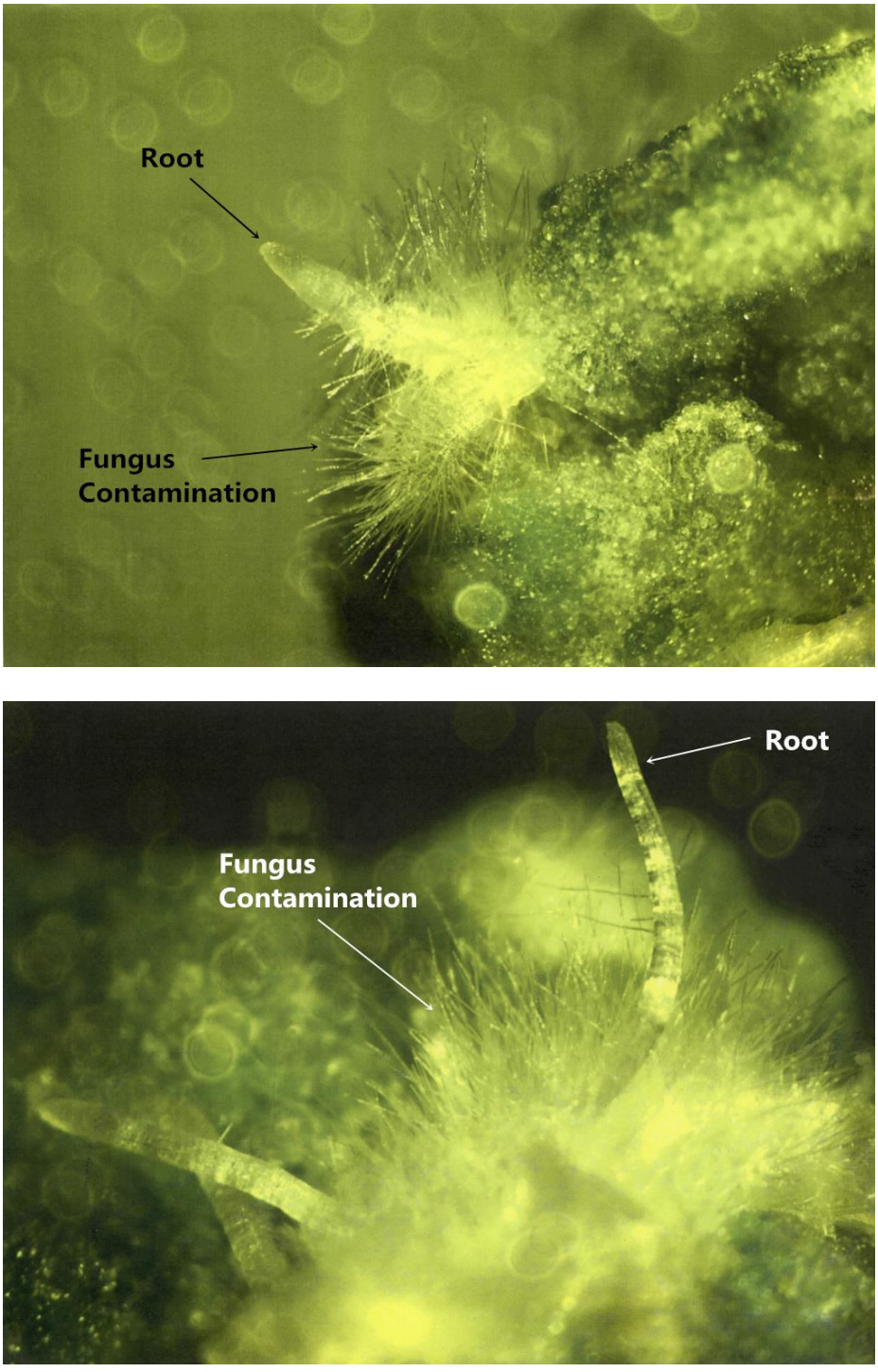

Figure 73: Rooted calluses under microscope with fungus contamination.

determined so that contamination could be avoided while rooting process is not significantly inhibited.

