THE ROLE OF PREZYGOTIC SELF-COMPATIBILITY IN FACILITATING INTERSPECIFIC COMPATIBILITY IN SOLANUM SECTION PETOTA

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ABSTRACT

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Domesticated potato (*Solanum tuberosum* L.) is the fourth most important food crop worldwide and critical food security crop. The high significance of this crop coupled with high production losses place potato at a high priority for genetic improvement, especially in the face of climate change. Potato breeding is undergoing a seismic shift from a tetraploid outcrossing crop towards a F1 hybrid system using diploid inbred lines as parents. This transformation has also changed how potato breeders access important traits from wild species. Pre- and post-zygotic barriers, such as self-incompatibility (SI) and endosperm failure, inhibit and complicate the use of the more valuable wild species in conventional and diploid breeding schemes. Traditionally plant breeders have employed the SC x SI rule, using self-compatibility (SC) to increase the success of interspecific crosses. In order to evaluate the potential of several different SC factors in facilitating broad interspecific compatibility: Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 knockout (KO) lines of *S-RNase*, *HT-B* and *HT-B* + *S-RNase* in *S. tuberosum* were evaluated along with the self-compatible species *S. verrucosum*, and *S-locus inhibitor* gene (*Sli)* from *S. chacoense*. Findings confirm the central role of *S-RNase* in interspecific pollination barriers and the inconsequential role of *HT-B* alone. *Sli* had no effect on interspecific barriers despite its prominent role as a source of SC. Finally, *S. verrucosum* styles displayed an absence of prezygotic barriers. Further analysis of the role of *HT-A*, as well as other mechanisms that potentially underlie the SC phenotype in *S. verrucosum* will further clarify the role of specific genetic factors that regulate interspecific compatibility in *Solanum* section *Petota*

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CHAPTER 1

INTRODUCTION

Potatoes feed the world

Cultivated potato, *Solanum tuberosum* L. Group *Tuberosum* (2n=4x=48), plays a significant role in the human diet as a primary source of carbohydrates (FAOSTAT, 2019). Potatoes are also the fourth most important food crop worldwide after wheat rice and maize (FAOSTAT, 2019). On a yield per unit area basis, potato is highly productive compared to other staple crops and provides substantially more protein per unit of land area than rice or wheat and is a significant source of carbohydrates, antioxidants and micronutrients (Kaldy, 1972; Brown 2005; Zehara 2012). The United States currently ranks as the world's fifth largest producer of potatoes where the crop generates 3.9 million dollars over 0.9 million acres (FAOSTAT, 2019; NASS 2020)

The role of diploid potato breeding in the advancement of potato breeding

The significance of the potato crop, coupled with the high production losses due to both biotic and abiotic forces, place potato as a high priority for genetic improvement, especially in the face of climate change (Raymundo et al., 2018). Nearly all cultivated potatoes are tetraploid (2n=4x=48), which impedes the fixation of desirable alleles in populations and new cultivars. Consequently, there is an effort to develop a F1 hybrid system using diploid inbred lines as parents. This would accelerate the generation of new varieties, and simplify efforts to incorporate better agronomic and abiotic resistance traits into existing germplasm (Jansky et al., 2016). The use of diploid germplasm also simplifies the introgression of traits from wild species that have been major sources for disease resistance and abiotic stress tolerance.

Importance of wild species and the barriers to their effective use

The diversity of wild species and their classification

There are currently 107 recognized species of wild and cultivated potato in *Solanum* section *Petota*; with natural ranges extending from the desert southwest of North America to the Southern Cone of South America (Spooner et al., 2014). The classification of this taxonomic group is complicated by differences in ploidy and interspecific hybridization, with taxonomists recognizing as many as 232 species to the current 107 (Hawkes, 1990; Huang et al., 2019; Spooner et al., 2018). The use of DNA analyses to aid classification has elucidated the presence of four main clades, with the first two clades later being recombined to form the larger 1+2 clade after further analysis (Spooner et al., 2018). Another classification system was also created to describe the relationships of sexual compatibility between species by their Endosperm Balance Number (EBN) as proposed by Johnston et al. (1980).

In the context of potato breeding both EBN and taxonomic classification play important roles. Different species are assigned an EBN number based on their ability to form normal endosperm when crossed to a species with a "known" EBN number (Johnston et al., 1980). As a general rule only individuals with the same EBN number are able to form viable seeds, and crossing individuals of different EBN numbers results in endosperm failure and aborted seeds (Johnston et al., 1980; Lafon-Placette and Köhler, 2016; Stadler et al., 2021). Of all the wild species available to potato breeders, the species in clade 1+2 which all have an EBN number of one are of special interest because of their high degree of disease resistance, pest resistance, and tuber quality traits (Lara-Cabrera and Spooner, 2004; Jansky and Hamernik, 2009). These "1EBN" species such as *S. bulbocastanum*, *S. pinnatisectum*, and *S. jamesii* are unfortunately difficult for plant breeders to access as all cultivated species have an EBN of two making

interspecific hybridization difficult and seed formation exceedingly rare (Jansky and Hamernik, 2009; Johnston and Hanneman, 1982).

Wild potato species, especially the 1EBN species, are important sources of robust disease resistance and valuable consumer traits (Bhaskar et al., 2010; Duan et al., 2012; Helgeson et al., 1998). Each species selected as pollen parents for this study have unique value in a potato breeding program. *S. bulbocastanum, S. pinnatisectum,* and *S. jamesii* exhibit robust levels of resistance to potato late blight (Karki et al., 2020; Zheng et al., 2020). *S. jamesii* is also source of Potato Virus Y (PVY) resistance and important long-term tuber storage traits (Singh et al., 1994; Bamberg 2010). The South American species *S. commersonii* has displayed resistance to bacterial wilt and frost tolerance (Laferriere et al., 1999; Esposito et al., 2019). Each of these four pollen donors have meaningful resistance to Colorado potato beetle (Jansky et al., 2009). Other species used in this study are important in the advancement of diploid breeding and introgression of wild germplasm. *S. chacoense* (2EBN) represented by the clones M6 and USDA8380-1 are important sources of *Sli*-based self-compatibility and leptine-mediated Colorado potato beetle (*Leptinotarsa decemlineata*) host plant resistance, respectively (Kaiser et al., 2020; Jansky et al., 2014). Finally, *S. verrucosum* (2EBN) is the primary component of bridge crosses and its unilateral incompatibility relationship with cultivated potato has been well documented (Eijlander et al., 2000; Jansky & Hamernik, 2009; Yermishin et al., 2014).

Unfortunately, the use of 1EBN species in conventional breeding programs is often impeded by pre- and postzygotic barriers (Novy & Hanneman, 1991; Jansky & Hamernik, 2009). The genetic basis for these interspecific barriers has not been fully explored in *Solanum* section *Petota*. Consequently, the contribution of each of these barriers to interspecific incompatibility is not clear. In the closely related *Solanum* section *Lycopersicon*, there are several barriers that

have been characterized, but these have not been confirmed in *Solanum* section *Petota*. Prezygotic barriers such as *S-RNase* dependent and independent mechanisms not only inhibit interspecific hybridization but also function in gametophytic self-incompatibility (Tovar-Méndez et al., 2014, 2017; Baek et al., 2015). Additionally, postzygotic barriers such as endosperm failure and sterile progeny are major impediments in the use of wild species in conventional breeding (Hermsen and Ramanna, 1976; Lafon-Placette and Köhler, 2016; Städler et al., 2021). The understanding of these barriers is crucial in the effort to introgress wild species into cultivated diploid germplasm.

Prezygotic interspecific barriers

The prezygotic barrier of gametophytic self-incompatibility (GSI) also presents a significant obstacle to interspecific crosses. The GSI system is controlled by the multiallelic S-locus (Porcher and Lande, 2005). This locus contains a set of tightly linked genes: *S-RNase*, expressed in the style, and *SLF* (S-locus F-box), expressed in the pollen (Kubo et al. 2010). SLF proteins recognize specific sets of *S-RNase* alleles, allowing self vs. non-self-pollen to be recognized. In compatible pollinations, SLF proteins degrade *S-RNase* proteins allowing the pollen tubes to make it to the ovary. In self-pollinations, SLF proteins do not degrade "self" *S-RNase* resulting in the cytotoxic activity of *S-RNase* and pollen tube inhibition. Other components such as *HT* and the 120kDa glycoprotein facilitate the function of *S-RNase*, thereby affecting self-incompatibility (Markova et al., 2016; Hua et al., 2008; Baek et al., 2015; Tovar-Méndez et al., 2014, 2017). Because *HT* mediates the action of *S-RNase*, it also exhibits and influence on interspecific crosses that are inhibited by *S-RNase* (Baek et al., 2015; Tovar-Méndez et al., 2014, 2017).

Interspecific crosses in Solanaceae often display unilateral incompatibility or incongruity (UI). UI usually follows the self-incompatible (SI) x self-compatible (SC) rule of Lewis and Crowe (1958). This rule states that SI species × SC species crosses are incompatible, but the reciprocal crosses are compatible (Bedinger et al., 2011; Baek et al., 2015; Murfett et al., 1996). This is because the same factors that affect pollen tube growth to prevent self-pollinations also prevent interspecific hybridization by inhibiting the pollen tubes of other species (Baek et al., 2015; Bedinger et al., 2011). It has been demonstrated that *S-RNase* partially mediates self-compatibility in *Solanum* section *Petota*, but has not been observed to mediate interspecific compatibility as it has in *Solanum* section *Lycopersicon* (Tovar-Méndez et al., 2014; Enciso-Rodrigues et al., 2019) Likewise, *HT* has been demonstrated to mediate *S-RNase* dependent and independent self and interspecific compatibility in section *Lycopersicon* but not in *Petota* (Tovar-Méndez et al., 2014, 2017) It is important to note that not only are section *Lycopersicon* and *Petota* close relatives phylogenetically, but that gene sequence and order are conserved between these two taxonomic sections (Bohs & Weese, 2007; Zhu et al., 2008). Because of this, it is likely that there will be similar findings regarding the role of *HT* and *S-RNase* in interspecific compatibility in section *Petota* as has been found in *Lycopersicon*.

The correlation between SC and interspecific compatibility in Solanum verrucosum

The interaction between post- and prezygotic barriers confounds and complicates interspecific crosses. *S. verrucosum* is an important species for creating bridge crosses with important 1EBN species. When used in interspecific crosses, *S. verrucosum* is used as the female parent as it exhibits unilateral compatibility with self-incompatible species (Dinu et al., 2005). As with *Solanum* section *Lycopersicon,* this follows the SI x SC rule since *S. verrucosum* is a self-compatible species (Tovar-Méndez et al., 2014; Jansky & Hamernik, 2009). Self-

compatibility in *S. verrucosum* has been attributed to the lack of a functional *S-RNase* protein (Eijlander, 1998). The absence of functional *S-RNase* alleles prevents the inhibition on pollen tube growth in what would otherwise be incompatible pollinations. While it has been demonstrated that there is no detectable *S-RNase* at the protein level (Eijlander, 1998), it is unknown if the genes for *S-RNase* in *S. verrucosum* are missing, non-functional, or inhibited in some way. The lack of this functional *S-RNase* protein in *S. verrucosum* is likely the reason that it is able to serve as a major bridge species in between 1EBN species and cultivated germplasm (Eijlander, 1998).

Sli in interspecific compatibility

The ability of *Sli* to overcome interspecific barriers was initially reported by Sanetomo et al. (2014). It was also observed that the pollen tubes from *Sli* carrying pollen donors were able to bypass stylar barriers, while pollen tubes from *S. megistacrolobum*, *S. demissum*, and *S. phureja* stopped growing at or near the stigma of 4x and 2x *S. pinnatisectum* (Sanetomo et al, 2014). The ability of *Sli* to overcome all prezygotic stylar barriers, not just those controlling gametophytic self-incompatibility, could be an important factor in accessing the sexually isolated species. Additionally, the widespread use of *Sli*-based SC in North American diploid potato breeding would mean that most public and private breeders would have access to these methods (Kaiser et al., 2021). The employment of *Sli* to overcome interspecific barriers could represent and powerful albeit underutilized method to access valuable traits from sexually isolated species.

Post-zygotic barriers Endosperm failure and Endosperm Balance Number

A significant barrier to interspecific hybridization is endosperm failure. Endosperm regulates the early development of the embryo and is a major food store for the developing seed (Lester & Kang, 1998). In angiosperms, the process of double fertilization occurs when one

sperm cell fuses with the egg cell to form an embryo, while a second sperm cell fuses with the polar cell to form the endosperm (Raghavan, 2003). The development and specifically the rate of cellularization of the endosperm must occur in concert with the development of the embryo; otherwise, endosperm failure occurs and embryo growth is arrested (Lafon-Placette and Köhler, 2016). In interspecific and interploidy crosses, the parental dosage between the two parents can be out of balance. This powerful post zygotic barrier maintains the ploidy level and integrity of species.

Endosperm Balance Number (EBN)

EBN is a measure of "effective ploidy" not actually ploidy (Johnston et al., 1980; Roth et al., 2019; Stadler et al., 2021). Endosperm development is a tightly regulated process, any increase or decrease in expression from the maternal or paternal genomes will result in endosperm failure (Lafon-Placette, and Köhler, 2016; Stadler et al., 2021). Sometimes this difference in expression occurs because of differences in actually ploidy, for example: crossing a tetraploid female to a diploid male of the same species results in endosperm failure (Johnston et al., 1980; Stadler et al., 2021). The extra copy of the female genome results in overexpression of maternally expressed genes, preventing normal seed development, since both parents are of the same species; this is an example of the phenomenon referred to as "triploid block" (Marks, 1966; Stadler et al., 2021). There are different levels of gene expression between species of the same ploidy as well. Because of this, crossing two different species can sometimes have the same outcome as crossing individuals of the same species but different levels of actual ploidy, this is where EBN comes in as a measure of "effective ploidy" (Jansky, and Hamernik, 2009; Lafon-Placette, and Köhler, 2016; Stadler et al., 2021).

