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Genetic Analysis of Self-Incompatibility in *Petunia* and *Schlumbergera*

By

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ABSTRACT

Self-incompatibility (SI) is a widespread genetic mechanism which prevent self-fertilization in angiosperms. The genus *Petunia* of the *Solanaceae* serve as a model for SI research due to its short life cycle and accessible floral morphology. In *Petunia*, SI is mediated by *S-RNase* dependent degradation of self-pollen RNA within the style, arresting pollen tube growth. Breakdown of SI confers self-compatibility (SC), with implications for breeding and yield optimization.

This study investigates the genetic basis of SI breakdown in hybrids of *P. inflata* × *P. hybrida*. For the first time, it provides a concise analysis of the novel *PiSd*-allele and its association with SC across five generations. Genotypic and phenotypic analyses, including genomic PCR, identification of *S*-alleles by sequencing, and controlled self-pollinations were performed on lines carrying *PhS3* and *PiSd* alleles.

The novel *PiSd* allele correlates with SI breakdown, suggesting functional alteration in associated *SLF* genes, which remain uncharacterized. The segregation ratios confirm the linkage between the *PiSd* and SI breakdown. Future molecular dissection of *PiSd*-linked *SLF* genes will firstly provide an understanding of the mechanism of SI breakdown and ultimately is warranted to inform targeted breeding strategies in *Petunia* and beyond.

The second stage of this research is focused on SI mechanism in *Schlumbergera truncata*. *S. truncata* is among the most widely cultivated cactus species notable for its diverse array of colourful flowers and various flower forms. SI in *S. truncata* is under GSI control. This study tests whether the GSI mechanism in *S. truncata* is of the *S-RNase* system and explores whether it is homologous to that in the rosids and the asterids, as it likely seems by the phylogenetic relationships of angiosperms. This was achieved by analysing the genetic and phenotypic characteristics of different lines, determining the presence and functionality of *S*-alleles within the study lines.

This study successfully identified three *S*-alleles in the *S. truncata* F1 progeny lines. All three are present in the five alleles identified previously. These *S* alleles identification combined with the data of the classic diallele self and cross-pollination and the formed incompatibility/compatibility groups strongly suggest the likelihood of a *S-RNase* mechanism under the control of a single locus occurring in the *S. truncata* population.

These findings in *S. truncata* combined contribute to a deeper understanding that the *S-RNase* mechanism is more widely functional and polymorphic among angiosperms, which adds to the insights of their evolutionary relationships. A question for future study is whether this mechanism is functional through self-recognition or non-self-recognition pathways.

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Petunia and False Christmas Cactus. I really feel thankful more to these plants as great creatures that we based our all-long learning years on. They are better than many humans these days.

Contents

LIST OF ABBREVIATIONS

SI Self-Incompatibility

PSC Pseudo Self-Compatibility

GSI Gametophytic Self-Incompatibility

SSI Sporophytic Self-Incompatibility

SC Self-Compatibility

C1-C5 Conserved domains (1 to 5) of solanaceous *S-RNases*

HVa and **HVb** Hypervariable domains of solanaceous *S-RNases*

PCR Polymerase Chain Reaction

QRT-PCR Quantitative Real Time-Polymerase Chain Reaction

RT-PCR Reverse Transcription-Polymerase Chain Reaction

SBP *S-RNase* binding protein

SFB S-haplotype-specific F-Box

SLF S-locus F-box

SCF Skp1-Cullin1-f-box complex

SCR S-locus cysteine-rich

SP11 S-locus Protein 11

S-RNase S-locus Ribonuclease

SRK S-locus Receptor Kinase

SLGs S-locus glycoproteins

PCPs Pollen coat proteins

PrpS Papaver rhoeas pollen S determinants

PrsS Papaver rhoeas stigma S determinant

CHAPTER 1; GENERAL INTRODUCTION

1.1 An overview of self-incompatibility (SI)

Self-incompatibility (SI) in flowering plants is identified as a genetic mechanism by which self-fertilization is prevented, thereby a high degree of heterozygosity is maintained (de Nettancourt, 1997; Silva and Goring, 2001). This phenomenon allows the plant to be able to recognize its own pollen and reject it by arresting pollen tube growth either at the stigma or through the style and consequently preventing inbreeding (Hiscock and Tabah, 2003; Sims and Robbins, 2009). Inbreeding can reduce the fitness of progeny, SI is one of reproductive strategies which are adopted by such hermaphroditic plants to promote outcrosses (de Nettancourt, 2001). SI has a significant role in shaping both the spatial and temporal distribution of genetic diversity among flowering plants as it maintains the genetic polymorphism within a population (Nettancourt, 1997; Li *et al*., 2017). Non-self-pollination can also promote the selection of favorable phenotypes within the species (Silva and Goring, 2001). SI is one of the most sophisticated and widespread mechanism of controlling outbreeding in flowering plants. It has been known in flowering plants for over a century since Darwin's description (Darwin, 1876).

"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, 1876). SI is widespread genetic mechanism that is utilized by many angiosperms (East, 1940; Brewbaker, 1957; de Nettancourt, 1977; Charlesworth *et al*., 2005; Richards, 1997; Hiscock and Kues, 1999; Franklin-Tong, 2008). SI exists in many families including *Leguminosae, Solanaceae, Brassicaceae, Compositae and Gramineae*. In addition, incompatibility is found in cultivated crops such as red, white and alsike clovers, alfalfa, ryegrass, rye, Sugar beets, sunflower, tobacco, potatoes, bermudagrass and others (Haring *et al*., 1990; de Nettancourt, 2001; Allen and Hiscock, 2008).

SI provides the pistil of a flower with the ability to distinguish between self and non-self-pollen and results either in the inhibition of germination of self-pollen on the stigmatic surface or in the inhibition of growth of self-pollen tubes in the style. It is a process of rejection or acceptance occurs between the pistil and the pollen. It is crucial mechanism in order to maintain the SI system over an extendable period of time (Williams *et al*., 2015). In early studies, there were challenges to identify the biochemical components of SI mechanisms in most families (Clarke and Newbigin, 1993). However, within the last four decades, researchers have achieved to complement Darwin's genetic observations with the use of advanced molecular and biochemical analyses, which have positively

contributed to the elucidation of the complex series of interactions occurring at the pollen-stigma level (Silva and Goring, 2001; Franklin-Tong and Franklin, 2003; Takayama and Isogai, 2005). The focus of such analyses of SI systems has been on identifying and characterizing the male and female components of the self-incompatible response as well as other proteins and events that result in pollen rejection.

The occurrence of SI breakdown can be due to non-functional/paternal aspects. This will lead to self-compatible (SC) plants where a full seed set is produced when self-pollinated (Sims and Robbins, 2009). There is also partial breakdown of SI. This is referred to as pseudo-self-compatibility (PSC). It is characterized from a variable level of seed set. It is a result of quantitative variation in SI reaction strength (Robbins *et al*., 2000). PSC was first described by Ascher (1984). It was defined as the capability of an otherwise SI individual to set seed when self-fertilized or crossed with an individual that have the same S-allele (Dana and Ascher, 1985).

There are two forms of SI; Gametophytic Self-Incompatibility (GSI) where incompatibility is controlled by the pollen haploid genotype and Sporophytic Self-Incompatibility (SSI) where incompatibility phenotype of the pollen is controlled by the diploid parental genome (Hiscock and Tabah, 2003). SSI is the less common system compared to GSI.

1.2 CLASSIFICATION OF SELF-INCOMPATIBILITY

1.2.1 Heteromorphic self-incompatibility

Floral morphological differences are the key aspects of self-incompatibility mechanisms. Self-incompatibility here is classified due to the length differences of both the style and the stamen. There are two types of SI on the Heteromorphic system. Distylous SI where the flower has two forms (short style with long anthers, and long style with short anthers). Lewis (1947) has shown that on these two types, the positions of the style and the anther are genetically controlled by the allelic dominance at the single *S*-locus. Tristylous SI there are three flower forms (1-short style with high and middle stamen. 2- middle style with high and low stamen. 3- long style with middle and low stamen) (de Nettancourt, 2001). In contrast with Distylous SI, on the tristylous SI positioning of the style and the stamen are genetically controlled by the relationship dominancy between alleles at two loci, S and M (Mather, 1943). Barrett (1998) and McCubbin (2008) described the biological and genetical aspects of this system.

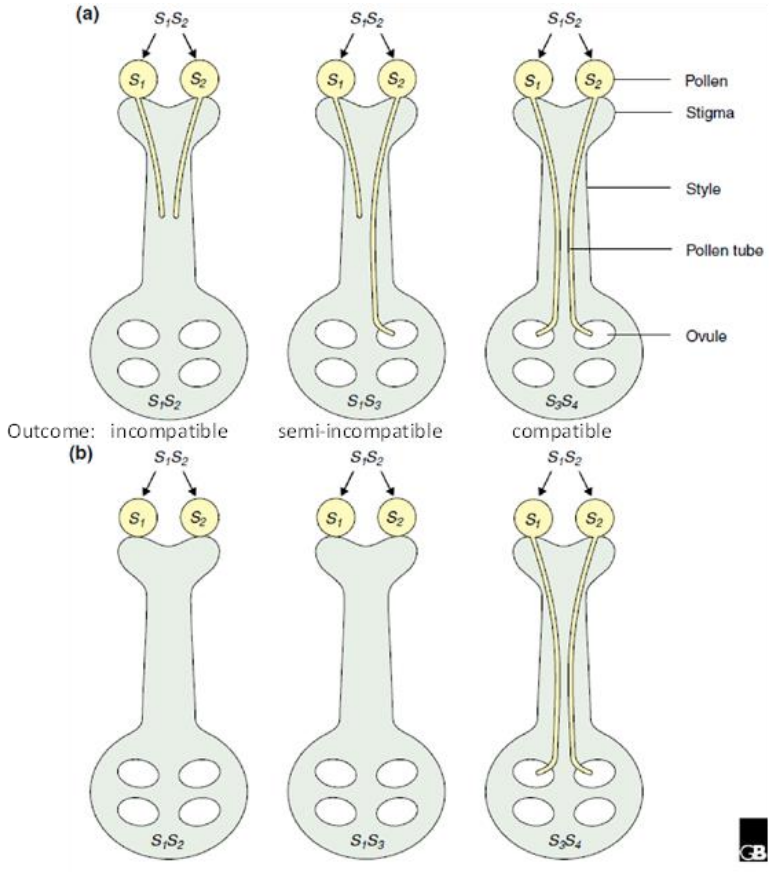
1.2.2 Homomorphic self- incompatibility

On this system, plants have no morphological flower differences (Bernatzky *et al.*,1988; Kao and McCubbin,1996). The Classification of SI on this system is based on how the pollen phenotype is genetically controlled. Thus, it is classified as either sporophytic SI (SSI) or gametophytic SI (GSI). In the early 1950s,

classical genetic studies revealed these two types of Self-incompatibility (Silva and Goring, 2001; de Nettancourt, 2001; McCubbin and Kao, 2000).

In the homomorphic SI system, SI is controlled by single or multi-S-loci (Hinata *et al*., 1993). The S-allele usually contains two genes. The first is the pistil S-gene which determines the pistil-specificity. The second is the pollen S-gene which determines the pollen-specificity (Robbins *et al*., 2000). This association of the pistil S genes and the pollen S genes constitute different S-haplotypes (Kubo *et al*., 2010). SSI is commonly present in *Brassicaceae* family such as *Brassica napus L.* (Stein *et al*., 1991) and *Brassica oleracea* (Trick and Flavell, 1989). GSI is widely spread in many other families such as the *Solanaceae* (Ioerger *et al*., 1990), *Rosaceae* (Sassa *et al*., 2010) and *Papaveraceae* (Steinbachs and Holsinger, 2002; Bosch and Franklin-Tong, 2008).

In the sporophytic self-incompatibility, the pollen SI phenotype is determined by the parents' diploid genome, whereas in the gametophytic SI the pollen phenotype is determined by its own haploid genome. Figure 1 demonstrates the differences between the SSI and GSI mechanisms.



Outcome: Incompatible Incompatible Compatible

Figure 1. Genetic controls of SSI and GSI systems (adapted from Hiscock, 2002). (A) The gametophytic SI. If the pollen *S1* and *S2* lands on a stigma of *S1S2*, pollen tubes growth will be inhibited. However, when *S1* and *S2* pollen lands on stigma of *S1S3*, the *S2* pollen tube only will develop normally while the *S1* pollen will not reach the ovary. In addition, if the *S1* and *S2* pollen reach the stigma of the *S3S4*, both pollen tubes will grow. (B): In the sporophytic SI system, if the pollen of the *S1S2* lands on a stigma that carries the haplotypes (*S1S2* or *S1S3*), pollen tube will be arrested. A successful pollination and fertilization will only occur when pollen from *S1S2* lands on pistil genotype of *S3S4*.

1.3 Sporophytic self-incompatibility (SSI)

First studies to reveal the genetics of the SSI were done in two species from the same family (*Asteraceae*). *Crepis foetida* (Hughes and Babcock, 1950) and *Parthenium argentium* (Gerstel, 1950). Following these, the SSI system was also described in the *Brassicaceae* family species (Bateman, 1955). The SSI system has been also found in other families including *Asteraceae, Auriculata, Betulaceae* and *Araucariaceae* (Charlesworth, 1988). SSI is well studied. This system has only been characterized at the molecular level in detail in *Brassicaceae* family (Stein *et al*., 1991; Ma *et al*., 2016: Ockendon, 1974; Dwyer *et al*., 1991; Stein *et al*., 1991; Suzuki *et al*., 1999; Schopfer *et al*., 1999; Nasrallah *et al*., 1991; Nasrallah *et al*., 1985; Hiscock and McInnis, 2003; Kachroo *et al*., 2002; Takayama and Isogai 2003; Watanabe *et al*., 2003).

The molecular mechanisms of the SSI system differ between families such as that of the *Convolvulaceae* and *Asteraceae*. Cellular and molecular studies in *Senecio squalidus* (Oxford ragwort) have highlighted the significance. The differentiation might be as a consequence of the *Asteraceae* which have a 'semi-dry' stigma, rather than the 'dry' stigma which is the typical of the *Brassicaceae* (Allen *et al.*, 2011). In the sporophytic self-incompatibility, the pollen SI phenotype is determined by the parents' diploid genome (Figure 1B). As a result, the pollen grain carries the two different alleles product instead of one.

Moreover, on the SSI, dominance relationships between allelic pairs dose exist. Therefore, complicated patterns of compatibility and incompatibility give either male or female the opportunity for homozygotes which holding the recessive S allele (Hiscock and Tabah, 2003). In this system, the stigma surface, as a recognition site, recognizes the outer coat proteins of the pollen grain.

1.3.1 SSI in *Brassicaceae* family

Bateman (1955) has interpreted the genetics of SI in the *Brassicaceae* family as a single Mendelian locus, the S (Sterility) locus. This was identified as multiple alleles or variants. These each encodes a distinct mating specificity. The S-locus alleles is usually of a large number. For instance, in *Brassica rapa* (syn. campestris) 30 S-haplotypes and in *B. oleracea* 50 S-haplotypes were identified (Nou *et al.*, 1993; Brace *et al*., 1994, and Ockendon, 2000). It was not till 2014 when the S-locus responsible for SI was investigated in distinct species. Leducq *et al* (2014) studies in *Brassica* and *Arabidopsis-Capsella*, have revealed SI in a third deep-branching lineage of *Brassicaceae* known as the *Tribe Biscutelleae*.

In self-incompatible plants of this family, the pollen proper development is prevented by the stigma which expresses the same S-haplotype. The Self-pollen rejection is triggered by invalidated pollen hydration, or an immediate arrest at the stigma surface (Hiscock and Tabah, 2003). S-locus region molecular analysis

studies (Boyes *et al.,* 1991; Casselman *et al*., 2000) have revealed the complexity of this locus. It is found spanning many kilobases containing several physically linked transcriptional units which co-segregate perfectly with SI phenotype.

Brassica S alleles have been classified into two classes through classical genetic analysis. This is based on their phenotypic effect on self-incompatibility. The high-activity alleles feature a strong self- incompatible phenotype. These are placed relatively high on the dominance scale. They have an average of 0 to 10 pollen tubes developing per self-pollinated stigma. The second class (low-activity) has a weak self-incompatible phenotypic effect. This is where 10 to 30 pollen tubes develop per self-pollinated stigma. These are postulated to be recessive (Nasrallah *et al.,* 1991). The group of genes within the S-locus complex (S-haplotype) are highly polymorphic. Furthermore, different SI specificities are defined by specific combinations of allelic forms of each of these genes. Consequently, the S-locus may be demonstrated as a major recognition component which encodes the stigma desirable function(s) to distinguish between self and non-self-related pollen (Jung *et al*., 2013).

1.3.1.1 The female determinant in *Brassica*

Studies of the female determinant were first initiated by an S-haplotype's immunological identification of the stigmatic specific antigen. This was then

followed by the biochemical exploration of stigma glycoproteins (S-locus glycoproteins, SLGs) which co-segregate with S-haplotypes. There are two closely linked genes which were identified. These are at the same S-locus (SLG for S-locus glycoprotein and SRK for S-locus receptor kinase). Studies consider these to be cooperatives in the perception of self-pollen by the stigma (Nasrallah *et al*., 1985; Stein *et al*., 1991). They are both highly polymorphic. Their expression is found to be specifically at the surface of mature stigmas. SRK mutations (Goring *et al*., 1993; Nasrallah *et al*., 1994) and expression loss of SLG (Toriyama *et al*., 1991; Nasrallah *et al*., 1992; Shiba *et al*., 1995) were found to be related to the loss of SI.

1.3.1.2 The male determinant in *Brassica*

Identification of the male S determinant was led by analytical studies of the pollen coat proteins (PCPs) in *Brassica* species. These are derived from the anther tapetum. They are found to be crucial for pollen development (Stephenson *et al*., 1997; Heslop-Harrison, 1975). Doughty *et al* (1993), initially sought SLG-interactive PCPs. They were later characterized as cysteine-rich small proteins (Hiscock *et al*., 1995; Stanchev *et al*., 1996; Toriyama *et al*., 1998; Doughty *et al*., 1998; Takayama *et al*., 2000).

Identification of the male determinant genes, named SP11 (S-locus protein 11) and SCR (S-locus cysteine-rich), was achieved by both the cloning and

sequencing of the S-locus region and by polymorphic gene experiments. Furthermore, it was confirmed that this gene (SP11/SCR) encodes the *Brassica* pollen S determinant using functional analysis in transgenic plants (Schopfer *et al*., 1999; Suzuki *et al*.,1999; Takayama *et al*., 2000). Jung *et al* (2012) illustrated that silencing of the SP11 gene resulted in self-compatible phenotype in a transgenic line of *Brassica rapa*. Moreover, it was confirmed that this trait was stable in subsequent generations. This was even after crossing with other commercial lines. These results confirm that, the consequent self-compatibility is possible to be transferred to commercial cultivars in *Brassicaceae* seed crops (Tantikanjana and Nasrallah, 2015). In crop plants which are of high economical value, SI system can be exploited to increase efficiency in the production of F1 hybrids (Eaves *et al*., 2014).

1.4 Gametophytic self-incompatibility (GSI)

In contrast to the SSI system, the gametophytic system (GSI) the phenotype of the pollen incompatibility is determined by its own haploid genotype. The growth of the self-pollen is rejected in the style not on the stigma. The GSI system is the most widespread and exists in more than 60 flowering plant families (Weller *et al*., 1995). GSI system has been well studied in the *Solanaceae* family (particularly in *Petunia*), *Plantaginaceae*, and *Rosaceae* (Sims and Robbins, 2009). GSI system has so far two forms that have been illustrated. These were studied in detail at the molecular level. In order to achieve self-pollen recognition, two different stigmatic S-genes were revealed. The first form was the stylar ribonuclease system (*S-RNase* system). This was initially investigated and characterized broadly in member of *Solanaceae*. Then it was also found in *Rosaceae, Plantagenaceae* (Franklin-Tong and Franklin, 2003a). Secondly, the GSI system was found in the *Papaveraceae, Papaver rhoeas* (Hiscock and McInnis 2003; Franklin-Tong and Franklin, 2003b). However, it was found totally different at the molecular level. Despite the first system, pollen rejection found to be at the stigma level not during its growth in the style as observed with *Solanaceae* (Franklin-Tong *et al.,* 1992).

1.4.1 Self-incompatibility in *Papaveraceae*

At the molecular and biochemical level GSI in *Papaveraceae* and *Solanaceae* family is totally different. in poppy, the self-incompatible pollen is rejected on the surface of stigma and not in the style as observed with the *Solanaceae* (Franklin-Tong *et al*., 1992), although in both families' pollen phenotype is determined by the pollen genotype. This SI system has been extensively studied in *Papaver rhoeas* (wild poppy). it is found that it is controlled by a single multi-allelic S-locus (Lawrence *et al*., 1978). Later studies identified the two tightly linked multi-allelic S-genes encoding the stigmatic and the pollen S proteins (Foote *et al*., 1994; Jordan *et al*., 1999; Wheeler *et al*., 2009).

Franklin-Tong *et al* (1991) doubted the idea that the stigmatic S proteins of *Papaver rhoeas* to be *S-RNase*. After extensive studies of GSI in *Papaver rhoeas*, several stigmatic S-proteins were isolated (Foote *et al*., 1994; Kurup *et al*., 1998). These stigmatic S genes were expressed in vitro and were able to inhibit pollen tube growth in an S-allele-specific manner (Foote *et al*., 1994; Walker *et al*., 1996). Thus, the multi-allelic stigmatic S gene suggested to be the pistil component of the *Papaver* SI system (Foote *et al*., 1994).

1.4.1.1 The female determinant in *Papaveraceae*

The stigmatic S proteins of *Papaver rhoeas* were initially predicted to be RNases. However, after several experiments which did not give any evidence, this idea was in doubt (Franklin-Tong *et al.*, 1991).

It was evident that when the first stigmatic *S1* gene was cloned and sequenced, it encodes something other than an RNase. Several other stigmatic S proteins corresponding to different S allele (*S3, S7, S8*) and (*Sn1*) from *Papaver rhoeas* and its close relative, *Papaver nudicaule* respectively have isolated (Foote *et al*., 1994 and Kurup *et al*., 1998). They were expressed in Escherichia coli and recombinant proteins were illustrated in vitro which show their ability to inhibit pollen tube growth in an S-allele-specific manner (Foote *et al*., 1994; Walker *et al*.,1996). It was concluded that the multi-allelic stigmatic S gene represents the pistil component of the *Papaver* SI system (Foote *et al*., 1994).

1.4.1.2 The male determinant in *Papaveraceae*

Hearn *et al* (1996) using biochemical studies has suggested that SBP (S-Protein binding protein) acts as a pollen receptor candidate which interacts with the specific stigmatic S-proteins. In vitro studies show that SBP binds specifically to stigmatic S-proteins although apparently in a non-S-haplotype-specific manner. This indicates that SBP may be an accessory receptor rather than the pollen S-

receptor. In contrast, site-directed mutagenesis have illustrated that, all S-protein mutants that have a decreased ability to inhibit incompatible pollen are also reduced in SBP binding activity. This has raised evidence that SBP could be the pollen S-receptor (Jordan *et al*., 1999). There was also a highly polymorphic pollen-expressed gene which was identified by Wheeler *et al* (2009). This is named PrpS (*Papaver rhoeas* pollen S determinants) and it encodes a novel protein which considered as the male S-determinant. This was a suggestion to rename the pistil S-gene from S to PrsS (*Papaver rhoeas* stigma S determinant). Although PrpS and PrsS proteins both exhibit similar levels of polymorphism, PrpS proteins have two conserved domains. It was found that one of these domains is overlapping with part of a predicted extracellular domain while the other is a part of hydrophobic region which surrounding the central part of the protein (Wheeler *et al*., 2009 and 2010).

In the genus *Petunia*, there is collaborative non-self-recognition model. There are at least three different *SLF* proteins that function as the pollen determinant. Each product in the haplotype interacts with a subset of non-self *S-RNases*. This *Petunia* model has been supported by the isolation of the *SCFSLF* complex (Kubo *et al*., 2010).

1.5 The stylar ribonuclease (*S-RNase*) *Solanaceae* type system

Anderson *et al* (1986) cloned the first cDNAs of the styler protein from self-incompatible *Nicotiana alata*. It was found as glycoproteins of ~30kDa protein which is located in the style that co-segregates with the S-haplotype in genetic crosses. It is shown that this protein contains a regional homology with the T2 fungal ribonuclease and therefore was renamed as '*S-RNase'*. These were later confirmed as extracellular proteins and were revealed to have ribonuclease activity (Bredemeijer and Blaas, 1981; Anderson *et al*., 1986).

Franklin-Tong and Franklin (2003) discussed that *Petunia* species such as *P. inflata*, share a female S-determinant with the S-determinant in the *Rosaceae* and *Plantaginaceae* families. In *Petunia*, the first cDNA sequences to be reported for S-proteins were isolated by Clark *et al* (1990) and Ai *et al* (1990) for *P. hybrida* and *P. inflata* respectively. Analysis of *S-RNases* by Ioerger *et al* (1991) in the *Solanaceae* family identified five conserved domains (C1, C2, C3, C4 and C5) plus two hypervariable regions (HVa and HVb) which are located between the C2 and C3 domains (Xue *et al*., 1996). In tobacco, the two HV regions are close to each other and are found exposed to the molecular surface (Ida *et al*., 2001).

These are suggested to form the domain that interacts with the male S-determinant (Matton *et al*., 1997). Mutagenesis study by Matton *et al* (1999) show that, the hypervariable regions play a key role in determining S-haplotype specificity. This was shown when the amino acid of HVa and HVb of *S11-RNase* altered to *S13-RNase*, the *S13* pollen was rejected in this transgenic line but not in *S11* pollen.

The *S-RNases* are found to be expressed with the highest concentration exclusively in the pistil (Clark *et al*., 1990). It was found that high level of *S-RNase* expression is required for S-haplotype-specific pollen rejection. Kachroo *et al* (2001) shows that 10-50 mg/ml is necessary.

Other stylar factors are shown to be essential for the *S-RNases* to function (Cruz-Garcia *et al*., 2003). The known HT-B is one of these. It is a small asparagine-rich protein which was initially identified in self-incompatible *Nicotiana alata* (McClure *et al*., 1999). In their studies in tomato, Kondo *et al* (2002a and b) also showed a correlation of HT-B with SI in tomato. In *P. inflata* three alleles in the S-locus encoding the S-proteins which confer self-incompatibility were identified by Ai *et al* (1990). These are found to be specific to the pistil and the amino-terminal sequence is found to show homology to the S-proteins found in *P. hybrida*, *Nicotiana alata* and *Lycopersicon peruvianum*. In *P. hybrida*, the first cDNA sequences for three *S-RNases* were reported by Sims and co-workers. These *P. hybrida* were originally derived from stocks which were

described by Ascher (Clark *et al*., 1990). It was sixteen years later when the first S-pollen gene was cloned (Lai *et al*., 2002).

Two genes comprise the S-locus. They encode a single female and multiple male S-determinants (*S-RNase* and *SLFs/SFB*, respectively). Endocytosis and membrane are supposed to take *S-RNase* into the pollen tube (Moscatelli *et al*., 2007; Chen *et al*., 2010). *S-RNase* is incorporated in a pollinated style into the pollen tubes and functions as a cytotoxin which degrades pollen RNA as a result inhibits its growth. In cross-pollination however, some members of *SLFs* interact with non-self *S-RNase*, which derived from a different S-haplotype, so *S-RNase* detoxified thus allowing pollen tube growth (Figure 2, Takayama and Isogai, 2005). It is believed that the *S-RNases* selection is controlled by the male determinant, which has been identified as S-locus F-box proteins (Sijacic *et al*., 2004), *SLF* proteins. These have been seen to be part of a skp1-Cullin1-f-box (SCF) complex (Williams *et al*., 2015). As a result, an E3 ubiquitin ligase complex is suggested to control the selective ubiquitin mediated degradation of non-self *S-RNase*. This is based on the interaction of self *S-RNase* and the *SCFSLF* complex which is thought to be weaker than that of non-self *S-RNases*, thus allowing it to escape degradation.

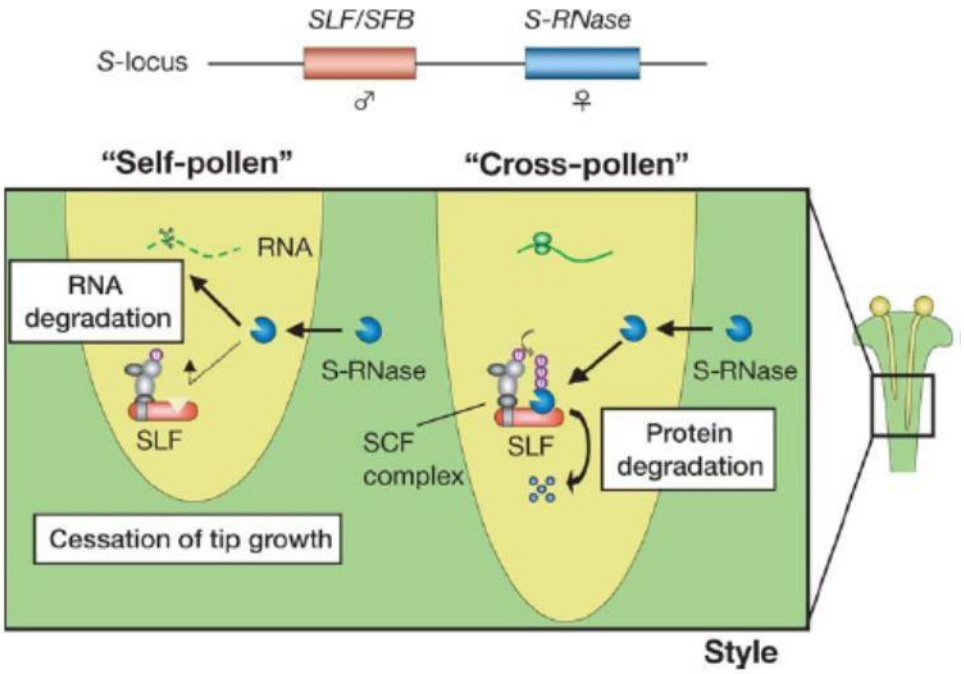


Figure 2. Molecular model of the GSI response in the families of S*olanaceae, Rosaceae* and *Plantaginaceae* (Takayama and Isogai, 2005). Two genes, *S-RNase* and *SLF/SFB* comprises the S-locus. They represent the female and male determinants respectively. *S-RNase* is secreted in large amounts into the extracellular matrix of the style. In a pollinated style, *S-RNase* is incorporated into the pollen tubes and functions as a cytotoxin to degrade pollen RNA. RNA degradation occurs only in self-pollen tubes and *S-RNase* are known to enter the pollen tubes regardless of their S-haplotypes. *SLF/SFB* is a member of the F-box family of proteins, which generally function as a component of an E3–ubiquitin ligase complex. Thus, *SLF/SFB* is expected to be involved in ubiquitin-mediated protein degradation of non-self-*S-RNases*.

1.5.1 *S-RNase*; the style-recognition component

The female determinant was initially revealed in self-incompatible *Nicotiana alata* as style glycoproteins of ~30 kDa that are secreted into the extracellular matrix of the stigma transmitting tract and inner epidermis of the ovary (Anderson *et al*., 1986; Cornish *et al*., 1987; McClure *et al*., 1993). These glycoproteins co-

segregate with the S-haplotype in genetic crosses which led to cloning and identification of related proteins from members of the *Solanaceae* and other families (Takayama and Isogai, 2005). The expression of *S-RNase* gene is found to be at high levels in later stages of the pistil development (Clark *et al*., 1990), by which a secreted protein is encoded and accumulates to high levels in the stylar transmitting tract (Anderson *et al*., 1989; Ai *et al*., 1990). *S-RNase* genes which isolated from number of different species in several comparative analyses (Anderson *et al*., 1989; Clark *et al*., 1990; Ioerger *et al*., 1991; Xue *et al*., 1996; Ishimizu *et al*., 1998) show that *S-RNase* proteins have a regular pattern of interspersion of highly conserved amino acid sequence with more variable sequence. The two conserved domains C2 and C3 have two histidine residues, His32 and His91, that is with Lys90 to make up the catalytic site of the ribonuclease (Ida *et al*., 2001). In the *Solanaceae* and *Plantaginaceae*, HVa and HVb are two highly variable sequences domains of *S-RNase* proteins (Ioerger *et al*., 1991; Xue *et al*., 1996).