Yeast Two-Hybrid (Y2H) Assays

In this dissertation, I intend to focus on the Bimolecular Fluorescence Complementation (BiFC) of protein-interaction studies *in vivo*. As an alternative *in vitro* technique studying protein-protein interactions, however, yeast two-hybrid (Y2H) has been applied in our lab for years. Although I did not officially use this technique in my project, I did finish constructing four SLF variants (SLF1, 2, 3, 5) in Y2H vectors, and they were ready for subsequent assays in yeast.

I designed the Y2H assays between SLF and S-RNase as follows: in addition to testing full-length SLF variants, SLF whose F-box is truncated will also be tested, as a previous study (Qiao et al. 2004) indicated that, in the case of AhSLF-S_2, removal of the F-box was necessary to demonstrate protein interactions by two-hybrid assays. In general, protein interactions were expected to be seen at least with partially overlapping patterns between SLF variants and different S-RNase alleles. Some SLF variants may interact with S_1-RNase and some with S_3-RNase. Considering the collaboration between SLF variants, it was also possible to find that SLF variants interact with more than one S-RNase allele.

Besides determination of the relationship between different SLF variants and the S-alleles of S-RNases, Y2H assays could be extended in several ways: (1) SLF variants and S-RNases had been cloned into both pAD and pBD vectors. As a control, and also for the purpose of confirming observed interactions, Y2H assays were carried out reciprocally for each SLF-S-RNase combination. Assuming a significant and strong binding is observed, reciprocal assays

should show the same. (2) To gather more information for SLF-collaboration hypothesis, the possibility of protein interactions existing among different subtypes of SLF variants should also be considered, especially those verified by Y2H assays showing interactions with multiple S-RNase alleles. Because Petunia SLF proteins appeared to act collaboratively (Kubo et al. 2010), it was reasonable to predict that different SLFs could form a higher-level complex and function as one unit. So partially overlapping patterns of protein interaction among various SLF variants should not be overlooked. (3) Interactions between different SLF variants and other proteins participating in pollen recognition and rejection as parts of SCF^{SLF} model would be examined. That was to test various combinations of SLF, SBP1, SSK1 and Cullin-1 in Y2H assays in order to gain a better understanding of SCF^{SLF} model. Based on previous studies (Hua et al. 2008, Zhang et al. 2009, Sims and Robbins 2009), as well as the data from our lab of SBP1 showing interaction between full-length SBP1 and S_1-SLF1 and S-RNase, above experimental arrangements should have solid theoretical support.

Last but not least, as I acquired sequence data from the Petunia Genome Project, I was able to isolate more SLF variants from both *Petunia hybrida* and *Petunia axillaris*. To clone those SLF genes into Y2H vectors, I was able to propose a new cloning method instead of conventional enzyme digestion and ligation. That was converting Y2H vectors into Gateway® vectors by digesting pDONR-Zeo vector, which was used in BP reaction, and Y2H vectors by the same restriction-enzyme combination, EcoR I and Xho I. In subsequent ligation, the Gateway® cassette containing attP sites isolated by EcoR I and Xho I was ligated with digested Y2H vectors. With the modified Y2H vectors, I should be able to use previous PCR products of SLF variants amplified by attB-flanking primers (Table 5, 6), and carry out one-step cloning, i.e. BP reaction, to build Y2H constructs that are ready for Y2H tests.

Bimolecular Fluorescence Complementation (BiFC) Assays

SLF has been shown to interact with S-RNase in transgenic experiments employing "competitive interaction" (Sijacic et al. 2004), in co-immunoprecipitation assays (Hua and Kao, 2006) and *in vitro* yeast two-hybrid assays (Sims et al. 2010). In addition, Diwa Malla (M.S. thesis, 2012) from our lab did demonstrate *in vivo* interaction between S_1-SLF1 and SBP1 by BiFC assays. To date, I have been able to identify and isolate 17 types of SLF variants from S_{ax1} *Petunia axillaris* whose S-RNase gene sequence was shown to be completely identical to the S_1-RNase from *Petunia hybrida*. Additionally, recent data indicated that previously identified SLFL (SLF-like) genes were actually true SLF genes, and SLF proteins may recognize S-RNase in a collaborative fashion (Kubo et al. 2010). Together, investigating the pattern of protein-protein interactions *in vivo* between SLF and S-RNase, SLF and SBP1, SLF and other proteins involved in pollen recognition in GSI will be significantly informative and certainly will provide insights into the mechanisms of gametophytic self-incompatibility.