Many misunderstandings surround EBN and effective ploidy. Among these being: the genetic basis and thereby the phylogenic relationships between different species of the same EBN, and the use of discrete categories to describe post-zygotic barriers. Despite early research by Ehlenfeldt and Hanneman (1988) detailing that genetic control was polygenic, the belief still persists in the potato breeding community that all species of the same EBN are related and intercrossable. The use of discrete categories to classify relationships of interspecific compatibility has limited value and lacks specificity; especially since it is clear that the genetic mechanisms than govern endosperm failure are complex and genome-wide (Ehlenfeldt and Hanneman, 1988; Lafon-Placette, and Köhler, 2016; Stadler et al., 2021). Additionally, individual species of the same EBN are not always closely related (Pritchard, 2005). Therefore, it would be inappropriate to surmise that the genetic mechanisms that result in the categorization of individual North American and South American species as "1EBN" to be the same (Pritchard, 2005).

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CHAPTER 2

BREAKING BARRIERS: THE ROLE OF PLANT ONTOGENY AND GAMETOPHYTIC SELF-INCOMPATIBILITY FACTORS S-RNASE AND HT-B IN INTERSPECIFIC POLLEN REJECTION IN SOLANUM SECTION PETOTA

Abstract

The relationships of interspecific compatibility and incompatibility in *Solanum* section *Petota* are complex and intricate. Inquiry into these relationships has elucidated the pleiotropic and redundant function of *S-RNase* and *HT* which tandemly and independently mediate both interspecific and intraspecific pollen rejection. The wild species *Solanum bulbocastanum, S. commersonii, S. jamesii,* and *S. pinnatisectum* were used as pollen donors to pollinate Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas9 gene knockouts of *S-RNase* and *HT-B* in the clone DRH195. Analysis of pollen tube growth was used to determine the role of *S-RNase* and *HT-B* in prezygotic stylar barriers that inhibit the pollen tube growth of these species. The findings presented here are consistent with previous work conducted in *Solanum* section *Lycopersicon* showing that *S-RNase* plays a central role in interspecific pollen rejection. Statistical analyses also demonstrated that *HT-B* alone was not a significant factor in these pollinations highlighting the potential function and importance of *HT-A* and other factors. *S. commersonii* was unique in comparison to the other pollen donors used as *S-RNase* did not appear to affect pollen rejection. Intra and interspecific compatibility is also heavily influenced by environmental and ontogenic factors. Although variable gametophytic self-incompatibility responses due to plant and floral age have been previously demonstrated, these findings have not been observed in interspecific pollinations. Analysis of pollen tube length across three time-points indicates that plant age significantly impacts interspecific pollen rejection.

Objective:

Apply findings from *Solanum* section *Lycopersicon* to section *Petota* by demonstrating that *S-RNase* and *HT* play major roles in interspecific compatibility. Knock-outs of *S-RNase* did increase interspecific compatibility. However, both *HT-A* and *HT-B* would need to be knocked out in order to significantly affect interspecific compatibility in these crosses as they serve the same function. These findings are consistent with those in *Solanum* section *Lycopersicon.*

Introduction

Gametophytic self-incompatibility (GSI) is the default condition in diploid cultivated potato and the vast majority of its wild relatives (Carson and Howard 1942; Whalen and Anderson, 1981; Fuji et al. 2016). GSI in Solanaceae is governed by the multiallelic S-locus on chromosome 1, containing *S-RNase* tightly linked to multiple *SLF* (S-locus F-box) genes (Enciso-Rodriguez et al. 2019; Kaiser et al., 2021; Kubo et al., 2010; McClure et al., 2011). This GSI system also serves the dual function of preventing interspecific pollinations as well by inhibiting the growth of pollen tubes from other species (Baek et al., 2015; Bedinger et al., 2011). Because of this general overlap in function, interspecific crosses in Solanaceae often display unilateral incompatibility or incongruity (UI). UI usually follows the self-incompatible (SI) x self-compatible (SC) rule reported by Lewis and Crowe (1958) where SC species can only act as the female, as the functional GSI system in the SI species prevents both self and interspecific pollen from reaching the ovary (Bedinger et al., 2011; Baek et al., 2015; Murfett et al,. 1996).

While the SI x SC rule proposed by Lewis and Crowe (1958) has been generally helpful, it is an oversimplification and does not fully capture the complex relationships of interspecific compatibility and incompatibility in *Solanum*. Interspecific pollen rejection by SC species has

been observed between members of the tomato and potato clades of *Solanum* (Baek et al., 2015; Pandey, 1962; Tovar-Méndez et al., 2017). This can be partially explained by the differences in gene presence and function between SC species. *S-RNase* and other components such as *HT* which facilitate the function of *S-RNase* are the primary components of GSI (Markova et al., 2016; Hua et al., 2008; Baek et al., 2015; Tovar-Méndez et al., 2014, 2017). The lack of functional *S-RNase* alleles will result in SC and greater acceptance of interspecific pollen (Tovar-Méndez et al., 2017; Enciso-Rodrigues et al., 2019). Additionally, *HT* has been demonstrated to mediate both SC and S-RNase-independent interspecific compatibility (O'Brien et al., 2002; Tovar-Méndez et al., 2014, 2017).

Differences in the response of GSI are also dependent on environmental conditions and plant and floral ontogeny. Environmental conditions such as temperature and humidity significantly impact fruit set even in compatible pollinations (Bienz, 1958; Henderson and LeClerg, 1943). Elevated temperatures and increased floral age has also been observed to significantly weaken the GSI response in *S. peruvianum* and *S. carolinense*, respectively (Webb and Williams, 1988; Travers et al., 2004). Because there is a substantial overlap between the mechanisms regulating GSI and interspecific pollen rejection, the impact of temperature and floral age on GSI are relevant to interspecific compatibility as well. It has also been speculated that plant ontogeny plays a role in the strength of the GSI response, independent of floral age (Kaiser, personal communication, 2020). Observational findings have led to the speculation that the GSI response weakens as the plant ages. This has led to the hypothesis that the weakening of GSI in older plants results in greater self-compatibility or that SC is only observable towards the very end of plant maturity. This hypothesis is generally supported by findings in *S. carolinense*

and *Nicotiana alata* where there is a noticeable deterioration of GSI in older plants (Travers et al., 2004; Liao et al., 2016).

Materials and Methods

Plant materials

The wild species *S. bulbocastanum*, *S. commersonii*, *S. jamesii*, and *S. pinnatisectum* were used as pollen donors for this study. Multiple plant introduction populations for each species were acquired from the USDA-ARS Potato Germplasm Introduction Station (Sturgeon Bay, WI) and individual genotypes were selected from their respective field-grown plant introduction populations based on their fertility and overall vigor (Table 1). In this study, *S-RNase* and/or *HT-B* mediated interspecific pollen rejection was investigated using CRISPR-Cas9 gene knockouts of *S. tuberosum* clone DRH195 and untransformed controls (Table 2) (Enciso-Rodriguez et al., 2019; Lee et al., 2021). Each genotype was maintained in tissue culture on Murashige and Skoog Basal Medium with Vitamins and Sucrose (PhytoTech Labs) and Phyto Agar (Research Products International) prepared with DI water and balanced with 1M HCL and 8N NaOH to a pH of 5.8, and cultured in growth chambers with 16-h-light/8-h-dark photoperiod at 22°C and average light intensity of 200 μmoles m-2s-1.

Greenhouse pollination assays

In October of 2020, two individuals from each genotype were transferred directly from tissue culture to 14L (3.8 gallon) plastic pots filled with Suremix Perlite peat and perlite soilless medium (Michigan Grower Products INC.). For the duration of the experiments, greenhouse conditions were maintained at 20° C with a 16-hr photoperiod under Philips GreenPower light-emitting diode (LED) DR/W-MB lights (Philips Lighting Holding B.V., Netherlands). In order to validate the male fertility of the pollen donors, pollen from 5-6 anthers was collected directly

onto a glass slide and immediately stained with acetocarmine-glycerol as described by Ordoñez (2014), covered with a cover slip, and sealed using clear nail polish. These slides were stored at room temperature in the dark and visualized the same day using a Leica DM750 binocular microscope (Leica Microsystems, Germany) with the associated Leica imaging software at 10x and 40x magnification. A minimum of 100 pollen grains were used to calculate the percentage of viable pollen. Stained, turgid pollen was classified as viable while any grains that were shriveled, unstained, or unusually large or small were classified as unviable (Figure 2.10).

Stylar squash assays

For the evaluation of stylar barriers in interspecific crosses, 6-8 newly opened flowers (within 24 hours after anthesis) from each of the female genotypes were carefully emasculated and pollinated with fresh pollen collected on a glass slide directly from the pollen donor. Styles were collected 48 hours post-pollination, by removing the remaining petals and sepals and storing the remaining intact style, ovary, and receptacle in 1.5mL microcentrifuge tube containing a 3:1 ethanol/acetic acid fixation solution. These styles were then kept in the dark at room temperature for at least 24 hours. Styles and ovaries were then softened using an 8N NaOH solution at 60C for 1 hour. Samples were then rinsed three times with distilled water and stained with 0.1% aniline blue in 0.1N K3PO4 keeping them in dark conditions with light shaking. Styles with attached ovaries were then placed on glass slides, gently squashed under a coverslip, and sealed with nail polish for subsequent visualization. Samples were visualized using a Nikon Eclipse Ni-U upright microscope (Nikon Instruments Inc., Melville, NY, United States) with a SOLA light engine (Lumencor, Beaverton, OR, United States), and photographed with the attached ANDOR Zyla sCMOS camera (Oxford Instruments, United Kingdom) and NIS-

Elements BR 5.02 software. Images of each stylar sample were stitched together using Image Composite Editor 2.0 software. Pollen tube growth measurements were made using ImageJ 1.53e software (Schneider et al., 2012). Measurements of pollen tube growth were calculated as a proportion of the total length of the style due to the variation of total style length with a single genotype. Pollinations were made at three discrete time points, separated by 30 days, with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse.

Data collection and analysis

Measurements of the pollen tube front, or the point where the majority of the pollen tubes stop, the longest pollen tube, and the total length of the style were collected using ImageJ software (see Figure 2.1 for example of measurement methods). From these measurements, distance from the surface of the stigma to the pollen tube front and length of the longest pollen tube were calculated based on the total length of the style in which they were measured. Significant differences between means ($\alpha = 0.05$) from replicated measurements where calculated using analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) using "R" software version 4.0.4.

Results

The role of S-RNase and HT-B in interspecific pollen rejection

S-RNase and *HT-B* play important roles in GSI by preventing self-pollination and function as primary components of S-RNase dependent and independent rejection of interspecific pollen in *Solanum* (Tovar-Méndez et al., 2014, 2017; Baek et al., 2015). In this study, the use of stylar squash assays were employed to visualize the growth and inhibition of

pollen tubes within the style. Results from statistical analysis of pollen tube growth are consistent with previous findings by Tovar-Méndez et al (2014) showing that *S-RNase* plays a central role in interspecific pollen rejection. This is readily observable in the majority of pollen tubes as well as the longest pollen tubes from *S. bulbocastanum* (Figures 2.2-2.3) and the longest pollen tubes for *S. pinnatisectum* (Figure 2.8). Pollen tubes from these two species were able to penetrate significantly further down the styles in *S-RNase* and *S-RNase/HT-B* knockouts. Additionally when *S. bulbocastanum* and *S. pinnatisectum* were the pollen donors, the longest pollen tubes were nearly able to traverse the entire length of the style in *S-RNase* knockouts (Figures 2.2 & 2.8). With *S. jamesii* (Figures 2.6-2.7) and *S. commersonii* (Figures 2.4-2.5) as the pollen donors the effect of *S-RNase* was not statistically significant, although the inhibitory effect of *S-RNase* was still evident for *S. jamesii* (Figure 2.6).

Results from the same analyses demonstrate that *HT-B* alone is not a significant factor in the inhibition of pollen tubes from these species. No statistically significant difference was observed between the ability of pollen tubes to penetrate the styles of wild-type plants compared to the *HT-B* knock-out line. Likewise, the differences between the *S-RNase* knock-out and the *S-RNase/HT-B* knock-out were insignificant; showing that knocking out both *S-RNase* and *HT-B* compared to *S-RNase* alone offered no advantage to pollen tubes attempting to traverse the style. For example, pollen tubes from *S. jamesii* were generally arrested closer to the stigma in the *HT-B* knock-out compared to the wild type DRH195 (Figure 6). These results do not contradict the findings from Tovar-Méndez et al (2014, 2017) that showed *HT* proteins played a significant role in interspecific pollen rejection. Due to the vicissitudes of gene editing, knock-outs of *HT-A* were not available for this study. Since *HT-A* remains functional and active with its expression in DRH195 shown by RT-PCR and cDNA sequencing in all of the female genotypes used, it can be

concluded that either: 1) *HT-A* plays a greater role in interspecific pollen rejection compared to *HT-B*; 2) there is a significant overlap in gene function between *HT-A* and *HT-B* such that the loss of *HT-B* is not significant; and/or 3) *HT* in general is not a significant component of interspecific pollen rejection in these combinations.