McCubbin *et al* (1997) reported the effect of the mutant *S3-RNase* levels where plants lost the ability of inhibiting *S3*-pollen. This was done by introducing a RNase- (H93R) variant of the *S3* SR-Nase of *P. inflata* into an *S2S3* background. In a following experiment, a dominant-negative phenotype where only the *S3* allele was affected in the transgenic *S2S3* (S3H93R) plant when self-pollinated. This shows that the transgenic plant was self-compatible. This suggests that the

*S3*H93R allele was capable to either block an interaction or prevents the *S3* allele normal function. It should be noted that only the style recognition was modified in all these studies while the pollen recognition specificity was not altered. This confirms that the "Pollen-S" component is encoded by a different gene product from that of *S-RNase*. There is correlation between the ribonuclease activity of the *S-RNase* and pollen rejection. *S-RNase* proteins are glycoproteins. The number, type, and fine structure of their glycan chains widely vary. Woodward *et al* (1989); Karunanandaa, Huang and Kao (1994) show that the carbohydrate group appear not to be essential for self-incompatibility.

1.5.2 *SLF*; The pollen-recognition component

The pollen S-gene (*SLF*) was initially identified as a F-box protein and called *SLF* (S-locus F-box) or *SFB* (S-haplotype-specific F-Box) gene (Lai *et al*., 2002; Entani *et al*., 2003). Following studies of mutants by Ushijima *et al* (2004); Sonneveld *et al* (2005), it was confirmed that the *SLF/SFB* are the pollen S-determinants. It is shown that *SLF* interact with *S-RNases* to elicit the SI response (Qiao *et al*., 2004a; Qiao *et al*., 2004b). In contrast, *SLFs* have been proposed to interact with *S-RNase* in compatible pollination and hence trigger *S-RNase* degradation as to prevent the cytotoxic effect of *S-RNase* (Goldraij *et al*., 2006).

Although *SLF* was identified to be the pollen-S gene, it was doubted due to insufficient polymorphism which was shown between S-haplotypes to determine S-specificity (McClure and Franklin-Tong, 2006). their low sequence diversity, which limits their ability to identify the pistil S-gene (Newbigin *et al*., 2008) This was problematic until Kubo *et al* (2010) proposed the action of multiple *SLFs* in each haplotype. This raises the possibility of pollen S-gene as to be a complex of genes. This is shown to be the case in apple (*Malus domestica*) and also in Japanese pear (*Pyrus pyrifolia*) (Minamikawa *et al*., 2010; Sassa *et al*., 2007). In the case of our experiment species *Petunia* (of the *Solanaceae*), the pollen-S gene is shown to be made up of multiple functional *SLF* genes (Kubo *et al*., 2010; Williams *et al*., 2014). In earlier studies in *Petunia* it was known that there were *SLF*-like genes at the S-locus (Hua *et al*., 2007). Comparison of *P. inflata* S-locus F-box protein (*Pi SLF*) with *Pi SLF*-like proteins reveals its unique function in *S-RNase*-based self-incompatibility.

1.6 The proposed models for pollen S-allele

Kao and McCubbin (1996) have proposed two possible models. First is known as the gatekeeper model (McClure *et al*., 1990), and the second is known as the inhibitor model (Luu *et al.,* 2000), Figure 3a and b.

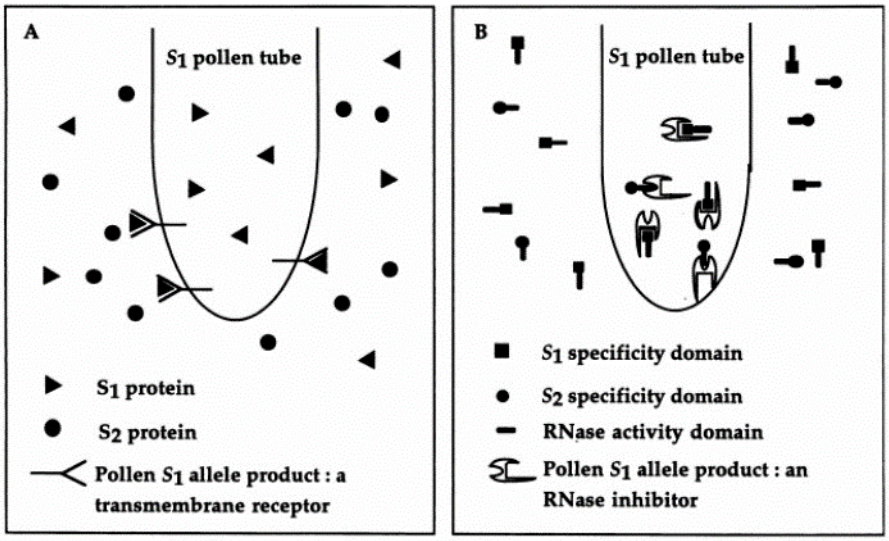


Figure 3, a and b. The pollen S-allele-specific suggested models. **(A)** The gatekeeper model. **(B)** The inhibitor model (Kao and McCubbin, 1996).

Figure 3a illustrates the first model. It suggests that the pollen S gene product functions as a receptor to allows S proteins to access the pollen tube. If for example *S1* proteins produced in *S1* pollen tube that is growing in a pistil carrying *S1S2* allele, *S1* proteins will be secreted into the extracellular space where *S1* pollen tube is growing. This pollen tube growth will be arrested. This is related to the pollen tube production of a receptor which allows *S1* protein to enter and resulting on the RNase activity of this protein to degrade the pollen tube RNA and consequently inhibiting the pollen tube growth (Kao and McCubbin, 1996).

The difference in the second model as shown on figure 3b is that the pollen S product is an RNase inhibitor which is present in the pollen tube. This inhibitor

functions as to obstruct all the RNase activity of non-self S proteins but not the self-protein. This is where non-self being ubiquitinated and as a result degraded (Williams *et al*., 2015). As shown, if *S1* pollen tube growing in *S1* and *S2* pistil, *S1* and *S2* both will be able to enter the pollen tube, but only *S1-RNase* will be fully functional as *S2-RNase* activity will be inhibited. As a result, the *S1* pollen tube will be arrested (Kao and McCubbin, 1996).

The cytotoxic model for pollen-tube rejection, in which pollen RNA is degraded by the *S-RNase*, is the most supported (Sims, 2005; Sims and Robbins, 2009). Therefore, the absence of *S-RNase* activity in pollen tubes, either by preventing the initial import of *S-RNase* or by inhibiting their activity inside pollen tubes, is necessary for compatible crosses. Although the accurate nature of the *S-RNase* inhibition has not yet been conclusively demonstrated, the model is supported to be correct by several different experimental approaches. Specifically, these provide evidence for non-selective uptake of *S-RNases* (Luu *et al*., 2000; Goldraij *et al*., 2006).

In advance to these observations, investigations of "competitive interaction" in diploid heteroallelic pollen from tetraploid plants present most of the support of the inhibition model. In several well-established observations (Crane and Lewis 1942; Lewis and Modlibowska 1942; de Nettancourt 1977), the GSI breaks down in tetraploid plants provided that the diploid parent is heterozygous at the S-locus.

There has been long discussion and several hypotheses have been suggested regarding the interact of *S-RNase* and the pollen S and several models were suggested. Efforts also have been made to identify *SLFs* specific functional features (Goldraij *et al*., 2006; Hua and Kao, 2006; Wheeler and Newbigin, 2007; Minamikawa *et al*., 2010; Hua *et al*., 2007; Hua *et al*., 2008). Hua and Kao (2006) carried out a yeast two-hybrid screening to confirm the key role of SBP1. They used three separate bait constructs of the *PiSLF2* F-box gene of *Petunia inflata*. It was confirmed that all of these three baits bound to a protein that is 98% identical to PhSBP1. This was named PiSBP1. In addition, they carried out protein interaction assays and showed that PiSBP1 was bound to *PiSLF1* of *P. inflata*, as well as to an unrelated F-box protein PiFBP2411.

Significantly, Luu *et al*., (2000) using electron microscopy plus immunogold labeling of *S-RNases* have shown that both compatible and incompatible *S-RNases* were imported into pollen tubes. Recently, cellular, biochemical, and molecular biology approaches have shown evidence to support the *S-RNase* degradation mechanism (Liu *et al*., 2014; Kubo *et al*., 2010). This mechanism is shown to be the most widely accepted one in *P. hybrida* (Liu *et al*., 2014).

1.7 THE GENUS *PETUNIA* AND SELF-INCOMPATIBILITY

Petunia is one of the most popular cultivated garden bedding flowering plants worldwide. It has large colorful flowers with well-defined organ morphology. The genus *Petunia* was established by Jussieuin in 1803, and it originates from the eastern side of South America including Brazil and Uruguay. It belongs to the *Solanaceae* family. *Petunia hybrida* named because of the commercial *Petunia* cultivars as well as the laboratory lines are known to have a hybrid origin. It is a consensus that *P. hybrida* has arisen from hybridization in early 19th century between the self-incompatible species *P. integrifolia* (purple bee-pollinated) and the self-compatible species *P. axillaris* (white hawkmoth pollinated) figure 4. Effective crosses can be achieved with each other and yield normal diploid offspring. This enables *P. hybrida* varieties to have the same 2n chromosome numbers as the parental species, therefore avoiding associated genetic complications that are found in tetraploid hybrids.

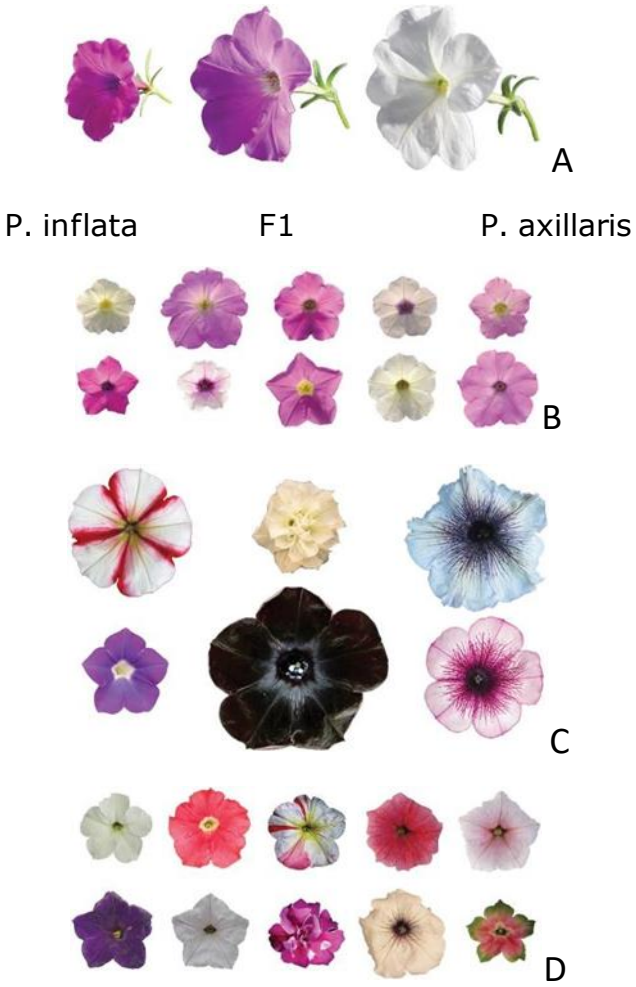


Figure 4. *P. hybrida* flowers origin and diversity. A, *P. inflata* S6, *P. axillaris* N and their F1. B, F2 population of *P. inflata* S6 × *P. axillaris*. C. shows some *P. hybrida* commercial accessions. D, *P. hybrida* accessions with mutants. Adapted from (Bombarely *et al*., 2016).

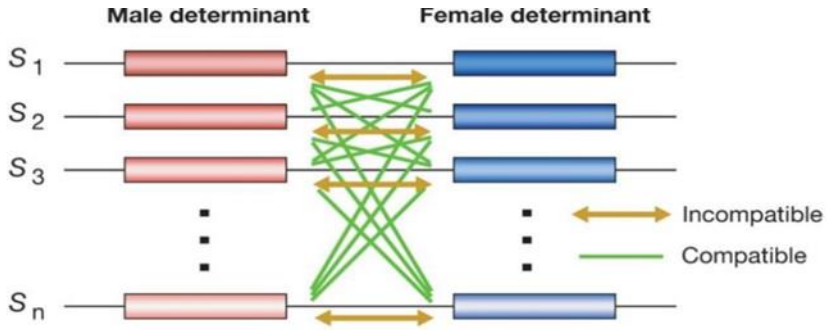
Petunia hybrida and its wild relatives have been described and focused on as a suitable model for SI research (Linskens 1975; Ascher 1984). This suitability is based on several biological features. First is the known strong SI reaction resulting on absence of seed set. This can be overcome conveniently by bud pollination by which homozygous stocks can be established and maintained adding a further advantage. Second, is the general ease of controlled

pollinations plus its suitability for biochemical and physiological studies. This all have eased the investigations of pollen tube growth rates in compatible and incompatible pollinations (Herrero and Dickinson 1980, 1981). Darwin (1981) described the essential features of self-sterility in *Petunia*; however, it was until later when Harland and Attack (1933) have established that a gametophytic mechanism controls pollen specificity. The *Petunia* materials used in these early studies were suggested to be *P. hybrida* rather than *P. violacea*, Mather (1943).

An important early discovery is the phenomenon of SI breakdown where some seed set was produced Ascher (1984). Ascher described this as "*Pseudo*-self-compatibility" (PSC) and defined it as the capability of an otherwise self-incompatible plant to set seed if either self-fertilized or been crossed to separate individuals carrying the same S-allele. This phenomenon has been the case of a study in *Nemesia* and in *Senecio* (Robacker and Ascher 1982; Hiscock 2000).

The style and pollen components together form a recognition "haplotype". The recognition and rejection responses of pollen occurs when there is a match of haplotypes between the growing pollen tube and the style (Figure 5) (Takayama and Isogai, 2005). This results in inhibition of the pollen tube growth in the transmitting tract, and fertilization rarely occurs. In most cases, seed capsule formation can be the distinct point between compatible and incompatible crosses. In compatible crosses, large seed capsules that may contain up to a few

hundred seeds are formed whereas in a fully incompatible cross no seed capsules can be formed so no seeds are produced.



Family	Type of SI	Male determinant	Female determinant
Brassicaceae	SSI	SP11/SCR	SRK
Solanaceae, Rosaceae, Scrophulariaceae	GSI	SLF/SFB	S-RNase
Papaveraceae	GSI	(Unknown)	S-protein

**

Figure 5. The S-locus contains at least two genes. One is carried by the pollen grain and encodes the male determinant. The second gene is expressed by the pistil and encodes a female determinant. These are both polymorphic and inherited as one segregating unit with no or little recombination known as multigene complex. The variants of the multigene complex are called the S-haplotypes. The recognition of self or non-self-pollination is controlled at the protein-protein interactions between the male and female determinants. If both the male and female determinants are from the same S-haplotypes, the incompatible response occur which result on pollen tube growth arrest. Adapted from (Takayama and Isogai, 2005).

(**) The female and male determinants in the *Papaveraceae* family have been shown to be an extracellular signaling S protein secreted on the stigma and found to induce programmed cell death in pollen tubes (Wheeler *et al*., 1999; Franklin-Tong and Franklin 2003). They were named *PrpS/PrsS* respectively (Wheeler *et al*., 2009).

1.7.1 RNase allelic diversity in *Petunia*

The allelic diversity in *Petunia hybrida* is significantly limited compared to its wild species; *P. axillaris* and *P. integrifolia*. Robbins *et al* (2000) reported that the number of S-alleles found in cultivated *P. hybrida* is suggested to be less than 10. This is far less than that of the wild populations in South America where there are 40 or more distinct S-alleles (Tsukamoto *et al*., 2003). Some of S-alleles in *Petunia* and *S-RNase* sequences were identified (Broothaerts *et al*., 1989 and 1991; Clark *et al*., 1990; Ai *et al*., 1992; Ai *et al*., 1991; Robbins *et al*., 2000; Entani *et al*., 1999; Wang *et al*., 2001; Ai *et al*., 1990; Ioerger *et al*., 1990; Tsukamoto *et al*., 2003).

The first three alleles known as Linsken's S-alleles reported as *S1, S2*, and *S3*. From these original alleles, the N-terminal sequences for purified *S-RNases* were reported by Broothaerts *et al* (1989). In 1991 Broothaerts and coworkers reported an S-allele which was derived from a commercial variety. This was named Sb. The first reported cDNA sequences for three *S-RNases* from *P. hybrida* were raised by Sims and coworkers. These were derived from stocks originally described by Ascher (Clark *et al*. 1990). Although Ascher S-alleles found to be distinct from the Linskens alleles, they were also named *S1–S3*. To avoid confusions, Robbins *et al* (2000), proposed the addition of suffix "L" to Linskens S-alleles. Ai *et al* (1992) reported the functional allele Sx, which was

derived from crosses between *P. hybrida* and *P. inflata*. The Sx allele was derived from the *P. hybrida* parent. Additionally, Harbord *et al* (2000) reported a functional allele which was identified from the self-incompatible stock V13 maintained by the Free University of Amsterdam. Entani and coworkers (1999) have also identified this allele in an independent cultivar. A single population of 100 individuals of *P. inflata* from Argentina found to contain 19 different S-haplotypes (Wang *et al*., 2001). Another study of a natural populations of *P. axillaris* has shown as many as 40 different haplotypes (Tsukamoto *et al*., 2003).

1.8 THE GENUS *SCHLUMBERGERA* AND SELF-INCOMPATIBILITY

Schlumbergera as well as the vast majority of flowering plants is co-sexual, with hermaphrodite flowers. This morphology aspect provides ample opportunity for both self-pollination and self-fertilisation. Despite this aspect, relatively few predominantly self-fertilise. Almost 50% of angiosperms are known to strictly avoid self-fertilisation (Raduski *et al*., 2012). That is, they are most commonly obligate to cross-fertilise. This is deployed by a genetic system 'self-incompatibility' (SI). This system causes fertile hermaphrodites to recognise then reject their self-pollen.

The genus *Schlumbergera* belongs to the Cactaceae family. The family has approximately 1800 species. The genus *Schlumbergera Lemaire* is comprised of six species which are indigenous to the south-eastern part of Brazil (Barthlott and Taylor, 1995). The *Cactaceae* family have four subfamilies (*Cactoideae*, *Maihuenioideae*, *Opuntinoideae* and *Pereskiodeae*) (Anderson, 2001). Self-incompatibility in the *Cactaceae* family has only been identified in three out of the four sub families (*Cactoideae, Opuntinoideae* and *Pereskiodeae*). Total of 28 genera in the *Cactaceae* family are expressing the self-incompatible phenotype. Self-incompatibility in the genus *Schlumbergera* has been identified as of being gametophytic type under the control of a single multiallelic *S*-locus (Boyle 1997).

There are past major reviews of the distribution of SI systems in angiosperms (Brewbaker 1957; de Nettancourt 1977; East 1940; Fryxell 1957; Sedgley 1994), however, there is no documentation of any cases in the *Cactaceae* family (Barthlott and Hunt 1993). This family has 98 genera and "1500 species of succulent perennials (Barthlott and Hunt 1993). SI has been reported to occur in at least 28 genera of the *Cactaceae* family (Boyle, 1997). This includes all three of the *Cactaceae* sub-families (*Pereskioideae*, *Opuntioideae*, and *Cactoideae)*. The *Cactoideae* consists of nine tribes. It is the largest and most evolutionarily advanced of the three sub-families, (Barthlott and Hunt, 1993). Studies indicate that SI is widespread in the *Cactaceae*.

The *Cactaceae* family is considered as one of these that are not well suitable for genetic studies of SI. This is because of the lengthy juvenile period which may last a decade or longer for some taxa (Cullmann *et al*., 1987). However, *Schlumbergera* in comparison can be considered speedy for two main reasons. Firstly, the first flowers can be obtained as early as 18 months post seed germination, although it is common that flowering is 24-month period after germination. Secondly, propagation of *Schlumbergera* cultivars is easily done from cuttings and flowering can be induced in 12 months period if appropriate environmental conditions are provided (Hansson, 1992).

False Christmas cactus (*S. truncata*) is an ornamental plant that is a popular winter-flowering houseplant. It is economically important member of

ornamentals that are marketed as Thanksgiving cacti. The Thanksgiving cactus (*Schlumbergera truncata*) flowers near thanksgiving, approximately one month before Christmas cacti, and the Easter cactus (*Rhipsalidopsis gaertneri*) which flowers mainly in spring. Thanksgiving cactus is predicted to be the most widely cultivated species of cacti (Anderson, 2001). It is indicated as vulnerable by the International Union for Conservation of Nature (IUCN) (Taylor and Zappi, 2017). This species can be easily grown from cuttings. Since the early 19th century, the species has been extensively hybridised throughout the western part of Europe as It produces a rich array of colourful flowers and various flower forms (Boyle, 2003). The most majority of the commercially grown plants are of uncertain provenance, despite dozens of named varieties.

In *Schlumbergera*, isozyme segregation ratios following semi-compatible crosses have been studied by O'Leary and Boyle (1998). The approach was to examine the inheritance of three enzyme systems: leucine aminopeptidase (LAP), phosphoglucomutase (PGM) and shikimate dehydrogenase (SKD). These were made in *Schlumbergera truncata* and *S. x buckleyi*. In addition, reciprocal crosses were done and subjected to heterogeneity chi-square tests. This is to determine the deviation significance of the segregation ratios. It was found that, few families exhibited anomalous segregation ratios of Pgm-1 or Skd-1. However, 1 in 14 of the 22 families that were examined, segregation ratios were identified for Lap-1. This distortion of segregation ratios confirmed the linkage

between Lap-1 and the S-locus which is responsible for self-incompatibility in *Schlumbergera*. Utilising the allelic diversity of the isozyme locus Lap-1, Boyle 2003 has identified a minimum of five S-alleles in 19 cultivars of both Christmas cactus and 10 cultivars of Easter cactus. Lap-1 phenotypes were determined earlier by polyacrylamide gel electrophoresis (O' Leary and Boyle, 2000). Boyle (1996) has found that SI is present in *Schlumbergera* showing that pollen tubes rarely extend beyond the upper third of the style when pistils are either selfed or incompatibly crossed, which resulted in preventing or greatly reducing fruit and seed set.

The angiosperm phylogenetical analysis outlined the interrelationships among many families including *Cactaceae*, *Plantaginaceae*, *Solanaceae* and *Rosaceae* families. It is suggested that there is a potential relationship and polymorphism among these families based on their gametophytic self-incompatibility system (Figure 6).

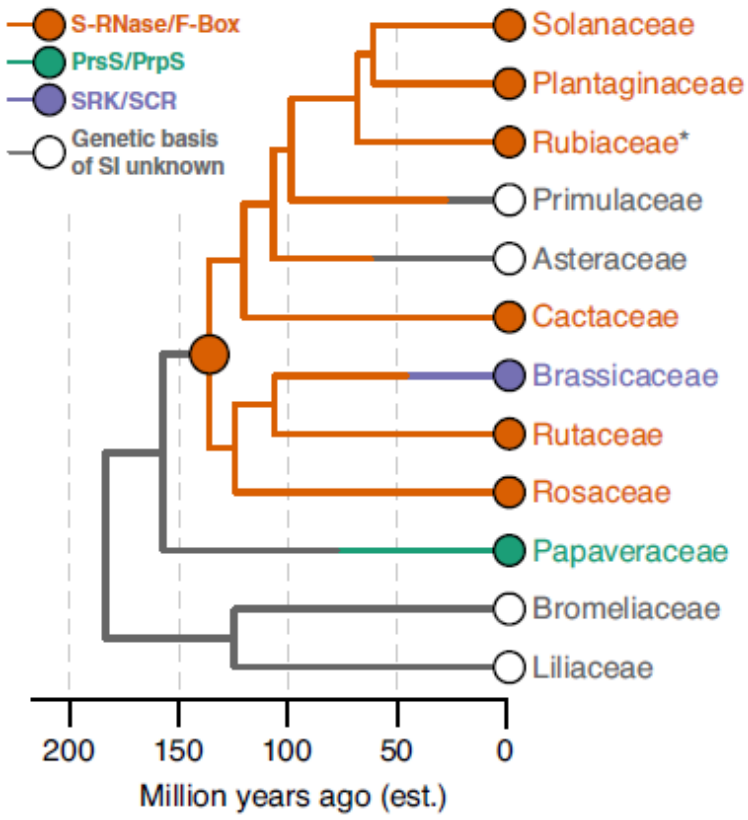


Figure 6. Summary of the current understanding of the distribution of SI systems across selected eudicot and monocot species. The coloured branches represent the key molecular components identified in each lineage. Accumulating evidence supports the homology of *RNase*-based SI mechanisms across most core eudicots, suggesting that this system was present in the common ancestor of approximately two-thirds of extant land plants. This hypothesis leads to the prediction that RNase-based SI should be found in a broader range of eudicot species than currently recognized. In the *Rubiaceae* family (denoted with an asterisk), some species exhibit heterostyly, and the molecular mechanisms underlying their SI remain to be elucidated (Adapted from Ramanauskas and Igić, 2021).

It is concluded that, with the recent finding of RSI in *Rutaceae* (Liang *et al*., 2020), weight of evidence was added to confirm that this mechanism is likely to be more common across core eudicots than generally appreciated.

S-RNase based phylogenetic relationship in *Cactaceae* family and others will be illustrated in the findings Chapter 5 of this study.

1.9 Previous studies; the novel *Petunia Sd* allele and breakdown of SI in *Petunia*

The novel *PiSd* allele from *P. inflata* was first identified here at the University of Nottingham by Dzidzienyo in 2011. In his study, a diallel cross design was performed to determine and characterize the possible S-phenotype of 4 *P. inflata* stock plants. He identified 2 incompatibility groups; group I consisted of two plants (P2 and P8), and group II consisted of two plants (P5 and P7). As illustrated in the table below where these are represented (-) as incompatible or (+) as compatible.

This grouping allowed the selection of plants from each group for S-allele cloning and sequencing. Subsequently the group I of these plants shown to carry *PiSk1PiSd* alleles whereas the group II found to be of *PiS3PiSd* genotype. It was observed that, crosses between P2 and P8 also between P5 and P7 resulted in no seed set in either direction (Table 1)**.** In contrast, crosses between the two groups of either P2 or P8 with P5 or P7 were found to be successful in either direction which resulted in seed set.

This indicates that, plants of the same group harbour the same S-alleles, therefore, crosses among them had resulted in the arrest of pollen tube growth which led to the eventual failure of fertilization. A compatible reaction will only occur resulting in a successful pollination and the ultimate growth of the pollen

tube when the haplotypes expressed in the pollen and the style are not the same.

Table 1. Incompatibility groups derived from diallel crosses of *P. inflata*. (+) indicates a compatible cross. and (-) indicates an incompatible cross. P2, PS, P7 and P8 represent the individual *P. infiata* plant IDs.

	Pollen Donor			
	Incompatibility Group I		**Incompatibility Group II**	
Plant ID	P2	P8	P5	P7
P2	-	-	+	+
P8	-	-	+	+
P5	+	+	-	-
P7	+	+	-	-

1.9.1 The *Sd* allele

Alleles *PiSK1* and *PiS3* were already in the public databases (Dzidzienyo, 2011) It was found that one allele revealed to be novel and hence provisionally called *Sd*. Using the 3' RACE technique as shown in Figure 7 below, Dzidzienyo (2011) was able to clone the *S-RNases* from pistils of group I and group II *Petunia* plants. The deduced S-genotype of P8 and all plants in Group I is *PiSkPISd* and that of P5 and plants in Group II is *PiS3PiSd*.

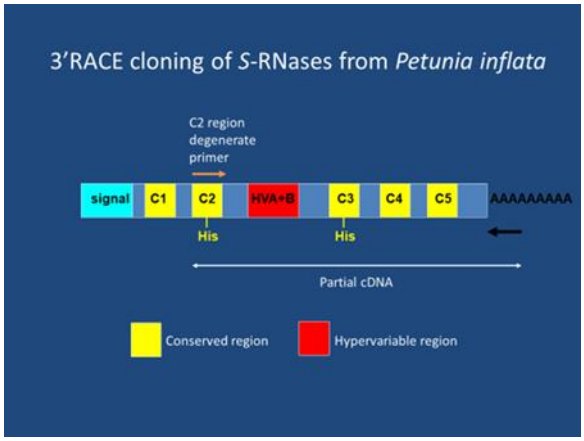


Figure 7. The two hypervariable regions (Hva and HVb) and three (C3, C4 and C5) of the five conserved domains found in all *Solanaceous S-RNases* are highlighted. The C3 conserved histidine residue involved in the ribonuclease activity of *S-RNases*. (Adapted from Robbins, 2015).

The sequencing results revealed that these *P. inflata* plants harbour three different S-alleles including the *Sd* allele. This allele shares homology with two *Nicotiana alata* S-alleles (*S7* and *SC10*) and one tomato S-alleles (*Sp16* from *Solanum peruvianum*) from the database rather than *Petunia* S-alleles. This

observation is typical for *Solanaceae* where inter-specific similarity among *S-RNases* is commonly observed feature (Ioerger *et al*., 1990).

1.9.2 *S*-alleles analysis

PSC and SC is widely seen in cultivated *P. hybrida* and previously shown to be associated with a specific S-allele, termed *So* (Ai *et al*., 1990; Sims and Robbins, 2009). In our own studies, self-compatibility arose in a cross between *P. hybrida* and *P. inflata* which was thought to be caused by the *PiSd* (*Sd*) S-allele. Sharef (2017) showed through her Ph.D. studies here at the UoN the role of the *Sd* allele in breaking down SI in a large population of *Petunia* hybrids. Reciprocal crosses showed that the loss of the *Sd* allele function was attributed to the pollen part. In some cases, intermediate seed production was detected and hence marked as pseudo self-compatible (PSC). Sharef (2017) through her study, has developed self-compatible *P. hybrida* cultivars from SI parents.

In that Ph.D. project, a female *P. hybrida* containing *PhS3PhS3* was crossed with male *P. inflata* containing *PiS3PiSd* genotype, a self-compatible phenotype arose in the F1 hybrids carrying the *PiSd* allele. This result was not expected as combining of different functional S-alleles usually result in a self-incompatible progeny. Therefore, this finding was further studied by analysing the progeny of these hybrids by genotyping and self-pollinating to investigate the roles of these alleles in the self- incompatibility system.

The F1 hybrid shown in Figure 8 is an example of the six possible genotype all of which show nearly the same appearance. The self-incompatibility phenotype of the F1 hybrids is indicated in parentheses (SI or SC). It was found that all progenies are SI except those carry the *PiSd* allele.