BiFC Assay – S_1-SLF vs. SBP1

S_1-SLF variants (SLF1, 3, 4, and 5) were included in the BiFC assays with SBP1. CNX6::CNX7 was previously proven as a reliable positive control (Gehl et al. 2009), and SLF1::CNX6 has not been observed to yield significant signal in previous BiFC assay (Malla, M.S. thesis, 2012), thus used as negative control. In all the BiFC assays that I have carried out so far, positive control was always the first to be scanned under confocal microscopy, in order to establish a base line of the pin-hole value and the detector-gain value, defined as "minimum positive", which is related to signal strength presented on images. The next scanned sample was

always the negative control SLF1::CNX6 which normally provided almost completely dark image when being scanned under the same condition, in terms of same pin-hole value and detector-gain value, as the positive control. According to preliminary data and my personal experience, however, I also raised the pin-hole value and the detector-gain value to the level as high as possible until negative control started to show unspecific noise in the background, defined as "maximum negative". Thus, when scanning other samples, i.e. the protein interactions to be investigated such as SLF::SBP1, confocal microscopy was set between minimum positive and maximum negative, which resulted in better presentation of fluorescent signals, as well as avoiding mistakenly recognizing background noise as the expected signal.

In the case of SLF::SBP1, images taken under the confocal microscopy (Fig. 50) showed that strong signals, i.e. at the comparable level with the signal observed in positive control, were found from SLF3/4dF/5/5dF::SBP1. Signal produced by SLF3dF::SBP1 was significantly visible but not as strong as those from other SLF::SBP1 pairs or the positive control. SLF1/1dF::SBP1, however, only provided weak signal in a limited scale where some fluorescence was likely unspecific noise in background but the expected signal on the edge of the epidermal cells. Together, results indicated strong protein interaction existed for S_1-SLF3::SBP1, S_1-SLF4dF::SBP1, S_1-SLF5::SBP1, and S_1-SLF5dF::SBP1 combinations; detectable, but not as strong, protein interaction was found for S_1-SLF3dF::SBP1. No specific protein interactions were observed in S_1-SLF1::SBP1 or S_1-SLF1dF::SBP1. In addition, a previous study (Qiao et al. 2004) indicated that, in the case of AhSLF-S_2, removal of the F-box was necessary to demonstrate protein interactions by two-hybrid assays, I did not, however, find significant difference, in terms of signal strength, from the fluorescence emitted from full-length SLF or from F-box-truncated SLF under confocal microscopy. These results indicated that the F-box

sequence did not appear to affect the ability of SLF to interact with SBP1, or the strength of SLF::SBP1 protein interactions

Since the BiFC assays of SLF::SBP1 were carried out before the acquisition of sequence data from the Petunia Genome Project, I only tested four S_1-SLF variants. To date, I have been able to identify and isolate 17 types of S_1-SLF variants, and finished constructing all of them for BiFC assays. In the future, more S_1-SLF variants need to be assayed with SBP1 before concluding the pattern of how SLF protein(s) interact(s) with SBP1 protein *in vivo*. As far as the four SLF variants tested, it has been confirmed that protein interactions between S_1-SLF variants and SBP1 can be and have been detected by BiFC assays *in vivo*.

BiFC Assay – S_1-SLF vs. S_1-RNase

S_1-SLF variants (SLF1, 3, 4, and 5) were also paired with S_1-RNase in BiFC assays to find out whether proteins interactions existed when S-RNase is recognized as self by S_1-SLF variants. Two versions of S_1-RNase constructs were employed: full-length S_1-RNase, and S_1-RNase mature. In S_1-RNase mature constructs, the signal peptide domain had been removed.