The contrast between *S. commersonii* and the other pollen donors used in this study highlights the diversity in interspecific pollen rejection systems. *S. commersonii* was unique as a pollen donor as both *S-RNase* and *HT-B* did not appear to affect the rejection of pollen in DRH195. As discussed previously, since *HT-A* is still functional, *HT-A* alone may mediate the *S-RNase/HT-B* independent rejection of *S. commersonii* pollen. If this is the case, this would be consistent with the findings of Tovar-Méndez et al (2017). The rejection or allowance of interspecific pollen is entirely dependent on the specific combination of species used as parents (Baek et al., 2015). This is also the case with the role of certain genes in interspecific pollen rejection. *HT-A*, *HT-B*, *S-RNase* and other factors such as the 120 kDa arabinogalactan protein or farnesyl pyrophosphate synthase (*FPS2*) may play significant or inconsequential roles during the rejection of interspecific pollen; and these roles are entirely dependent on the specific species and genotypes present in these interactions (Bedinger et al, 2011; Baek et al, 2015; Qin and Chetelat, 2021).

Effects of plant age on interspecific pollen rejection

It has been speculated that plant age plays a role in the GSI response in *S. tuberosum* and its hybrids. It has been hypothesized that in greenhouse pollinated plants, the GSI response to pollen weakens as a plant ages. Resulting in the hypothesis that the weakening of GSI with age would result in greater self-compatibility in older plants. Outside of *Solanum* section *Petota*, this deterioration of GSI with plant age, as well as the weakening of the GSI response, has been

observed in *S. carolinense* and *Nicotiana alata* (Travers et al., 2004; Liao et al., 2016). In order to evaluate the effect of plant age alone, pollinations were only made on newly opened flowers, within 24 hours of anthesis. Pollinations were made at three discrete time-points, with the first pollination occurring at the initial onset of flowering and each time-point being separated by 30 days.

Different conclusions can be drawn from the analysis of pollen tube front and longest pollen tube measurements. Examination of the pollen tube front, the majority of pollen tubes were able to penetrate further down the style in older plants, however, this is not consistent. In contrast, analysis of the longest pollen tubes yielded different results. Individual pollen tubes were able to penetrate significantly further down the style at the third time-point in almost every case, dependent on the pollen donor and the female used. When *S. bulbocastanum* (Figure 2.3) or *S. jamesii* (Figure 2.7) were the pollen donors, the highest mean values for the longest pollen tubes were always found in the styles collected at the oldest plant stage. With *S. commersonii* as a pollen donor, the mean values for the longest pollen tubes were always among the lowest at the first time point (Figure 2.5). For *S. pinnatisectum*, pollen tubes always traveled further down the style in older plants with the exception of the *HT-B* knock-out where this trend was reversed (Figure 2.9). Amongst all the pollen donors, individual pollen tubes penetrated significantly further down the style at the third time-point compared to the first; the only exception being the combination of *S. pinnatisectum* as the pollen donor and the *HT-B* knock-out being the female (Figure 2.9).

Discussion

The relationships of interspecific compatibility and incompatibility in *Solanum* section *Petota* are complex and intricate. Inquiry into these relationships has elucidated the pleiotropic

and redundant function of *S-RNase* and *HT* which tandemly and independently mediate both interspecific and intraspecific pollen rejection (Baek et al., 2015; Tovar-Méndez et al., 2014, 2017). The outcome of interspecific pollinations is specific to the individual species involved and direction of the cross due to the direct and indirect involvement of multiple factors such as *HT* and *S-RNase*, 120 kDa, Cullin1 (*CUL1*), and farnesyl pyrophosphate synthase (*FPS2*) (Bedinger et al., 2011; Li and Chetelat, 2015; McCormick, 2018; Tovar-Méndez et al., 2017). This recent advancement partially explains the inconsistencies to the SI x SC rule found by Pandey (1962) and Dionne (1961) where interspecific pollen was rejected by the SC species *S. polyadenium*.

The findings presented here are consistent with previous work conducted in *Solanum* section *Lycopersicon* showing that *S-RNase* plays a central role in interspecific pollen rejection (Baek et al., 2015; Tovar-Méndez et al., 2014). Statistical analyses also demonstrated that *HT-B* alone is not a significant factor in these pollinations. These results do not contradict the findings from Tovar-Méndez et al. (2014, 2017) where *HT* proteins played a significant role in interspecific pollen rejection. Since *HT-A* remained functional in all of the female genotypes used these findings demonstrate the overlap in gene function between *HT-A* and *HT-B*, the greater importance of *HT-A*, and/or that *HT* is not a significant factor in these combinations implicating other mechanisms (Baek et al, 2015; Qin and Chetelat, 2021).

In comparison to the other species used as pollen donors, *S. commersonii* was unique as both *S-RNase* and *HT-B* did not appear to affect the rejection of pollen in DRH195. Since the rejection or allowance of interspecific pollen is entirely dependent on the specific combination of species used as parents, *HT*, *S-RNase* and other factors may play significant or inconsequential roles during the rejection of interspecific pollen (Bedinger et al, 2011; Baek et al., 2015). In this instance *HT-A* alone may mediate the *S-RNase* independent rejection of *S. commersonii* pollen in

the *HT-B*/*S-RNase* knock-out. If this is the case, this would be consistent with the findings of Tovar-Méndez *et al* (2017).

Intra- and interspecific compatibility is also heavily influenced by environmental and ontogenic factors such as temperature, humidity, flower age, and plant age (Bienz, 1958; Henderson and LeClerg, 1943; Travers et al., 2004; Liao et al., 2016; Webb and Williams, 1988). Although variable GSI responses due to plant and floral age have been demonstrated in *S. carolinense* and *N. alata*, these findings have not been observed in interspecific pollinations (Travers et al., 2004; Liao et al., 2016). In order to examine the effect of plant age on interspecific pollen tube growth, pollinations were made only on newly opened flowers, within 24 hours of anthesis, at three discrete time-points. The first time-point for pollinations occurred at the initial onset of flowering, with each of the time-points being separated by 30 days.

The results from statistical analyses indicate that plant age does have a significant impact on interspecific pollen rejection, consistent with the findings published by Travers et al., (2004) and Liao et al., (2016). Analysis of the longest pollen tubes revealed that individual pollen tubes were able to travel significantly further down the style at the third time-point in almost every case. Amongst all the pollen donors, individual pollen tubes penetrated significantly further down the style at the third time-point compared to the first; the only exception being the combination of *S. pinnatisectum* as the pollen donor and the *HT-B* knock-out being the female where this trend was reversed. Across all females and pollen donors, the third time point yielded the only cases where pollen tubes were nearly able to travel completely down the style.

Future assessment of the role of *HT-A* in interspecific pollen rejection is essential to inform our understanding of interspecific pollination barriers. Efforts are being made to make these gene edits and further assessment will be made after the edits are confirmed. There is still a

lot to understand about plant ontogeny and the degradation of interspecific and GSI barriers with age. Since all of the plants in this study were planted at the same time, it is unknown whether the age of the pollen donor or the female parent are more or less responsible for the break-down in prezygotic barriers. Further inquiry into these questions will aid in the identification of the causative mechanisms.

APPENDICES

APPENDIX A: Chapter 2 Tables

Pollen Donors		
ID	Species	PI#
SBGG505-A	*S. bulbocastanum*	275197
Scmm320266-02	*S. commersonii*	320266
SPGG544-A	*S. pinnatisectum*	275232
SJGG520-A	*S. jamesii*	592417

Table 2.1. List of potato-related species used as pollen donors. *PI# indicates the source plant introduction.*

Female Genotypes	
Line	Gene Edit
DRH195	WT (wild type)
DRH195.158	*S-RNase*
DRH195.121_009	*HT-B*
DRH195.124_001	*S-RNase/HT-B*

Table 2.2a. Genotypes used as female parents and respective gene edit.

```
Line          Edit                                                    sgRNA-HTB1        PAM
DRH195                 5'  ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAGTTATTGCAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGG  3'
121_009    -15(bp)     5'  ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAGTTATTG---------------AGCCTTCACTTCCATTGCTTGAGG  3'
124_001     -6(bp)     5'  ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAG------CAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGG  3'

Line          Edit                          sgRNA-1          PAM
DRH195                 5'  ATGGGGATTTCGACAAATTGCAACTGGTATTAACATGGCCACCATCATTT  3'
DRH195.158  -1(bp)     5'  ATGGGGATTTCGACAAATTGCAACTGGTATT-ACATGGCCACCATCATTT  3'
124_001     -2(bp)     5'  ATGGGGATTTCGACAAATTGCAACTGGTAT--ACATGGCCACCATCATTT  3'
```

Table 2.2b. Description of gene edits in female genotypes used. *The first alignment depicts HT-B gene edits for lines 121_009 and 124_001 compared to the wild-type DRH195 sequence. Line 121_009 has a 15bp deletion flanking the PAM (protospacer adjacent motif) sequence while line 124_001 shows a 6bp deletion upstream from the PAM sequence. The second alignment depicts the gene edits in S-RNase for DRH195.158 and 124_001 which have 1bp and 2bp deletions respectively, upstream from the PAM sequence. The single guide RNA sequences are also shown: sgRNA-HTB1 (single guide RNA targeting the first exon of the HT-B gene), sgRNA-1 (single guide RNA targeting the first exon of the S-RNase gene.*

APPENDIX B: Chapter 2 Figures

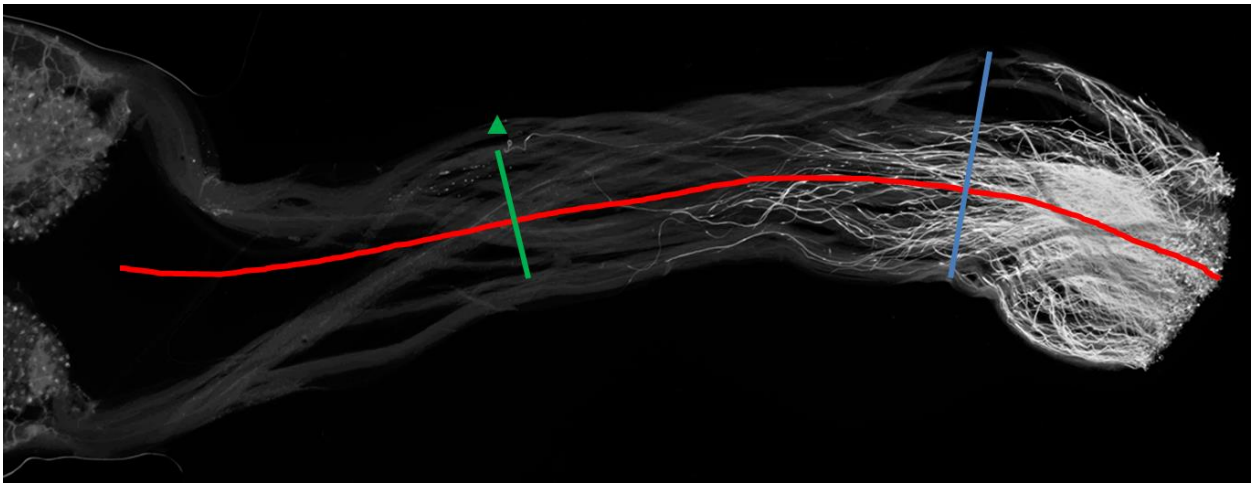

Figure 2.1. Pollen tube measurement description *(style of DRH195 with S. pinnatisectum as pollen donor). Total length of the style was measured along the midline of the style from the stigma surface to the base of the style, following the curvature if present (depicted by the red line). The pollen tube front was measured along the midline, at the point where the majority of the pollen tubes stopped (Blue line). The measurement would be taken at the point where the blue and red lines intersect. The longest pollen tube (identified by the green arrow) was measured along the midline and would be taken where the green and red line intersect.*

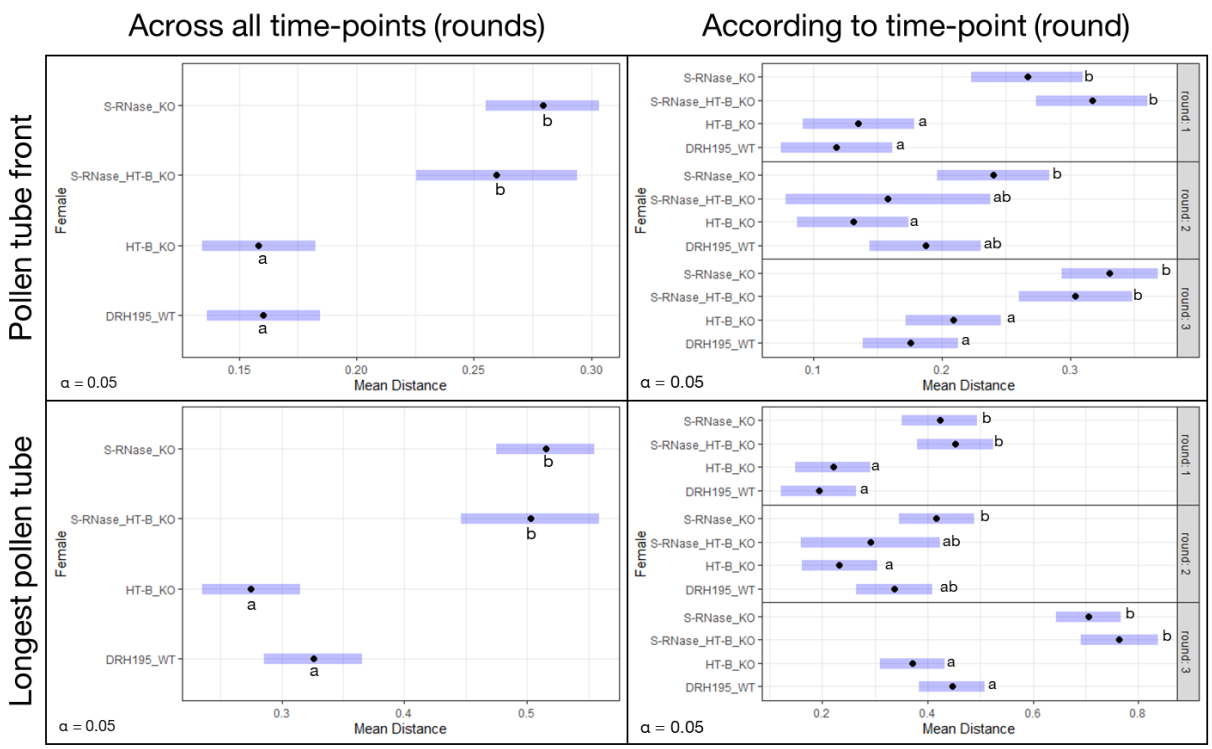

Figure 2.2. Measurement of the longest pollen tube as well as the pollen tube front with *S. bulbocastanum* as a pollen donor. *Blue bars depict the estimated marginal pollen tube means (emmean) as a proportion of total style length at 48h post pollination. Female knock-outs and wild type DRH195 (S. tuberosum) genotypes are depicted on the left vertical axis, and time-points or rounds are depicted on the right vertical axis. Pollinations were made at three discrete time points (rounds), separated by 30 days, with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse.*