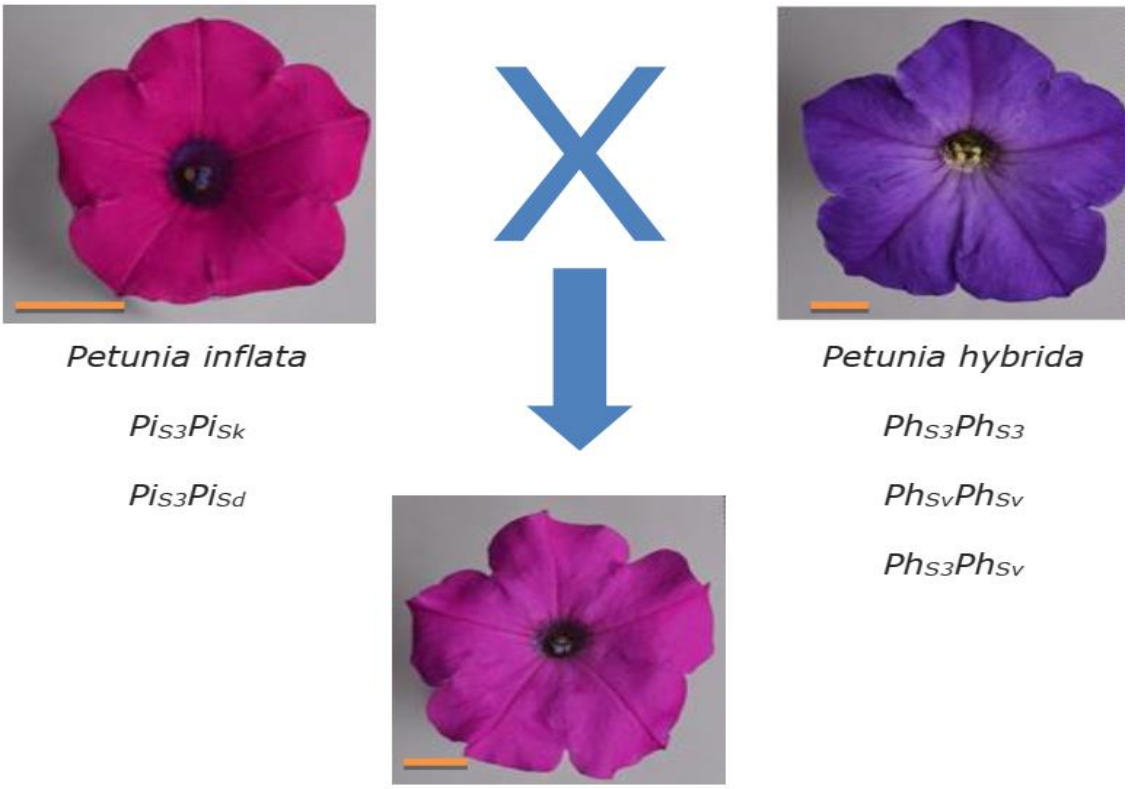


Figure 8. Crosses between *Petunia inflata* and *Petunia hybrida* to create F1 hybrids. Sharef (2017). The F1 hybrid shown is an example of the 6 possible genotype all of which show nearly the same appearance. The phenotype of the F1 hybrids is indicated in parentheses (SI or SC). The purple shading highlights the lines which carry the *Sd* allele, and they all are self-compatible whereas lines lacking this allele are SI.

In the F1 hybrid it was found that, seven plants out of thirty-two inherited the *PiSd* allele and all of them are self-compatible. This indicates the relation between compatibility and this particular allele as the remaining plants that lack this allele are incompatible.

As the breakdown of SI was noticed with plants carrying *PiSd* allele in F1 hybrid different self and cross pollination between F1 plants were done, Sharef (2017).

The collected seeds were used to produce F2 hybrid various families. Four families; N463, N464, N465 and N467 were generated from crossing between semi compatible plants in F1 hybrid in order to obtain the alleles for segregation of the SC phenotype with *PiSd* allele to be tested in a segregation family.

In a subsequent BSc project at the UoN by L. Milnes (2017. Unpublished), the plant N453.2 of the F1 hybrid as shown on the Table 2 below was self-pollinated to produce another F2 family which was named N461 as shown in Table 3 below.

Table 2. Background of the N453.2 family. The parents' phenotypes and genotypes are shown.

F1			Parents	Parents' Phenotype	Parents' Genotype	
N453.2	**Phenotype**	**SC**	**N440.4**	**SI**	(***PhS3PhS3***)	**A. Sharef (2017)**
	Genotype	(***PhS3PiSd***)	**N445.1**	**SI**	(***PiS3PiSd***)	

Table 3. The F2 generation parents. The parents' phenotypes and genotypes are shown.

F2			Parent	Parent' Phenotype & Genotype	
N461.4	**Phenotypes**	**SC/PSC**	**N453.2** ⊗	(***PhS3PiSd***) – SC	**L. Lesley (2017)**
	Genotypes	(***PhS3PiSd***)			

1.9.3 Evolution of SI and SC in the plant kingdom

Self-incompatible plants, such as *Petunia*, can sometimes breakdown their own SI mechanism, which will lead to these plants losing their ability to reject self-pollen (Tsukamoto *et al*., 2003). many potential reasons are causing this phenomenon. Sometimes the SI determinants have been mutated, resulting in lost functions, Furthermore, these mutations may not only occur in the male and female determinants but also in other factors that influence the SI process. For instance, the mutation of the HT-B influences the SI function of pistils render the plant self-compatibility (O'Brien *et al*., 2002; Kondo *et al*., 2002; McClure, 2006). In addition, a collapse of the SI process, which can result from a duplication of part or whole genome, will often lead to competitive interaction (Golz *et al*., 2000; Golz *et al*., 2001; Sims and Robbins, 2009).

In nature, studies have found that some self-incompatible plants produce a limited number of seeds after self-pollination. This is termed pseudo-self-incompatibility (PSC) (Flaschenreim and Ascher, 1979). PSC is likely caused by the reasons mentioned above; however, it lacked a reasonable interpretation at the molecular level at that time, as *S-RNase* and *SLF* genes had not yet been identified.

The breakdown of SI occurs recurrently throughout the evolutionary history in plants (Wright *et al*., 2013). SC in angiosperms represents a recurrent outcome

of convergent evolution (Shimizu and Tsuchimatsu, 2015). Numerous SC species have independently arisen from SI progenitors (Vekemans *et al*., 2014; Shimizu and Tsuchimatsu, 2015). Human selections practices between outcrossing and selfing have been proposed as the cause of the recurrent loss and re-establishment of SI (Kohn, 2008). Despite the genetic heterozygosity underpinning the shift from SI to SC, polyploidy is frequently implicated in a substantial proportion of such transitions (Miller and Venable, 2000; Stone, 2002). In *Solanaceae*, the breakdown of SI is mainly caused by polyploidization (Pandey, 1968). In *Solanaceae* and others including *Fabaceae* and *Rosaceae*, both induced and naturally occurring tetraploids consistently demonstrate that polyploidy almost invariably disrupts GSI (Stone, 2002).

Phylogenetic relationship studies indicate a single common ancestor of SI in *Solanaceae*, *Rosaceae*, *Plantaginaceae* and *Rutaceae* (Zhao *et al*., 2021) supported by the conserved use of *S-RNase* as the stylar determinant across these families (McClure *et al*., 1989; Sassa *et al*., 1996, 2007; Liang *et al*., 2020). Furthermore, the ability of the SLF proteins from diverse eudicots to detoxify *P. hybrida S-RNases* reinforces the ancestral nature of this SI system.

During angiosperm evolution, in some lineages, SI has been re-established through deletion or functional inactivation of a duplicated S-locus (Zhao *et al*., 2021). The build-up of nonfunctional S-haplotypes is suggested to cause loss of SI in SC cultivars (Hauck *et al*., 2006). Population subdivision is one of the most

common characteristics of natural populations (Slatkin, 1987). Population subdivision is a common feature of natural populations (Slatkin, 1987) and is considered a significant factor influencing the breakdown of SI. This subdivision can impact the evolution of mating systems, particularly self-fertilization and SI.

Increased inbreeding depression within SI populations may trigger the invasion of SC mutants. The likelihood of this incursion increases when the number of segregating SI alleles is low, as SI pollen is more frequently rejected by individuals sharing the same SI allele. In contrast, SC mutants possess universal compatibility, thereby benefiting from male reproductive success (Charlesworth and Charlesworth, 1979). The conservation of SI was strongly arranged when a population was subdivided under conditions that enabled a subdivided population with GSI to counter invasion by an SC mutant.

As SI follows Mendel's laws, both the male and female S-determinants must reside at the same locus. Isolated plant populations experience pollen limitation (Elam *et al*., 2007), Under such genetic and demographic circumstances, selection may favour the breakdown of SI, as SC individuals can maintain fertility in low-density environments, a principle known as Baker's Law (Baker, 1967).

In *A. thaliana*, SC is believed to have evolved at least three times (Shimizu *et al*., 2012). *A. thaliana* is thought to have become SC through the loss of SI

genes. Diverse loss-of-function mutations are suggested to underlie the emergence of SC across independently selfing populations. Distinct S-locus haplogroups exhibit multiple, independent inactivating mutations associated with this breakdown of Si (Shimizu *et al*., 2012). These mutations have been shown to be generally recessive (Nanjundiah, 1993). SI is expected to be restored in such cases as genetic complementation between two self-compatible (SC) individuals carrying distinct recessive loss-of-function mutations. SI can be reinstated by several mechanisms, such as deletion of duplicated S-loci, reactivation of the expression of *S-RNase*, or an evolution of a novel SI system (Fujii *et al*., 2016).

1.10 Aims of the project

This project explores the genetics and evolution of SI in two species: *Petunia* of the *Solanaceae* family and *Schlumbergera truncata* of the *Cactaceae* family. The aims of each stage of this study are outlined in the following sections.

1.10.1 SI in *Petunia*

This study investigates self-compatibility in *Petunia* plants derived from crosses between *Petunia inflata* and *Petunia hybrida*, which typically exhibit strong self-incompatibility (SI). The novel *Sd* allele of *P. inflata*, is a focal point of this study. Preliminary earlier work by Sharef (2017) demonstrated that plants carrying the *PiSd* allele exhibited self-compatibility (SC) and pseudo self-compatibility (PSC). Sharef (2017) suggested that the *PiSd* allele contributes to the breakdown of SI in *Petunia* F1 hybrid and a small F2 progeny. The current work aims to shed light on the identity and function of this novel allele across three subsequent generations for the first time.

The hypothesis of this study proposes that the novel *Sd* allele is associated with the observed self-compatibility in these *Petunia* plants. To test this hypothesis further, plants from each generation will be cultivated, genotyped to identify the S-alleles present using allele-specific PCR techniques. Subsequent phenotypic analysis will be conducted through extensive controlled self-pollination trials.

Seed pods will be collected and analyzed for capsule production and seed count to determine the compatibility status. Analysis also will include sequencing of the novel *Sd* allele and the *P. hybrida S3* allele to determine their structure and their phylogenetic interrelationship and evolutionary insights in linkage to SI and SC phenotypes. Also, tests will include allele segregation patterns and ratios.

1.10.2 SI in *Schlumbergera truncata* (False Christmas Cactus)

In the second phase of this research, SI mechanism in the species *Schlumbergera truncata* of the family *Cactaceae* (known as False Christmas Cactus), within the genus *Schlumbergera*, is investigated. The aim here is to test further whether S-RNase mechanism is functional in *S. truncata* and to explore its presence across angiosperms. The initial aim was to conduct bulk mRNA sequencing to identify *S-RNases* on this F1 progeny. However, a study by Ramanauskas and Igić (2021) at University of Illinois, Chicago had identified five S-RNases in the same species. Following this, our study aimed to further explore the hypothesis that *S-RNase* homology exists across the eudicots, including the *families Cactaceae, Scrophulariaceae, Solanaceae*, and *Rosaceae*. To achieve this, we aimed to identify the *S*-alleles that are functional in the F1 progeny of *S. truncata* and explore their genetic divergence and, their phylogenetic

relationship with other eudicots. This will help us to gain evolutionary insights on *S-RNases*.

To achieve these aims, six lines of *S. truncata*, derived from the F1 progeny of a previous study conducted in 2006, were utilized. Phenotypic analyses were performed including PCR and DNA sequencing to identify *S-RNases,* by means of large-scale diallelic self- and cross-pollination.

CHAPTER 2; MATERIALS AND METHODS;
2.1 materials and methods; *Petunia*

In the previous studies, *Petunia inflata* stocks used are obtained here at the University of Nottingham, Plant Science Division. In this current research, I use the subsequent seed set in order to produce several offset *Petunia* generations aiming to study the presence and frequency of SC phenotypes in *Petunia* hybrids derived from stocks containing the *PiSd* allele, as well as the *PhS3* allele. Previous research of these stocks found self-pollination of plants carrying the novel *PiSd* allele produced SC and PSC individuals (Chapter 1). This project will use self-pollinations and subsequent seed set as well as phenotyping and genotyping to determine the inheritance of these alleles and aid in the ongoing research into SC inheritance and the *PiSd* allele in *Petunia*.

2.1.1 *Petunia* alleles

The first allele investigated in this study is the *Petunia hybrida* (*PhS3*) allele. It was sequenced by Robbins *et al* (2000) as *S-RNase* allele. This was done by degenerate PCR cloning procedure using the known conserved region of other *S-RNase* proteins and a primer was created based on the C2 conserved domain (Ioerger *et al*., 1991). Robbins *et al* (2000) have isolated this using this approach. Then using allele specific primer based on the hypervariable domain of

the S-alleles. This is the same primer we use in this study. This primer pair features to correctly identify the target allele alone and not S-like RNases. The second allele which is tested in this study is *Petunia inflata* kind (*PiSd*). It was identified by Dzidzienyo (2011). *PiSd*-RNase is found to be a novel allele (Dzidzienyo, 2011).

2.1.2 *Petunia* primers

The *P. inflata* primers used on this study were previously designed by Dzidzienyo (2011) based on hypervariable regions (Figure 9 below shows the sequence alignment where the *Sd* specific primers are highlighted).

```
Sd-RNase ---------------TGATAACGTCAGTACACCGCTGAATTTTTGTGGTGCCAAAGAAGA
S3-RNase TCATGGGCTTTGGCCCGAAAAGGAGCACTTTCGTCTGGAGTTTTGCGATGGAGATAAGTT
Sk1-RNase TCACGGGCTTTGGCCGGAAAAAAAGGGGTTTCGTCTGGAGTTCTGTAGTGGCGGTACCAA

Sd-RNase AAACTACAAAGACTTAACGGATGATAACAAAAAGAATAATTTGTATAAACGTTGGCCTGA
S3-RNase TGTGTCGTT---CAGCTTAAAAGATAGAATTGTCAATGATTTGGAGCGCCACTGGGTTCA
Sk1-RNase GT---ATAA---GATTTTCGAGGACAATATGGTCAATGATCTGGAACGCCACTGGTTACA

Sd-RNase ATGTAAACCGCTACGATAATCA----GTACAATCAAATAAAATTTTATTTGAAAATT---
S3-RNase TGGTTCTTTCTAA-------------GAAAAAGAAAA---------------TGCTGAGC
Sk1-RNase TTTTTCCTTCTGTTGGCTCTTACTAAGAAAAAGTGTAGTCGAGGTCATCTAAAAATAAAA
```

Figure 9. The *PiSd-RNase* part of the multiple sequences' alignment (adapted from Shareef 2017). *Sd* primers are highlighted in red.

In addition, the *Petunia hybrida PhS3*-R1 primer was designed by Robbins *et al* (2000). A degenerate primer based on the C2 conserved domain of S-RNases from the *Solanaceae* (loerger *et al*., 1991) was used to amplify partial *S-RNase* sequences by RT-PCR of pistil RNA (Xue *et al*., 1996). Several identical cloned

PCR products with homology to other *S-RNases* were obtained for each allele. Confirmation that these cDNA sequences corresponded to the Sv and *S3 RNases* was obtained by designing allele specific primers based on the hypervariable domain and the 3' untranslated region. The *P. hybrida PhS3*-F2 was designed by Dr. Upendra Devisetty (unpublished). All primers that were used are shown in Table 4.

Table 4. Primers pairs used in this study. The *Sd*-F and *Sd*-R primers amplify *Petunia* plants carrying the *PiSd*-RNase. *PhS3* amplify *Petunia hybrida* plants segregating for *PhS3*-RNase. Tm °C represents the annealing temperature of each pair. Also, the sequences of the primers used are shown.

Allele	Primer Name	Primer Direction	Sequence (5'-3')	Length	Tm °C
PiSd	Sd-F	Forward	AGAAAACTACAAAGACTTAACGG	22mer	57.3
	Sd-R	Reverse	TACTGATTATCGTAGCGGTTTAC	23mer	58.8
PhS3	PhS3-F2	Forward	ATACGCCTGCAGTACTGCAAGCC	23mer	70.1
	PhS3-R1	Reverse	CGCATGTATCACTTTGACGACA	24mer	65.9

2.1.3 *Petunia* genetic stocks

In this study, I have generated three subsequent *Petunia* generations (F3, F4 and F5). The N453.2 family was derived from a past study by the Ph.D. student here at The University of Nottingham (A. Sharef, Ph.D. Thesis, 2017). In that Ph.D. project, female *P. hybrida* parent N440.4 of genotype (*PhS3PhS3*) was crossed with male *P. inflata* parent N445.1 of genotype (*PiS3PiSd*), a self-compatible phenotype arose in the F1 hybrids carrying the *PiSd* allele (Chapter

1, section 1.9). This result was not expected as combining of different functional S-alleles usually result in a self-incompatible progeny. Therefore, I am studying this finding further by analyzing the subsequent generations (F3, F4 and F5). This is by genotyping and self-pollinating to investigate the roles of these alleles in the self-incompatibility system. The collected seeds are used to generate a large population of each generation. F3 and F4 have four families each while the F5 includes six families.

2.1.4 *Petunia* plant methods

Seeds of *Petunia* generations used in this project were sown on Levington F2+S compost using 15*10cm trays. Each family had a separate tray filled with compost, tapped to settle large pockets of air, then seed sprinkled thinly across the soil surface. These were kept at the allocated glasshouse room where temperature is (22/24 °C) and day/night period (16/8hours). These were watered regularly and left for to germinate. After about 3 weeks seedlings were then pricked out carefully and removed from the tray and moved into a new cell tray. These new cell trays were filled with M3 compost. They were given and labelled with specific ID number for each individual.

After about 4 weeks the plants were potted into individual 2L pots with M3 Levington compost Figure 10. Baskets were added to the pots to support side-branching. Plants with excessive side branching or height were pruned regularly

to avoid damage and also secure the stability of the plant. In addition, pesticides were applied as propriate, and nutrients supplied when deemed necessary.

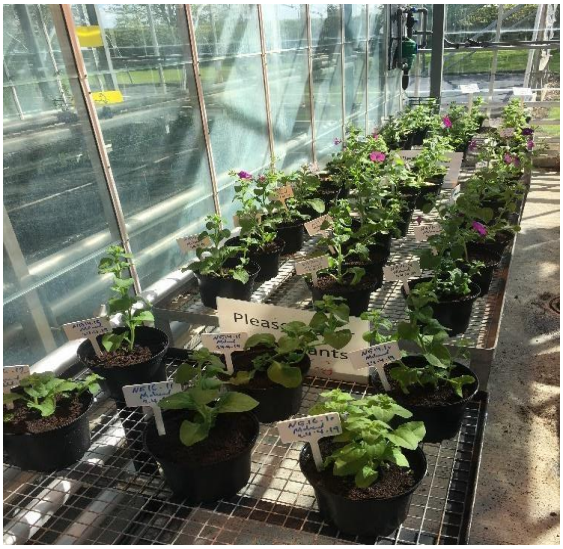


Figure 10. *Petunia* Juvenile plants after being potted into 2L pots at the glasshouse, Sutton Bonnington Campus, The University of Nottingham.

2.1.5 *Petunia* pollination

Controlled pollinations were done manually. Mature flowers were used by removing a flower and peeling back the petals for easy access to the stamens. Using a sterilised tweezers (washed in 70% ethanol) the stamens were carefully removed. The anthers were then rubbed against the stigma of another flower from the same plant. This is to transfer the pollen grains onto the stigma. Then each pollinated flower was tagged with a label which was marked as a self-pollination and dated. Usually total of ten self-pollinations were conducted per

each plant. Pollinated plants were set aside and left to develop seed pods and produce seeds.

2.1.6 *Petunia* seed harvesting

Usually about 3 weeks after pollination the start of seed harvest was conducted. Before pods are open and senesce usually characterised of them starting to change colour into brown, seed pods were manually removed from each plant and crushed into glassine bags to release seeds. The collection bags were marked with the plant ID and indication of self-pollination. Each bag was sealed with a paper clip. These were then moved into the lab and stored in an open tray in a cool, dry area. Seed harvest continued until all prospective seed capsules had been harvested. Then the total seed produced from each plant was weighed and recorded.

2.1.7 *Petunia* seed weight

An empty glassine bag was first weighted. The bag weight was noted, which was later deducted from the total weight of each bag. As a weight guide, 100 seeds were counted by eye and weighted. This was to generate a conversion factor to allow for the estimation of the seed number produced by each individual plant. Each individual plant produced seeds were weighed in the same bag in which they were harvested and stored. As seed harvests were continuous over several

dates, all seed per plant was transferred into one bag before weighing. Each individual bag was then weighed, and this was recorded. For accuracy, each bag's weight was measured twice.

2.1.8 *Petunia* DNA extraction

Using the GenElute TM Plant Genomic DNA Miniprep Kit (Sigma, UK), DNA was extracted from the leaf tissues of *Petunia hybrida* plants. This was by removing two medium young leaves using a 1.5 ml pre-labelled microfuge tube which then immediately flash frozen in liquid nitrogen. The frozen leaf tissues were then firmly ground by micropestle until they turned into fine powder. After that 350 µl Lysis Solution A and 50 µl Lysis Solution B were added to the fine powder. These were then incubated at 65°C for 10 minutes in order to dissolve the leaf tissues. Following this, 130 µl precipitation solution was added to the mixture, left on ice for 5 minutes and centrifuged at 13,000rpm for 5 minutes to precipitate the debris from the leaf tissue. Then the debris were filtered by pipetting the supernatant onto GenElute filtration column which were centrifuged at 13,000 rpm for 1 minute. Meanwhile, the binding columns were prepared. A volume of 500 µl of column preparation solution was added to the binding columns which functions as to maximise the binding of the DNA to the column. Following this, 700 µl of the Lysate was loaded onto the Binding Column and centrifuged at 13,000rpm for 1 minute. This makes the DNA to bound to the column. Then the

flow through liquid was discarded and the column was transferred to a new collecting tube. After this, the column was washed twice by using 500 µl Wash Solution which contains ethanol. The column was then transferred to another new collecting tube and a volume of 100 µl of pre-warmed Elution Solution was applied. The tube was centrifuged for 1 minute. The DNA samples quality was then analysed using a NanoDropTM 1000 Spectrophotometer (Thermo Scientific).

2.1.9 *Petunia* genomic PCR

The reaction mix was assembled as follows: 12.5 µl of the master mix (OneTaq® 2X Master Mix with Standard Buffer, M0482S) supplied by (New England BioLabs) was added to 0.5 µl of primer mix (0.5 µl of each of the primers at 10 uM concentration). Then 9.5 µl of distilled water was added until the final volume was 23 µl, this was then added to a 2 µl of DNA sample. The PCR reaction was conducted using 35 cycles comprising 3 steps as illustrated on Table 5. Conditions of the PCR 3 steps differ from primer to another as shown in Table 5.

Table 5. The conditions of the genomic PCR. Annealing temperatures are shown for each primer pair.

Allele	Step	Temperature	Time	Cycle
PiSd	Initial Denaturation	94 °C	3 minutes	1
	Denaturation	94 °C	30 seconds	35
	Annealing	52 °C	30 seconds	
	Extension	72 °C	1 minute	
	Final Extension	72 °C	5 minutes	1
PhS3	Initial Denaturation	94 °C	2 minutes	
	Denaturation	94 °C	1 minute	35
	Annealing	64 °C	30 seconds	
	Extension	72 °C	1 minute	
	Final Extension	72 °C	5 minutes	1

2.1.10 *Petunia* gel electrophoresis

To prepare the gel, 1.5 grams of agarose was mixed with 150ml of TBE buffer (see Appendix 1). Per each 100ml of TBE buffer (121.2g of Tris base, 61.8g of boric acid and 7.4g of ethylenediaminetetraacetic acid, made up to 1L with RNase-free H2O). This mixture was then dissolved using a microwave for 2-3 minutes. Then the mixture was cooled under cold water, 7 µl of ethidium bromide (500µl/ml) was added and mixed before the mixture was poured into

the electrophoresis tray which was prepared in advance. The tray containing the mixture was left in the fume hood for about 15 minutes for the gel to settle. While waiting for the gel to set, 10 µl of each sample was mixed with 2 µl of Thermo Scientific 5X DNA loading Dye. After the gel was ready the PCR product samples were pipetted into the each well in the gel. In addition, 5 µl of Hyper Ladder II (Bioline, London, UK) was added to the first well for size calibration. This Hyper Ladder II produces a pattern of 15 regularly spaced bands ranging from 50 to 2000bp. The bands, 300, 1000 and 2000bp have the highest intensity where the clearest band is shown. Also, the last two wells were used for controls, the samples were substituted with the pre-prepared control stocks. Then the gel was run at 98 volts for 90 minutes. After that the electrophoretic gel was visualised under ultraviolet light and photographed using the digital imaging system (Syngene, Cambridge, UK). For sequencing total of 50 µl of the PCR product was run on gel using two wells per sample. In total 4 samples were run for sequencing two for the *S3* allele and two for the *Sd* allele.

2.1.11 Gel purification, DNA extraction for sequencing

In order to confirm the size of each allele in Petunia, two samples were chosen for each allele. For the *S3* allele samples N514.1 and N515.9 of the *Petunia* F3 generation were chosen. For the *Sd* allele two samples also were chosen one from the F3 generation (N514.1) and the second belongs to the F5 generation (N534.1). 50 µL of each of these sample was applied divided in two wells in the

gel. After running the gel at a constant voltage of 98V for 90 minutes, the DNA extraction from the gel centrifuge protocol was followed using the Thermo Scientific GeneJET Gel Extraction Kit.

The two well of gel slice containing the DNA fragment was excised using a clean scalpel. It was cut as close to the DNA as possible to minimize the gel volume. The gel slice was placed into a pre-weighed 1.5 mL tube and weigh again. The weight of the gel slice was recorded. Then 1:1 volume of Binding Buffer was added to the gel slice (volume: weight). This was Incubated the at 60 °C for 10 min until the gel slice was completely dissolved. Every few minutes this was mixed by inversion to facilitate the melting process till the gel was completely dissolved. This followed by vertexing the gel mixture briefly before loading it on the column. After this, 800 µL of the solubilized gel solution was transferred to the GeneJET purification column. Then centrifuged for 1 min. The flow-through was discard and the column was placed back into the same collection tube. When the total volume exceeded 800 µL, the solution was added to the column in stages and centrifuged for 60 s then the flow-through was discarded after each spin. Then 100 µL of Binding Buffer was to the GeneJET purification column. This was centrifuged for 1 min then the flow-through was discard and the column placed back into the same collection tube. 700 µL of the Wash Buffer was added to the GeneJET purification column, and centrifuged for 1 min. Then the flow-through discarded and the column placed back into the same collection

tube. To completely remove residual wash buffer, the empty GeneJET purification column was centrifuged for an additional 1 min. The GeneJET purification column transferred into labelled and clean 1.5 mL microcentrifuge tube. Then 50 µL of Elution Buffer was added to the centre of the purification column membrane. The final step was to centrifuge for 1 min and the purification column was discard and the purified DNA was stored at -20 °C. The extracted DNA concentration was then assessed using NanoDropTM 1000 Spectrophotometer (Thermo Scientific). The labelled samples tubes along with their forward and reverse primers were sent to Source Bioscience for sequencing.

2.1.12 *Petunia* DNA sequencing

For the *PhS3* allele, DNA sequenced from two samples. F3 sample 14.1 and F5 sample 15.9. Sequencing was done using both primer pair. *S3* forward primer 5' ATACGCCTGCAGTACTGCAAGCC 3', and *S3* reverse primer 5' CGCATGTATCACTTTGACGACA 3'.

The *PhS3* primer pair and their intended targets are shown below.

>AJ271065.1 *Petunia hybrida* partial mRNA for *S3L*-ribonuclease (*S-RNase* gene)

```
product length = 439
Forward primer  1    ATACGCCTGCAGTACTGCAAGCC  23
Template        202  ......................  224

Reverse primer  1    CGCATGTATCACTTTGACGACA  22
Template        640  ......................  619
```

For the *PiSd* allele, also DNA sequenced from two samples. F3 sample 14.1 and F5 sample 34.1. Sequencing performed using both primer pair. *Sd* forward 5' AGAAAACTACAAAGACTTAACGG 3', and *Sd* reverse 5' TACTGATTATCGTAGCGGTTTAC 3'. This allele is not published so primers designed based on the original sequence identified by Dzidzienyo (2011) as shown below.

```
product length = 477
Forward primer  1    ATACGCCTGCAGTACTGCAAGCC  23
Template        43   .......................  65

Reverse primer  1    CGCATGTATCACTTTGACGACA  23
Template        519  ......................  497
```

As to confirm the expected sizes of *Petunia* alleles in this study, all samples DNA sequences for both the *S3* and *Sd* alleles were checked using the Biotechnology Information (NCBI) database Global Sequence Alignment Tool . (http://blast.st-va.ncbi.nlm.nih.gov) and also the SnapGene software. These sequence analysis will be illustrated in the results chapter.

2.1.13 Phylogenetic and multiple sequence alignment analyses of *S-RNase* nucleotide and amino acid sequences

The alignment, comparison and phylogenetic analyses of the novel *Sd-RNase* and *PhS3-RNase* along with all *Solanaceae's S-RNases* sequences were performed using the sequence data analyses tools. A total of 100 homologous nucleotide sequences corresponding to the novel *Sd* and *S3 RNases* from *P.*

inflata, *P. hybrida*, *P. integrifolia* and *P. axillaris* were employed for phylogenetic tree construction and motif analysis along with all *Solanaceae's S-RNases* sequences. All *S-RNase* sequence were obtained from the NCBI database with accession numbers listed in Supplementary Table S2**.** Multiple sequence alignments (MSAs) of the nucleotide sequences were generated using the MUSCLE algorithm (Edgar, 2004). The optimal model of nucleotide substitution was determined via MEGA 6.0, which performs tree topology optimization beginning with a Neighbor-Joining tree and utilizes maximum likelihood estimation in conjunction with model selection criteria including the Bayesian Information Criterion (BIC), Akaike Information Criterion (AIC), and log-likelihood (LnL) values to find the best fitting model of amino acid substitution. The General Time Reversible model with gamma-distributed rate variation and a proportion of invariant sites (GTR + G + I) was identified as the best-fitting substitution model, where G denotes the gamma shape parameter and I represents the proportion of invariant sites. Phylogenetic inference was subsequently conducted using the maximum likelihood method implemented in PhyML 3.0, accessible through the phylogeny.fr platform. Parameters for gamma shape and proportion of invariant sites were estimated, branch lengths were optimized, and branch support was evaluated using SH-like approximate likelihood ratio tests (SH-aLRT). The resulting phylogenetic tree was visualized with the Interactive Tree of Life (iTOL) tool.

Additionally, an amino acid alignment was constructed with the novel *Sd-RNase*, *PhS3-RNase* sequences together with selected *Solanaceous S-RNases* retrieved from the NCBI database BLAST (http://blast.ncbi.nlm.nih.gov).

2.2 MATERIALS AND METHODS;

Schlumbergera truncata genetic stocks

The False Christmas cactus plants used in this study are obtained from the stock here at The University of Nottingham. They are kept generating by the mean of cuttings in the previous 14 years. These plants were kept here since the last research project done by Tumusiime in 2006. These are from F1 progeny which was derived following a cross between two different parent plants which were bought from a commercial stock at Home Base, Nottingham. One of the parents had white flowers and was designated (White Parent; WP) whilst the other parent had pink flowers and was designated (Pink Parent; PP). The PP was used as a pistillate parent, and the WP was the pollen parent (S. Tumusiime 2006).

2.2.1 *Schlumberger* plant material and growth conditions

In this project, cacti plants were grown using the M3 Levington compost filles in 1L pots. A total of six lines were obtained which are available from original eight as two were lost. Three of these have pink flowers whilst the other three have white flowers. The white flowered progeny was designated (W1, W2 and W4) and the pink flowered progeny was designated (P1, P2 and P4). Then four clones were obtained from each line (W1*4, W2*4, W4*4) (P1*4, P2*4, P4*4). In total 24 plants. During the vegetative stage of development, plants were grown in the

glasshouse and exposed to long days (16h) at temperature ranging between 18°C/22°C. In order to induce flowering, the plants were then put under a shade as the daytime shorten (8h) and at a lower temperature round 15°C /19°C.