Results indicated that the protein interactions between S_1-SLF and S_1-RNase were either weak or undetectable. Weak fluorescence was observed from S_1-SLF3/3dF/4dF/5/5dF::S_1-RNase, and from S_1-SLF3/3dF/4dF/5::S_1-RNase mature. According to the mechanism of gametophytic self-incompatibility and the putative SCF^{SLF} E3 ubiquitin ligase model, S-RNase recognized as self will not be targeted for ubiquitination leading to the activity of degrading pollen-tube RNA. Results from BiFC assays of S_1-SLF versus S_1-RNase were consistent with GSI mechanism and findings in previous studies.

BiFC Assay – S_1-SLF versus S_3-RNase

Besides the self-combination of SLF::S-RNase, BiFC assays were also carried out between non-self SLF variants and S-RNase, in this case S_1-SLF versus S_3-RNase. Three versions of S_3-RNase constructs were employed: full-length S_3-RNase, S_3-RNase mature, and N-terminal S_3-RNase. The S_3-RNase N-terminal construct was prepared by truncating the C-terminal part from the full-length S_3-RNase but still including two hypervariable regions (HVa and HVb), for the purpose of asking whether the proposed S_1-SLF::S_3-RNase binding was domain specific. In addition, in middle of preparing to test interactions between non-self SLF::S-RNase combinations, I obtained sequence data from the Petunia Genome Project and therefore was able to isolate more SLF variants (PCR primers are listed in Table 6) from *Petunia axillaris* for BiFC tests.

At first, the results obtained from BiFC assays of S_1-SLF versus S_3-RNase were surprising. That was, most of the combinations were found to be as dark as negative control without giving any signal under confocal microscopy, such as SLF13/14/16:: S_3-RNase mature (Fig. 45), even if some pairs did emit detectable fluorescence, for example SLF1/3/4/5:: S_3-RNase N-terminal (Fig. 39), or SLF1:: S_3-RNase mature (Fig. 40), or SLF10/10dF:: S_3-RNase mature (Fig. 47).The signals, however, were only at the comparable level with those from self SLF::S-RNase pairs, not indicating strong binding as SLF::SBP1 pairs did.

It was unexpected because results were contrary to either the generally accepted mechanism of GSI, or to the result from previous “pull-down” assays (Hua and Kao 2006) suggesting strong interactions between non-self SLF::S-RNase combination, which resulted in a further hypothesis: only SLF and S-RNase were not powerful enough to hold the protein

interactions in between, and there might be a third, fourth, or even fifth protein acting as bridge(s) to stabilize non-self SLF::S-RNase protein interactions. Such bridge protein(s) would have been present, but not specifically detected in previous transgenic or pull-down assays. In addition, this hypothesis was also inspired by the SCF^{SLF} E3 ubiquitin ligase complex which implied a network of numerous proteins collaborating to recognize S-RNase. Similar to the patterns found by Kubo et al. (2010), not only SLF variants, but other components of SCF^{SLF} E3 ubiquitin ligase complex were also required for a stable non-self SLF::S-RNase binding.

Another interesting observation was that among those SLF variants cloned from S_{ax1} *Petunia axillaris*, only SLF10/10dF was able to bind N-terminal S_3-RNase and give a relatively strong signal. SLF10 gene was known as the variant that is located nearest to the S_{ax1}-RNase gene in the genome (Fig. 74). Both genes were in the same scaffold, as well as in the same orientation. In the future researches, it might be worth testing the hypothesis that the local distance in genome between SLF gene and S-RNase gene affects the affinity to each other resulting in different protein interactions *in vivo*.