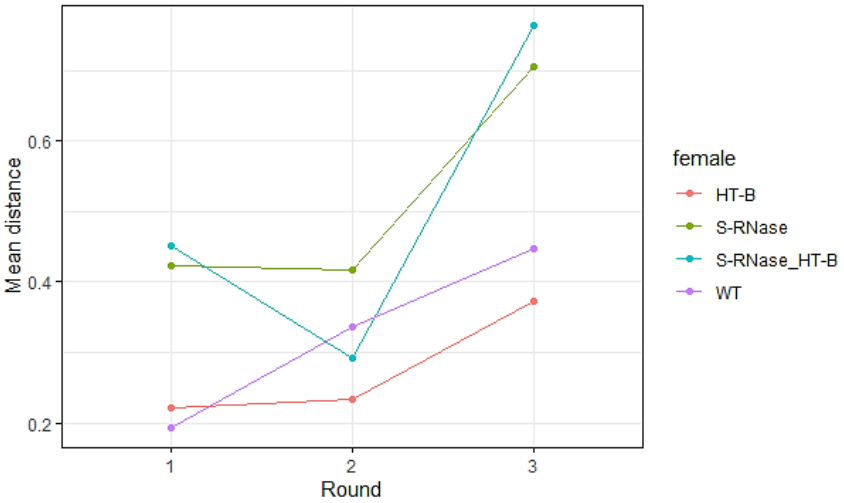

Figure 2.3. Estimated marginal means (Linear prediction), plotted against the different time-points (rounds) used in this study with *S. bulbocastanum* as the pollen donor, *showing effect of plant age on interspecific pollen tube inhibition. The vertical axis depicts the estimated marginal means (Linear prediction) of the longest measured pollen tube as a proportion of the total length of the style. The horizontal axis plots the three pollination time-points (rounds) used in the study with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse with subsequent pollination rounds separated by 30 days.*

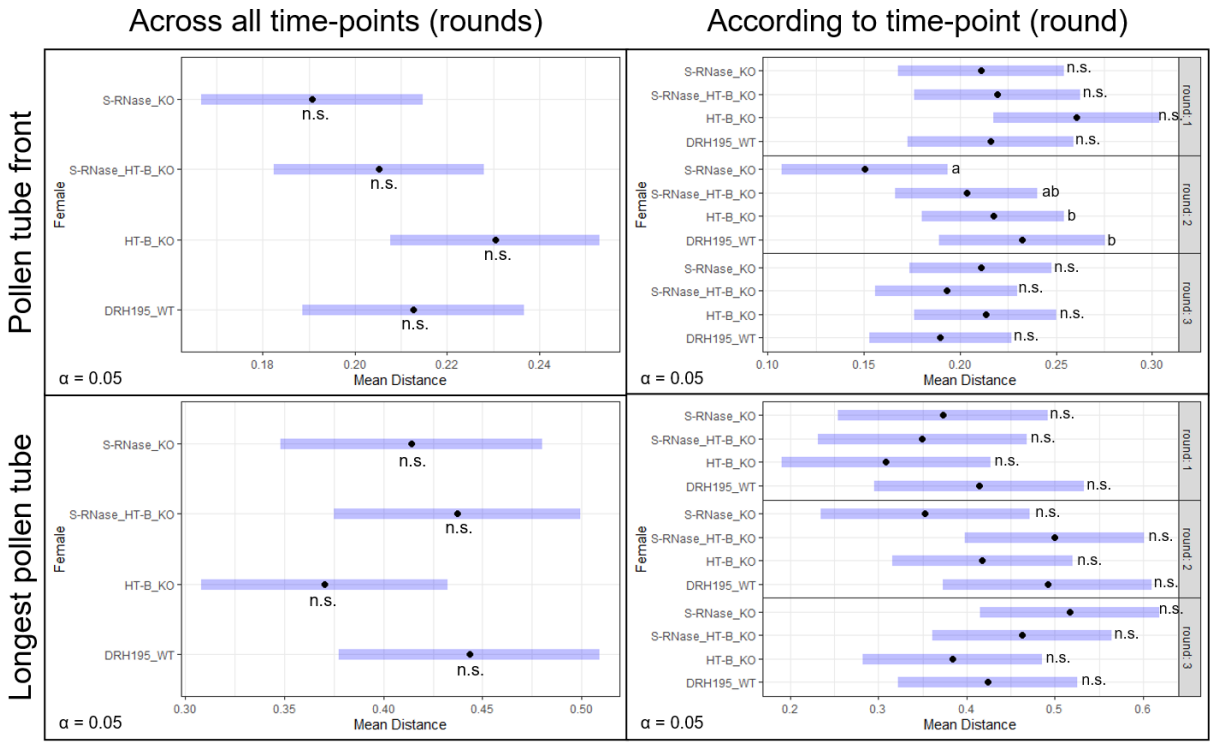

Figure 2.4. Measurement of the longest pollen tube as well as the pollen tube front with *S. commersonii* as a pollen donor. *Blue bars depict the estimated marginal pollen tube means (emmean) as a proportion of total style length at 48h post pollination. Female knock-outs and wild type DRH195 (S. tuberosum) genotypes are depicted on the left vertical axis, and time-points or rounds are depicted on the right vertical axis. Pollinations were made at three discrete time points (rounds), separated by 30 days, with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse.*

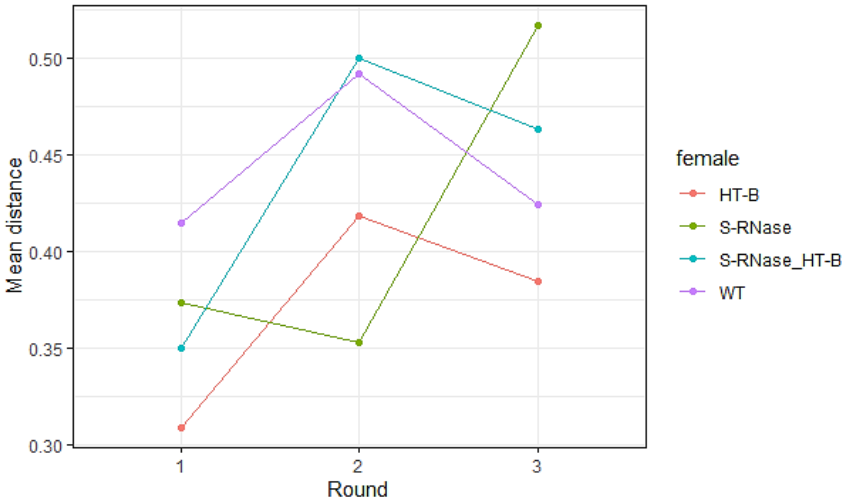

Figure 2.5. Estimated marginal means (Linear prediction), plotted against the different time-points (rounds) used in this study with *S. commersonii* as the pollen donor, *showing effect of plant age on interspecific pollen tube inhibition. The vertical axis depicts the estimated marginal means (Linear prediction) of the longest measured pollen tube as a proportion of the total length of the style. The horizontal axis plots the three pollination time-points (rounds) used in the study with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse with subsequent pollination rounds separated by 30 days.*

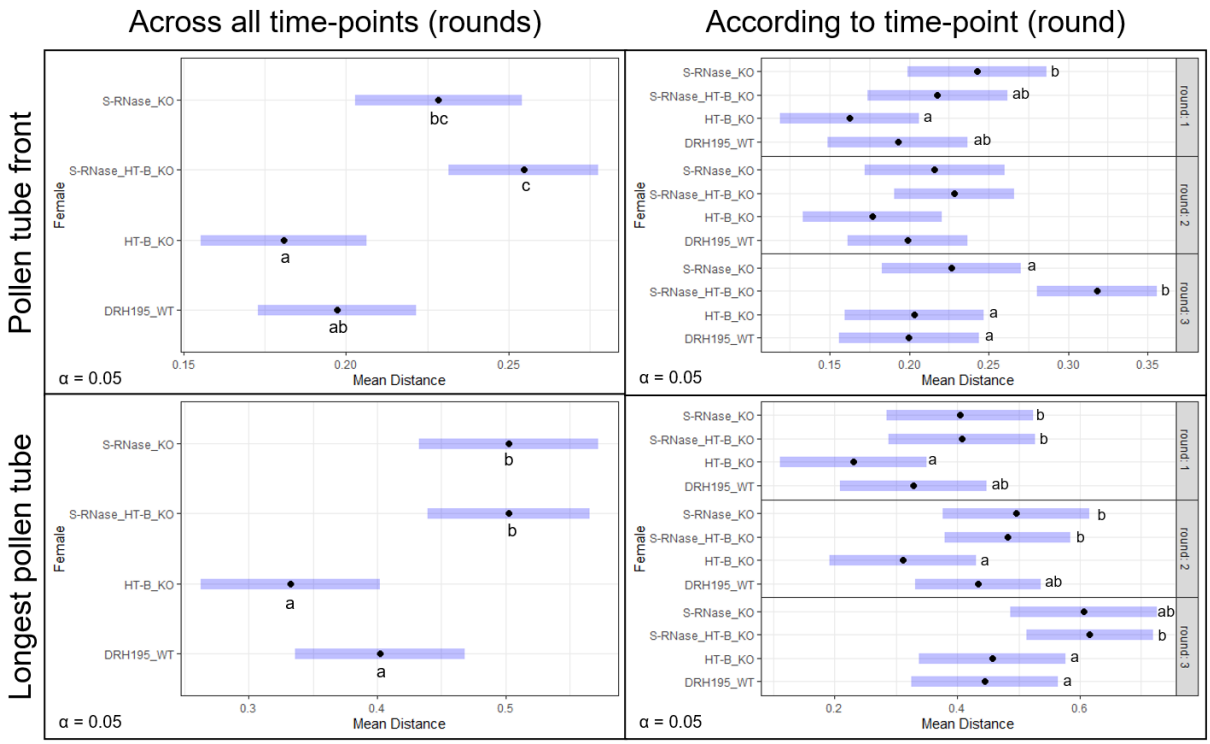

Figure 2.6. Measurement of the longest pollen tube as well as the pollen tube front with *S. jamesii* as a pollen donor. *Blue bars depict the estimated marginal pollen tube means (emmean) as a proportion of total style length at 48h post pollination. Female knock-outs and wild type DRH195 (S. tuberosum) genotypes are depicted on the left vertical axis, and time-points or rounds are depicted on the right vertical axis. Pollinations were made at three discrete time points (rounds), separated by 30 days, with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse.*

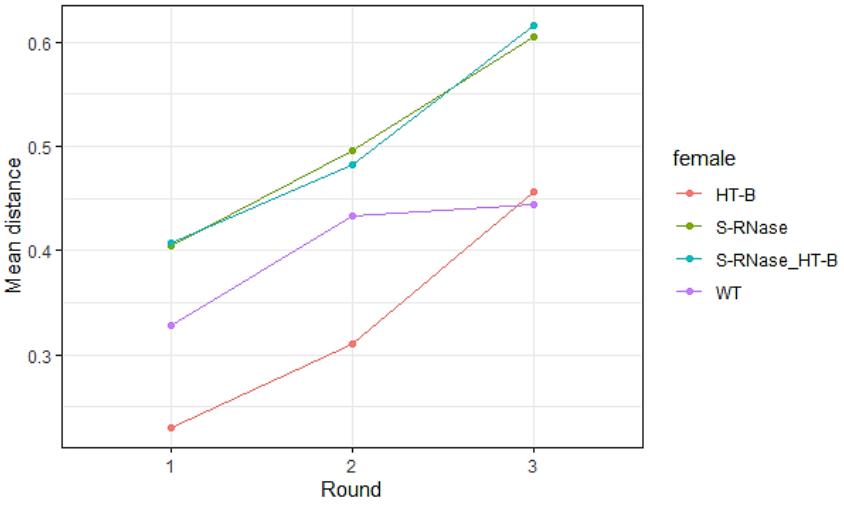

Figure 2.7. Estimated marginal means (Linear prediction), plotted against the different time-points (rounds) used in this study with *S. jamesii* as the pollen donor, *showing effect of plant age on interspecific pollen tube inhibition. The vertical axis depicts the estimated marginal means (Linear prediction) of the longest measured pollen tube as a proportion of the total length of the style. The horizontal axis plots the three pollination time-points (rounds) used in the study with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse with subsequent pollination rounds separated by 30 days.*