2.2.2 Harvesting of pistils

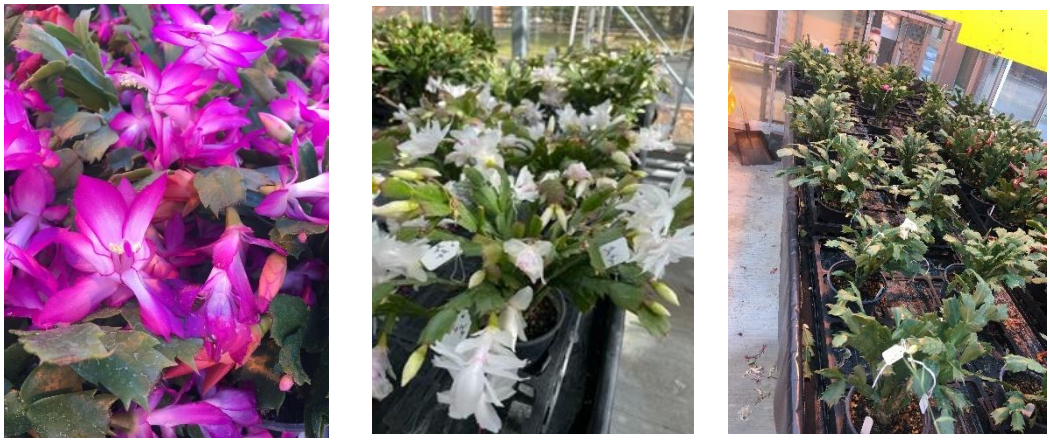
At the first flowering season (Autumn/Wintr 2019) as the plants still young only limited number of flowers were produced. Therefore, flowers of this season were used to harvest pistils. Mature flowers were selected. Flowers were opened using a blade then pistils were taken off. At least 10 pistils were harvested from each line's four clones. These were put in foil envelops and flash frozen in Liquid Nitrogen. Pistils were then stored at -80 °C ready for RNA extractions.

2.2.3 Pollination; classic complete diallele pollination of the F1 progeny of *Schlumbergera*

The F1 progeny of 24 individuals belonging to six lines (P1, P2, P4, W1, W2, W4) on this study were crossed in a complete classic diallele composed of self-pollinations and reciprocal crosses between mature flowers (Figure 11). Pollination was performed by rubbing dehisced anthers on the stigmatic lobules.

Figure 11. Flowers from *Schlumbergera truncata*. Flowers are from two different *Schlumbergera* plants forming part of the collection at The University of Nottingham, The School of Plant Sciences.

Throughout our cacti plants flowering seasons, large scale of self and cross-pollination was performed. Across the six lines, 360 pollinations were carried out. On each line we have four clones. Out of the total 360 pollinations, 60 were self-pollinations. The rest 300 were cross-pollinations. On each line 10 self and 50 cross-pollinations were achieved. Photographs below shows pink and white individuals intense flowering in the cacti progeny and some tagged pollinations done in this experiment. The classic complete diallele pollination exercise results are represented in more details in the coming results chapter number 5.



A B C

Figure 12. A. represents one of the twelve Pink flowered individuals. B. represents one of the twelve White flowered individuals. C. Shows pollinated flowers tagged.

At the second flowering season (Autumn/Winter 2020), the covid 19 pandemic lockdown has major impact on our research plan as we have very limited access to both the glasshouse and the labs. At the start of their flowering season 2020/2021 the aim was to carry out self and cross-pollination, however because of the Covid-19 lockdown this was not possible. Therefore, the pollination aimed was not possible. As a result, a full classic diallel crossing design was postponed to the following flowering season 2021/2022.

During the 2021/2022 flowering season, pollination was performed in the glasshouse. Each flower was pollinated once using anthers collected from one flower. The flowers were then tagged indicating the date of pollination and the parents involved in the pollination exercise. Records of flowers that abscised

from the plant following the pollination exercise were made and the parents involved were recorded. Ten self and fifty cross pollinations per line were carried out where possible. Floral abscission is a feature of the Cactus plants that are self-pollinated as they are SI. Flowers that abscised from the plant following the self-pollination exercise were recorded. Flowers remaining on the plants and forming fruit were monitored for a period of not less than 6 months before seeds' harvest. These self and cross pollinations are shown in Table 6 below.

Table 6. Design of the complete classic diallele pollination exercise. The number of self- and cross-pollinations performed. P indicates the pink flower line, and W represents the white flowering line (P1, P2, P4 and W1, W2, W4). The self-pollination events are indicated in red.

♀ \ ♂	P1	P2	P4	W1	W2	W4
P1	**10**	10	10	10	10	10
P2	10	**10**	10	10	10	10
P4	10	10	**10**	10	10	10
W1	10	10	10	**10**	10	10
W2	10	10	10	10	**10**	10
W4	10	10	10	10	10	**10**

2.2.4 *S. truncata* Seed harvesting

Successful cross-pollinations forming fruits were monitored for a period of not less than 6 months, then were manually harvested. The seed were extracted by

hand squeezing onto a Whatman filter paper and were allowed to dry. These then were stored in envelopes at room temperature.

2.2.5 *S. truncata* DNA extraction

Using the GenElute TM Plant Genomic DNA Miniprep Kit (Sigma, UK), DNA was extracted from the corolla tissues of the false Christmas Cactus's plants. This was by removing manually and using a 1.5 ml pre-labelled microfuge tube which then immediately flash frozen in liquid nitrogen. The frozen flower's tissues were then firmly ground by micropestle until they turned into fine powder. After that 350 µl Lysis Solution A and 50 µl Lysis Solution B were added to the fine powder. These were then incubated at 65 °C for 10 minutes in order to dissolve the flower's tissues. Following this, 130 µl precipitation solution was added to the mixture, left on ice for 5 minutes and centrifuged at 13,000rpm for 5 minutes to precipitate the debris from the flower's tissue. Then the debris were filtered by pipetting the supernatant onto GenElute filtration column which were centrifuged at 13,000 rpm for 1 minute. Following that the same protocol steps in the *Petunia* section (2.1.8) above were applied. The DNA samples quality was then analysed using a NanoDropTM 1000 Spectrophotometer (Thermo Scientific).

2.2.6 *S. truncata* primers design

Following the study by Ramanauskas and Igić (2021) which identified five S-alleles in false Christmas Cactus, I have requested the identified S-alleles' sequences data from the author Boris Igic (Department of Biological Sciences, University of Illinois at Chicago, Chicago, USA) in order to design the specific primers based on hypervariable regions. The designing of the forward primers is based on HV region whereas the reverse primers based on 3'UTR. A multi sequence alignment was performed using CLUSTAL alignment tool program https://www.ebi.ac.uk/Tools/msa/clustalo/. This alignment is shown in the appendix. Following this five sets of primers were designed using the NCBI primer design program at https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome.

All primers that were used are shown in Table 7.

Table 7. Primers pairs used in this study. Tm °C represents the annealing temperature of each pair. Also, the sequences of the primers used are shown.

Allele	Primer Name	Primer Direction	Sequence (5'-3')	Length	Tm °C
S1	S1-F	Forward	ACTGCACTGTGGTCGTTAGG	20mer	62.8
	S1-R	Reverse	GGATTTCCAGCGAAGTCCCA	20mer	68.4
S2	S2-F	Forward	TCAACCGTTGTTCGATGCCT	20mer	67.8
	S2-R	Reverse	AGCCTGTTCTTCCAACCGAG	20mer	65.5
S3	S3-F	Forward	AAGCTTACAACAGTCGGGGG	20mer	65.1
	S3-R	Reverse	TTGGATTGCCGTTTGCATCG	20mer	71.1
S4	S4-F	Forward	TGCTTAGAGCCATTCGTGGA	20mer	65.7
	S4-R	Reverse	ACGACACGAATTGTGAGGGA	20mer	65.6
S5	S5-F	Forward	TTCAGCCGAACTATGGTGGC	20mer	67.2
	S5-R	Reverse	CCCCAACTGGATGGCATTTC	20mer	68.5

2.2.7 Genomic PCR

The reaction mix was assembled as follows: 12.5 µl of the master mix (OneTaq® 2X Master Mix with Standard Buffer, M0482S) supplied by (New England BioLabs) was added to 0.5 µl of primer mix (0.5 µl of each of the primers at 10 uM concentration). Then 9.5 µl of distilled water was added until the final volume was 23 µl, this was then added to a 2 µl of DNA sample. The PCR reaction was conducted using 35 cycles comprising 3 steps as illustrated on table 8. Conditions of the PCR three steps and each primer annealing temperature are shown in Tables 8 and 9 respectively.

Table 8. The conditions of the genomic PCR. Temperatures used and number of cycles of each step.

Step	Tem	Time	Cycle
Initial Denaturation	94 °C	2 min	1
Denaturation	94 °C	1 min	35
Annealing	variable	30 Sec	
Extension	72 °C	45 sec	
Final Extension	72 °C	5 Min	1

Table 9. Annealing Tm °C used for each primer pair.

Primer	Annealing Temperature
S1	62 °C
S2	62 °C
S3	60 °C
S4	60 °C
S5	62 °C

2.2.8 Gel electrophoresis for DNA sequencing

For this stage the same conditions used in *Petunia* were applied here. For sequencing total of 50 µl of the PCR product was run on gel using two wells per sample. Of each allele 2 samples were run for sequencing except for the S2 allele which is present in one sample only.

2.2.9 Gel purification and DNA extraction for sequencing

In order to confirm the size of the *S. truncata* alleles identified in this study, two samples were chosen for each allele except for the *S2* allele. For *S1* allele samples W2 and P1 were chosen. For the *S2* allele, sample W1. For the *S3* allele samples W1 and P1 were chosen. 50 µL of each of these samples were applied divided in two wells in the gel. After running the gel at a constant voltage of 98V for 90 minutes, the DNA extraction from the gel centrifuge protocol was followed using the Thermo Scientific GeneJET Gel Extraction Kit (see protocol, section 2.1.12). The extracted DNA concentration was then assessed using NanoDropTM 1000 Spectrophotometer (Thermo Scientific). The labelled samples tubes along with their forward and reverse primers were sent to Source Bioscience for sequencing.

For the *S1* allele, DNA sequenced from two samples. W2 and P1. Sequencing was done using both primer pair. S1 forward primer 5' ACTGCACTGTGGTCGTTAGG 3' and S1 reverse primer 5' GGATTTCCAGCGAAGTCCCA 3'

The S1 primer pair and their intended targets are shown below.

```
product length = 305
Forward primer  1   ACTGCACTGTGGTCGTTAGG  20
Template        41  ....................  60

Reverse primer  1    GGATTTCCAGCGAAGTCCCA  20
Template        345  ....................  326
```

For the *S2* allele, DNA sequenced only from W1 sample as it is the only sample carry this allele. Sequencing performed using both primer pair. S2 forward 5’ TCAACCGTTGTTCGATGCCT 3’ and S2 reverse 5’ AGCCTGTTCTTCCAACCGAG 3’.

The S2 primer pair and their intended targets are shown below.

```
product length = 286
Forward primer  1   TCAACCGTTGTTCGATGCCT  20
Template        50  ....................  69

Reverse primer  1    AGCCTGTTCTTCCAACCGAG  20
Template        335  ....................  316
```

For the *S3* allele, DNA sequenced from two samples W1 and P1. Sequencing performed using both primer pair. *S3* forward 5’ AAGCTTACAACAGTCGGGGG 3’ , and *S3* reverse 5’ TTGGATTGCCGTTTGCATCG 3’.

The *S3* primer pair and their intended targets are shown below.

```
product length = 317
Forward primer  1   AAGCTTACAACAGTCGGGGG  20
Template        107 ....................  126

Reverse primer  1    TTGGATTGCCGTTTGCATCG  20
Template        423  ....................  404
```

2.2.10 DNA sequences analyses

As to confirm the expected sizes of these three identified alleles in this study, all samples DNA sequences were checked using the Biotechnology Information (NCBI) database Global Sequence Alignment Tool . (http://blast.st-va.ncbi.nlm.nih.gov) and also SnapGene software. These analysis findings will be represented in the results chapter.

Chapter 3. Results

3.1 SI analysis of *Petunia* F3 generation

The analysis of this generation focus is on the linkage of the *Sd* allele in terms of its role in breaking down self-incompatibility in *Petunia* and also testing the segregation ratio of both examined alleles. This generation had two families and 24 individuals in total. In this chapter, the results of the F3 generation will be represented with a following discussion.

3.1.1 *Petunia* F3 generation

The genotypic analysis was conducted to identify the alleles potentially associated with the breakdown of self-incompatibility (SI) in the F3 *Petunia* generation. A total of 24 plants from two distinct families were genotyped using allele-specific primers, specifically *PhS3* and *PiSd*.

At this stage, the F3 generation's four families raised from self-seeds of their four parents from the F2 family (N461) analyzed on previous study (chapter 1). These F3 families, their parents' genotype and phenotype are shown in Table 10**.**

Table 10. The F3 *Petunia* generation's 4 families and their parents' genotype and phenotype. Each family was assigned an ID.

F2 Family members	Genotype	Phenotype	F3 Families' IDs
N461.4	(*PiSdPhS3*)	SC	N514
N461.9	(*PiSdPhS3*)	PSC	N515
N461.10	(*PiSdPhS3*)	PSC	N516
N461.12	(*PiSdPhS3)*	SC	N517

The population of these four families of the F3 have 48 plants in total. Twelve seedlings were pricked from each family. Each individual was given ID as illustrated in Table 11 below.

Table 11. The F3 *Petunia* generation. Parents genotype and phenotype are shown in the first column. These raised four families have given 12 plants each, each was given unique identification number.

F3							
Parents' phenotype & genotype	F3 Family ID	F3 Individuals' ID					
N461.4(*PhS3PiSd*) – SC ⊗	N514	N514.1	N514.2	N514.3	N514.4	N514.5	N514.6
		N514.7	N514.8	N514.9	N514.10	N514.11	N514.12
N461.9(*PhS3PiSd*) – PSC ⊗	N515	N415.1	N415.2	N415.3	N415.4	N415.5	N415.6
		N415.7	N415.8	N415.9	N415.10	N415.11	N415.12
N461.10 (*PhS3PiSd*) – PSC ⊗	N516	N416.1	N416.2	N416.3	N416.4	N416.5	N416.6
		N416.7	N416.8	N416.9	N416.10	N416.11	N416.12
N461.12(*PhS3PiSd*) – SC ⊗	N517	N517.1	N517.2	N517.3	N517.4	N517.5	N517.6
		N517.7	N517.8	N517.9	N517.10	N517.11	N517.12

3.1.2 DNA quality checks

Two families of the F3 were analyzed at this stage, N514 and N515. The other two were used in a subsequent MSc research project by another student. These will be commented on later in the discussion section where relevant to provide correlation of results. The extracted DNA from all 24 samples of both families

N514 and N515 was assessed using the NanoDropTM 1000 Spectrophotometer (Thermo Scientific). Two readings of each sample DNA concentration (ng/ul) were recorded as two elution's were done. However, only the first elution was used unless shortage of DNA quantity. This approach was followed also in all coming generations.

The result of the first elution has shown on average concentration for all the samples of N514 family of 27.35 µg/µl, and for the N515 family of 22.55 µg/µl. Other recorded readings are the sample absorbance at 260 nm and 280 nm. The quality of the DNA considered based on the ratio 260/280. Based on this, any ratio of more than 1.8 is accepted as good quality DNA.

3.2 Identification of specific alleles (S-RNases) in the F3 generation

All plants of each F3 family were screened using allele-specific primers (Chapter 2), in order to test the inheritance and segregation of both S-alleles (*PhS3* and *PiSd*) in the N514 and the N515 families. The primers which were used each has its PCR conditions as shown in Chapter 2.

3.2.1 Identification of the *PhS3-RNase* in the N514 family individuals

The PCR products obtained show the presence of the tested *PhS3* allele in all individuals of the N514 *Petunia* family as shown in Figure 13. The presence of the allele was shown by a band when the gel electrophoresis was observed under UV light and seen to be approximately 550 bp as identified by the HyperLadder II (Bioline, UK). The two arrows on the HyperLadder indicate key markers for estimating DNA fragment sizes in base pairs (bp) using the provided size guide. The top arrow corresponds to the 1000 bp marker, while the bottom arrow represents the 300 bp marker. The DNA samples are positioned at approximately 550 bp. The size of the *S3* allele was further checked by sequencing which will be illustrated in a coming section.

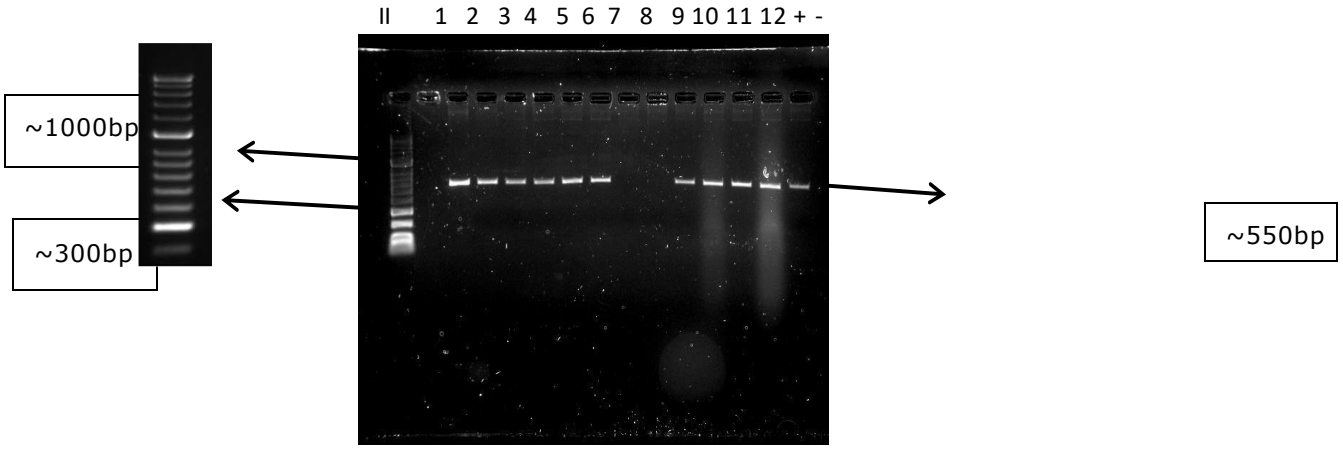


Figure 13. PCR amplification of the N514 *Petunia* family with *PhS3* forward and reverse primers. Lane (II) represents HyperLadder II; (1) N514.1; (2) N514.2; (3) N514.3; (4) N514.4; (5) N514.5; (6) N514.6; (7) N514.7; (8) N514.8; (9) N514.9; (10) N514.10; (11) N514.11; (12) N514.12; (+) positive control and (-) negative control. It is clear that all samples gave + band except 7 and 8 which means they have inherited the *PhS3* allele.

3.2.2 Identification of the *PiSd*-RNase in the N514 family individuals

PiSd-RNase specific primers were used to screen the 12 plants of the N514 family of the F3. As illustrated in Figure 14 total of ten plants out of the twelve found holding the *PiSd*-RNase. The HyperLadder has been annotated showing the main markers used, with the size guild, to identify the size in bp of the DNA. The top arrows show the 1000 bp marker and the bottom arrow shows the 300 bp marker. The DNA lies at ~600 bp.

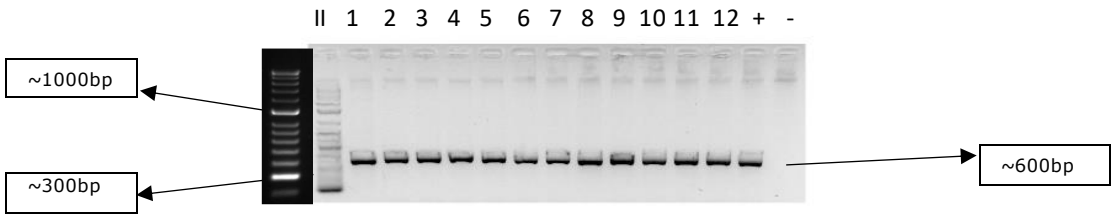


Figure 14. PCR amplification of the N514 *Petunia* 12 samples with *PiSd* forward and reverse primers. Lane (II) represents HyperLadder II; (1) N514.1; (2) N514.2; (3) N514.3; (4) N514.4; (5) N514.5; (6) N514.6; (7) N514.7; (8) N514.8; (9) N514.9; (10) N514.10; (11) N514.11; (12) N514.12; (+) positive control and (-) negative control. In this family all samples gave positive band for the *PiSd* allele.

3.2.3 *S*-alleles genotype summary for *Petunia* N514 family

All individual plants of the N514 family have inherited the *P. inflata Sd* allele, meanwhile ten individuals out of twelve carry the *P. hybrida S3* allele (Table 12). The segregation ratio in this family is found to be as 2:10.

Table 12. Shows the presence and/or absence of both tested S-alleles which are represented + or – respectively.

Plant ID	*PhS3*	*PiSd*	Genotype
N514.1	+	+	(*PhS3PiSd*)
N514.2	+	+	(*PhS3PiSd*)
N514.3	+	+	(*PhS3PiSd*)
N514.4	+	+	(*PhS3PiSd*)
N514.5	+	+	(*PhS3PiSd*)
N514.6	+	+	(*PhS3PiSd*)
N514.7	-	+	(*PiSdPiSd*)
N514.8	-	+	(*PiSdPiSd*)
N514.9	+	+	(*PhS3PiSd*)
N514.10	+	+	(*PhS3PiSd*)
N514.11	+	+	(*PhS3PiSd*)
N514.12	+	+	(*PhS3PiSd*)

3.2.4 Identification of the *PhS3*-RNase in the N515 family individuals

All twelve plants were screened for the presence/absence of the *PhS3*-RNase using the specific primers. As can be seen in Figure 15 of the obtained image which shows the presence of the *PhS3* allele in five individuals. The Hyper Ladder has been annotated with arrows showing two main markers use, from which, along with the size guild, the size in bp of the DNA samples can be estimated. The top arrow shows 1000 bp while the bottom arrow shows 300 bp. The DNA lie at ~550 bp.

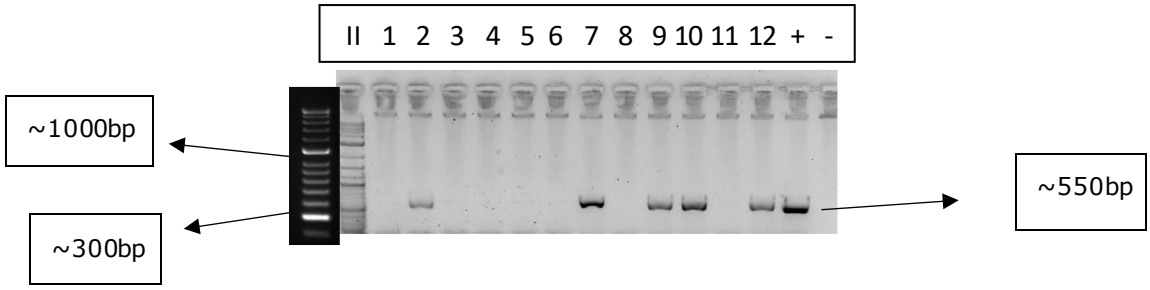


Figure 15. PCR amplification of the N515 *Petunia* 12 samples testing for the *PhS3* allele. Lane (II) represents HyperLadder II; (1) N515.1; (2) N515.2; (3) N515.3; (4) N515.4; (5) N515.5; (6) N515.6; (7) N515.7; (8) N515.8; (9) N515.9; (10) N515.10; (11) N515.11; (12) N515.12; (+) positive control and (-) negative control. Samples 2,7,9,10 and 12 show a band so all have the *PhS3* allele.

3.2.5 Identification of the *PiSd*-RNase in the N515 family individuals

As shown in the Figure 16 below, eight plants have inherited the *PiSd* allele in this family. The two arrows indicate main markers on the HyperLadder from which, with the help of the size guild provided with the HyperLadder, the size in bp can be estimated; the top arrows shows the marker at 1000 bp and the bottom show the marker at 300 bp. The DNA samples lie at ~600 bp.

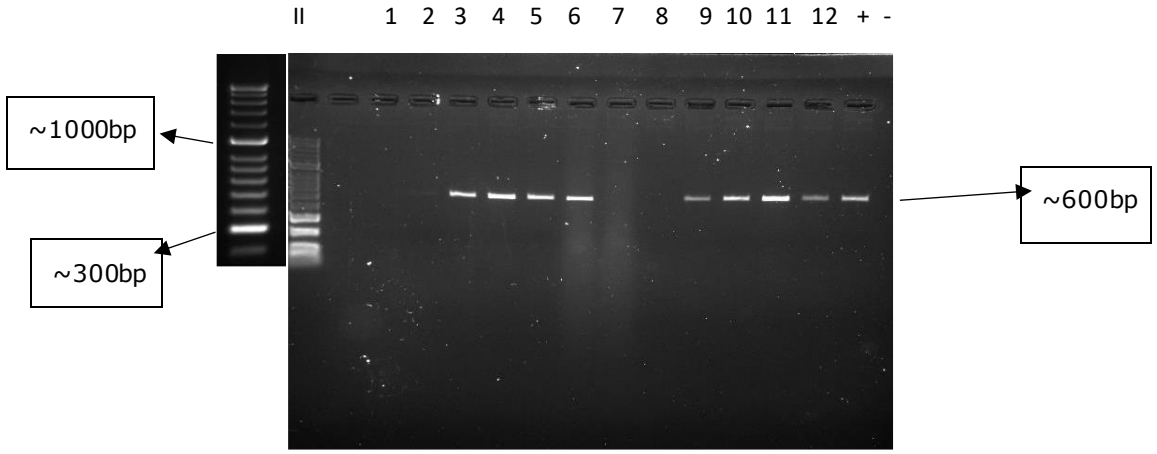


Figure 16. PCR amplification of the N515 *Petunia* 12 samples with *PiSd* forward and reverse primers. Lane (II) represents HyperLadder II; (1) N515.1; (2) N515.2; (3) N515.3; (4) N515.4; (5) N515.5; (6) N515.6; (7) N515.7; (8) N515.8; (9) N515.9; (10) N515.10; (11) N515.11; (12) N515.12; (+) positive control and (-) negative control. Samples 3,4,5,6,9,10,11 and 12 show positive band which means they carry the *PiSd* allele.

3.2.6 *Petunia S*-alleles sequencing analyses; confirmation of size and identity

As to confirm the expected sizes of *Petunia* alleles in this study, all samples DNA sequences for both the *S3* and *Sd* alleles were checked using the Biotechnology Information (NCBI) database Global Sequence Alignment Tool . (http://blast.st-va.ncbi.nlm.nih.gov) and also the SnapGene software to illustrate the primers positions in the targeted sequence. As of assurance and as described before two samples DNA per allele were sequenced. Forward and reverse sequences were obtained per sample.

In regard to the first allele *PhS3* a reference made to its data published in the NCBI database. The DNA sequence obtained at this study was found 100% identical to the subject *PhS3* sequence using its concession number >AJ271065.1 as shown below. Also this confirms the expected product size that

illustrated in the gels above of ~550 bp

```
>Petunia hybrida partial mRNA for S3L-ribonuclease (s-RNase gene)
Sequence ID: AJ271065.1 Length: 746
Range 1: 249 to 641

Score:726 bits(393), Expect:0.0,
Identities:393/393(100%),  Gaps:0/393(0%), Strand: Plus/Plus

Query  83   GGGTAAGATGCTCAATGATCTTGACAAACACTGGATTCAGTTGAAGTATAAAGAAGCCTA
142
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  249  GGGTAAGATGCTCAATGATCTTGACAAACACTGGATTCAGTTGAAGTATAAAGAAGCCTA
308

Query  143  TGCTCGACGGGAGCAACCGACATGGAAATATCAATATCAAAAGCATGGATCCTGTTGCCA
202
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  309  TGCTCGACGGGAGCAACCGACATGGAAATATCAATATCAAAAGCATGGATCCTGTTGCCA
368

Query  203  GACAAAATACAAGCAAATCCCGTATTTTAGTTTAGCCTTGCGCTTGAAAGATAGATTTGA
262
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  369  GACAAAATACAAGCAAATCCCGTATTTTAGTTTAGCCTTGCGCTTGAAAGATAGATTTGA
428

Query  263  CCTTTTGACAACTCTGCGAACTCATCACATTGTCCCTGGATCAAGTTATACATTTGATGA
322
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  429  CCTTTTGACAACTCTGCGAACTCATCACATTGTCCCTGGATCAAGTTATACATTTGATGA
488

Query  323  TATCTTTGATGCCGTCAAGACAGTTACTCAAATGAATCCTGATCTCAAGTGCACTGAAGT
382
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  489  TATCTTTGATGCCGTCAAGACAGTTACTCAAATGAATCCTGATCTCAAGTGCACTGAAGT
548

Query  383  GACTAAAGGAACACAGGAACTAGATGAGATAGGCATATGTTTCACTCCTAAAGCAGATAA
442
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  549  GACTAAAGGAACACAGGAACTAGATGAGATAGGCATATGTTTCACTCCTAAAGCAGATAA
608

Query  443  AATGTTTCCCTGTCGTCAAAGTGATACATGCGA  475
            ||||||||||||||||||||||||||||||||
Sbjct  609  AATGTTTCCCTGTCGTCAAAGTGATACATGCGA  641
```

For the *PiSd* allele a reference made to its sequence found on the cited thesis and also it shows 100% identical as shown below. Also the expected size of ~600bp is the right product size.

```
Sequence ID: Query_5543513 Length: 607
Range 1: 64 to 519

Score:843 bits(456), Expect:0.0,
Identities:456/456(100%),  Gaps:0/456(0%), Strand: Plus/Plus

Query  70   GGATGATAACAAAAAGAATAATTTGTATAAACGTTGGCCTGACTTGACCACCGATGAAGC
129
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64   GGATGATAACAAAAAGAATAATTTGTATAAACGTTGGCCTGACTTGACCACCGATGAAGC
123

Query  130  TGTATGTTTGGAAAAGCAAGATTTCTGGAGACATGAGTATAATAAGCATGGAACGTGTTG
189
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124  TGTATGTTTGGAAAAGCAAGATTTCTGGAGACATGAGTATAATAAGCATGGAACGTGTTG
183

Query  190  TTTAGGTAGCTACAATGAAGATCAATACTTTCATTTAGCCATGGCCCTAAAAGACAAGTA
249
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184  TTTAGGTAGCTACAATGAAGATCAATACTTTCATTTAGCCATGGCCCTAAAAGACAAGTA
243

Query  250  TGATCTTCTAACATCTTTGAGAAAGCATGGAATTAGTCCTGGCTGGCAATATACCGTTCA
309
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244  TGATCTTCTAACATCTTTGAGAAAGCATGGAATTAGTCCTGGCTGGCAATATACCGTTCA
303

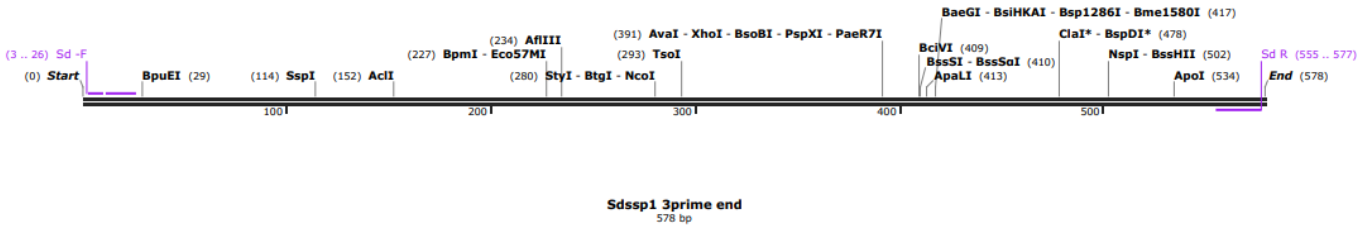
Query  310  GAAAATCAATAGCACCATCAAGACAATAACTCGAGGGTATCCTAACCTCTCGTGCACTAA
369
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304  GAAAATCAATAGCACCATCAAGACAATAACTCGAGGGTATCCTAACCTCTCGTGCACTAA
363

Query  370  GAAAATGGAACTATGGGAGATAGGAATATGTTTCGACTCGACGGTACAAAAGGTGATCGA
429
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364  GAAAATGGAACTATGGGAGATAGGAATATGTTTCGACTCGACGGTACAAAAGGTGATCGA
423

Query  430  TTGTCCTCATCCTAAGACATGCGCGCCGATGAAAATTATGTTTCCATAAATAAAATTTCA
489
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424  TTGTCCTCATCCTAAGACATGCGCGCCGATGAAAATTATGTTTCCATAAATAAAATTTCA
483

Query  490  TTTTCTCTCTTATGTAAACCGCTACGATAATCAGTA  525
            ||||||||||||||||||||||||||||||||||||
Sbjct  484  TTTTCTCTCTTATGTAAACCGCTACGATAATCAGTA  519
```

The *Sd* sequence map was created using SnapGene software as shown below. The forward and reverse primers are highlighted and also it shows the size of the obtained sequence.