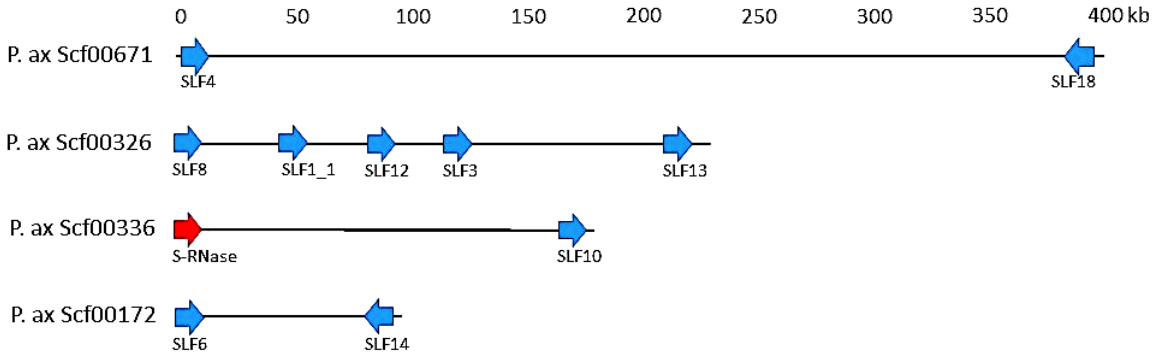

Figure 74. S-locus linkage groups in S_{ax1} *Petunia axillaris.* SLF10 and S-RNase genes are in the same scaffold and the same orientation.

BiFC Assay – S_1-SLF vs. S_3-RNase + Hypothesized "Bridge" Protein(s)

Based on the results of BiFC tests between non-self SLF and S-RNase, putative "bridge" proteins to test were selected from those though to form the SCF^{SLF} E3 ubiquitin ligase complex, i.e. SBP1 (or RBX1 in the typical SCF-type E3 ubiquitin ligase complex), SSK1 (or SKP1 in the typical SCF-type E3 ubiquitin ligase complex), and Cullin-1.

Since it was not known if either one or more than one bridge proteins were actually required, I designed three-way, four-way and five-way BiFC tests by co-infiltrating one, two, or three bridge proteins. Before co-infiltration, the cell resuspension of bridge-protein BiFC construct(s) was/were mixed equally with S_1-SLF and S_3-RNase, and all the subsequent procedures were the same as regular two-way Agroinfiltration.

Before I started testing with bridge proteins, the Zeiss confocal microscopy broke down, so that I had to switch to Nikon E600 microscope. To eliminate the deviation caused by different equipment, I carried out the BiFC tests between SLF1/3/4/5 and S_3-RNase mature again. Results were consistent with those obtained from confocal microscopy. That was only weak signal observed from SLF1:: S_3-RNase mature, while others were as dark as negative control.

Signal improvement was indeed observed from several addition of bridge protein(s): (1) signals from SLF1/3/5::S_3-RNase mature (Fig. 56) were significantly amplified by adding bridge SBP1; (2) signals from SLF1dF/3dF/4dF/5dF::S_3-RNase mature (Fig. 56) were slightly amplified by adding bridge SBP1; (3) signal from reverse SLF2::S_3-RNase mature (Fig. 61) was amplified by adding bridge SSK1; (4) signal from SLF3dF:: S_3-RNase mature (Fig. 62) was slightly improved by adding bridges SBP1+SSK1; (5) signals from SLF3/3dF::S_3-RNase mature (Fig. 64) were significantly amplified by adding bridges SBP1+SSK1+Cullin-1, and such

amplification was comparable to that of adding bridge SBP1 alone; (6) signal from SLF3dF::S_3-RNase mature (Fig. 66) was significantly strengthened by adding bridges RBX1+Cullin-1. Together, co-infiltrating bridge-protein construct(s) did improve the fluorescent signal from certain non-self SLF::S-RNase pairs. Considering the protein interactions between SBP1/SSK1 and S_1-SLF variants observed in previous BiFC assays (Figs. 50, 52), bridges SBP1 and SSK1 appeared to be playing more critical roles in signal enhancement than others like bridge Cullin-1 or RBX1. In the future researches, more BiFC tests of S_1-SLF variants cloned from *Petunia axillaris* need to be carried out to demonstrate the potential roles of bridge proteins.