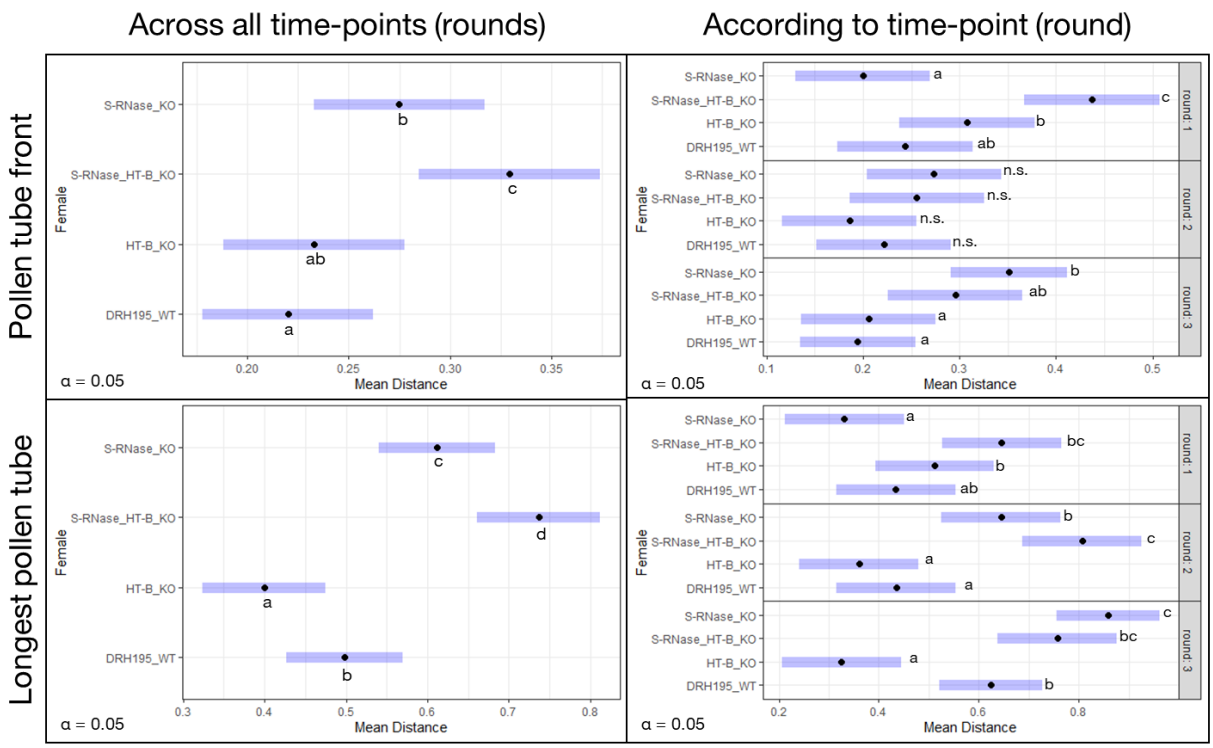

Figure 2.8. Measurement of the longest pollen tube as well as the pollen tube front with *S. pinnatisectum* as a pollen donor. *Blue bars depict the estimated marginal pollen tube means (emmean) as a proportion of total style length at 48h post pollination. Female knock-outs and wild type DRH195 (S. tuberosum) genotypes are depicted on the left vertical axis, and time-points or rounds are depicted on the right vertical axis. Pollinations were made at three discrete time points (rounds), separated by 30 days, with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse.*

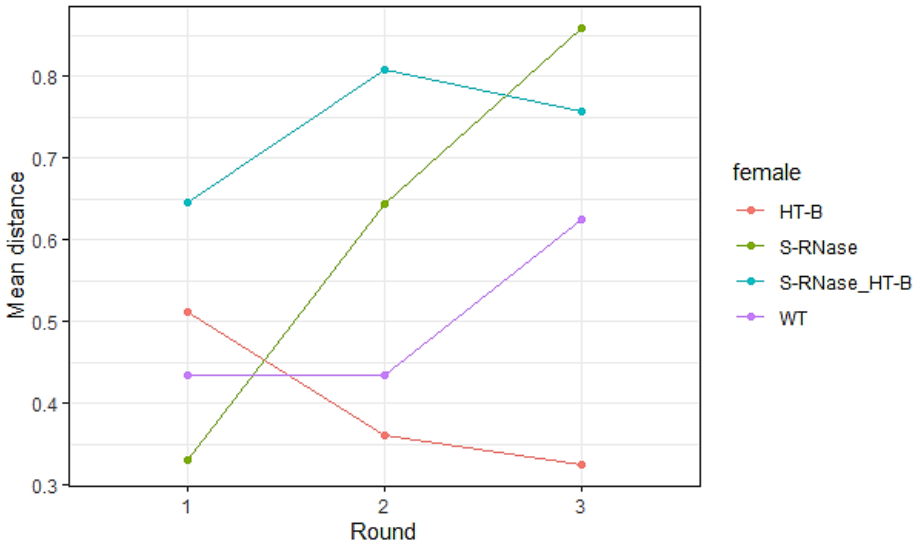

Figure 2.9. Estimated marginal means (Linear prediction), plotted against the different time-points (rounds) used in this study with *S. pinnatisectum* as the pollen donor, *showing effect of plant age on interspecific pollen tube inhibition. The vertical axis depicts the estimated marginal means (Linear prediction) of the longest measured pollen tube as a proportion of the total length of the style. The horizontal axis plots the three pollination time-points (rounds) used in the study with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse with subsequent pollination rounds separated by 30 days.*

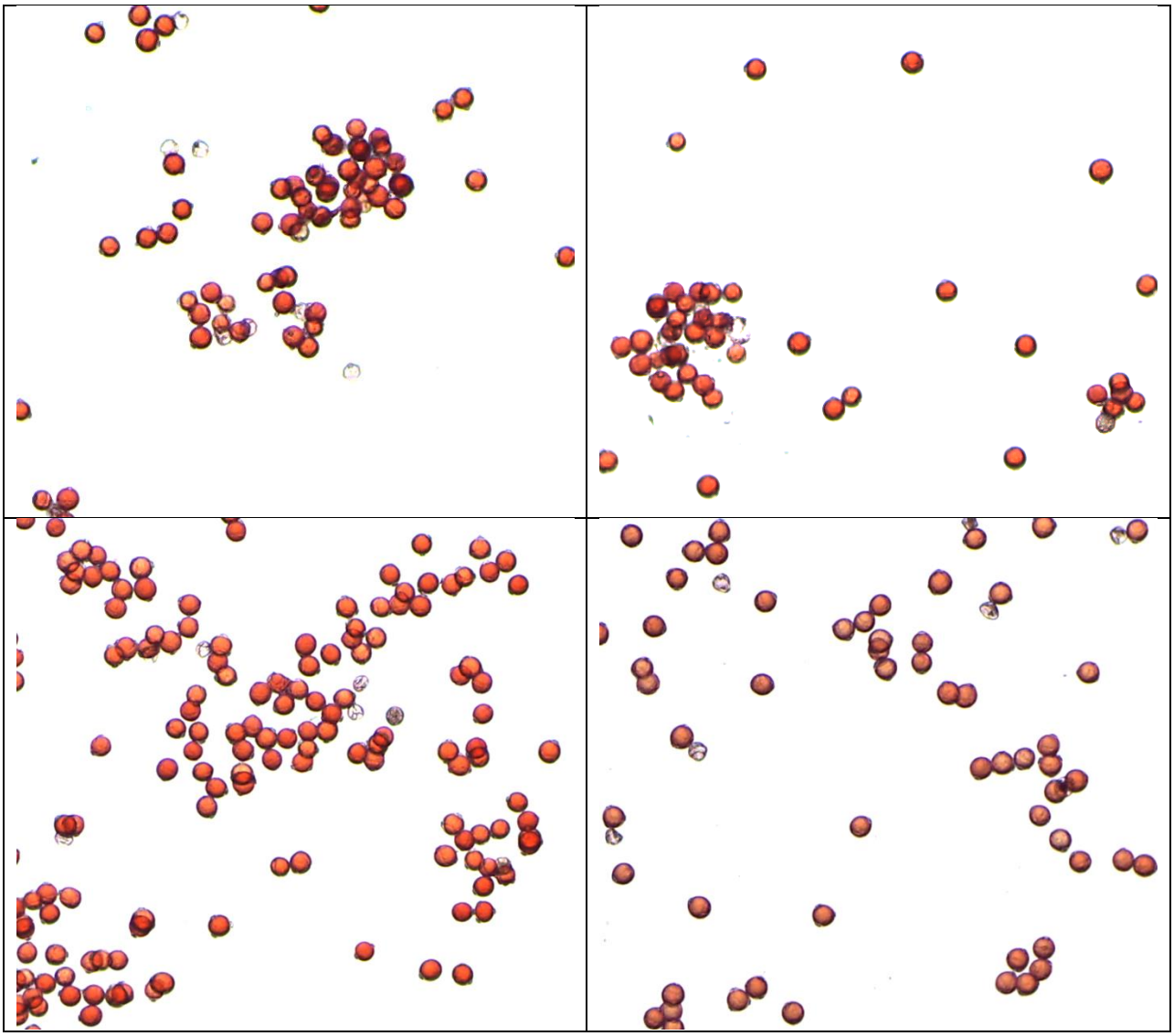

Figure 2.10. Example images of pollen viability assays *using acetocarmine-glycerol as described by Ordoñez (2014). Starting in the upper left-hand corner and moving in a clockwise are pollen grains from each of the four pollen donors: S. bulbocastanum (SBGG505-A), S. commersonii (Scmm320266-02), S. jamesii (SJGG520-A), and S. pinnatisectum (SPGG544-A). Viable pollen is turgid and stained red while shriveled, unstained, or unusually large or small grains were classified as unviable.*

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CHAPTER 3

EVALUATION OF SLI AS A GENETIC RESOURCE TO OVERCOME INTERSPECIFIC PREZYGOTIC BARRIERS IN SOLANUM SECTION PETOTA

Abstract

The ability of *Sli* to overcome interspecific barriers was reported by Sanetomo *et al*. (2014). Because *Sli* based self-compatibility (SC) is widespread in North American diploid germplasm, the use of *Sli* to overcome interspecific barriers could represent a both underutilized and widely accessible resource. Statistical analysis of pollen tube growth in four wild species *S. pinnatisectum, S. bulbocastanum, S. commersonii,* and *S. jamesii* demonstrated that there was no practical significance between pollen donors carrying *Sli* and pollen donors without *Sli*. These findings directly contradict previous findings of Sanetomo *et al*. (2014) using the same *S. pinnatisectum* PI (275232). These inconsistencies are discussed and can be largely explained by differences in pollen donors used.

Objective:

Demonstrate the ability of *Sli* to overcome prezygotic barriers in interspecific pollinations. Despite previous research claiming otherwise, *Sli* was found to be an insignificant factor in these interspecific crosses.

Introduction

The 107 wild species of potato represent a rich and diverse source of disease resistance and tuber quality traits for cultivated potato (Spooner and Bamberg, 1994; Jansky and Hamernik, 2009). However, in the century long history of potato breeding the enormous value of wild species traits has been realized only occasionally (Bethke et al., 2017). Among all the species characterized, the diploid 1EBN species are of particular interest as they exhibit exceptional resistance to economically important pests such as early blight (*Alternaria solani*), Colorado

potato beetle (*Leptinotarsa decemlineata*), and late blight (*Phytophthora infestans*) (Jansky et al., 2008, 2009; Zlesak and Thill 2004; Zheng et al., 2020). However, due to prezygotic stylar barriers and differences in effective ploidy between 1EBN species and cultivated germplasm, accessing the traits from these species is extraordinarily difficult (Novy and Hanneman, 1991; Jansky and Hamernik, 2009; Städler et al., 2021). If *Sli* is able to overcome barriers between diploid cultivated germplasm and the 1EBN species, it would be an enormous breakthrough.

The ability of *Sli* to overcome interspecific barriers was reported by Sanetomo *et al*. (2014). Using inbred 2x pollen donors carrying *Sli*, 388 total pollinations were made with 4x *S. pinnatisectum* (PI275232) yielding nine 3x hybrid seedlings. It was also observed that the pollen tubes from *Sli* carrying pollen donors were able to bypass stylar barriers, while pollen tubes from *S. megistacrolobum*, *S. demissum*, and *S. phureja* stopped growing at or near the stigma of 4x and 2x *S. pinnatisectum* (Sanetomo et al., 2014). The ability of *Sli* to overcome all prezygotic stylar barriers and not just those controlling gametophytic self-incompatibility could be an important factor in accessing the sexually isolated Mexican diploid species (Sanetomo *et al*., 2014). Additionally, the widespread use of *Sli*-based self-compatibility (SC) in North American diploid potato breeding would mean that most public and private breeders would have access to these methods (Kaiser et al., 2021).

Currently, North American diploid potato breeding largely relies on the dominant SI inhibitor gene *Sli* on chromosome 12 as a source of SC (Hosaka and Hanneman 1998a, b; Kaiser et al., 2021). The inbred *S. chacoense* clone M6 is currently a primary source of *Sli* based SC, and an alternative source to the other *S. chacoense Sli* donor clones that harbor recessive deleterious and lethal alleles linked to *Sli* (Endelman et al., 2019; Jansky et al., 2014; Kaiser et al., 2021). The employment of *Sli* to overcome interspecific barriers could represent a powerful

albeit underutilized method to access valuable traits from sexually isolated species. In order to better capture the value of *Sli*, this study was designed to confirm the findings of Sanetomo *et al*. (2014) and determine if these findings can be applied to other *Solanum* species.