3.2.7 S-alleles genotype summary of *Petunia* N515 family

The *P. hybrida S3* allele is found present in 6 individuals while the *P. inflata Sd* allele is inherited by 8 out of the total 12 N515 family's members as shown in Table 13 below. As a result, the segregation ratio in this family is 4:4:2.

Table 13. Shows the presence and/or absence of both tested S-alleles which are represented + or – respectively.

Plant ID	*PhS3*	*PiSd*	Genotype
N515.1	-	-	DNA quality
N515.2	+	-	(*PhS3PhS3*)
N515.3	-	+	(*PiSdPiSd*)
N515.4	-	+	(*PiSdPiSd*)
N515.5	-	+	(*PiSdPiSd*)
N515.6	-	+	(*PiSdPiSd*)
N515.7	+	-	(*PhS3PhS3*)
N515.8	-	-	DNA quality
N515.9	+	+	(*PhS3PiSd*)
N515.10	+	+	(*PhS3PiSd*)
N515.11	+	+	(*PhS3PiSd*)
N515.12	+	+	(*PhS3PiSd*)

3.3 F3 phenotyping; pollination and seed sets

Pod sizes can be one of the differentiation factors for phenotyping. Self-pollination can result in a small size pod which indicates no seeds are produced, suggesting self-incompatibility or a large size pod which indicates fully self-compatible or semi-compatible phenotypes. An intermediate size pod shows pseudo-self-compatible phenotype.

3.3.1 Phenotyping of the N514 family members; self-pollination

As result of the self-pollinations, it is found that all members are self-compatible (SC) Table 14. These all carry the *PiSd* allele (Table 14). The highest seed number per capsule is recorded in the individual number eight which has *PiSdPiSd* genotype.

Table 14. Individuals phenotypes of the N514 family plants. Seed production is high in almost all individuals.

Plant ID	Number of Capsules	Total Weight (g)	Weight per Capsule	Approximate number of seeds per capsule	Phenotype
N514.1	7	0.17	0.024	400	SC
N514.2	3	0.061	0.020	380	SC
N514.3	4	0.083	0.020	380	SC
N514.4	4	0.085	0.021	390	SC
N514.5	13	0.156	0.012	200	SC
N514.6	1	0.015	0.015	250	SC
N514.7	10	0.276	0.027	450	SC
N514.8	8	0.226	0.028	460	SC
N514.9	13	0.225	0.017	285	SC

N514.10	9	0.136	0.015	250	SC
N514.11	13	0.173	0.013	215	SC
N514.12	1	0.009	0.009	150	SC

3.3.2 Phenotyping of the N515 family members; self-pollination

It can be seen in Table 15 below, all members of this family are found self-compatible (SC) except individual number three which is PSC and member four which shows SI phenotype although number of pollinations achieved is low.

Table 15. Individuals phenotypes of the N515 family plants. Seed production here is noted with variable rates showing differences between PSC and SC phenotypes.

Plant ID	Number of Capsules	Total Weight (g)	Weight Per Capsule	Approximate number of seeds per capsule	Phenotype
N515.1	10	0.193	0.019	315	SC
N515.2	3	0.054	0.018	300	SC
N515.3	6	0.023	0.003	50	PSC
N515.4	3	-	-	5	SI
N515.5	6	0.065	0.010	160	SC
N515.6	7	0.05	0.0071	115	SC
N515.7	11	0.183	0.016	250	SC
N515.8	10	0.124	0.0124	200	SC
N515.9	6	0.125	0.020	330	SC
N515.10	13	0.301	0.023	350	SC
N515.11	3	0.018	0.006	100	SC
N515.12	8	0.052	0.006	100	SC

3.3.3 Summary of the F3 population genotypes and phenotypes

The genotypic and phenotypic analyses were conducted to identify the alleles potentially associated with the breakdown of self-incompatibility (SI) in the F3 *Petunia* generation. A total of 24 plants from two distinct families were genotyped using allele-specific primers, specifically *PhS3* and *PiSd*. A summery of results of the F3 generation analyses is illustrated in Table 16 below.

Table 16. The F3 population summery of individuals' genotypes and phenotypes. All plants that inherited the *Sd* allele are self-compatible (highlighted in green) except two individuals highlighted in blue.

F3 N514 family	Genotype	Phenotype	F3 N515 Family	Genotype	Phenotype
N514.1	*(PhS3PiSd)*	SC	N515.1	*DNA quality*	SC
N514.2	*(PhS3PiSd)*	SC	N515.2	*(PhS3PhS3)*	SC
N514.3	*(PhS3PiSd)*	SC	N515.3	*(PiSdPiSd)*	PSC
N514.4	*(PhS3PiSd)*	SC	N515.4	*(PiSdPiSd)*	SI
N514.5	*(PhS3PiSd)*	SC	N515.5	*(PiSdPiSd)*	SC
N514.6	*(PhS3PiSd)*	SC	N515.6	*(PiSdPiSd)*	SC
N514.7	*(PiSdPiSd)*	SC	N515.7	*(PhS3PhS3)*	SC
N514.8	*(PiSdPiSd)*	SC	N515.8	*DNA quality*	SC
N514.9	*(PhS3PiSd)*	SC	N515.9	*(PhS3PiSd)*	SC
N514.10	*(PhS3PiSd)*	SC	N515.10	*(PhS3PiSd)*	SC
N514.11	*(PhS3PiSd)*	SC	N515.11	*(PhS3PiSd)*	SC
N514.12	*(PhS3PiSd)*	SC	N515.12	*(PhS3PiSd)*	SC

3.3.4 The segregation of the two tested specific *S*-alleles (*PhS3* and *PiSd*) in the F3 generation

The result of the F3 generation phenotypes and genotypes indicates that almost all population individuals which inherited the *PiSd* allele are SC. The segregation ratios found to be 2:10 and 4:8 for N514 and N515 families respectively.

The genotypes and phenotypes analyses of the plants included in the F3 generation in this study are listed in Table 16. The genotypic analysis performed on N514 revealed 2 plants with the genotype *SdSd* and 10 for the genotype *SdS3*. For the N515 the genotypic analysis showed 4 plants each for genotype *SdSd* and 4 of *S3Sd* genotype as 2 plants DNA was excluded. The prediction was 1:2:1 (*SdSd*: *SdS3:S3S3*) for both families. Table 16 shows that the ratios are closer to this. Distortion might result from the SI systems still functioning during the initial self-pollination, which could prevent some pollen from successfully fertilizing the ovum. This supports the hypothesis that if the *Sd* allele were associated with compatibility, the initial self-pollination would not produce any offspring lacking the *Sd* allele, as observed. Enhanced predictions and validation of the *Sd* allele's role in self-compatibility (SC) can be attained by employing a larger sample size across both families. Although the preliminary self-pollination experiment provides further insights into the hypothesis, it is posited that the *Sd* allele confers self-compatibility. Therefore, progeny resulting from the self-

pollination of the parental plants are expected to inherit at least one *Sd* allele. This expectation is corroborated by the results presented in Table 16, which supports the hypothesis that the *Sd* allele is associated with self-compatibility. Therefore, this is explored further in this study by generating the F4 and F5 generations which will be presented in the coming chapter.

3.3.5 Chi square analyses of segregation ratio

In the F3 generation, the parental plant genotypes give rise to two families, each possessing two alleles. In both families (N514 and N515), the potential alleles for the progeny were *S3* and *Sd*. Self-pollination of N514 and N515 was predicted to yield offspring with a genotypic ratio of 1:2:1 (*SdSd:SdS3:S3S3*), as illustrated by the Punnett square in Table 17.

Table 17. The Punnett square illustrates the expected 1:2:1 (*SdSd:SdS3:S3S3*) genotypic ratio resulting from the self-pollination of heterozygous plants, which produced the seeds used in this study. It represents the N461 family Punnett square, predicting one homozygous genotype for both the *S3* and Sd alleles and two heterozygous genotypes for the alleles.

N461.4 and N461.9 (*PhS3PiSd* x *PhS3PiSd)*	*S3*	*Sd*
S3	*S3S3*	*S3Sd*
Sd	*S3Sd*	SdSd

The data presented in Table 15 above indicates that ten plants from family N514 possess the *PhS3* allele, whereas only five plants from family N515 exhibit this allele. Based on theoretical expectations, the segregation ratio should conform to

a 1:2:1 Mendelian pattern, as previously mentioned. Given a total of 24 plants, the expected ratio for this experiment would therefore be 6:12:6. However, two plants from family N515 were excluded from the analyses due to DNA degradation.

According to the Chi-square (χ^2) statistical test and referencing the χ^2 distribution table, the critical value for χ^2 at a significance level of 0.05 with 2 degrees of freedom ($\chi^2(2, p < 0.05)$) is 5.99. The observed χ^2 value of 0.999 indicates that there is no significant deviation between the observed segregation ratio and the expected ratio, as the observed value falls below the critical threshold. The segregation ratio was determined to be 6:14:2, as presented in Table 18.

Table 18. The chi squared test comparing the expected ratio (6:12:6) for the self-pollination and the observed ratios of the F3 generation as (6:14:2). The results show that the ratios are not significantly different.

N514 and N515	O	E	O-E	(O-E)2	$(O-E)_2/E$
SdSd	6	6	0	0	0
SdS3	14	12	2	4	0.333
S3S3	2	6	-4	4	0.666
$\chi_2=\Sigma(O-E)_2/E$			0.999		

3.3.6 The novel *Sd-RNase* exhibit allelic diversity and intraspecific sequence polymorphism

The phylogenetic analyses using the tools and methods described in Chapter 2 revealed the evolutionary aspects of the novel *Sd-RNase* and its interrelationship within the genus *Petunia* and more broadly within the *Solanaceae* family.

The *S-RNases Petunia* phylogeny tree (Figure 17) below shows that our novel *Sd-RNase* is closely related to its ancestral parent *P. integrifolia SI-RNases*. It also appears that the *Sd-RNase* shows similarity with the non-*S-RNase* (rnx2), however; this is referred to as S-like-*RNase* which also been identified in many other plant species (Kao and McCubbin 1996; Hugot *et al*., 2002). Although these S-like-*RNases* share domain structure with real *S-RNases*, they have no function in the SI reaction. The phylogenetic analyses also show the *P. hybrida S3-RNase* investigated in this study. It is clear that *S-RNase* is identical to the *SB2-RNase*. These two identical alleles were reported in two different stocks at different labs (Robbins *et al*., 2000 and Entani *et al*., 1999).

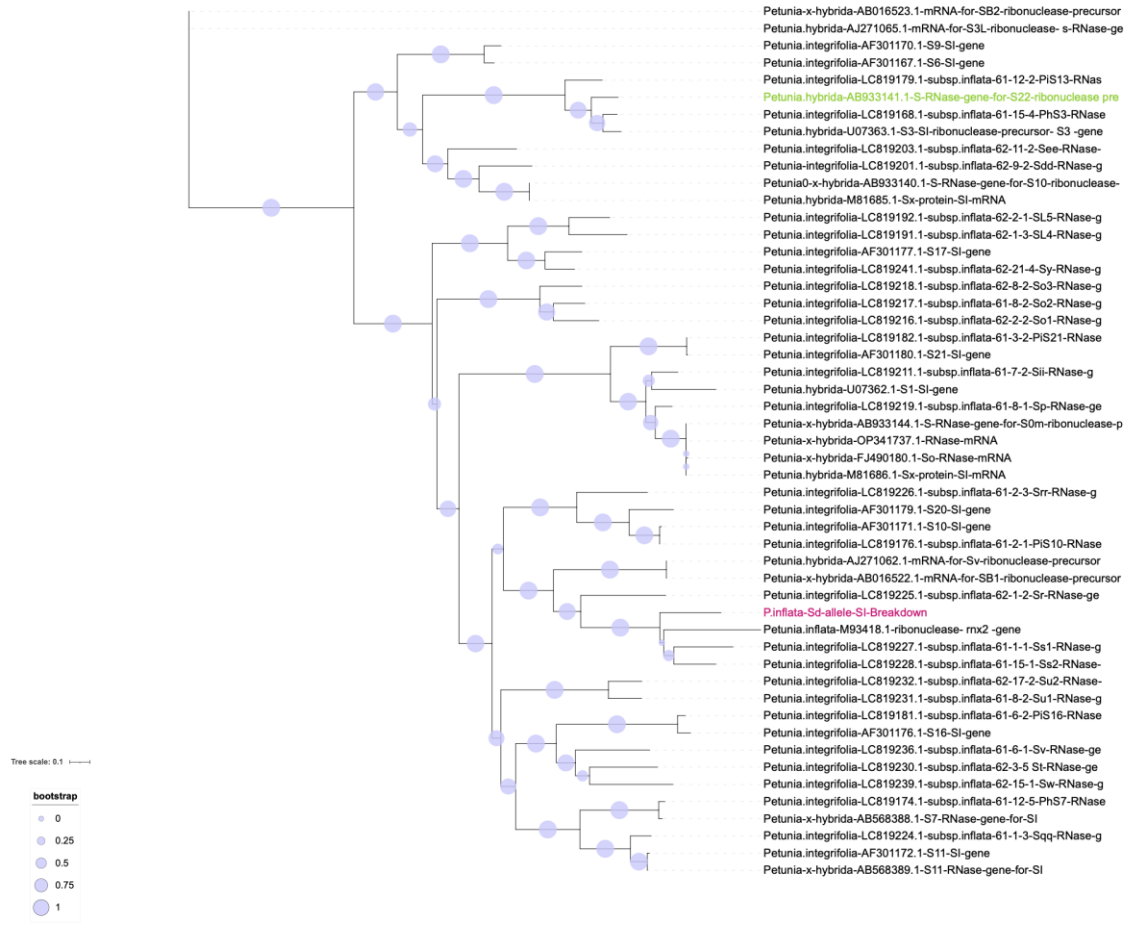


Figure 17. Maximum likelihood phylogeny of *S-RNase* genes within the *Petunia* genus. Interrelationships among *Petunia* previously identified *S-RNases,* along with the current study novel *Sd-RNase,* and the *P. hybrida S3-RNase*. Tip labels list species binomials, GenBank accession IDs and gene names (if given). Numbers at nodes indicate branch support according to the nonparametric SH-like branch test. The evolutionary distance between the *Petunia* alleles *Sd* and *S3* reveals the allelic diversity of the SI genes across the genus.

Following our phylogenetic analyses of the novel *Sd-RNase* within its genus *Petunia*, it reveals an interest to expand phylogenetic analyses to cover a more comprehensive sample of *S-RNases* in the family *Solanaceae* as shown below. This phylogenetic tree (Figure 18) is obtained from the alignment involving the

sequences of the novel *PiSd-RNase* and all *Solanaceous S-RNases* retrieved from the NCBI database (See the Supplementary table S2).

The resulting phylogenetic tree below shows that the *Petunia* alleles are found in all clades. In particular, our novel *Sd-RNase* shows similarity to *N. alata S6-RNase* and *L. australe S1-RNase*. This shows consistency with previous observations of trans-specific polymorphism, a key observation focused on the age of S-allelic polymorphism and their evolutionary origin (Ioerger *et al*. 1990).

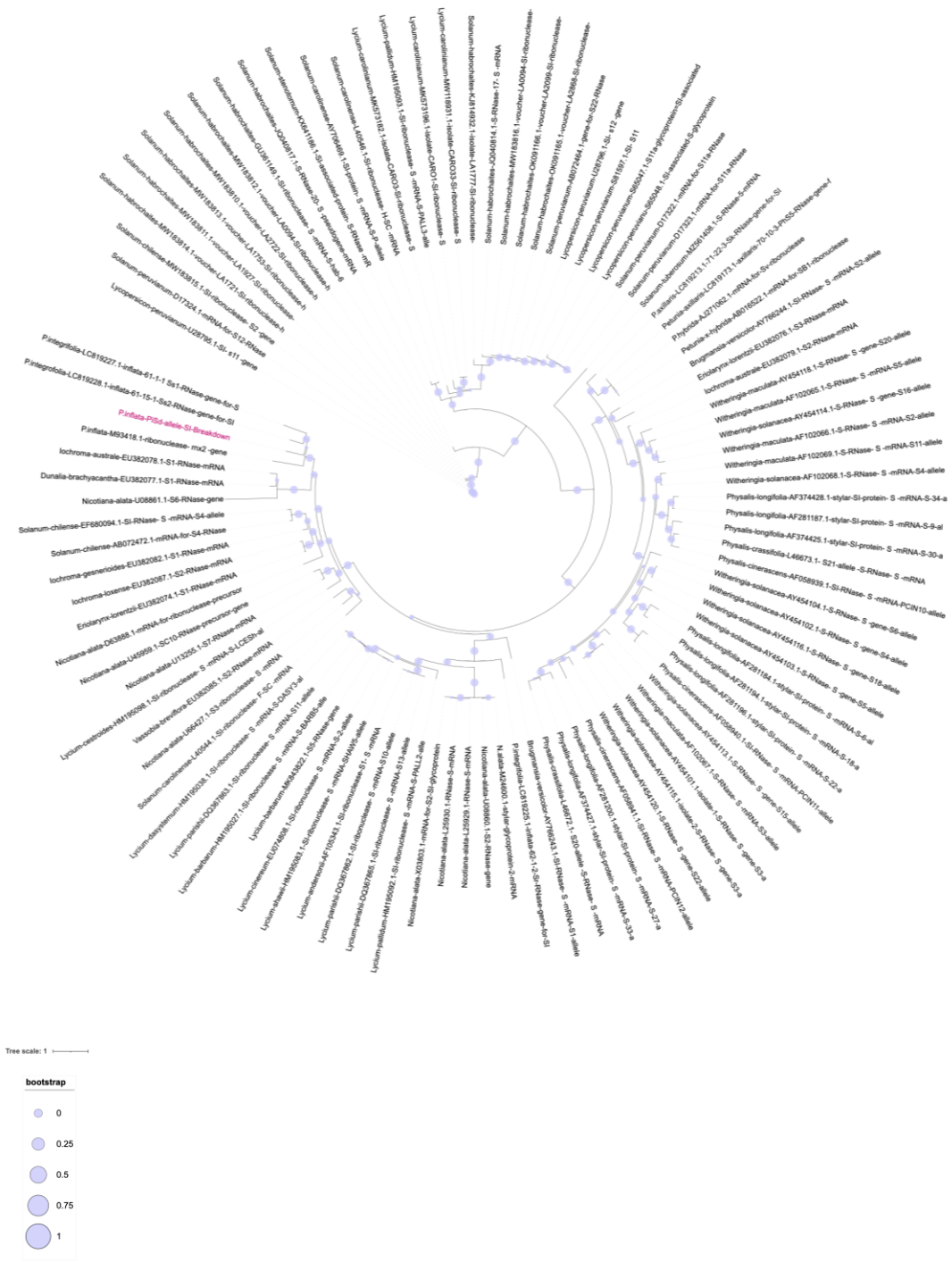


Figure 18. Maximum likelihood phylogeny of *Solanaceae S-RNase* genes. The phylogenetic relationships were inferred using the sequences of our novel *P. inflata Sd-RNase*. Tip labels list species binomials, GenBank accession IDs and gene names (if given). Numbers at nodes indicate branch support according to the nonparametric SH-

like branch test. All *S-RNase* within the family show they are SI functional. As highlighted in the tree, the novel *PiSd-RNase* allele groups with *S-RNases* from *Nicotiana alata*, *Dunalia brachyacantha*, *Lochroma australe* and two of its parents, *Petunia integrifolia*.

The amino acid MSA revealed that the novel *PiSd-RNase* appears to be most similar to other *Solanaceae* species *RNases* such as *N. alata S9-RNase*. The *Sd* amino acid sequence level of similarity observed in the alignment below is consistent with earlier observations made for *Solanaceous S-RNases* (Ioerger *et al*., 1990; McCubbin and Kao 2000). These are known to have conserved regions C1, C2, C3, C4 and C5.

Figure 19. Amino acid MSA of the deduced 10 amino acid sequences. Alignment shows the *Petunia inflata* novel *Sd-RNase*, *P. hybrida PhS3-RNase*, *Nicotiana. alata S6-RNase*, *Dunalia brachyacantha S1-RNase*, *Lochroma australe S1-RNase*, and also *S-RNases* from *P. integrifolia*. The hypervariable regions (HVa and HVb) and conserved regions (C3–C5) are boxed. Gaps in the alignment are indicated by (−). The conserved C3 region contains highly conserved histidine residues that are involved in the ribonuclease activity of *S-RNases*. Conserved amino acids across the 10 sequences are marked with asterisks (*).

3.4 Discussion

As shown in the summary Table 16 above, almost all of the F3 population's plants that inherited the *PiSd* allele are self-compatible as confirmed by the production of seeds. The F3 population phenotyping and genotyping analyses in the first stage of this study indicate that, there is linkage between the genotype of this *Petunia* population and their self-compatibility. These outcomes provide an indication of the validity of the research hypothesis. That is the *Sd* allele is the allele that conveys self-compatibility phenotype. That result is consistent with the past Ph.D. project by Sharef (2017) here at the UoN (Chapter 1). In that study also almost all plants which carried the *PiSd* allele become SC in the F1 and the F2 generations. It was confirmed that the *PiSd* allele lost its function while all other tested alleles (*PiS3, PiSk1, PhSv, PhS3*) are all maintained in stable self-incompatibility. This confirms the relationship between the *PiSd* allele and self-compatibility. Further, the F2 population studied by L. Lesley (2017, UoN. Unpublished) see section (1.9) chapter 1, show support of the findings. In addition, in the MSc research by A. Coban (2019 UoN, unpublished), show consistent results. In her study, A. Coban used two of my F3 generation families (N516 and N517).

As shown on the F3 generation genotyping results (section 3.1.12 above), 20 plants out of 24 have inherited the *PiSd* allele. All F3 generation plants of the

N514 family have inherited the *PiSd* allele while in the N515 family 8 plants have carried the *PiSd* allele out of 11. The segregation ratio of the three possible genotypes *PiSdPiSd, PhS3PiSd, PhS3PhS3* for the 24 of the F3 generation plants should be 6:12:6. However, the segregation ratio here is found to be 6:14:2 (22 in total as plant N515.1 DNA degraded). Interpretation of this is that, in a such a small F population it is expected that the segregation ratio is different. This also can be attributed to the genetic preference of the alleles. This result is consistent with the past Ph.D. project by Sharef (2017) in which 16 plants out of 24 inherited the *PiSd* allele in the F2 generation. The segregation ratio for the *PiSd* allele was twice of the *PhS3*. In addition, in the MSc study by Cuban and Robbins (2019), in which 24 individuals were tested, the segregation ratio was also different (9:15:0).

In addition, in the F3 generation six plants N514.7, N514.8 and N515.3, N515.4, N515.5 and N515.6 are identified as *PiSd* homozygous. However, plants N515.3 and N515.4 are PSC and SI respectively whereas the other four homozygous are SC. This was also the case in the past Ph.D. project by Sharef (2017) where 3 plants out of 16 inherited the *PiSd* allele but were self-incompatible. It was interesting plan here to sow this plant self seeds along with the *Sd* homozygous (SC) and cross pollinate them. It was planned to use the SI plant as pollen donor in the first experiment and then to use the SC plant as pollen donor in the second. The aim of this is to identify the loss of function whether it is in the

pollen part or in the style part and to confirm further if the SC phenotype is linked with the *Sd* allele. Unfortunately, this individual plant very limited seed number was sown but lost later because of the Covid-19 pandemic lockdown where there was no access to the research facilities.

As of the study hypothesis, it can be thought that the *SdSd* genotype can result in a higher level of self-compatibility than the *S3Sd* genotype, however, the few homozygous plants in the F3 population, show similar compatibility with others of the *S3Sd* genotype. This suggests that the level of compatibility is not correlated to the number of the S-alleles present. In addition, the *S3* allele neither helps nor hinders the capability of the *Sd* allele to convey self-compatibility. This aspect of SI will be discussed more in the F4 *Petunia* generation result section.

Our phylogenetic analyses provide further characterization of *S-genes*. These provide insights into their evolutionary origins (Allen and Hiscock, 2008). Such advances of the molecular phylogenetic approaches have facilitated the construction of well-defined phylogenetic trees. This has been instrumental in identifying the most basal lineages within the angiosperms. For example, the use of such phylogenetic approaches by Igic and Kohn (2001) has led to propose that GSI represents the ancestral origin in approximately 75% of eudicots, and

that the *RNase*-based SI system likely constitutes the ancestral form of SI within most dicotyledonous lineages.

A general pattern emerging from our as well as others' is that *S-RNases* forming of clades is not restricted to individual species. Instead, interspecific clades indicative of sequence similarity across species boundaries are commonly observed as shown in the trees above. Original findings by Ioerger *et al*., (1990) of *S*-locus in three *Solanaceae* species has revealed greater similarity among alleles across species than within species. These results strongly support the hypothesis that polymorphism at the *S*-locus predates species divergence within the *Solanaceae*. The extraordinary allelic diversity observed at this locus has been maintained by long-term balancing selection.

Additionally, our phylogenetic analyses reveal the presence of trans-generic clades, particularly among *Petunia* and *Nicotiana*. This suggests an extensive diversification and retention of *S-alleles* across these genera. In contrast, limited trans-generic lineage sharing demonstrated between *Witheringia* and *Physalis* implies decreased allelic diversity across genera. This has been suggested to be due to historical population bottlenecks that reduced *S*-allele diversity (Richman *et al*., 1996). For the genus *Witheringia,* limited trans-generic allele sharing is thought to reflect analogous demographic constraints (Stone and Pierce, 2005).

Chapter 4. Results of SI analysis of the *Petunia* F4 and F5 generations

The aim at this analyses stage is to further investigate the behavior of the novel *Sd* allele in conveying the SC haplotype through generations. It is also aimed to test the segregation of both tested alleles *Sd* and *S3*. The results of these analyses will be represented hereafter.

4.1 *Petunia* F4 generation

At the second research stage, the F4 generation established with four families. These are from self-seeds of their F3 generation parents analyzed on the previous phase (Section 3.1) They were chosen based on the highest seed production plus the interest of more studies based on their genotype. These F4 families, their F3 parents' phenotype and genotype plus their identification numbers are shown in the Table 19 below.

Table 19. The F4 *Petunia* generation's 4 families, their parents' genotype, and phenotype. Their given IDs are shown as well.

F3 Family members	Genotype	Phenotype	F4 Families' IDs
N514.1	(*PhS3PiSd*)	SC	N520
N514.2	(*PhS3PiSd*)	SC	N521
N515.2	(*PhS3PhS3*)	SC	N522
N515.7	(*PhS3PhS3*)	SC	N523

The F4 population have 4 families with a total of 40 plants. Their parents' sown seeds have generated 10 healthy plants per family. Each of these 4 families' individuals was given ID as illustrated in Table 20 below.

Table 20. The F4 *Petunia* generation. Parents' genotype and phenotype are shown in the first column. These raised 4 families of the F4 generation each comprising 10 individual plants the F4 each with its unique identification number.

F4							
Parents' Phenotype & Genotype	F4 Family ID	F4 Individuals' ID					
N514.1 (*PhS3PiSd*)- SC ⊗	N520	N520.1	N520.2	N520.3	N520.4	N520.5	N520.6
		N520.7	N520.8	N520.9	N520.10		
N514.2 (*PhS3PiSd*)–SC ⊗	N521	N521.1	N521.2	N521.3	N521.4	N521.5	N521.6
		N521.7	N521.8	N521.9	N521.10		
N515.2 (*PhS3PhS3*)-SC ⊗	N522	N522.1	N522.2	N522.3	N522.4	N522.5	N522.6
		N522.7	N522.8	N522.9	N522.10		
N515.7 (*PhS3PhS3*)-SC ⊗	N523	N523.1	N523.2	N523.3	N523.4	N523.5	N523.6
		N523.7	N523.8	N523.9	N523.10		

4.1.1 Identification of specific alleles (*S-RNases*) in the F4 generation

The aim here was to further analyze this generation phenotypes and genotypes. The total population of 40 plants was to be screened using the S-alleles specific primers as shown in Chapter 2. However, it was unfortunate that this research phase was significantly affected by the Covid-19 pandemic lockdown. Regulations in place had ceased access to the research facilities. As a result, the whole F4 population at the glasshouse was lost. As access to the glasshouse was eased, the F4 population was grown again from seeds stocks. This had severely delayed the research phases. Consequently, much more focus was aimed at F4 population phenotyping approaches at the glasshouse as there was limited access to the facilities. As a result, the only data generated regarding the F4 population are the phenotyping results. The genotyping of this population was not possible as the tissue samples stored in the lab were lost as well because of freezer fault during lockdown which caused them melting and rotting. Thus, the priority was given to the phenotyping route which worked well despite very limited access to the glasshouse. Particularly, a significant effort was made to achieve all possible pollinations and capsules harvesting of the whole F4 population of 40 plants before we experienced another issue of glasshouse ventilators failure during a very hot weekend resulting in the death of the whole population of 40 individuals.

4.1.2 F4 generation phenotyping; pollination and seed sets

A limited glasshouse access was permitted as of Covid-19 pandemic lockdowns. Therefore, as shown in the following sections there is limited numbers of self-pollinations that were carried out which can affect the reliability of the phenotypic analysis.

4.1.3 Phenotyping of the N520 family members; self-pollination

Self-pollination of this family plants show that all members are self-compatible as shown in Table 21 below. The highest seed number per capsule is produced in member number 7 and 8. It is notable that there is a significant variation of number of seeds produced (220-620). However, the pollination number was very small as the ideal target should be 10 self-pollinations.

Table 21. Individuals' phenotype of N520 family. All individuals produced large numbers of seeds as a result they show SC phenotype.

N520	Number of Capsules	Total Weight (g)	Weight Per Capsule	Approximate number of seeds per capsule	Phenotype
N520.1	2	0.046	0.023	390	SC
N520.2	2	0.027	0.013	220	SC
N520.3	3	0.087	0.029	400	SC
N520.4	1	0.029	0.029	480	SC
N520.5	1	0.030	0.030	510	SC
N520.6	1	0.016	0.016	280	SC
N520.7	1	0.038	0.038	620	SC
N520.8	1	0.038	0.038	620	SC
N520.9	3	0.063	0.021	350	SC
N520.10	1	0.018	0.018	300	SC

4.1.4 Phenotyping of the N521 family members; self-pollination

It is again here there is a variation in the number of seeds produced (200-650).

All individuals show SC phenotype (Table 22).

Table 22. Individuals' phenotype of N521 family. Individual number 10 was dropped because of no flower's formation.

N521	Number of Capsules	Total Weight (g)	Weight Per Capsule	Approximate number of seeds per capsule.	Phenotype
N521.1	2	0.057	0.028	480	SC
N521.2	1	0.013	0.013	200	SC
N521.3	1	0.021	0.021	350	SC
N521.4	2	0.031	0.015	250	SC
N521.5	1	0.028	0.028	480	SC
N521.6	1	0.035	0.035	590	SC
N521.7	1	0.039	0.039	650	SC
N521.8	1	0.016	0.016	280	SC
N521.9	3	0.072	0.024	430	SC
N521.10	-	-	-	-	-

4.1.5 Phenotyping of the N522 family members; self-pollination

It is found that all members of this family are self-compatible (Table 23). The seeds number variation gap here is greater (190-700).

Table 23. Individuals' phenotype of N522 family. Plant 10 was lost because of death.

N522	Number of Capsules	Total Weight (g)	Weight Per Capsule	Approximate number of seeds per capsule.	Phenotype
N522.1	1	0.042	0.042	700	SC
N522.2	2	0.071	0.035	600	SC
N522.3	3	0.096	0.032	530	SC
N522.4	2	0.056	0.028	480	SC
N522.5	1	0.020	0.020	330	SC
N522.6	2	0.057	0.028	480	SC

N522.7	2	0.052	0.026	440	SC
N522.8	2	0.076	0.038	630	SC
N522.9	1	0.011	0.011	190	SC
N522.10	-	-	-	-	-

4.1.6 Phenotyping of the N523 family members; self-pollination

All members of this family found to be self-compatible as well (Table 24).

Table 24. Individuals' phenotype of N523 family. Plants 4 and 10 were dropped because of no flower formation.