Summary

I was able to employ BiFC assays to prove the existence of strong protein interactions between S_1-SLF variants and SBP1. Although I could not observe the expected strong signal from non-self S_1-SLF::S_3-RNase combination, I was able to provide evidence that bridge proteins, especially SBP1 and SSK1, could stabilize the protein interactions within non-self SLF::S-RNase to different extent. Further experiments need to be carried out to involve more S_1-SLF variants in the future.

Western Blot and Agroinfiltration with MG132

The purpose of the Western Blot assay was to determine if either S-RNases have been poorly expressed *in vivo* or the non-self SLF::S-RNase interactions have led to ubiquitination and degradation of the S-RNase, leading to no visible signal in BiFC assays. Nevertheless, results from the blotted membranes indicated that only the positive control CNX6::CNX7

expressed normally in leaves. Other controls, for example SLF3::SBP1, which had been found to yield strong BiFC signals were not expressed at the level of protein, as assayed on these blots. In addition, there was no sign of expression of S-RNase, and interestingly no expression of SLF was observed either.

Based on preliminary data, I propose two potential possibilities. First, S-RNase was truly degraded so that no protein interactions were seen in BiFC or no expression was detected in Western Blot. Second, in previous BiFC tests, SLF3::SBP1 showed strong signal that was also comparable to positive control, but was not as massively distributed as positive control. Fluorescence from SLF3::SBP1 could only be found in a certain area of epidermal cells, which indicated much less protein expressed than positive control CNX6::CNX7. Same issues, or even worse situations, were found when assaying with SLF::S-RNase. Poorly expressed proteins may not be concentrated enough to be detected via Western Blot, even if they did express or interact as expected. To testify the second possibility, I can arrange Western Blot in future as following: (1) Carrying out Agroinfiltration as regular protocol; (2) Harvesting leaves and preparing microscopy slides by sectioning leaves into small strips; (3) Observing under confocal microscopy looking for fluorescence; and (4) Collecting all the strips emitting fluorescence and carrying out Western Blot. Based on this method, the total protein extracted from leaves will be significantly reduced, and target proteins will be more concentrated so that they will be more likely detected in Western Blots. If it is not the concentration issue that is preventing protein interactions from being observed, S-RNase is much more likely degrade *in vivo* while being recognized as non-self.

As an alternative method, I can combine Western Blot with Agroinfiltration involving the 26S proteosome inhibitor MG132. If non-self S-RNase is actually degraded, the addition of

MG132 should be able to inactivate the proteasome and terminate such degradation, leading to signal enhancement in non-self SLF::S-RNase BiFC assays, as well as detectable expression of S-RNase in Western Blot. In addition, newly published research (Sun et al. May 2015) indicated even SLF itself was also subject to degradation via the ubiquitin–26S-proteasome pathway, which indicates that tests including the action MG132 should have a high priority.

Bioinformatic Analysis of SLF Sequences

Based on the results provided by MAFFT multiple sequence alignments performed so far, it is known that same-subtype of Petunia SLF variants, no matter what species they are from or what type of S-locus genes they contain in their genomes, are greatly more conserved than those of different subtypes but carrying the same S haplotype. Considering the hypothetical collaborative recognition of non-self S-RNase (Kubo et al. 2010), one way to expand the Bioinformatic works is to review previous reports to identify and align either the haplotypes of SLF that do interact with the same S-RNase or the subtypes of SLF from the same haplotype that do not interact with the same S-RNase. The alignment of SLF interacting with the same S-RNase may provide information, such as recognition domain, in the conserved areas. The alignment of SLF from the same haplotype but not interacting with the same S-RNase will be informative by investigating the conserved regions that may prevent those SLF variants from recognizing the same non-self S-RNase. Another analysis that can be completed in the future is to investigate the existence of non-synonymous substitutions within the alignments of the same-subtype SLF variants that are mostly conserved but with spots of amino-acid variations. Such analysis must be

coordinating with the SLF-collaboration analysis, and may provide further evidence of specific domain(s) required during recognition of non-self S-RNase.

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