Relationships of interspecific compatibility and incompatibility in *Solanum* are complex and are mediated by many different characterized and uncharacterized factors (Bedinger et al., 2011; Pandey, 1962; Tovar-Méndez et al., 2017). To eliminate variables associated with other species, only *S. chacoense* pollen donors were selected for this study.

Materials and Methods

Plant Materials

The *S. chacoense* clone USDA8380-1 (PI 458310, 80-1) and the *S. chacoense* self-compatible inbred line M6 were selected as pollen donors selected for this study (Jansky et al., 2014). The M6 clone was selected as it is a homozygous, well characterized, and widely used source of *Sli* based SC (Jansky et al., 2014; Kaiser et al., 2021). In order to reduce confounding variables the self-incompatible clone 80-1 was selected as a control as it is largely homozygous, and also the same species as M6 (Kaiser et al., 2020). Individual clones were selected as representatives of genetically valuable wild species (*S. bulbocastanum*, *S. commersonii*, *S. jamesii*, and *S. pinnatisectum*) and were used as females. These female clones were selected from multiple plant introduction populations of each species acquired from the USDA-ARS Potato Germplasm Introduction Station (Sturgeon Bay, WI) with individual genotypes selected from their respective field-grown plant introduction populations based on their fertility and overall vigor (Table 1). Each genotype was maintained in tissue culture on Murashige and Skoog Basal Medium with Vitamins and Sucrose (PhytoTech Labs) and Phyto Agar (Research Products International) prepared with DI water and balanced with 1M HCL and 8N NaOH to a pH of 5.8,

and cultured in growth chambers with 16-h-light/8-h-dark photoperiod at 22°C and average light intensity of 200 μmoles m-2s-1.

Greenhouse propagation and pollinations

In October of 2020, two individuals from each genotype were transferred directly from tissue culture to 14L (3.8 gallon) plastic pots with filled with Suremix Perlite peat and perlite soilless medium (Michigan Grower Products INC.). For the duration of the experiments greenhouse conditions were maintained at 20°C with a 16-hr photoperiod under Philips GreenPower light-emitting diode (LED) DR/W-MB lights (Philips Lighting Holding B.V., Netherlands). The pollen donors 80-1 and M6 are known to exhibit high levels of male fertility, pollen from these individuals displayed high percentage of pollen stainability as well as high percentages of *in vitro* and *in vivo* pollen tube growth (Alsahlany et al., 2021; Jansky et al., 2014). Male fertility was validated by collecting pollen from 5-6 anthers directly onto a glass slide and immediately stained with acetocarmine-glycerol as described by Ordoñez (2014), covered with a cover slip, and sealed using clear nail polish. These slides were stored at room temperature in the dark and visualized the same day using a Leica DM750 binocular microscope (Leica Microsystems, Germany) and the associated Leica imaging software at 10x and 40x magnification. A minimum of 100 pollen grains were then used to calculate the percentage of viable pollen. Stained, turgid pollen was classified as viable while any grains that were shriveled, unstained, or unusually large or small were classified as unviable.

Stylar squash: assay of pollen tube growth

For the evaluation of stylar barriers in interspecific crosses, 6-8 newly opened flowers (within 24 hours after anthesis) from each of the female genotypes were carefully emasculated

and pollinated with fresh pollen collected on a glass slide directly from the pollen donor. Styles were collected 48 hours post-pollination, by removing the remaining petals and sepals and storing the remaining intact style, ovary and receptacle in 1.5mL microcentrifuge tube containing a 3:1 ethanol/acetic acid fixation solution. These styles were then kept in the dark at room temperature for at least 24 hours. Styles and ovaries were then softened using an 8N NaOH solution at 60°C for 1 hour. Samples were then rinsed three times with distilled water and stained with 0.1% aniline blue in 0.1N K3PO4 keeping them in dark conditions with light shaking. Styles with attached ovaries were then placed on glass slides, gently squashed under a coverslip, and sealed with nail polish for subsequent visualization. Samples were visualized using a Nikon Eclipse Ni-U upright microscope (Nikon Instruments Inc., Melville, NY, United States) with a SOLA light engine (Lumencor, Beaverton, OR, United States), and photographed with the attached ANDOR Zyla sCMOS camera (Oxford Instruments, United Kingdom) and NIS-Elements BR 5.02 software. Images of each stylar sample were stitched together using Image Composite Editor 2.0 software. Pollen tube growth measurements were made using ImageJ 1.53e software (Schneider et al., 2012). Due to the variation of style length with a single genotype, measurements of pollen tube growth were calculated as a proportion of the total length of the style. Pollinations were made at three discrete time points, separated by 30 days, with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse.

Data collection and analysis

Measurements of the pollen tube front, or the point where the majority of the pollen tubes stop, the longest pollen tube, and the total length of the style were collected using ImageJ software. From these measurements, distance from the surface of the stigma to the pollen tube

front and length of the longest pollen tube were calculated based on the total length of the style in which they were measured. Significant differences between means ($P<0.05$) from replicated measurements where calculated using analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) using "R" software version 4.0.4 (R Foundation for Statistical Computing, 2012, available at www.r-project.org).

Results

The role of Sli in interspecific pollen rejection

A statistically significant difference was observed between *Sli* +/+ and *Sli* -/- pollen donors in the styles of *S. bulbocastanum* and to a lesser extent *S. commersonii*, with pollen tubes of the *Sli*+/+ clone M6 traveling further down the style (Figures 3.1 and 3.2). However, findings from the same analysis failed to find any statistical significance between pollen donors in the styles of *S. jamesii* or *S. pinnatisectum,* which is inconsistent with previous findings (Figures 3.1 and 3.2). Pollen tubes from M6 generally traveled further down the style in all of the four species compared to pollen tubes from the self-incompatible *Sli* -/- clone 80-1 (Figure 3.2). However, even the statistically significant differences between pollen donors failed to have any practical significance and did not change the outcome of these interspecific pollinations.

In the styles of *S. commersonii* and *S. bulbocastanum,* the difference between where the majority of the pollen tubes stopped was not significant in the first time point. In the subsequent time-points, the growth of the majority of pollen tubes from 80-1 was arrested closer to the stigma while the majority of pollen tubes from M6 penetrated much further down the style. As noted above, this difference lacked significance as the longest pollen tubes from both parents were able to traverse the entirety of the style in *S. commersonii*, and all pollen tubes were arrested in the first third of the style in *S. bulbocastanum* (Figure 3.4).

As with *S. bulbocastanum* and *S. commersonii*, *Sli* presence or absence did not have a significant impact on interspecific pollen-tube inhibition in the styles of *S. jamesii* and *S. pinnatisectum*. Pollen tubes from M6 generally grew further down the style in *S. pinnatisectum* with the opposite occurring for *S. jamesii* (Figures 3.1 and 3.2). No pollen tubes from either pollen donor entered the ovary in *S. jamesii* (Figure 3.2), while the majority of pollen tubes from both pollen donors traversed the entire style in *S. pinnatisectum* (Figure 4). Pollinations of *S. pinnatisectum* by both M6 and 80-1 also formed fruit although all the seeds were aborted.

Effects of plant ontogeny

It is clear that plant maturity plays a role in the interspecific pollen-tube rejection response in all the species evaluated in this study (Figure 3.3). In *S. pinnatisectum* and *S. jamesii,* no statistical difference was observed between pollen tube measurements taken at different time-points. Despite this, it is clear that pollen tubes from both parents were more likely to travel further down the style at the third time-point compared to the first time-point. This trend aligns with the results observed in *S. commersonii* that is statistically significant (Figure 3.3). Analysis of pollen tubes in *S. bulbocastanum* styles displayed dynamic genotype by environmental effects. At the first time-point, pollen-tubes from 80-1 penetrated further down the style compared to M6. At the second and third time-points, pollen-tube growth of 80-1 was arrested closer to the stigma, while M6 pollen-tubes traveled significantly further down the style (Figure 3.3).

Discussion

Although the difference between pollen donors was statistically significant in the styles of *S. commersonii* and *S. bulbocastanum*, the differences between pollen donors lacked any practical significance. The presence or absence of *Sli* did not change the outcome of any of the pollinations made. Additionally, no significant difference was observed in pollinations made

with *S. pinnatisectum*, which is inconsistent with the findings of Sanetomo *et al.* (2014) despite the use of the same *S. pinnatisectum* PI (273232). This study suggests that *Sli* is not a significant factor in interspecific pollen rejection in these pollinations.

The lack of consistent findings between these experiments and those of Sanetomo *et al*. (2014) is likely attributable to some unknown factors in *S. chacoense* besides *Sli*. The pollinations conducted in this study demonstrated that the clones M6 and 80-1 were comparable in their ability to bypass stylar barriers, or in their uniform inhibition in the styles of *S. bulbocastanum*. Unlike the pollen donors used by Sanetomo *et al*. (2014), these pollen donors were from a single species, *S. chacoense* while the pollen donors used by Sanetomo *et al.* (2014) came from interspecific crosses between *S. chacoense* and *S. phureja*. The difference between pollen donors is the likely source of inconsistencies as shown in the introduction of unknown genetic variables from *S. phureja* presenting a confounding variable in the studies carried out by Sanetomo *et al*. (2014).

The ability to form viable seeds cannot be attributed to the presence or absence of *Sli* either, as postulated by Sanetomo et al. (2014). Hosaka and Hanneman (1998a, b), observed that *Sli* inhibits *S*-gene expression in the pollen, thus overcoming the prezygotic GSI barrier. Recent findings have identified *Sli* as a non-*S-locus* F box protein capable of interacting with a diverse array of *S-RNase* alleles (Eggers et al.,2021; Ma et al.. 2021). Failure to produce viable seed after fertilization is a post-zygotic barrier that is exhibited commonly as endosperm failure. These two interspecific barriers are independent even though the outcome is the same. Because the genetic and molecular mechanisms surrounding endosperm failure and effective ploidy are complex and poorly characterized it would be inappropriate to conclude that a genetic factor effecting pre-

zygotic stylar barriers also plays a role in post-zygotic barriers without significant substantial evidence.

The effect of plant ontogeny plays a fascinating role in interspecific pollen rejection. No statistical significance was observed between pollen tube measurements taken at different time points was found in *S. pinnatisectum* or *S. jamesii*. However, it is clear that pollen tubes from both parents were more likely to travel further down the style at the third time-point compared to the first time-point. The differences between the growth pollen tubes in the styles of *S. bulbocastanum* over time also show the dynamic nature of prezygotic barriers over time.

Although *Sli* played no significant role in these pollinations, it is possible that *S. chacoense* as a pollen donor is better able to bypass stylar barriers in EBN1 species such as *S. pinnatisectum* compared to *S. megistacrolobum*, *S. demissum*, and *S. phureja* (*S. tuberosum group phureja*) as reported by Sanetomo et al. (2014). Consequently, *S. chacoense* may be a valuable resource in accessing these EBN1 species regardless of *Sli* status. Currently, other methods such as bridge crossing with *S. verrucosum* are more reliable and feasible in their ability access EBN1 species (Jansky, and Hamernik; 2009). Unlike the methods proposed by Sanetomo et al. (2014) and Carputo et al. (1997), the use of *S. verrucosum* bridge crosses is also more accessible for most plant breeders as it does not require ploidy manipulation, interploidy crosses, or the use of advanced techniques (Jansky, and Hamernik; 2009).

APPENDICES

APPENDIX A: Chapter 3 Tables

Female Genotypes			Male Genotypes		
ID	Species	PI#	ID	Species	PI#
SBGG505-A	*S. bulbocastanum*	275197	M6	*S. chacoense*	(Jansky et al., 2014)
Scmm320266-02	*S. commersonii*	320266			
SPGG544-A	*S. pinnatisectum*	275232	USDA8380-1	*S. chacoense*	458310
SJGG520-A	*S. jamesii*	592417			

Table 3.1. List of potato-related species used as females in this study. *PI number indicates the source plant introduction.*

APPENDIX B: Chapter 3 Figures

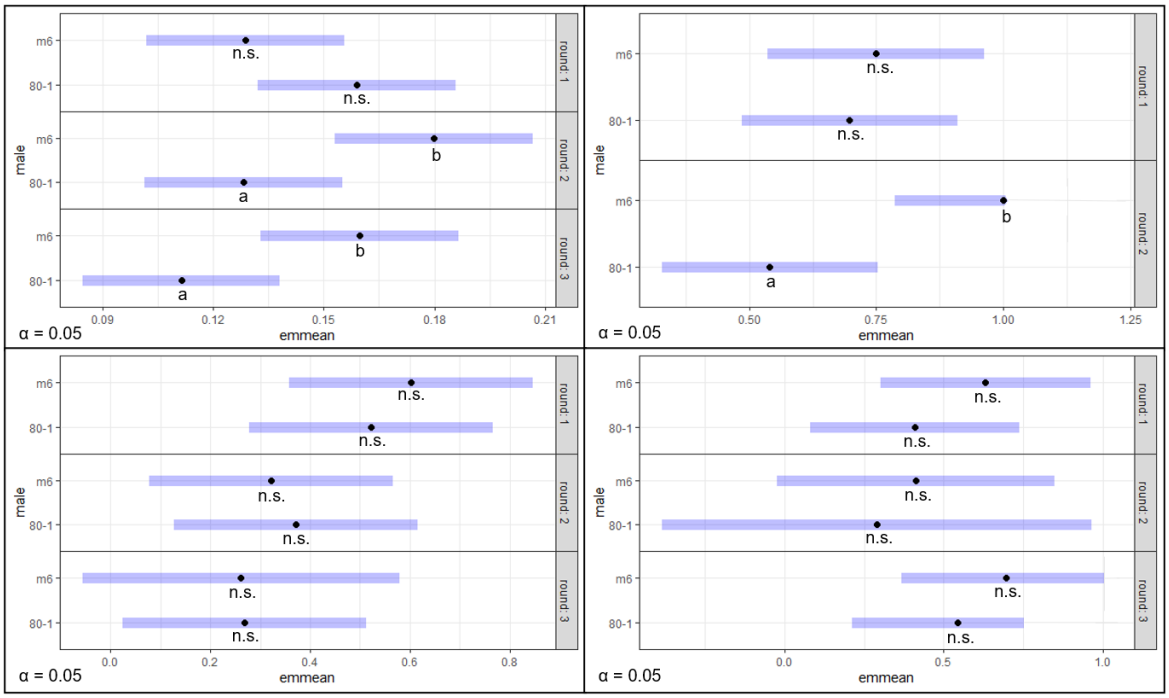


Figure 3.1. Measurement of pollen tube front according to female. *Starting from the top left and moving clockwise: S. bulbocastanum, S. commersonii, S. pinnatisectum, and S. jamesii. Blue bars depict the marginal pollen tube means (emmean) as a proportion of total style length at 48h post pollination. Female genotypes are depicted on the left vertical axis, and time-points or rounds are depicted on the right vertical axis. Pollinations were made at three discrete time points (rounds), separated by 30 days, with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse.*