N523	Number of Capsules	Total Weight (g)	Weight Per Capsule (g)	Approximate number of seeds per capsule.	Phenotype
N523.1	1	0.026	0.026	430	SC
N523.2	1	0.030	0.030	500	SC
N523.3	1	0.042	0.042	700	SC
N523.4	-	-	-	-	-
N523.5	1	0.016	0.016	280	SC
N523.6	1	0.027	0.027	450	SC
N523.7	1	0.041	0.042	680	SC
N523.8	1	0.010	0.010	180	SC
N523.9	1	0.031	0.031	550	SC

4.1.7 Summary of the F4 population's phenotypes

As stated above, this population analysis was carried out during very difficult times because of the Covid-19 pandemic lockdowns. Therefore, it was only possible with limited access to the glasshouse but not to the lab, to investigate the phenotypes of all 40 individuals across this population based on its self-pollinations data as shown in Table 25 below. Unfortunately, due to the Covid-19 lockdown impacts and the loss of all plant tissues stored in the freezer; it was not possible to genotype the F4 population's 40 individuals.

Table 25. Shows the F4 population summary of individuals' seed productions per pod and their phenotypes. All plants that are self-pollinated have produced a high number of seeds which show they are self-compatible (highlighted in green). Four plants are excluded as no flowers are produced (highlighted in yellow).

F4 N520 family	Seeds per pod	Phenotype	F4 N521 Family	Seeds per pod	Phenotype	F4 N522 Family	Seeds per pod	Phenotype	F4 N523 Family	Seeds per pod	Phenotype
N520.1	390	SC	N521.1	480	SC	N522.1	700	SC	N523.1	430	SC
N520.2	220	SC	N521.2	200	SC	N522.2	600	SC	N523.2	500	SC
N520.3	400	SC	N521.3	350	SC	N522.3	530	SC	N523.3	700	SC
N520.4	480	SC	N521.4	250	SC	N522.4	480	SC	N523.4	-	-
N520.5	510	SC	N521.5	480	SC	N522.5	330	SC	N523.5	280	SC
N520.6	280	SC	N521.6	590	SC	N522.6	480	SC	N523.6	450	SC
N520.7	620	SC	N521.7	650	SC	N522.7	440	SC	N523.7	680	SC
N520.8	620	SC	N521.8	280	SC	N522.8	630	SC	N523.8	180	SC
N520.9	350	SC	N521.9	430	SC	N522.9	190	SC	N523.9	550	SC
N520.10	300	SC	N521.10	-	-	N522.10	-	-	N523.10	-	-

A collective discussion of the F4 and F5 generations analysis and findings will follow after representing the F5 results in the section below.

4.2 *Petunia* F5 generation

As the previous *Petunia* F4 generation has given limited data as of lockdown impacts, it is aimed that this F5 generation to generate more results and analysis of both its genotypic and phenotypic aspects. At this research phase, *Petunia* F5 generation of 6 families were raised from seeds of F4 generation (Chapter 2). These were chosen again based on their phenotype or/and genotype data. Figure 20 below provides an overview of *Petunia* generations tree that are used on this study and their F1 and F2 parents.

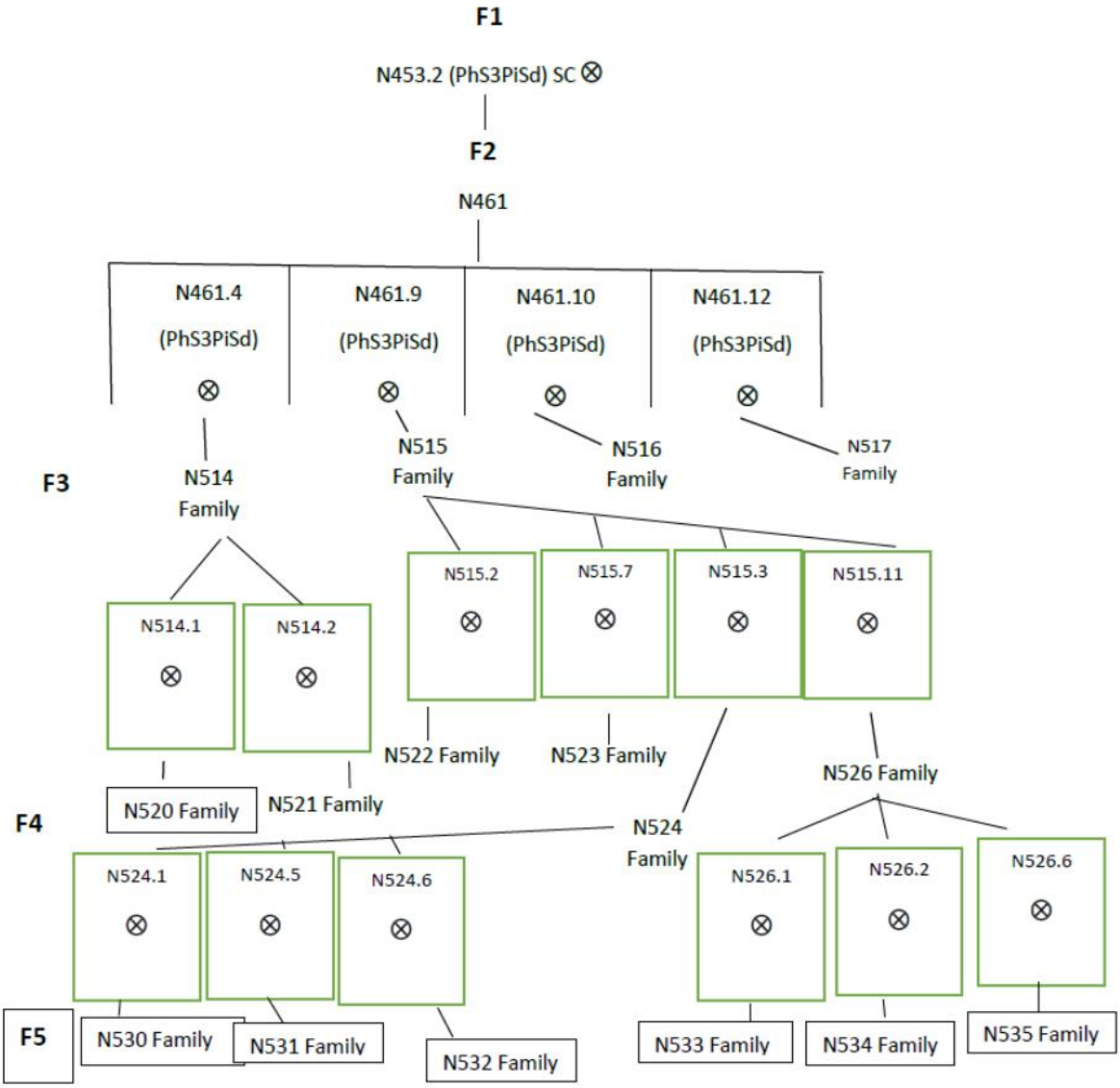


Figure 20. A tree of the *Petunia* generations created on this study. Their past parents are also shown.

The F5 generation 6 families' parents and their phenotype and genotype plus their identification numbers are shown in Table 26 below.

Table 26. The F5 *Petunia* generation families. Their Parents' genotype and phenotype if known and their IDs.

Parent ID	Genotype	Phenotype	F5 families ID
N524.1	Unknown	PSC	N530
N524.5	(*PhS3PiSd*)	SC	N531
N524.6	-	PSC	N532
N526.1	-	PSC	N533
N526.2	(*PhS3PiSd*)	SC	N534
N526.6	Unknown	PSC	N535

The F5 population has 6 families with a total of 44 individuals. However, because of their unhealthy plants and/or no flowering, families N530 and N532 dropped. The other four families are illustrated in Table 27 below.

Table 27. The F5 *Petunia* generation. Parents genotypes and phenotypes are shown in the first column. These 4 families on F5 have 8 plants each except the N533 family where only there are 4 individuals. They are all with their unique identification number.

F5							
Parents' Phenotype & Genotype	F5 Family ID	F5 Individuals' ID					
N524.5 (*PhS3PiSd*) (SC) ⊗	N531	N531.1	N531.2	N531.3	N531.4	N531.5	N531.6
		N531.7	N531.8				
N526.1 (PSC) ⊗	N533	N533.1	N533.2	N533.3	N533.4		
N526.2 (*PhS3PiSd*) (SC) ⊗	N534	N534.1	N534.2	N534.3	N534.4	N534.5	N534.6
		N534.7	N534.8				
N526.6 (PSC) ⊗	N535	N535.1	N535.2	N535.3	N535.4	N535.5	N535.6
		N535.7	N535.8				

4.2.1 Identification of specific alleles (*S-RNases*) in the F5 generation

The F5 generation of all four families were screened using allele-specific primers (Chapter 2).

4.2.2 Identification of the *PhS3-RNase* in the N531 family individuals

All of these family samples show positive band which means they carry the *PhS3* allele except individuals 1 and 7. The two arrows on the HyperLadder indicate key markers for estimating DNA fragment sizes in base pairs (bp) using the provided size guide. The top arrow corresponds to the 1000 bp marker, while the bottom arrow represents the 300 bp marker. The DNA samples are positioned at approximately 550 bp. The size of the *S3* allele was further checked by sequencing which will be illustrated in a coming section.

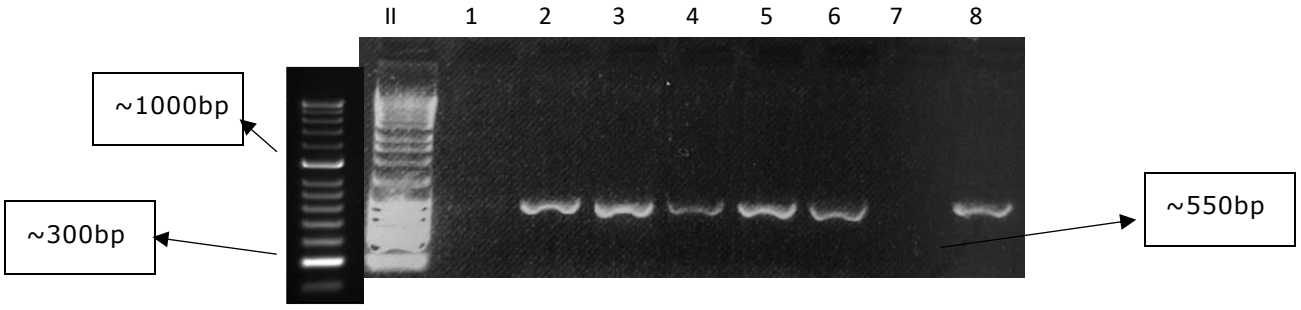


Figure 21. PCR amplification of the *PhS3* allele on the N531 *Petunia* family. Lane (II) represents HyperLadder II; (1) N531.1; (2) N531.2; (3) N531.3; (4) N531.4; (5) N531.5; (6) N531.6; (7) N531.7; (8) N531.8.

4.2.3 Identification of the *PiSd*-RNase in the N531 family individuals

All of this family have inherited the *PiSd* allele. The Hyper Ladder has been annotated with arrows showing two main markers use, from which, along with the size guild, the size in bp of the DNA samples can be estimated. The top arrow shows 1000 bp while the bottom arrow shows 300 bp. The DNA lie at ~600 bp. The size of the *Sd* allele was further checked by sequencing which will be illustrated in a coming section.

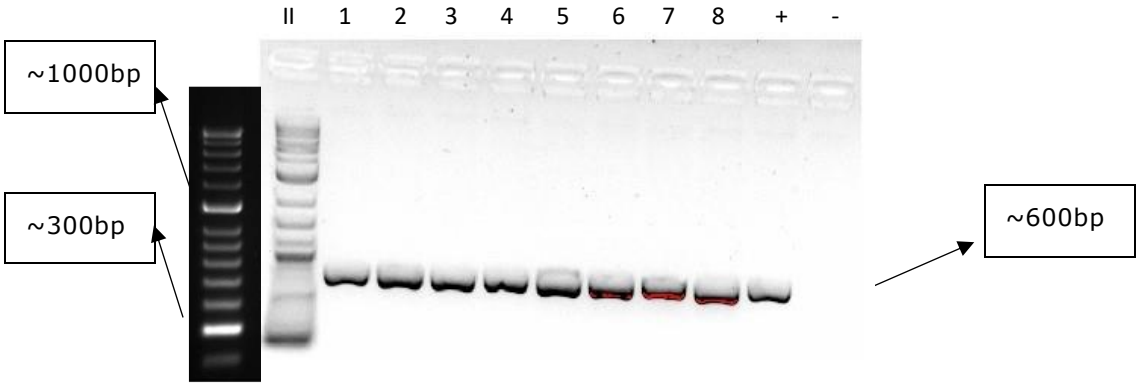


Figure 22. PCR amplification of the *PiSd* allele on the N531 *Petunia* family. Lane (II) represents HyperLadder II; (1) N531.1; (2) N531.2; (3) N531.3; (4) N531.4; (5) N531.5; (6) N531.6; (7) N531.7; (8) N531.8; (+) positive control and (-) negative control.

4.2.4 Identification of the *PhS3-RNase* in the N533 family individuals

All of this family samples show that they carry the *PhS3* allele. The HyperLadder indicated with two arrows that help estimate DNA fragment sizes in base pairs (bp) with the aid of the size guide provided. The top arrow points to the 1000 bp marker, and the bottom arrow indicates the 300 bp marker. The DNA samples are situated around the 550 bp mark.

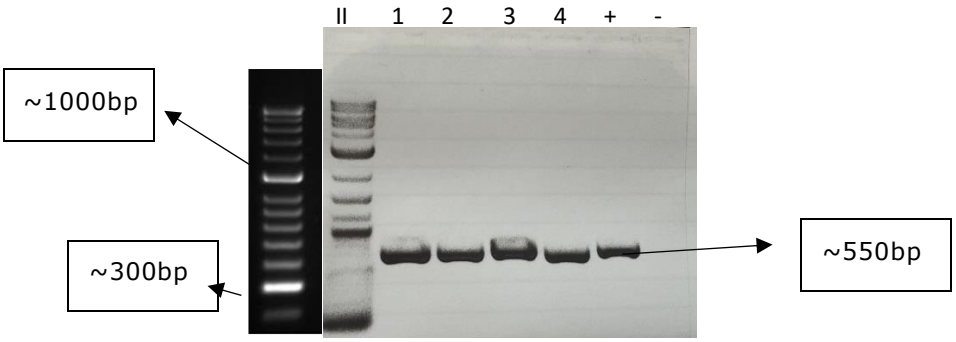


Figure 23. PCR amplification of the *PhS3* allele on the N533 *Petunia* family. Lane (II) represents HyperLadder II; (1) N533.1; (2) N533.2; (3) N533.3; (4) N533.4; (+) positive control and (-) negative control.

4.2.5 Identification of the *PiSd*-RNase in the N533 family individuals

All of this family samples show positive bands, so they carry the *PiSd* allele. The Hyper Ladder is marked with arrows indicating two primary reference points, which, together with the size guide, allow for the estimation of DNA sample sizes in base pairs (bp). The top arrow represents 1000 bp, while the bottom arrow

denotes 300 bp. The DNA sample is approximately 600 bp. The size of the *Sd* allele was verified by sequencing, and the results will be presented in a subsequent section.

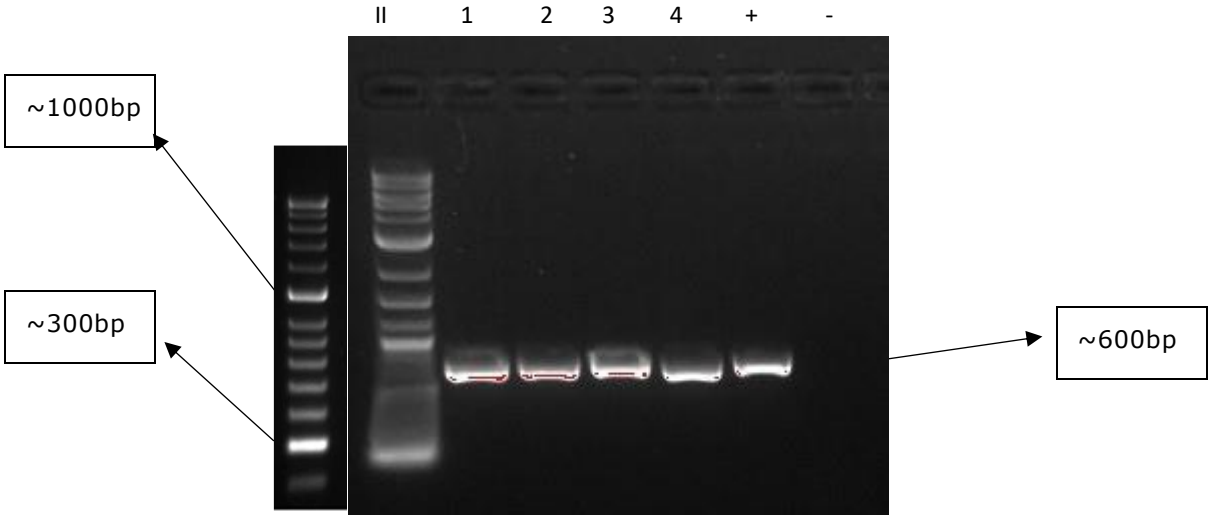


Figure 24. PCR amplification of the *PiSd* allele on the N533 *Petunia* family. Lane (II) represents HyperLadder II; (1) N533.1; (2) N533.2; (3) N533.3; (4) N533.4; (+) positive control and (-) negative control.

4.2.6 Identification of the *PhS3-RNase* in the N534 family individuals

Samples 1,3,4,5,7 and 8 carry the *PhS3* allele. The two arrows on the HyperLadder indicate key markers for estimating DNA fragment sizes in base pairs (bp) using the provided size guide. The top arrow corresponds to the 1000 bp marker, while the bottom arrow represents the 300 bp marker. The DNA samples are positioned at approximately 550 bp. The size of the *S3* allele was further checked by sequencing which will be illustrated in a coming section.

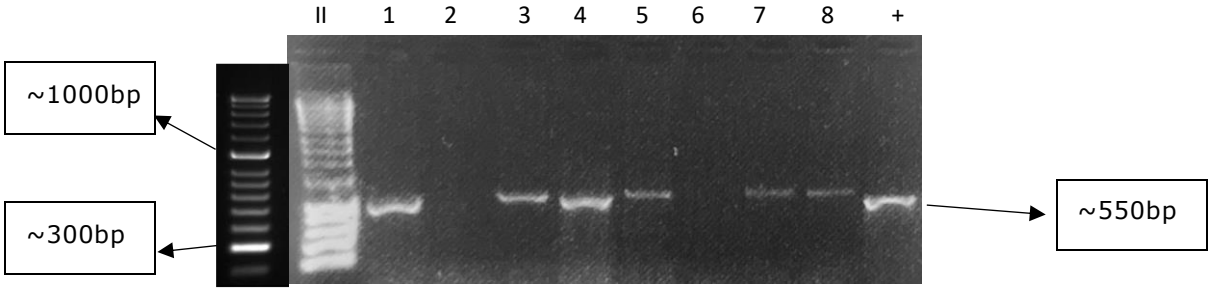


Figure 25. PCR amplification of the *PhS3* allele on the N534 *Petunia* family. Lane (II) represents HyperLadder II; (1) N534.1; (2) N534.2; (3) N534.3; (4) N534.4; (5) N534.5; (6) N534.6; (7) N534.7; (8); N534.8; (+) represents the positive control.

4.2.7 Identification of the *PiSd*-RNase in the N534 family individuals

All samples have the *PiSd* allele. As expected, the size of the *Sd* allele is approximately 600 bp. This will be analyzed more in a coming section.

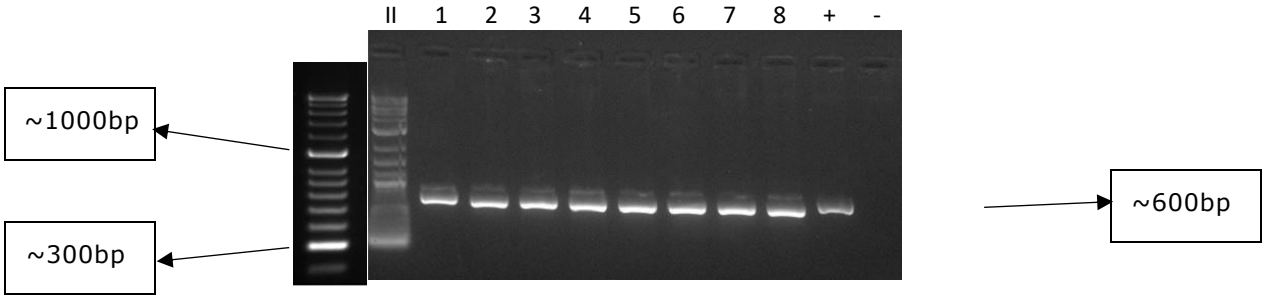


Figure 26. PCR amplification of the *PiSd* allele on the N534 *Petunia* family. Lane (II) represents HyperLadder II; (1) N534.1; (2) N534.2; (3) N534.3; (4) N534.4; (5) N534.5; (6) N534.6; (7) N534.7; (8); N534.8; (+) positive control and (-) negative control.

4.2.8 Identification of the *PhS3*-RNase in the N535 family individuals

All samples carry the *PhS3* allele. As stated in the previous figures, the *S3* allele size was found as expected and will be discussed in coming section with sequences analysis.

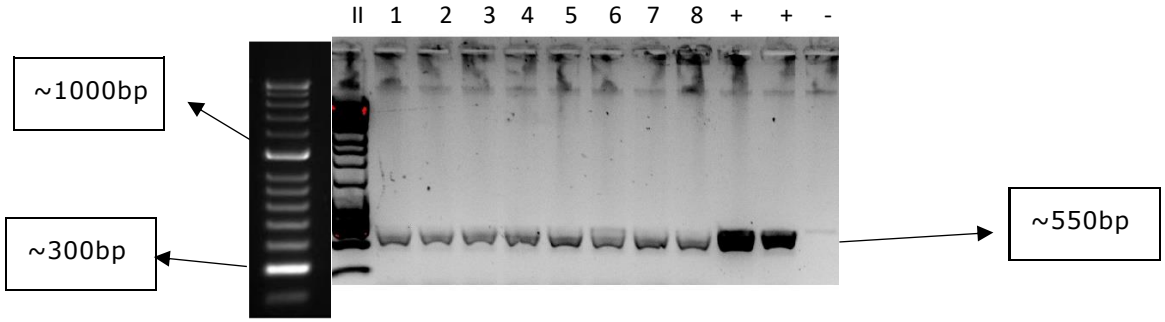


Figure 27. PCR amplification of the *PhS3* allele on the N535 *Petunia* family. Lane (II) represents HyperLadder II; (1) N535.1; (2) N535.2; (3) N535.3; (4) N535.4; (5) N535.5; (6) N535.6; (7) N535.7; (8); N535.8; (+) represents the positive control.

4.2.9 Identification of the *PiSd*-RNase in the N535 family individuals

All samples carry the *PiSd* allele. The Hyper Ladder is marked with arrows indicating two primary reference points, which, together with the size guide, allow for the estimation of DNA sample sizes in base pairs (bp). The top arrow represents 1000 bp, while the bottom arrow denotes 300 bp. The DNA sample is approximately 600 bp. The size of the *Sd* allele was verified by sequencing, and the results will be presented in a subsequent section.

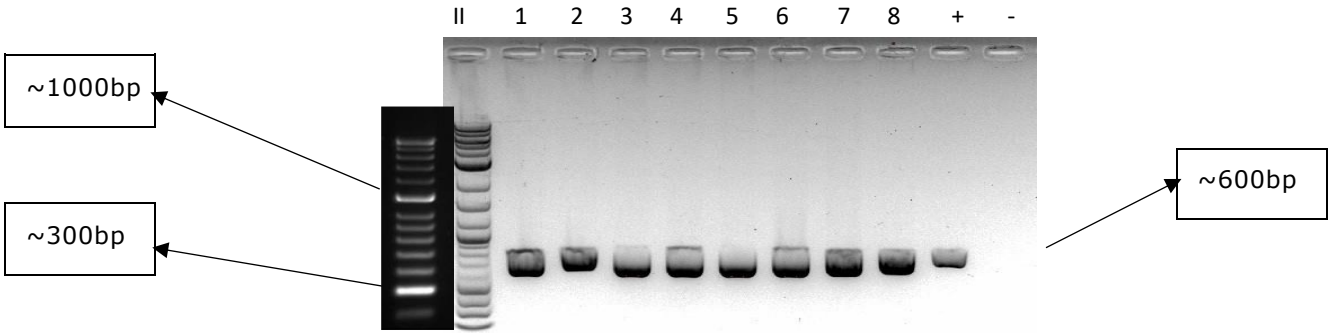


Figure 28. PCR amplification of the *PiSd* allele on the N535 *Petunia* family. Lane (II) represents HyperLadder II; (1) N535.1; (2) N535.2; (3) N535.3; (4) N535.4; (5) N535.5; (6) N535.6; (7) N535.7; (8); N535.8; (+) represents the positive control.

4.2.10 F5 phenotyping; pollination and seed sets

This section here represents the phenotypic analysis by the means of self-pollination exercises carried out in this *Petunia* generation. This will add up further confirmation of the *Sd* allele linkage to the breakdown of SI. Self-pollination may result in a small size pod which indicates no seeds are produced, suggesting self-incompatibility or a large size pod which indicates fully self-compatible or semi-compatible phenotypes. An intermediate size pod shows pseudo-self-compatible phenotype.

4.2.11 Phenotypes of the N531 family members; self-pollination

The aimed number of pollinations in each individual of this family was achieved. From Table 28 below all individuals show self-compatibility, although seed production varies from one plant to another. The segregation ratio here is 1:7.

Table 28. Shows the N531 family members seed production and their phenotype. As illustrated, all plants are self-compatible.

N531	Number of Capsules	Total weight (g)	Weight per capsule (g)	Approximate number of seeds per capsule.	Phenotype	Genotype
N531.1	10	0.19	0.019	300	SC	*(PiSdPiSd)*
N531.2	10	0.07	0.007	110	SC	*(PhS3PiSd)*
N531.3	10	0.15	0.015	240	SC	*(PhS3PiSd)*
N531.4	10	0.12	0.012	200	SC	*(PhS3PiSd)*
N531.5	10	0.08	0.008	130	SC	*(PhS3PiSd)*
N531.6	10	0.13	0.013	210	SC	*(PhS3PiSd)*
N531.7	10	0.07	0.007	110	SC	*(PiSdPiSd)*
N531.8	10	0.07	0.007	115	SC	*(PhS3PiSd)*

4.2.12 Phenotypes of the N533 family members; self-pollination

In this family plant number 1 is lost. Only 3 plants were self-pollinated at the rate of 10 pollinations each. They all show self-compatible phenotype as shown in the Table 29 below.

Table 29. Shows seed productions of three individuals as well as their phenotype.

N533	Number of Capsules	Total weight (g)	Weight per capsule (g)	Approximate number of seeds per capsule.	Phenotype	Genotype
N533.1	Sick	-	-	-	-	*(PhS3PiSd)*
N533.2	10	0.07	0.007	115	SC	*(PhS3PiSd)*
N533.3	10	0.11	0.011	190	SC	*(PhS3PiSd)*
N533.4	10	0.10	0.010	180	SC	*(PhS3PiSd)*

4.2.13 Phenotypes of the N534 family members; self-pollination

Ten self-pollinations were done in each individual of this family except plant number 6 where there were no flowers. As shown in Table 30 below they all have self-compatible phenotype. The segregation ratio here is 2:6.

Table 30. Shows seed productions post self-pollinations in the N534 family individuals. They all produced an average or high number of seeds, so they are self-compatible plants.

N534	Number of Capsules	Total Weight (g)	Weight per capsule (g)	Approximate number of seeds per capsule.	Phenotype	Genotype
N534.1	10	0.08	0.008	130	SC	*(PhS3PiSd)*
N534.2	10	0.17	0.017	290	SC	*(PiSdPiSd)*
N534.3	10	0.116	0.011	200	SC	*(PhS3PiSd)*
N534.4	10	0.13	0.013	210	SC	*(PhS3PiSd)*
N534.5	10	0.116	0.011	200	SC	*(PhS3PiSd)*
N534.6	No flowers	-	-	-	-	*(PiSdPiSd)*
N534.7	10	0.064	0.006	100	SC	*(PhS3PiSd)*
N534.8	10	0.06	0.006	100	SC	*(PhS3PiSd)*

4.2.14 Phenotypes of the N535 family members; self-pollination

Plant number 8 was sick so was excluded. All 7 other plants produced seeds when self-pollinated, which indicate they are self-compatible.

Table 31. Production of seeds post self-pollinations in the N535 family individuals. Their phenotypes are also shown.

N535	Number of Capsules	Total Weight (g)	Wight per capsule (g)	Approximate number of seeds per capsule.	Phenotype	Genotype
N535.1	10	0.09	0.009	150	SC	*(PhS3PiSd)*
N535.2	10	0.082	0.008	130	SC	*(PhS3PiSd)*
N535.3	10	0.06	0.006	100	SC	*(PhS3PiSd)*
N535.4	10	0.10	0.010	170	SC	*(PhS3PiSd)*
N535.5	10	0.06	0.006	100	SC	*(PhS3PiSd)*
N535.6	10	0.06	0.006	100	SC	*(PhS3PiSd)*
N535.7	10	0.06	0.006	100	SC	*(PhS3PiSd)*
N535.8	Sick/Excluded	-	-	-	-	*(PhS3PiSd)*

4.2.15 Chi Square analysis of F5 segregation ratio

In the F5 generation, genotypic analysis of the N531 family revealed two plants with the genotype *SdSd* and eight plants with the genotype *SdS3* as presented in Table 28. Similarly, for the N534 family, two plants exhibited the *SdSd* genotype and eight plants exhibited the *SdS3* genotype as shown in Table 30. Based on Mendelian inheritance, the predicted genotypic ratio for both families (N531 and N534) was expected to be 1:2:1 (*SdSd:SdS3:S3S3*), given that the parental genotypes were *PhS3PiSd*.

A Chi-square (χ^2) test was performed to assess the deviation between the observed and expected ratios (Table 32). The critical value from the chi-square distribution table for 2 degrees of freedom (df) at a significance level of $p<0.05$ is 5.99. The calculated χ^2 value of 0.833 indicates that the observed segregation does not significantly deviate from the expected 1:2:1 ratio ($\chi^2(2, p<0.05) = 5.99$). Although the limited sample size may affect the precision of the observed values—particularly since some expected values are less than 5 the P-value remains solid, suggesting that the observed segregation is consistent with the null hypothesis of normal Mendelian segregation.

Table 32. The chi squared test comparing the expected ratio (4:8:4) for the self-pollination and the observed ratios of the F5 generation as (4:10:2). The results show that the ratios are not significantly different.

N531 and N534	O	E	O-E	(O-E)2	(O-E)2/E
SdSd	4	4	0	0	0
SdS3	10	8	2	4	0.333
S3S3	2	4	-2	2	0.5
$\chi 2=\Sigma(O-E)2/E$			0.833		

4.3 Discussion

Although all four families' individuals of the F4 generation considered SC based on their seed sets productions, there is wide range of variations in terms of number of seeds produced per capsule as illustrated on the summary Table 25 above where seeds number widely range from 180 seeds per capsule up to 700 seeds per capsule. Unluckily the number of self-pollinated flowers was very limited due to lockdowns effects. However, the phenotypic obtained results in terms of seed productions can be viewed as of epigenetics effects.

In general, the majority of self-incompatible (SI) plants exhibit a typical self-incompatibility mechanism that prevents fertilization by self-pollen, theoretically leading to the absence of seeds through self-pollination. However, certain self-incompatible plants display intermediate traits between self-incompatibility and self-compatibility, a phenomenon referred to as pseudo-self-incompatibility (PSC) (Flaschenreim and Ascher, 1979). Specifically, PSC describes cases where self-incompatible plants, despite possessing functional SI determinants, are still capable of accepting self-pollen and producing seeds (Ascher, 1984). PSC is prevalent among various self-incompatible species (Levin, 1979), including Petunia integrifolia (Dana and Ascher, 1985) and more importantly in very high valuable crops such as Olive (Alagna et al., 2019).

Typically, PSC is associated with self-compatibility due to phenotypic similarities. Self-compatible plants produce robust seed sets following self-pollination, whereas pseudo-self-incompatible plants may generate two distinct types of seed sets: a plump seed set with numerous seeds and a slender seed set devoid of seeds (Levin, 1979; Ascher, 1984; Czyzyk, 2010. unpublished).

PSC/SC in SI plants has several distinct causes (Chapter 1). The first is linked to mutations affecting pollen or stigma function, potentially resulting from polyploidy induced by spontaneous mutations. Normally, self-incompatible plants reject self-pollen at the stigma or exhibit pollen tube arrest at approximately the upper third of the style. However, mutations in pollen, stigma, or pollen tube growth can disrupt this SI mechanism, leading to a failure in pollen rejection (Flaschenriem and Ascher, 1979). The second type of PSC is attributed to partial breakdown of SI within the flower without direct mutation effects on pollen, stigma, or pollen tube function (Clark et al., 1990). The third type of PSC is induced by environmental factors or developmental changes (Takahashi, 1979; Breton et al., 2016). For instance, *Campanula rapunculoides* flowers are initially self-incompatible but become self-compatible after a period post-anthesis, classifying them as PSC (Richardson et al., 1990; Good-Avila and Stephenson, 2003; Good-Avila et al., 2008).

Current hypotheses on PSC mechanisms suggest that it may be caused by environmental-induced mutations in S-locus genes (Takahashi, 1973) or by

interactions among these genes affecting normal SI function, leading to reduced or lost pollen rejection capabilities (Stevens and Kay, 1989). Additionally, PSC might result from mutations in downstream genes within the SI pathway or the presence of an extra S-allele, which disrupts SI (Murase et al., 2004; Baldwin and Schoen, 2017). The precise molecular mechanisms underlying PSC remain unresolved, and studies involving PSC in *Petunia* could provide further insights.

To classify a plant as pseudo-self-incompatible, self-compatible, or self-incompatible, the percentage of self-incompatible seed sets relative to total pollinations is used as a criterion (Flaschenreim and Ascher, 1979; Litzow and Ascher, 1983). *Petunia integrifolia* is generally recognized as self-incompatible (Dell'Olivo et al., 2011). Dana and Ascher (1985) investigated wild Petunia integrifolia, finding that 80% of samples from the population displayed self-incompatibility after self-pollination. Similarly, *Petunia hybrida* Sb-haplotype stocks were screened, with over 80% of individuals showing no seed production post-self-pollination, indicating self-incompatibility in this haplotype (Wright, 2004; Cyzyk, 2010). Additionally, Litzow and Ascher (1983) demonstrated that increased generational cycles could lead to a higher proportion of seed sets in PSC plants.

The F5 generation investigated here has a population of 28 plants in total among its four families (N531, N533, N534 and N535). All plants inherited the novel *Sd* allele show self-compatibility. The segregation analysis shown in Table 31 in

families N531 and N534 demonstrate closer ratios to the expected at 1:7 and 2:6 respectively.

Interpretation of these outcomes can be linked to the loss of function either in the female part of the novel allele (*PiSd-RNase*) or in the male multiple *SLFs*. If the loss of function occurred in the female part, PiSd-RNase would have not degraded pollens RNA which hold *PiSd* allele. In contrast, if the male part (*SLF*)s had lost its function, pollen would have escaped from the degradation effects of *PiSd*-RNase and grow successfully. In either case, the pollen tube was able to grow successfully through the style and leads to successful fertilization and the observed production of seeds. Through reciprocal crosses between SI and SC plants carrying the same genotype, Sharef (2017) confirmed that the phenomenon of PSC in Petunia is due to a loss of function that in the pollen part (*SLF* genes).

4.4 Conclusion; *Petunia* study findings

Self-incompatibility has different mechanisms depending on its different systems, SI is the genetic mechanism that functions in flowering plants in order to protect the genetic diversity. This is by the recognition of self-pollen and the rejection of the interaction between that self-pollen and the pistil (Sims and Robbins, 2009; Li *et al*., 2017).

First SI species studied in this research, *Petunia*, which is a genus of the *Solanaceae* family, its SI responses are controlled by two important determinants: the male determinant (*SLFs*) and the female determinant (*S-RNase*) both located in one S-locus (Kubo *et al*., 2015). It is understood that, during pollination and fertilization, when self-pollen lands on the self-stigma, the *SCFSLF* start functioning in order to identify *S-RNase* in the pollen tube. The *SLFs* of *SCFSLF* complex and the *S-RNase* are produced from the same S-allele. Therefore, the *SCFSLF* complex is unable to target the *S-RNase*. This results in the *S-RNase* to remain in the pollen tube and hence being able to degrade growth-related RNA to stop the growth of the pollen tube. However, If the cross-pollen lands on the stigma, the *SLFs* from the cross-pollen will recognize the *S-RNase* which is from different S-alleles. This will lead to the *SCFSLF* complex to target the *S-RNases* (ubiquitination). As a result, this will cause *S-RNases* degradation thus allowing the pollen tube to continue its growth (Takayama and Isogai, 2005).

One of the most important aims of this study was to investigate further the inheritance of the SC phenotype and the linkage to our novel allele *PiSd*. That is the segregation of the *PiSd* allele throughout several *Petunia* generations. This was carried out as I generated three generations of *Petunia* one after another chosen based on their parental phenotype/genotype characteristics. The finding of this aim is adding to the previous results that the SC phenotype is caused by

the *Sd* allele. This is to state that the *Sd* allele causes breakdown of SI throughout this study large population hence conveying the SC phenotype throughout the three *Petunia* generations that have 10 families and 96 individuals in total.

This finding is consistent with the past Ph.D. study by Sharef (2017) and the BSc study by Lesley (2017) which was under supervision of Sharef and Robbins. In addition, later the MSc research to this study by Cuban and Robbins (2019), in which two families of this project F3 generation were used, found that all plants inherited the *Sd* allele become SC. This study outcome of the SC phenotype and its linkage to the *Sd* allele is of similar finding of the past study in *P. hybrida* by Ai *et al* (1991). Ai *et al* (1991) observed the link of the S-allele *So* to SC phenotype. The *So* allele conveys self-compatibility in most cultivars of *P. hybrida* (Robbins *et al*., 2000).

In addition, a Ph.D. research project by Chen (2022) here at The University of Nottingham has found an indication of change in the *SLF* expressions between *So* and *Sb* alleles in *Petunia* which was detected by qRT-PCR. Chen (2022) has shown that the majority of *SLFs* in *SoSo* have significantly higher expression level in comparison to the *SbSb*. The expression level of 9 *SLFs* in *SoSo* is found to be double of the *SbSb*. This can be seen as of a similar effect of the behavior of the *PiSd* allele, but future studies can investigate this.

The significance of this study findings and the wider implications of SI and SC will be covered more in the coming chapter number 6.

Chapter 5. Results of SI in *S. truncata* F1 progeny

The previous study of *Schlumbergera* progeny by Tumusiime (2006) here at UoN has concluded from the preliminary findings that the mechanism of SI in *Schlumbergera* is likely to be a ribonuclease based. In this project, it is aimed to further test the hypothesis further.

5.1 Pollination analysis in the F1 Progeny

5.2 Self-pollinations results

As expected, none of the 60 attempts of the self-pollinations have formed fruit. All the self-pollinated flowers abscised approximately 2-3 days post pollination. Floral abscission is a feature of the Cactus plants that are self-pollinated and a sign of SI. Flowers that abscised from the plants following the self-pollination exercises were recorded as shown in Table 6 Chapter 2 above highlighted in red, which is an indication that they have failed to set fruit.

5.3 Cross-pollinations results

As a result of fruit set failure following selfing, there was no need to emasculate the flowers prior to performing crosses. A total of 300 cross-pollinations resulted in successful fertilization and hence fruit formations. The fruit matured and were only ready to harvest after turning pink following 5-to-6-month period post

cross-pollination. An example of the production of fruit following cross-pollination is shown in Figure 29 below.

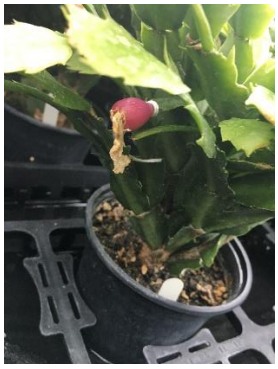


Figure 29. shows a cacti fruit as a result of successful cross pollination between two individuals. (P1 Pink flowered as male parent crossed to W1 white flowered as female parent). The fruit is at its maturity and turned pink in color. Photo taken after 6 months of cross-pollination. Fruit size is about 2cmL*1cmW.

All the fruits produced contained approximately 30-40 seeds. Fruit formation was not 100% in all cases as is indicated in Table 33. The outcome of these are shown as percentages of fruit set following the cross-pollination exercises as represented in Table 33.

Table 33. Percentage of fruit-set less than 100% indicates that pollination resulted in the loss of some flowers included in the pollination exercise. P1- P4 – Pink Flowered. W1 – W4 – White Flowered.

♀ \ ♂	P1	P2	P4	W1	W2	W4
P1	**0**	0	0	40	0	0
P2	0	**0**	0	50	0	0
P4	0	10	**0**	100	0	0
W1	100	100	90	**0**	90	90
W2	0	0	?	100	**0**	0
W4	0	0	0	100	0	**0**

5.4 Identifications of three *S-RNases* in *S. truncata* F1 progeny

Three S-alleles were identified in the *S. truncata* F1 progeny of this study.

5.4.1 Identification of the *S1*-allele in the six lines of the cacti plants

Bands on the obtained images show the presence of the *S1*-allele in all six lines of the cacti population except W1. The Hyper Ladder is marked with arrows indicating two primary reference points, which, together with the size guide, allow for the estimation of DNA sample sizes in base pairs (bp). The top arrow represents 1000 bp, while the bottom arrow denotes 300 bp. The DNA sample is approximately 350bp. The size of the *S1* allele was verified by sequencing, and

the results will be presented in a subsequent section. These are shown in Figure 30 below.

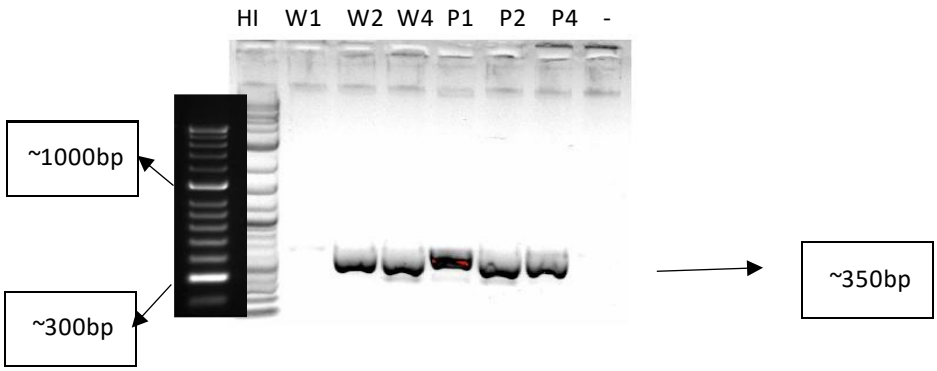


Figure 30. PCR amplification of the six cacti lines using the *S1* forward and reverse primers. Lane (HL) represents HyperLadder II; (1) W1 (2) W2 (3) W4 (4) P1 (5) P2 (6) P4. It is clear that all samples gave + band apart from W1 which means they have the *S1*-allele.

5.4.2 Identification of the *S2*-allele in the cacti six lines plants

The S2 specific primers were used to screen the six samples. As illustrated in Figure 31 below, only line W1 has the *S2*-allele. The two arrows on the HyperLadder indicate key markers for estimating DNA fragment sizes in base pairs (bp) using the provided size guide. The top arrow corresponds to the 1000 bp marker, while the bottom arrow represents the 300 bp marker. The DNA samples are positioned at approximately 400 bp. The size of the S2 allele was further checked by sequencing which will be illustrated in a coming section.

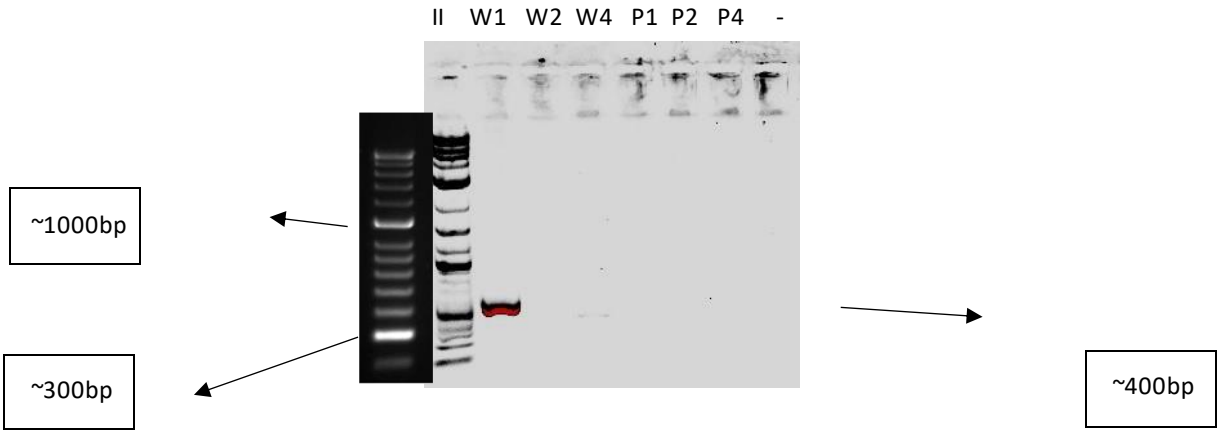


Figure 31. PCR amplification of the six cacti lines using the *S2* forward and reverse primers. Lane (HL) represents HyperLadder II; (1) W1 (2) W2 (3) W4 (4) P1 (5) P2 (6) P4. It is clear that only the sample of the W1 line gives positive band meaning it carries the *S2*-allele.

5.4.3 Identification of the *S3*-allele in the cacti six lines

Positive bands on the obtained image below show the presence of the *S3*-allele in all six lines of the cacti population. The two arrows on the HyperLadder indicate key markers for estimating DNA fragment sizes in base pairs (bp) using the provided size guide. The top arrow corresponds to the 1000 bp marker, while the bottom arrow represents the 300 bp marker. The DNA samples are positioned at approximately 400 bp. The size of the *S3* allele was further checked by sequencing which will be illustrated in a coming section.

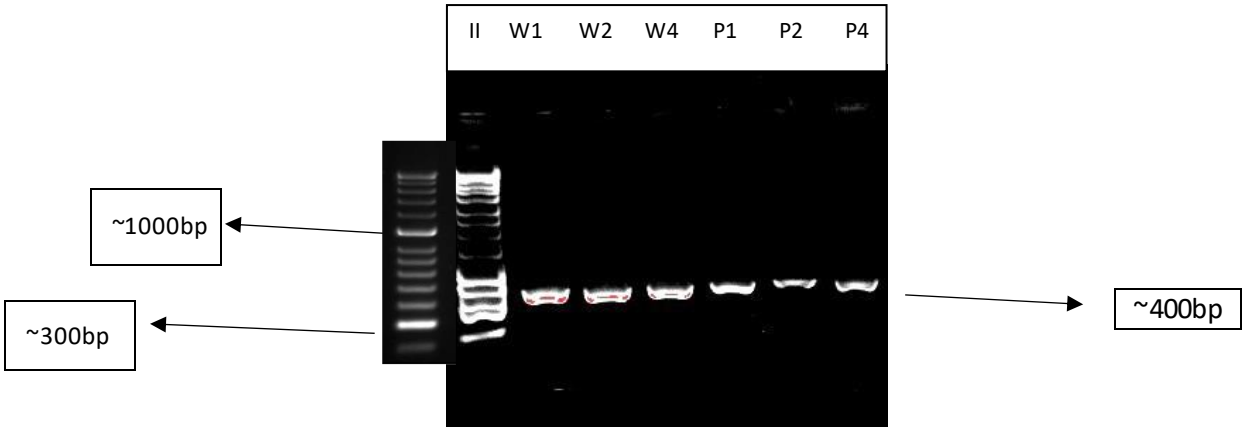


Figure 32. PCR amplification of the six cacti lines using the *S3* forward and reverse primers. Lane (HL) represents HyperLadder II; (1) W1 (2) W2 (3) W4 (4) P1 (5) P2 (6) P4. It is clear that all samples gave + band which means they have the *S3*-allele.

5.4.4 Identification of the *S4*-allele in the cacti six lines

As shown in the Figure 33 below, none of the six lines has inherited the *S4*-allele.

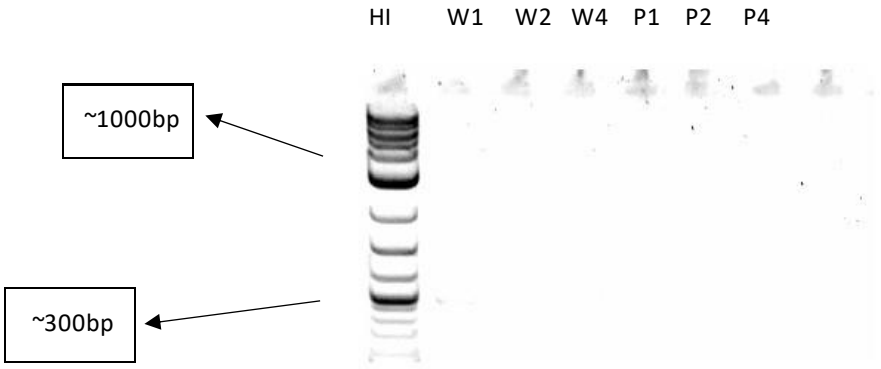


Figure 33. PCR amplification of the six cacti lines using the *S4* forward and reverse primers. Lane (HL) represents HyperLadder II; (1) W1 (2) W2 (3) W4 (4) P1 (5) P2 (6) P4. It is clear that *S4*-allele is not present on any line.

5.4.5 Identification of the *S5*-allele in the cacti six lines

Figure 34 below shows that none of our cacti six lines have the *S5*-allele.

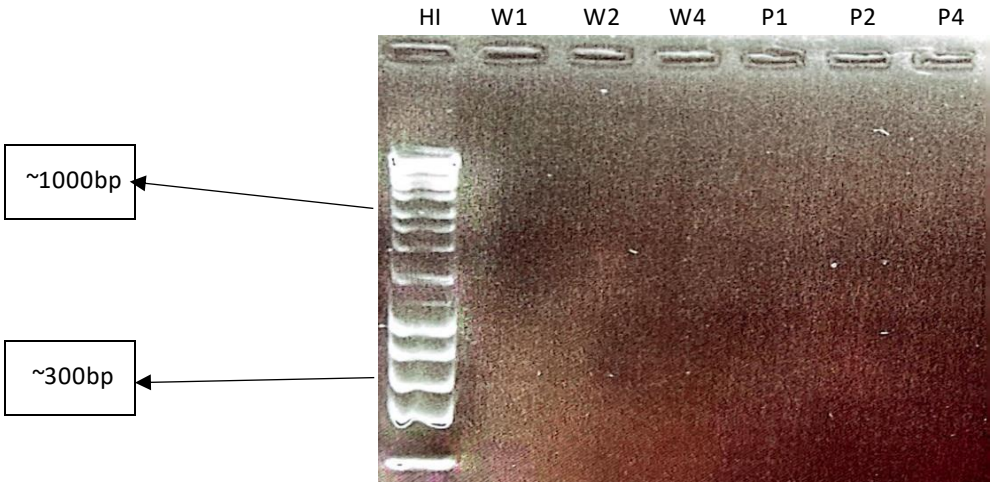


Figure 34. PCR amplification of the six cacti lines using the *S5* forward and reverse primers. Lane (HL) represents HyperLadder II; (1) W1 (2) W2 (3) W4 (4) P1 (5) P2 (6) P4. All cacti six lines give negative band results meaning none of them carry the *S5*-allele.

5.5 *S-RNases* sequences analysis of *S. truncata* show homology with that identified by Ramanauskas and Igić (2021)

As to confirm the sizes of the three identified alleles in this study, all samples DNA sequences were checked using the Biotechnology Information (NCBI) database Global Sequence Alignment Tool . (http://blast.st-va.ncbi.nlm.nih.gov) and also SnapGene software.

As example the *S3-RNase* sequence shown below illustrates 100% similarity to that provided by Igić from University of Chicago as shown below.

```
Sequence ID: Query_1171469 Length: 591
Range 1: 168 to 365

Score:366 bits(198), Expect:1e-105,
Identities:198/198(100%),  Gaps:0/198(0%), Strand: Plus/Plus

Query  132  GGTAGCCCACTTGGAGCGGCAATTGAATTGGCATTGGCCCTCGTTGAAGAATCCAAGGAA
191
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  168  GGTAGCCCACTTGGAGCGGCAATTGAATTGGCATTGGCCCTCGTTGAAGAATCCAAGGAA
227

Query  192  CAACCGGAGGTTTTGGGAGCATGAATGGAGTATACATGGCATATGCTTGCAACCGGTATT
251
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  228  CAACCGGAGGTTTTGGGAGCATGAATGGAGTATACATGGCATATGCTTGCAACCGGTATT
287

Query  252  TAATGTAACTACATATTTCCGGCGGGGACTTGATTTAATGAACCAATACAACTTGCTTGA
311
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  288  TAATGTAACTACATATTTCCGGCGGGGACTTGATTTAATGAACCAATACAACTTGCTTGA
347

Query  312  CATCCTGACTTCTAGCGG  329
            ||||||||||||||||||
Sbjct  348  CATCCTGACTTCTAGCGG  365



Range 2: 364 to 424

Score:108 bits(58), Expect:7e-28,
Identities:61/62(98%),  Gaps:1/62(1%), Strand: Plus/Plus

Query  438  GGCCATGATCCAGACCCTCAAGTCACTTACAATTTAGCCTCGATGCAAACGGCCAATCCA
497
            |||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbjct  364  GGCCATGATCCAGACCCTCAAGTCACTTACAATTTAGCCTCGATGCAAACGGC-AATCCA
422

Query  498  AA  499
            ||
Sbjct  423  AA  424
```

The table below shows how the identified S alleles of *S. truncata* in this study are identical to that used as reference identified on the study by Ramanauskas and Igić (2021). The data analysis applied using the NCBI alignment tool.

Table 34. The three identified S alleles of *S. truncata* in this study show identity to that shared by the study of Ramanauskas and Igić (2021).

Gene Name	Reference Seq	Query Cover	Identities
S1	Igic S-RNase 1	48%	98.81%
S2	Igic S-RNase 2	46%	100%
S3	Igic S-RNase 3	51%	100%

5.5.1 Summary of *S*-alleles presence in this study Cactus six lines

Table 35 below shows the presence and absence of the five Cactus *S*-alleles that found on this study Cacti six lines population.

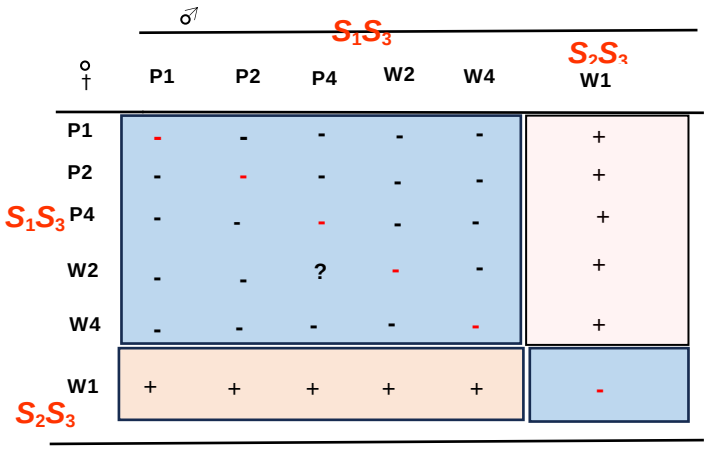
Table 35. The presence and/or absence of the five tested cactus *S*-alleles in the six lines of the population used on this study**.**

Cactus Line	*S1*	*S2*	*S3*	*S4*	*S5*	+/-
W1	-	+	+	-	-	*(S2S3)*
W2	+	-	+	-	-	*(S1S3)*
W4	+	-	+	-	-	*(S1S3)*
P1	+	-	+	-	-	*(S1S3)*
P2	+	-	+	-	-	*(S1S3)*
P4	+	-	+	-	-	*(S1S3)*

5.6 Incompatibility and compatibility groups based on the identification of the *S-RNases* and on the pollination data.

Compatibility versus incompatibility designations is based on the following stringencies: Incompatible pollinations resulted in no (0%) fruit-set whereas compatible pollinations resulted in between 50-100% fruit set as shown below.

Table 36. Compatibility versus incompatibility grouping of the *S. truncata* population. Pollen parents are at the top of each column and pistillate parents are at the left of each row. Pink boxes show the predominantly compatible crosses whereas blue boxes highlight the predominantly incompatible crosses. + denotes successful crosses. - denotes failed crosses. ? denotes crosses that are yet to be performed or need repeating.



One possible interpretation of the crosses between the cactus six lines in this study is that two incompatibility groups occur in the *S. truncata* population that was studied. One incompatibility group comprises pink-flowered lines P1, P2, and P4 and white-flowered progeny (W2 and W4). Line W1 form another incompatibility group.

5.7 Discussion

5.7.1 The combined findings of this study confirm *S-RNase* mechanism controls SI in the *Cactaceae* family

This study has achieved a large-scale of self and cross-pollination in the false Christmas Cactus population of the six lines of which there were four clones. Based on the self-pollination data (section 5.2) and as expected, all *Schlumbergera truncata* individuals displayed SI when self-pollinated. That is, they all failed to set fruit following their self-pollination. This confirms that SI is functional in the *Schlumbergera* progeny. This finding is consistent with the past research by Tumusiime in 2006 from which we obtained the Cactus plants. Tumusiime (2006) and this study confirmed that none of the self-pollinations across all lines resulted in fruit formation, indicating that all the cactus stock lines we have at UoN exhibit SI.

The cross-pollination results of this study (section 5.3) show that line W1 is the most productive when used as the pistil parent (Table 33). It resulted in nearly 100% fruit set when crossed with all lines. It is also the most productive line, yielding fruit set percentages ranging from 40 to 100% when used as a pollen donor to all other lines. This finding contradicts previous research by Tumusiime (2006), which noted that the W1 line produced fruits only when crossed with lines P1 and W4, but not with all other lines.

Tumusiime (2006) found that all F1 progeny show two distinct PCR bands, whilst this study, lines P1, P2, P4 and W4 displayed bands 1 and 2. In addition, lines W1 and W2 seemed to have band 2 plus a distinctive band which was named band 3.

My findings support Tumusiime's (2006) on the assumption that the WP (White-flowered parent) carries alleles S_2S_3 and the PP (Pink-flowered parent) incompatibility alleles-S_1S_3. The resulting progeny post-cross pollination would represent two likely different *S*-genotypes: *S1S3* and *S2S3*. This is confirmed by the *S*-allele identifications of this study (section 5.4). In contrast, Tumusiime (2006) assumed that band 3 represents the same *S*-allele in the two parents. Since the *S1S3* genotype is found in five lines in this study, *S3* allele shows it has segregated from the parents to the current F1 progeny.

My *S*-allele identifications indicate that the putative *S1S3* genotype for lines (P1, P2, P4, and W4) is correct. Additionally, the previous study predicted that lines W1 and W2 exhibit a putative *S2S3* genotype. This prediction is true for line W1, but not for line W2, which has an *S1S3* genotype (Table 35).

In this study, based on the observed incompatibility and compatibility groups following selfing and cross-pollination, as shown in Table 33, it is found that these results are consistent with the likelihood of a stylar ribonuclease mechanism being controlled by a single locus in this population of *S. truncata*. As confirmed by the *S*-alleles identifications (section 5.4), lines P1, P2, P4, W2 and W4 are *S1S3* genotype. Whereas the W1 line is found to carry the *S2S3* alleles. As the results of cross-pollination show (Table 33), lines that carry heterozygous alleles produce fruits after cross-pollination. This is the case when all lines are crossed to the W1 line. The W1 line results in high compatibility rates when used as the pistil parent (Table 33). The W1 line largely shows compatible crosses when used as the pistil parent with pollen sourced from the S1S3 heterozygous. However, lower compatibility rates are observed when the W1 line serves as the pollen donor crossed with lines P1 and P2. The compatibility rate of the cross with the P1 line is 40 while the P2 line is 50. These findings confirm the function of such alleles under the GSI control mechanism as illustrated on Figure 1 (Chapter 1). As the pollen *S1S3* lands on a stigma of *S2S3*, only the *S1* pollen tube will develop normally while the S3 pollen will not reach the ovary. This confirmation shows semi-compatible haplotypes which are illustrated by the low levels of successful cross pollination shown in lines P1 and P2.

This study's findings reinforce the results of the zymogram performed on the past study by Tumusiime (2006), the observed bands are likely representing our identified *S-RNases*. This is because there was a distinct polymorphism in pI between the representative bands. This feature is anticipated for a heterozygote. It is well defined that in species where the SI phenotype is under control of an *S-RNase* based mechanism, molecular weight polymorphisms are one of the defining features.

The combined evidence of the analysis in this study including phylogenetic relationships, large scale diallele crosses, genetic analysis, the identified and sequencing of three *S-RNases* indicates that *S. truncata* enforces *S-RNase* mechanism found across eudicots.

The study by Ramanauskas and Igić (2021) has identified five *S-RNases* in the same species of this study. Three of these *S-RNases* (*S1*, *S2* and *S3*) identified in this study. The combined evidence from our study and that of Ramanauskas and Igić (2021) indicates that *S. truncata* enforces SI through a genetic mechanism found across eudicots. This all adds to the knowledge of the evolutionary dynamics of the *S-RNase* system.

SI has been identified in numerous genera within the *Cactaceae* family (Boyle, 1997). It is likely that the recurrent SI observed in this family is driven by a shared mechanism of reproductive self-incompatibility (RSI), as seen in other

plant families exhibiting RSI. The molecular pathways governing SI tend to be conserved across closely related taxa, with members of the same family typically utilizing similar mechanisms (Igić *et al*., 2008). Research on the site and genetic expression pattern of pollen rejection, which involves gametophytic control, has been limited to a small number of cactus species. In each of these cases, the findings align with the RSI mechanism (Boyle, 1997; Martínez-Peralta *et al*., 2014). The identification of putative *S-RNase* alleles in *Matucana madisoniorum* and *Stenocereus thurberi*, along with evidence of preserved ancestral polymorphism, suggests that this mechanism has persisted since the common ancestor of most *Cactaceae*.

Chapter 6. Conclusion

6.1 SI in *Petunia*

The findings of this study first stage which focuses on the species *Petunia* concluded that, the novel *PiSd* allele causes breakdown of SI mechanisms throughout the three *Petunia* generations studied here. It will be of high significance for future research to conduct a comprehensive analysis of the *Sd-SLF* genes in terms of their identifications and expression analysis. Williams *et al* (2014b) identified a complete suite of *SLF* genes of *P. inflata* for two haplotypes, by the use of a de novo RNA-seq approach analysing both pollen and leave transcriptomes for cloning all *SLF* genes haplotype. Williams studies were in different *P. inflata* alleles which are different from this study *Sd* allele. This can be followed but it needs considerable efforts. Moreover, in order to test whether a change of *SLF* expression is observed in *SdSd* of SC phenotype and in comparison, with *SdSd* of SI phenotype, all *SLFs* in self-compatible and incompatible lines needs to be compared to identify any change. Additionally, further research into the *Sd* allele could involve assessing the degree of self-compatibility (SC) in larger progeny populations to precisely determine its effects in terms of SC segregation. This study findings contribute to a future direction which may include expanding the investigation to encompass a wider spectrum

of *S*-haplotypes as well as to explore potential universal mechanisms for overcoming SI.

Our study findings add insights into the SI breakdown in *Petunia* and the linkage to the novel *Sd* allele which resulted in majority of the population individuals being of SC phenotype and minor number of them being PSC. This is a multi-allelic system which is not just linked to a singular allele but also the genetic background of the parent providing the allele that could be resulting in PSC offspring. Ascher found that, PSC arisen because of a breakdown in the SI system in both parts; the pollen and the style (Dana and Ascher, 1986a and 1986b). It was confirmed that the PSC was linked to the S allele but that crossing over between the two was found rare.