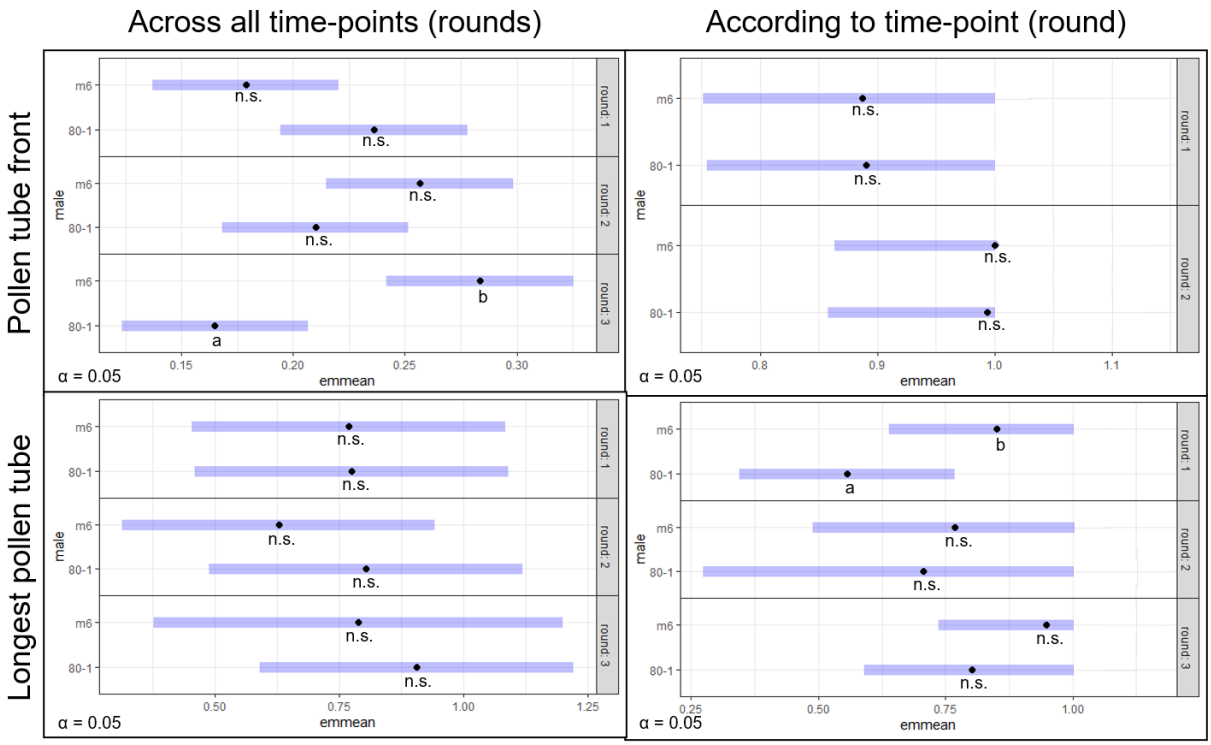


Figure 3.2. Measurement of the longest pollen tube according to female. *Starting from the top left and moving clockwise: S. bulbocastanum, S. commersonii, S. pinnatisectum, and S. jamesii. Blue bars depict the marginal pollen tube means (emmean) as a proportion of total style length at 48h post pollination. Female genotypes are depicted on the left vertical axis, and time-points or rounds are depicted on the right vertical axis. Pollinations were made at three discrete time points (rounds), separated by 30 days, with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse.*

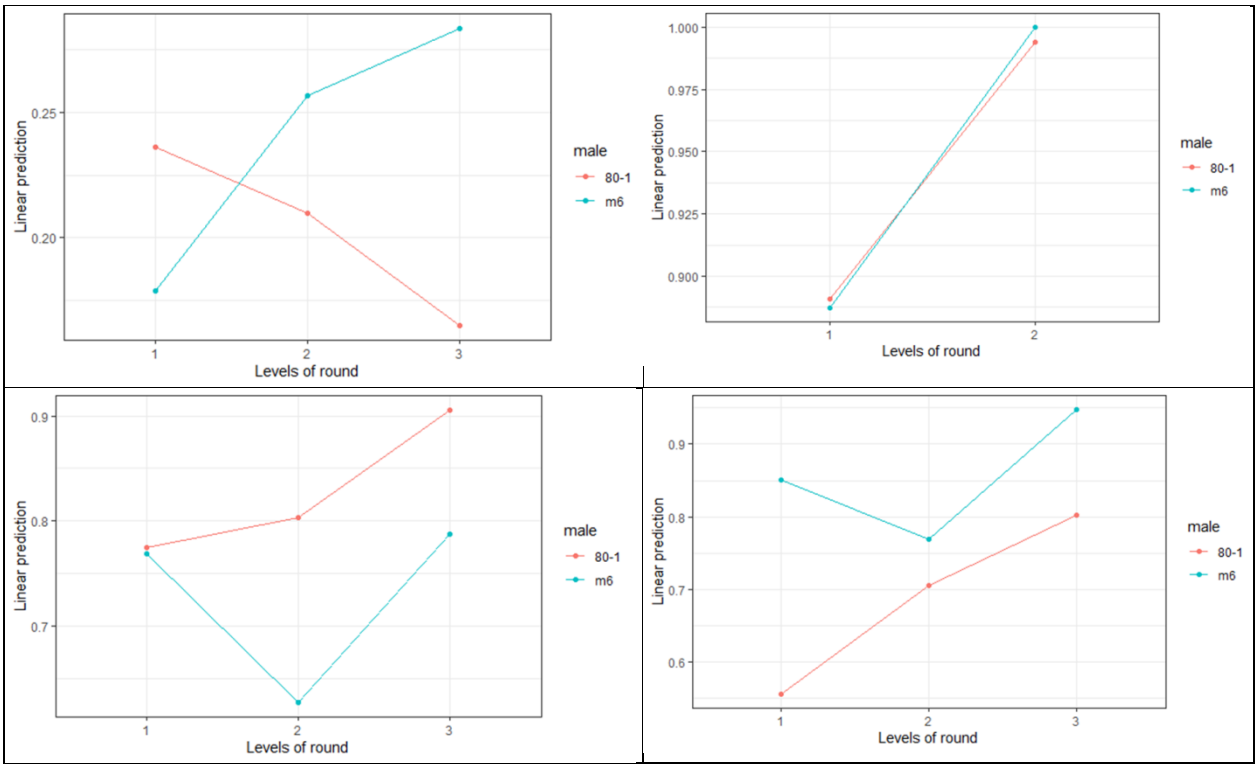
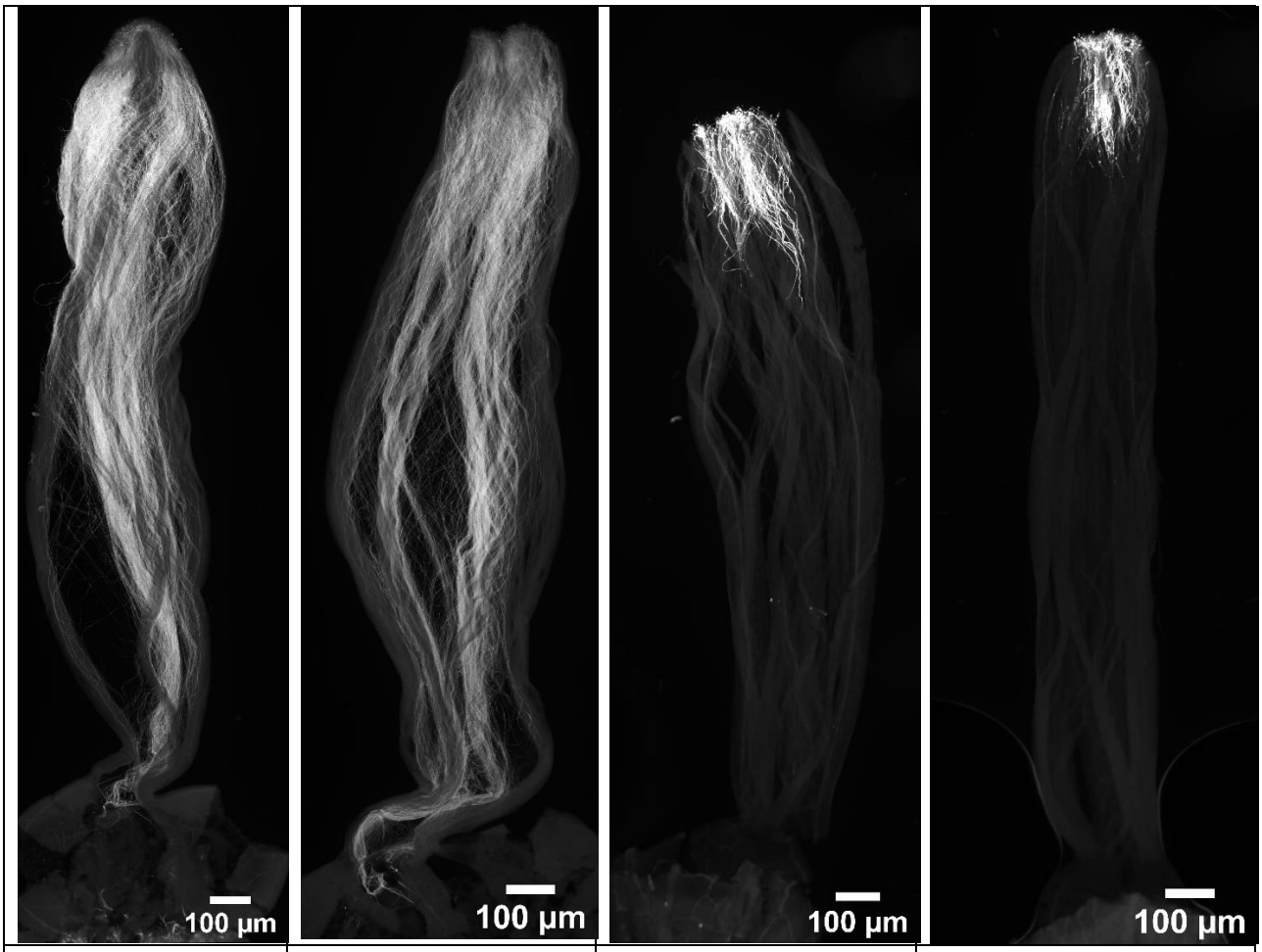


Figure 3.3. Estimated marginal means (Linear prediction), plotted against the different time-points (rounds) used in this study according to female. *Starting from the top left and moving clockwise: S. bulbocastanum, S. commersonii, S. pinnatisectum, and S. jamesii showing the effect of plant age on interspecific pollen tube inhibition. The vertical axis depicts the estimated marginal means (Linear prediction) of the longest measured pollen tube as a proportion of the total length of the style. The horizontal axis plots the three pollination time-points (rounds) used in the study with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse with subsequent pollination rounds separated by 30 days.*



***S. pinnatisectum* x 80-1**	***S. pinnatisectum* x M6**	***S. bulbocastanum* x 80-1**	***S. bulbocastanum* x M6**

Figure 3.4. Examples of styles pollinated with 80-1 and M6, *showing no difference between pollen donors despite the presence of Sli in M6. Styles of S. pinnatisectum are displayed in the two images on the left side, and styles from S. bulbocastanum are displayed in the two images on the right side. Pollen tubes from both pollen donors are uniform in their ability to penetrate the full length of the styles in S. pinnatisectum. In S. bulbocastanum, pollen tubes from both pollen donors are arrested in the first third of the style.*

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CHAPTER 4

ASSESSING THE ROLE OF S-RNASE IN SELF_COMPATIBILITY AND INTERSPECIFIC COMPATIBILITY IN SOLANUM VERRUCOSUM

Abstract

Solanum verrucosum (2x=2n=24 (2EBN)) is a unique wild potato species originating from central Mexico. Primary traits of interest include self-compatibility (SC) and the ability to act a parent in as a bridge crosses to access traits from genetically isolated 1EBN wild species. The postzygotic barrier of endosperm failure is a large gap that separates cultivated potato from the valuable traits in the 1EBN wild species. The use of *S. verrucosum* bridge crosses is a simple and valuable technique that offers direct access to these trait as bridge crosses using *S. verrucosum* require less time and resources compared to other methods used to access valuable traits from 1EBN species despite its low efficiency. In order to increase the efficiency of this method, characterization of interspecific barriers and *S-RNase* expression was necessary. Stylar pollen tube growth from four 1EBN pollen donors were used to visualize prezygotic barriers; no significant stylar barriers were observed. The use of primers designed to amplify conserved regions of *S-RNase* were able to capture two partial *S-RNase* alleles; however, RT-PCR expression analysis indicated that these were not expressed. Further inquiry into the SC and interspecific compatibility mechanisms in *S. verrucosum* is required to further characterize these mechanisms.