This gene linkage which causes SC is possible to be due to the novel *Sd* allele and a link *SLF* gene that is able to detoxify (Entani *et al*., 2014, Li *et al*., 2014, Sun and Kao, 2013) the *Sd S-RNase* which in result leads to the successful growth of the *Sd* pollen. In addition, the segregation ratios that are found in the three *Petunia* generations of this study can be possible indication of inactivation of the *Sd* allele in the pollen part which resulted on the *Sd* pollen's ability to convey SC throughout this study *Petunia* generations. This is well supported by the finding of the previous study by Sharef (2017) here at the UoN. A. Sharef has found that the breakdown of SI in the F2 progeny individuals which were of

SC phenotype was due to the pollen part of the *Sd* haplotype. This study findings of the *Sd* allele linkage to the breakdown of SI, has also show few cases that are SI or PSC. In such instances, this can be related to the SI loss mechanisms which can cause SI to be reinstated by the means of either a deletion of duplicated S-loci, the evolutionary aspects of a novel SI system, or possibly by the reactivation of the *S-RNase* (Fujii *et al*., 2016).

In this study there are results of a few *Petunia* individuals of PSC phenotype and yet they have inherited the *PiSd* allele. Such variable levels of SI observed in this study have been studied in the past by Ascher in several studies including (Ascher, 1984, Dana and Ascher,1982, Dana and Ascher, 1985, Dana and Ascher, 1986a, Dana and Ascher, 1986b, Flaschenriem and Ascher, 1978, Flaschenriem and Ascher, 1979a, Flaschenriem and Ascher, 1979b). This phenomenon was named pseudo-self-compatibility (PSC). There have been since several developed theories of many different approaches linked to PSC and its appearance as well as a quantitative analysis in order to differentiate the partial breakdown of SI phenotype that was seen in PSC plants and the general impacts of the fertility effecting the production of seeds in plants that have no SI systems. This investigation can be a next aim to be performed in future studies. Ascher was successful to identify different mechanisms by which PSC can arise.

Differing levels of PSC can be also a result of a reduced expression or activity of stylar-localized *S-RNase* proteins. If self-compatibility level seen in the study is due to a stylar-based PSC system, then it is possible to suggest that the molecular method by which this occurs might be similar. Regardless the cause of the alterations to the *S-RNase* concentration (suggested by Sims and Robbins, 2009; Flaschenriem and Ascher, 1979b; Dana and Ascher, 1986b), it is possible that this might also has an effect on the levels of *S3 S-RNase* that was active or produced, which could be to a greater extent than for *Sd S-RNase*, which in turn would result in the *S3Sd* genotype to be more self-compatible than the *SdSd* genotypes. It was also noted that the threshold accumulation levels can be capable to stop the growth in incompatible pollen tubes. In a stylar-based system, if the style ability to reach this threshold concentration is affected, PSC may arise.

Many past researchers claimed that the SI breakdown was caused by changes in S-allele. Several mutation studies in S-allele in either female, male or both of them had led to pseudo-Self-compatibility (Lewis, 1949; Lewis, 1951; Dana and Ascher, 1985; Dana and Ascher, 1986a; Denward, 1963). A study by Takahashi (1973) has suggested that there is a substance in the style self-incompatible plant which result in pollen tube growth prevention after self-pollination. Pseudo Self-compatibility can appear once the activity of this substance is decreased.

Other factors which were claimed by several classical breeding experiments are modifier loci. These were shown to be cooperating with S-locus in order to determine SI. They were found unlinked to the S-locus and can influence pistil or pollen components (Mather, 1943; Martin, 1961, 1968; Townsend, 1969; Takahashi, 1973; Kaothien *et al*., 2010). They were classified to three groups. Group 1 factor directly affect the S-locus expression. Groups 2 and 3 are required for pollen rejection (McClure *et al*., 2000). (Cruz-Garcia *et al*., 2003) show that mutations in Groups 1 or 2 change the phenotype from SI to SC. In addition, the well-known two pistil modifiers (HT-B and the 120kDa glycoprotein) were found to be required for self–incompatibility (McClure *et al*., 1999; Hancock *et al*., 2005).

6.2 SI in *Schlumbergera* and GSI evolution insights

In the second phase of this study, investigation was focused on the self-incompatibility (SI) mechanism in *Schlumbergera truncata* (*Cactaceae*). *S. truncata* is a widely cultivated cactus species, renowned for its diverse range of flower colours and morphologies. The study's combined results, based on the identification of the three distinct S-alleles and the large-scale diallelic cross-pollination experiments involving F1 progeny, indicate the presence of an *S-RNase*-based self-incompatibility mechanism in *S. truncata*. Furthermore, this study confirms that the identified S-alleles (*S1*, *S2* and *S3*) surprisingly are shared with other *S. truncata* specimens which were identified by the study of Ramanauskas and Igić (2021). These findings support the hypothesis that the ribonuclease-based self-incompatibility system is more broadly conserved across *Schlumbergera* species than previously recognized. Further research, potentially involving analyses of an F2 progeny from the F1 lines used in this study and the inclusion of additional species, could provide additional evidence to support the notion of a common ancestor for the *S-RNases* across angiosperms. This would strengthen the evidence for the role of such mechanisms in this genus particularly if more species of this genus can be included in such study. The exact mechanism by which rapid systemic induced GSI responses function in

cacti, whether through self-recognition or non-self-recognition pathways, remains undetermined.

More than 100 plant families and approximately 40% of plant species have been documented to utilize self-incompatibility (SI) mechanisms to prevent self-pollination and promote cross-pollination (Igic *et al*., 2008). Self-incompatibility (SI) is one of the most important mechanisms employed by flowering plants to prevent inbreeding and maintain high genetic diversity within a species by encouraging the generation of new genetic combinations (Ferrer and Good, 2012).

All self-incompatibility *RNases* from the *Plantaginaceae*, Solanaceae, *Rosaceae* and *Rubiaceae* form a monophyletic clade (Igic and Kohn 2001; Asquini *et al*., 2011). This pattern was interpreted to be an indication of homology of *S-RNases* from these three families. A common ancestor has been suggested for these three sub-classes. Richman and coworkers (1996) suggested that *S-RNases* from *Scrophulariaceae* (renamed as the *Plantaginaceae*) and *Solanaceae* are likely to have a common ancestry. The *Plantaginaceae* and the *Solanaceae* in fact, both belong to the subclass Asteridae and therefore are more closely related to one another than either is to the *Rosaceae* which is a member of the Rosidae subclass (Cronquist, 1981). It is probable that a majority number of angiosperm families might share this form of gametophytic self-incompatibility if

S-RNases from the *Solanaceae*, *Plantaginaceae* and *Rosaceae* are homologous. This possibly including the *Cactaceae*.

This study findings and its exploration of the GSI underpinning mechanisms in the distinct species of *Schlumbergera truncata* adds further evaluation to others in several plant families, such as *Solanaceae, Plantaginaceae* and *Rosaceae*, where a shared female determinant, specifically a T2-type ribonuclease (RNase), has been identified (McClure *et al*., 1989; Sassa *et al*., 1996, 2007; Liang *et al*., 2020). Phylogenetic analyses suggest a singular common origin of SI within these families (Zhao *et al*., 2022). Furthermore, the male S-determinant (S-locus F-box) from a wide range of eudicots has been shown to detoxify *S-RNase* from *Petunia hybrida*, indicating that this represents an ancestral system conducive to outcrossing.

6.3 Implications of SI and SC in practice

In fruit crops, self-incompatibility (SI) is a valuable trait as it promotes the development of seedless fruits, which are highly sought after due to their higher market value. For instance, in commercial pineapple cultivation, all cultivars exhibit SI, as self-compatible (SC) clones would lead to fruits with numerous hard, inedible seeds, reducing their commercial viability. Additionally, SI has been utilized in *Brassica* species for the commercial production of hybrid seeds (Shen *et al*., 2008). In SC crops, generating hybrid seeds requires efficient control of self-pollination, typically through mechanical emasculation, chemical treatments or male sterility. More widespread than using SI. The use of SI lines eliminates the need for these labour-intensive and expensive methods. SI lines also facilitate forward and reverse crosses, as well as double or three-way hybrid crosses, enhancing breeding efficiency.

In contrast, SI can present significant challenges in horticulture and agriculture, especially in breeding programs. In certain olive cultivars, such what we experience in Libya, SI results in reduced fruit set and high rates of fruit abortion (Seifi et al., 2015). This issue is exacerbated when the availability of compatible pollen and opportunities for cross-pollination are limited by factors like unfavourable weather conditions or low pollinator activity. Consequently, successful pollination can be impeded (Quinet et al., 2016), leading to seasonal

variability in pollination success. Such inconsistencies contribute to erratic fruit set, posing economic risks to growers. For SI-dependent crops, including sunflower, European pear, apple, and sweet cherry, the presence of effective pollinizers is crucial to ensuring successful cross-pollination (Woodcock and Initiative, 2012).

On the other hand, SC provides several advantages by removing the requirement for pollinizers, allowing for monoculture of a single cultivar to achieve consistent crop production. In perennial grasses SI is commonly utilized in hybrid breeding strategies; however, SC is favoured by breeders as it promotes genetic uniformity and simplifies the propagation of inbred parental lines (Thorogood et al., 2005).

In the context of plant reproduction, SI is beneficial for generating hybrid seeds, particularly in crops such as citrus. In contrast, SC facilitates production uniformity and simplifies breeding programs but reduces genetic variability. Thus, the selection between SI and SC systems depends on the specific cultivation aims, with each offering distinct advantages and limitations for breeding and crop improvement.

To conclude, the novel *PiSd-RNase* in particular and all *S-RNases* of *Petunia* and *Schlumbergera* studied here represent unique and useful additions to the knowledge of *S-RNase* gene sequence database.

The identified novel *Sd* allele can be used for further studying the diversity and phylogenetic relationship of *S*-alleles, particularly its role in conveying the SC phenotype. Our findings in both *Petunia* and *Schlumbergera* also show that although domestication of such ornamentals popular flowering plants has been for far long years, their *S-alleles* divergence appears to be limited by selections when compared to their wider wild populations. For future, what I gained knowledge of here may have application for the maintenance and application of my interest of SI in Olive in my country Libya and the Mediterranean basin in general for crop yield improvement.

6.4 The breakdown and regain of SI

Self-incompatible plants such as *Petunia*, can sometimes breakdown its own SI mechanism which will led to such plants to lose their ability to reject self-pollen (Tsukamoto *et al*., 2003). In fact, there are many potential reasons causing this phenomenon, sometimes the SI determinants have been mutated and so lost functions. Further, these mutations may not only happen in the male and the female determinants but also in other factors that play a role in the SI process. For instance, the mutation of the HT-B influences the SI function of pistils to render the plant self-compatibility (O'Brien *et al*., 2002; Kondo *et al*., 2002; McClure, 2006). In addition, a collapse of the SI process which can result from a duplication of part or whole genome will often lead to competitive interaction (Golz *et al*., 2000; Golz *et al*., 2001; Sims and Robbins, 2009).

In nature, studies have found that some self-incompatible plants are able to produce a limited number of seeds after self-pollination. This is as discussed before was termed pseudo-self-incompatibility (PSC) (Flaschenreim and Ascher, 1979). PSC is probably caused by the reasons described above, but it still has no reasonable interpretation at the molecular level at that time because the molecular tools were not available such as access to *S-RNase* or *SLF* genes.

To conclude, SI has been and will still to be a critical trait of interest for both breeders and researchers, as it represents a vital cell-to-cell signalling event influencing fertilization and, ultimately, food production and security. In practice, efforts have been made in crop plants which are of high economical value manipulating SI system in order to increase efficiency in the production of F1 hybrids. This is due to SI role as a barrier to the establishment of self-propagating lines. For further reader interest, wide range of methods ranging from molecular techniques, CRISPR-Cas9 technology and specific chemical treatments, have been employed to overcome this barrier (Scutt *et al*., 1993; Chen *et al*., 2018; Yu *et al*., 2021; Eggers *et al* 2021; Indriolo *et al*., 2012; Aloisi *et al*., 2020; Huu *et al*., 2022). The choice of method is contingent upon the specific characteristics of the crop in question.

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Appendix 1

TBE Buffer

108g Tris base

55g Boric acid

9.12g EDTA PH 8

Appendix 2

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CLUSTAL O(1.2.4) multiple sequence alignment OF s. truncata S alleles

Schlumbergera_truncata_S1-RNase 1    ---TATGACTACATGCAGTTTGTGCAAACTTGGCCACTAA
Schlumbergera_truncata_S2-RNase 1    ---TATGACTACATGCTCTTCGCGCAAATATGGCCGCCAG
Schlumbergera_truncata_S3-RNase 1    ---CATGACTATATGAAGTTTGTCCAGACATGGCCTCCAA
Schlumbergera_truncata_S4-RNase 1    GTACATGACTACCTGATCTTTGTGCAAACATGGCCACCAT
Schlumbergera_truncata_S5-RNase 1    GTACATGACTACCTGATCCATGTGGAGACATGGCCACCAT

Schlumbergera_truncata_S1-RNase 38   CTCACTGCACTGTGGTCGTTAGGAGATGTTCAACTCCCAT
Schlumbergera_truncata_S2-RNase 38   GCTATTGTAGCGTCAAC------CGTTGTTCGATGCCTAT
Schlumbergera_truncata_S3-RNase 38   CCTATTGTACTGTGAAC------AATTGTTCGCGTCCTAT
Schlumbergera_truncata_S4-RNase 41   CCTTTTGCAGGATTGAT------CGTTGTTTTGGTCCTGG
Schlumbergera_truncata_S5-RNase 41   CGTACTGTCGGATTAAC------CGTTGTTCAGGCCCAAA

Schlumbergera_truncata_S1-RNase 78   CATACGTGACAATTTCACTATCCACGGTGTCTGGGAAGAG
Schlumbergera_truncata_S2-RNase 72   ACC---TAACGATTTCACTATTCACGGTATCTGGGATCAA
Schlumbergera_truncata_S3-RNase 72   CATAAATGATAGATTCACTATTCACGGTATTTGGCAAGCT
Schlumbergera_truncata_S4-RNase 75   CATCAATAATCAATTCACAATTCACGGTGTTTGGGATGGC
Schlumbergera_truncata_S5-RNase 75   CATTAATAATCGATTCACAATTCACGGTGTTTGGGATGCC

Schlumbergera_truncata_S1-RNase 118  TTTAACAGGTTCACC---GGTCGTCGTAATTGCAATACGC
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Schlumbergera_truncata_S2-RNase 109 TACAACAGTGGCAAACGTGGTCCGGGTAACTGCACTG---
Schlumbergera_truncata_S3-RNase 112 TACAACAGTCGGGGG---GCCGCTCCTCCTTGCATGG---
Schlumbergera_truncata_S4-RNase 115 TACTACAACCGCAGGCATGCTAATGGCCCCTGCACCT---
Schlumbergera_truncata_S5-RNase 115 TATTACAATGGGCAGTATGGCGTTGGTCCTTGCACCT---

Schlumbergera_truncata_S1-RNase 155 CGACTAGTAGTGCATTTAATCGATCATTGGTGGCCCATTT
Schlumbergera_truncata_S2-RNase 145 ---GTACAC---CGTTCAATCTGACACTGGTGGCCCAATT
Schlumbergera_truncata_S3-RNase 145 ---CAACTAACTTTTCCTTCAGG---GCGGTAGCCCACTT
Schlumbergera_truncata_S4-RNase 151 ---CAACTAA---TTTCAGCCGAGCTCTGGTTGCCCATAT
Schlumbergera_truncata_S5-RNase 151 ---CAAACAA---TTTCAGCCGAACTATGGTGGCCAATAT

Schlumbergera_truncata_S1-RNase 195 GGAGCATCAATTGAATGCTAGCTGGCCGTACCTGGCCAAT
Schlumbergera_truncata_S2-RNase 180 TGAGACAGCATTGAATGCTAGCTGGCCCAACTTGAAGAAC
Schlumbergera_truncata_S3-RNase 180 GGAGCGGCAATTGAATTGGCATTGGCCCTCGTTGAAGAAT
Schlumbergera_truncata_S4-RNase 186 TGAGAGGCAATTGAATGCAAGTTGGCCTAATCTAAAAGAT
Schlumbergera_truncata_S5-RNase 186 AGAGAGGCGACTAAATATAAGTTGGCCCAACCTGAAAGAT

Schlumbergera_truncata_S1-RNase 235 CGAAATAACAACGTGTACATATGGACTCATGAGTGGAATA
Schlumbergera_truncata_S2-RNase 220 CAAAGGAACAACCAGAAATTTTGGAAGCATGAATGGGAAG
Schlumbergera_truncata_S3-RNase 220 CCAAGGAACAACCGGAGGTTTTGGGAGCATGAATGGAGTA
Schlumbergera_truncata_S4-RNase 226 CAAAACAACAACGACATAATATGGACGCATGAATGGAAAA
Schlumbergera_truncata_S5-RNase 226 CAAAGCAATAACGAAGAACTATGGATCCACGAATGGGATA

Schlumbergera_truncata_S1-RNase 275 GCCATGGCAAATGCTTGGAACCAACCTTTAATGTGGTTTC
Schlumbergera_truncata_S2-RNase 260 CACATGGCACATGCTTGGAACCTACCGTCACTGTTCCTAG
Schlumbergera_truncata_S3-RNase 260 TACATGGCATATGCTTGCAACCGGTATTTAATGTAACTAC
Schlumbergera_truncata_S4-RNase 266 AACATGGAACATGCTTAGAGCCATTCGTGGATGTAACAAC
Schlumbergera_truncata_S5-RNase 266 GACATGGAACGTGCTTAGAACCGTTCGTCAATGTAACTAC

Schlumbergera_truncata_S1-RNase 315 ATATTTTCAGCTGGGACTTCGCTGGAAATCCAGATATAAT
Schlumbergera_truncata_S2-RNase 300 TTATTTCAAGTTGGCACTCGGTTGGAAGAACAGGCTCAAC
Schlumbergera_truncata_S3-RNase 300 ATATTTCCGGCGGGGACTTGATTTAATGAACCAATACAAC
Schlumbergera_truncata_S4-RNase 306 TTATTTTGAGCTAGGACTTGCATTAATGCGCCGATATCCC
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Schlumbergera_truncata_S5-RNase 306 TTATTTCGAGTTGGGTCTAGATTTAAAGCACCGGTATCCC

Schlumbergera_truncata_S1-RNase 355 ATACTTGAAATCCTGACAAGACACGGCTTTG------CTC
Schlumbergera_truncata_S2-RNase 340 ATACTTGGCTTCTTGACAACGCAG------GGATACGCTC
Schlumbergera_truncata_S3-RNase 340 TTGCTTGACATCCTGACTTCTAGCGGCCATGATCCA----
Schlumbergera_truncata_S4-RNase 346 TTATACGACTTCCTCAGA------AGCCATGGTTACCCCC
Schlumbergera_truncata_S5-RNase 346 ATACTTAACTTCCTGAGA------GGAAATGGTATCAACC

Schlumbergera_truncata_S1-RNase 389 CAGGTGCG------GTTTATGATGTAACTTTAATGAAAGA
Schlumbergera_truncata_S2-RNase 374 CGGGTCGTGGAGTGAACTACCCTAGTTCCTCGATACGAAA
Schlumbergera_truncata_S3-RNase 375 --GACCCTCAAGTCACTTACAATTTAGCCTCGATGCAAAC
Schlumbergera_truncata_S4-RNase 380 CTGGGCCAGGAAAAACGTATGATACAAATCTGATGAGAAA
Schlumbergera_truncata_S5-RNase 380 ACGGTCAGAGG------TACTTAACAAGCAGGATGAGAAA

Schlumbergera_truncata_S1-RNase 423 TGCAATAAAGAACGAGACTGGCGGCATAGACCCAATCATG
Schlumbergera_truncata_S2-RNase 414 TGCAGTCATTGCCACGGTTAA---GGTTGAGCCGGTGCTA
Schlumbergera_truncata_S3-RNase 414 GGCAATCCAAACGGCGATTGGTGGTGTTACTCCGCTGCTG
Schlumbergera_truncata_S4-RNase 420 CGCAATCCGCTCAATGACCGG---CAGCTTTCCTGTGCTG
Schlumbergera_truncata_S5-RNase 414 TGCCATCCAGTTGGGGATTGGGGGCTCTGCTCCTGTGCTG

Schlumbergera_truncata_S1-RNase 463 AGTTGTAAGCCTTCACCGAACCGGCCAC------ATTACC
Schlumbergera_truncata_S2-RNase 451 CACTGTTTGGGGAATG------GTTCAC---ATACCTTCC
Schlumbergera_truncata_S3-RNase 454 AAATGTGTCCAGAAGTCTAATTCGACTAATTACCT---GC
Schlumbergera_truncata_S4-RNase 457 TTCTGTAAGCATGGCACCAATCGGACGGGAAACCCTTTGC
Schlumbergera_truncata_S5-RNase 454 ATCTGCAAGAACGTTCCCAATCAAACAG---ACCCGTTTC

Schlumbergera_truncata_S1-RNase 497 TATATGAAGTTCGCCTTTGTCTAGACAGTCGAGGACAGCA
Schlumbergera_truncata_S2-RNase 482 TGCTTGAAGTTCGCCTTTGCATGGATTACCAGGGGCAAAA
Schlumbergera_truncata_S3-RNase 491 TGTATGAGGTTGTGGTATGTCTTGGTTATCATGGGCAACC
Schlumbergera_truncata_S4-RNase 497 TGTTGGAAGTCCGCTTCTGTTTTGATTCTCAAGGCTTGCA
Schlumbergera_truncata_S5-RNase 491 TCATAGAAGTTCGCCTATGTTTTGATAGTAGGGGGTCACA

Schlumbergera_truncata_S1-RNase 537 ACTCATCTCATGCGCTTCCAAGGGATTGGCAGACAAGTGT
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Schlumbergera_truncata_S2-RNase 522 ACTCATAAGTTGTGCTG----------GCAAGAAGTTGA
Schlumbergera_truncata_S3-RNase 531 ACTTATACCTTGTCTTGGT---------ACCGGCTCTTGT
Schlumbergera_truncata_S4-RNase 537 CCCAATAAATTGTA---CT---------GCCGGAAGTTGT
Schlumbergera_truncata_S5-RNase 531 GCCTATACGTTGTA---CT---------GCCAGAAGTTGT

Schlumbergera_truncata_S1-RNase 577 CCAACAAACA-----CCACCACCGTCTTCTTCCCCTAA--
Schlumbergera_truncata_S2-RNase 551 ACGACACTTGCAAATCCAACTCGATCTATTTCCCCCAATA
Schlumbergera_truncata_S3-RNase 562 CCACCACAAA-----ACATCAG---CTG-GTCGCTCTAA-
Schlumbergera_truncata_S4-RNase 565 CCCTCACAAT-----TCGTGTC---G---TTTCCTTGA--
Schlumbergera_truncata_S5-RNase 559 GGATCCCGGT-----ACATCAT---G---TTCCCTTGA--

Schlumbergera_truncata_S1-RNase 609 -
Schlumbergera_truncata_S2-RNase 591 A
Schlumbergera_truncata_S3-RNase 591 -
Schlumbergera_truncata_S4-RNase 591 -
Schlumbergera_truncata_S5-RNase 585 -
```

Appendix 3

Supplementary Table S1, *Petunia* and *S. truncata* sequencing data obtained from SourceBioscience

Quality Value	> 35	20 - 35	< 20
LOR Value	> 600bp	301-600bp	< 300bp

Reaction	Template Name	Primer Name	N-Base Calls	Length of Read	Quality Value
8	Cactus_S3W1	S3_RV	2 %	518 bp	53
9	Cactus_S3P1	S3_FW	3 %	522 bp	53
10	Cactus_S3P1	S3_RV	2 %	519 bp	52
11	P_S3_14_1	P_S3_FW	2 %	495 bp	53
12	P_S3_14_1	P_S3_RV	2 %	500 bp	52
13	P_S3_15_9	P_S3_FW	3 %	496 bp	51
14	P_S3_15_9	P_S3_RV	3 %	502 bp	50
15	P_SD_14_1	P_SD_FW	2 %	541 bp	54
16	P_SD_14_1	P_SD_RV	2 %	540 bp	54

Appendix 4

Supplementary Table S2 - *Solanaceous S-RNases* used for the phylogenetic studies

S-RNase	Accession #	Species
Sd	Unpublished	*P. inflata*
S3L	AJ271065.1	*P. hybrida*
Sx	M81686.1	*P. hybrida*
Sx	M81685.1	*P. hybrida*
S22	AB933141.1	*P. hybrida*
Ss2	LC819228.1	*P. integrofolia*
Rnx2	M93418.1	*P. inflata*
Ss1	LC819227.1	*P. integrifolia*
PhS3	LC819168.1	*P. integrifolia*
Sy	LC819241.1	*P. integrifolia*
So1	LC819216.1	*P. integrifolia*
See	*LC819203.1*	*P. integrifolia*
SL4	*LC819191.1*	*P. integrifolia*
S17	*AF301177.1*	*P. integrifolia*
So3	*LC819218.1*	*P. integrifolia*
SL5	*LC819192.1*	*P. integrifolia*
S11	*AF301172.1*	*P. integrifolia*
PiS13	*LC819179.1*	*P. integrifolia*
Sdd	*LC819201.1*	*P. integrifolia*
S6	*AF301167.1*	*P. integrifolia*
Sqq	*LC819224.1*	*P. integrifolia*
S9	*AF301170.1*	*P. integrifolia*
PiS10	*LC819176.1*	*P. integrifolia*
Sv	*LC819236.1*	*P. integrifolia*
Sw	*LC819239.1*	*P. integrifolia*
S21	*AF301180.1*	*P. integrifolia*
PiS21	*LC819182.1*	*P. integrifolia*
S10	*AF301171.1*	*P. integrifolia*
Sp	*LC819219.1*	*P. integrifolia*
Srr	*LC819226.1*	*P. integrifolia*
Sii	*LC819211.1*	*P. integrifolia*
St	*LC819230.1*	*P. integrifolia*
PhS7	*LC819174.1*	*P. integrifolia*
S20	*AF301179.1*	*P. integrifolia*
S16	*AF301176.1*	*P. integrifolia*
Su2	*LC819232.1*	*P. integrifolia*
PiS16	*LC819181.1*	*P. integrifolia*
Sr	*LC819225.1*	*P. integrifolia*
Ss2	*LC819228.1*	*P. integrifolia*
Sk	LC819213.1	*P. axillaris*
Sr	LC819225.1	*P. integrifolia*
Sv	AJ271062.1	*P. hybrida*
S1	U07362.1	*P. hybrida*
Sv	AB568389.1	*P. hybrida*

SB1	AB016522.1	*Petunia-x-hybrida*
S0m	AB933144.1	*Petunia-x-hybrida*
mRNA	OP341737.1	*Petunia-x-hybrida*
So	FJ490180.1	*Petunia-x-hybrida*
SB1	AB016522.1	*Petunia-x-hybrida*
S7	AB568388.1	*Petunia-x-hybrida*
S10	AB933140.1	*Petunia-x-hybrida*
SB2	AB016523.1	*Petunia-x-hybrida*
PhS5	LC819173.1	*P. axillaris*
mRNA	D63888.1	*N. alata*
S7	U13255.1	*N. alata*
S6	U08861.1	*N. alata*
S3	U66427.1	*N. alata*
SC10	U45959.1	*N. alata*
2mRNA	M24600.1	*N. alata*
S2	X03803.1	*N. alata*
SmRNA	L25930.1	*N. alata*
SmRNA	L25929.1	*N. alata*
S2	U08860.1	*N. alata*
S1	KJ814932.1	*Solanum-habrochaites*
S17	JQ040814.1	*Solanum-habrochaites*
S16	OK091165.1	*Solanum-habrochaites*
SRN17	OK091166.1	*Solanum-habrochaites*
S9	MW183816.1	*Solanum-habrochaites*
S20	JQ040817.1	*Solanum-habrochaites*
S6	GU361149.1	*Solanum-habrochaites*
S6	MW183811.1	*Solanum-habrochaites*
S6	MW183814.1	*Solanum-habrochaites*
S6	MW183812.1	*Solanum-habrochaites*
S6	MW183813.1	*Solanum-habrochaites*
S6	MW183810.1	*Solanum-habrochaites*
S4	AB072472.1	*Solanum-habrochaites*
S4	EF680094.1	*Solanum-chilense*
S2	MW183815.1	*Solanum-chilense*
S5	MZ561408.1	*Solanum-tuberosum*
FSc	*L40544.1*	*Solanum-carolinense*
SP	AY706469.1	*Solanum-carolinense*
HSC	L40546.1	*Solanum-carolinense*
S11a	*D17323.1*	*Solanum-peruvianum*
S11a	D17322.1	*Solanum-peruvianum*
S22	AB072464.1	*Solanum-peruvianum*
S12	D17324.1	*Solanum-peruvianum*
S11	U28795.1	*Solanum-peruvianum*
Ss7	*KX641186.1*	*Solanum-stenotomum*
SPALL3	*HM195093.1*	*Lycium-pallidum*
SPALL2	HM195092.1	*Lycium-pallidum*
SLCESh	*HM195098.1*	*Lycium-cestroides*
CARO3	*MW118931.1*	*Lycium-carolinianum*
CARO1	MK573196.1	*Lycium-carolinianum*
CARO33	MK573182.1	*Lycium-carolinianum*
S5	*MK843822.1*	*Lycium-barbarum*
SBARB5	HM195027.1	*Lycium-barbarum*
DASY3	*HM195038.1*	*Lycium-dasystemum*
S5	*AF105343.1*	*Lycium-andersonii*
SHAWS	*HM195083.1*	*Lycium-shawii*
S11	*DQ367863.1*	*Lycium-parishii*
S10	DQ367862.1	*Lycium-parishii*
S13	*DQ367865.1*	*Lycium-parishii*
S2	*EU074808.1*	*Lycium-cinereum*
S-glycoprotein	*S65048.1*	*Lycopersicon-peruvianu*

S12	U28796.1	*Lycopersicon-peruvianu*
S11a	S65047.1	*Lycopersicon-peruvianu*
S11	S81597.1	*Lycopersicon-peruvianu*
S11	U28795.1	*Lycopersicon-peruvianu*
S1	*EU382082.1*	*Iochroma-gesnerioides*
S1	*EU382078.1*	*Iochroma-australe*
S2	*EU382079.1*	*Iochroma-australe*
S2	*EU382087.1*	*Iochroma-loxense*
S1	*EU382074.1*	*Eriolarynx-lorentzii*
S1	*EU382077.1*	*Dunalia-brachyacantha*
S1	*AY766243.1*	*Brugmansia-versicolor*
S2	*AY766244.1*	*Brugmansia-versicolor*
S2	*EU382085.1*	*Vassobia-breviflora*
S22	*AY454120.1*	*Witheringia-solanacea*
S4	AY454102.1	*Witheringia-solanacea*
S18	AY454116.1	*Witheringia-solanacea*
S3	AY454101.1	*Witheringia-solanacea*
S15	AY454113.1	*Witheringia-solanacea*
S4	AF102068.1	*Witheringia-solanacea*
S3	AY454115.1	*Witheringia-solanacea*
S6	AY454104.1	*Witheringia-solanacea*
S16	AY454114.1	*Witheringia-solanacea*
S5	AY454103.1	*Witheringia-solanacea*
S11	*AF102069.1*	*Witheringia-maculata*
S3	*AF102067.1*	*Witheringia-maculata*
S20	*AY454118.1*	*Witheringia-maculata*
S5	*AF102065.1*	*Witheringia-maculata*
S2	*AF102066.1*	*Witheringia-maculata*
S3	*EU382076.1*	*Eriolarynx-lorentzii*
S33	*AF374427.1*	*Physalis-longifolia*
S34	*AF374428.1*	*Physalis-longifolia*
S27	*AF281200.1*	*Physalis-longifolia*
S9	*AF281187.1*	*Physalis-longifolia*
S22	*AF281196.1*	*Physalis-longifolia*
S18	*AF281194.1*	*Physalis-longifolia*
S30	*AF374425.1*	*Physalis-longifolia*
S20	*L46672.1*	*Physalis-crassifolia*
S21	*L46673.1*	*Physalis-crassifolia*
PCIN12	*AF058941.1*	*Physalis-cinerascens*
PCIN11	AF058940.1	*Physalis-cinerascens*
PCIN10	AF058939.1	*Physalis-cinerascens*