Objective:

Demonstrate and validate that self- and broad interspecific compatibility in *S. verrucosum* is primarily rooted in its lack of functional *S-RNase*. Although genomic S-RNase sequences were discovered and sequenced, not expression was detected.

Introduction

The wild species *Solanum verrucosum* (2x=2n=24 (2EBN)) is unique among the wild species of potato for several reasons. Geographically speaking, *S. verrucosum* is an anomaly as it is closely related to species from southern South America and only distantly related to the other wild species in central Mexico where it is found (Lara-Cabrera and Spooner, 2004; Spooner et al., 2004; Huang et al., 2018). *S. verrucosum* is also unusual as it is self-compatible unlike the vast majority of species within *Solanum* section *Petota* (Abdalla, and Hermsen, 1973; Spooner et al., 2014).

Of primary interest to potato breeders is the ability of *S. verrucosum* to act as a bridging species to access genetically isolated wild species (Hermsen and Ramanna, 1976; Hamernik et al., 2001). Although a large portion of diploid wild potato species are intercrossable, there are important barriers that prevent the use of certain species. When two species used in interspecific crosses have different levels of "effective ploidy" or EBN, these crosses are rarely successful (Stadler et al., 2021: Johnston et al., 1980). Interspecific crosses of two diploid species of differing EBN usually results in endosperm failure which represents a strong postzygotic barrier to interspecific hybridization (Johnston et al., 1980; Lafon-Placette, and Köhler, 2016). This is a significant barrier to the genetic improvement of potato as diploid and tetraploid cultivated potatoes have an EBN of two and four respectively while the Mexican diploid species which have exceptional disease and pest resistance traits have an EBN of one (Jansky and Hamernik, 2009). Because of this difference in effective ploidy, introgressing traits from these species is exceedingly difficult.

The postzygotic barrier of endosperm failure is a large gap that separates cultivated potato from the valuable traits in the 1EBN wild species. The use of *S. verrucosum* bridge

crosses are a simple and valuable technique that offers direct access to these traits (Jansky and Hamernik, 2009: Yermishin et al., 2014). Although this technique has relatively low efficiency, it is still less time and resource intensive than previous attempts to access valuable traits from 1EBN species using complex strategies of ploidy manipulation, somatic fusion, embryo rescue, and transgenesis (Carputo et al., 1997, Helgeson et al., 1998; Orbegozo et al., 2016; Valkonen et al., 1995).

The ability of *S. verrucosum* to form interspecific hybrids with species of differing EBN is partially rooted in its lack of a functional *S-RNase* protein (Eijlander, 1998). The absence of functional *S-RNase* protein prevents the inhibition on pollen tube growth in what would otherwise be incompatible pollinations. While it is known that there is no detectable *S-RNase* at the protein level (Eijlander, 1998), it is unknown if the genes for *S-RNase* in *S. verrucosum* are missing, non-functional, or inhibited in some way. The lack of a functional *S-RNase* protein in *S. verrucosum* is likely the reason it serves as the major bridge species in between EBN1 species and cultivated germplasm. Endosperm failure is the main barrier that impedes the use of certain species. Prezygotic barriers (e.g. *S-RNase* and/or *HT* proteins) conceal relationships that might otherwise be compatible.

Materials and methods

Plant materials

The wild species *S. bulbocastanum*, *S. commersonii*, *S. jamesii*, and *S. pinnatisectum* were used as pollen donors for this study. Multiple plant introduction populations for each species were acquired from the USDA-ARS Potato Germplasm Introduction Station (Sturgeon Bay, WI) and individual genotypes were selected from their respective field-grown plant introduction populations based on their fertility and overall vigor (Table 1). In this study

interspecific compatibility in *S. verrucosum* was investigated using the clone SV607845.02. This *S. verrucosum* clone (Table 1) was selected based on its previous ability to create interspecific hybrid offspring with *S. bulbocastanum*. Each genotype was maintained in tissue culture on Murashige and Skoog Basal Medium with Vitamins and Sucrose (PhytoTech Labs) and Phyto Agar (Research Products International) prepared with DI water and balanced with 1N HCL and 8N NaOH to a pH of 5.8 and cultured in growth chambers with 16-h-light/8-h-dark photoperiod at 22°C and average light intensity of 200 μmoles m-2s-1.

Greenhouse pollination assays

In October of 2020, two individuals from each genotype were transferred directly from tissue culture to 14L (3.8 gallon) plastic pots with filled with Suremix Perlite peat and perlite soilless medium (Michigan Grower Products INC.). For the duration of the experiments greenhouse conditions were maintained at 20°C with a 16-hr photoperiod under Philips GreenPower light-emitting diode (LED) DR/W-MB lights (Philips Lighting Holding B.V., Netherlands). In order to validate the male fertility of the pollen donors, pollen from 5-6 anthers was collected directly onto a glass slide and immediately stained with acetocarmine-glycerol as described by Ordoñez (2014), covered with a cover slip, and sealed using clear nail polish. These slides were stored at room temperature in the dark and visualized the same day using a Leica DM750 binocular microscope (Leica Microsystems, Germany) and the associated Leica imaging software at 10x and 40x magnification. A minimum of 100 pollen grains were then used to calculate the percentage of viable pollen. Stained, turgid pollen was classified as viable while any grains that were shriveled, unstained, or unusually large or small were classified as unviable.

Stylar squash assays

For the evaluation of stylar barriers in interspecific crosses, 6-8 newly opened flowers (within 24 hours after anthesis) from each of the female genotypes were carefully emasculated and pollinated with fresh pollen collected on a glass slide directly from the pollen donor. Styles were collected 48 hours post-pollination, by removing the remaining petals and sepals and storing the remaining intact style, ovary and receptacle in 1.5mL microcentrifuge tube containing a 3:1 ethanol/acetic acid fixation solution. These styles were then kept in the dark at room temperature for at least 24 hours. Styles and ovaries were then softened using an 8N NaOH solution at 60°C for 1 hour. Samples were then rinsed three times with distilled water and stained with 0.1% aniline blue in 0.1N K3PO4 keeping them in dark conditions with light shaking. Styles with attached ovaries were then placed on glass slides, gently squashed under a coverslip, and sealed with nail polish for subsequent visualization. Samples were visualized using a Nikon Eclipse Ni-U upright microscope (Nikon Instruments Inc., Melville, NY, United States) with a SOLA light engine (Lumencor, Beaverton, OR, United States), and photographed with the attached ANDOR Zyla sCMOS camera (Oxford Instruments, United Kingdom) and NIS-Elements BR 5.02 software. Images of each stylar sample were stitched together using Image Composite Editor 2.0 software. Pollen tube growth measurements were made using ImageJ 1.53e software (Schneider et al., 2012). Measurements of pollen tube growth were calculated as a proportion of the total length of the style due to the variation of total style length with a single genotype. Pollinations were made at three discrete time points, separated by 30 days, with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse.

Pollen tube data analysis

Measurements of the pollen tube front, or the point where the majority of the pollen tubes stop, the longest pollen tube, and the total length of the style were collected using ImageJ software (Figure 1 for an example of measurement methods). From these measurements, distance from the surface of the stigma to the pollen tube front and length of the longest pollen tube were calculated based on the total length of the style in which they were measured. Significant differences between means ($\alpha = 0.05$) from replicated measurements where calculated using analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) using "R" software version 4.0.4

Assessment of S-RNase gene expression in Solanum verrucosum

Amplification and sequencing of S-RNase

DNA was isolated from young leaves of SV607845.02 using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Two sets of primers were used to capture the *S-RNase* sequences of both alleles. The SP3 primer set designed by Ye et al. (2018) was only able to capture one allele sequence (Table 1). A second set of primers were designed using reported S-RNase sequences from the Solanaceae family. For this, a T-BLASTN was performed against the M6 genome assembly using BLAST v2.2.31 (Altschul et al., 1990) with default parameters. Primers were designed from the conserved *S-RNase* regions using the top blast hit (Table 1). Both alleles were amplified using the following thermocycler conditions: one initial cycle of denaturation for 5 min at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 56°C, 1 min at 72°C and then a final extension of 5 min at 72°C. Amplicons were gel purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and then cloned into the pGEM T-Easy cloning vector (Promega, Madison, WI, United States) and transformed into DH5α competent cells (Thermo Fisher,

Carlsbad, CA, United States). A total of 22 colonies were Sanger sequenced and sequences were aligned manually using BioEdit version 7.2.5 (Hall, 1999).

S-RNase expression analysis via RT-PCR

A total of 100 styles were collected from newly opened flowers of *S. verrucosum* clone SV607845.02 and RNA was isolated using the Qiagen RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA Samples were then treated using Turbo DNase (Thermo Fisher Scientific, Waltham, MA, USA) to remove DNA contamination. *S-RNase* expression was assessed using the SuperScript III First-Strand Synthesis system for RT-PCR (Thermo Fisher Scientific, Waltham, MA, USA). Genomic DNA and cDNA generated from the RT-PCR were then amplified using the aforementioned primer sets and thermocycler schedule. Resultant PCR products were then visualized on 1% agarose genes and assessed for the presence or absence of the bands correlating with the respective primer sets.

Results

Prezygotic interspecific barriers in S. verrucosum

Pollen tubes from all of the pollen donors except for *S. bulbocastanum* were able to easily travel down the full length of the *S. verrucosum* style (Figures 4.1 and 4.2). The effect of plant age was minimal in these pollinations with the longest pollen tubes from *S. jamesii* and *S. pinnatisectum* present in the ovary at 48h post pollination in all three time-points (Figure 4.2). The majority of pollen tubes from *S. bulbocastanum* did not penetrate the full length of the style at any of the time-points, and the majority of pollen tubes for *S. commersonii* and *S. jamesii* only reached the ovary in the second and third time-points (Figure 4.1). Overall, no significant stylar barriers were observed and pollen tubes from all four pollen donors were observed in the vicinity

of the ovules. Fruit formation was observed for all four pollen donor species and hybrid progeny between *S. verrucosum* and *S. bulbocastanum* and *S. commersonii* were recovered.

S-RNase in S. verrucosum

Two *S-RNase* alleles were amplified and partially sequenced (Figure 4.5) despite the absence of S-RNase at the protein level as reported by Eijlander et al., (2000). Results from RT-PCR indicated a lack of S-RNase expression (Figure 4.7).

Discussion

The use of *S. verrucosum* as a bridge to access sexually isolated 1EBN species has been demonstrated several times (Jansky and Hamernik, 2009; Yermishin et al., 2014). Despite its recent and growing use, our understanding of this method is poor. If the EBN hypothesis is correct, no hybrids should result from interspecific crosses between *S. verrucosum* and any 1EBN species (Johnston et al., 1980). However, diploid interspecific hybrids are obtainable, making this bridging method a valuable technique to access EBN1 species.

Demonstrations of the *S. verrucosum* bridging technique by Jansky and Hamernik, (2009) and Yermishin *et al*. (2014) have a low efficiency. Only a few seeds are produced after a large number of pollinations, and often, hybrid progeny lack sufficient fertility for further use (Hermsen and Ramanna, 1976; Jansky and Hamernik, 2009; Yermishin et al., 2014). The use of mentor pollen (Yermishin et al., 2014) and careful or no emasculation (Bamberg, 2020) may increase seed set, but only to a marginal degree. Certain *S. verrucosum* clones may also vary in their ability to form fertile inter-EBN hybrids, and current research to identify elite clones is ongoing. The use of *S. verrucosum* bridge crosses remains a viable option despite its low efficiency rate, as it is less labor and resource intensive compared to other methods such as embryo rescue, complex ploidy manipulations, and somatic fusion (Ramon and Hanneman,

2002; Carputo et a., 1997; Polzerová et al., 2011; Jansky and Hamernik, 2009). Nevertheless, improving the efficiency of this method is necessary for it to gain greater use.

Prezygotic stylar barriers are not an important barrier in these pollinations as demonstrated in this study (Figure 4). The lack of prezygotic stylar barriers in *S. verrucosum* is comparable to *S. lycopersicum* (Baek et al., 2015). The lack of interspecific stylar barriers is a very valuable phenotype which allows *S. lycopersicum* to not only be SC, thus allowing the development of inbreds and F1 hybrids, but also serve as a parent for wide interspecific crosses allowing the introgression of valuable traits from genetically distant wild relatives (Baek et al., 2015; Canady et al., 2005: Ji et al., 2004). The lack of SC and interspecific incompatibility in *S. tuberosum* is a significant barrier to the effective use of wild species; and the ability to replicate the phenotype observed in *S. verrucosum* and *S. lycopersicum* within *S. tuberosum* would be a significant advancement in potato breeding (Novy and Hanneman, 1991; Enciso-Rodriguez et al., 2019). Understanding the causative genetic mechanisms behind SC and interspecific compatibility in *S. verrucosum* is necessary to replicate this phenotype. Additionally, stylar barriers are not the only significant factors barring the use of wild potato species in potato breeding. Therefore, further inquiry and manipulation of the genetic mechanisms underlying effective ploidy and endosperm balance number are likely to result in the largest increases in efficiency.

APPENDICES

APPENDIX A: Chapter 4 Tables

Primer	Sequence (5'-3')	Purpose
SP3	F: GGGGAAACTGGAAAATGGTT R: ATGTGAATGTGTTCAGCGAAA	Amplifying partial sequence of allele 1
M6	F: ACGATTCACGGGCTTTGG R: AATATTGATAGAGCCATAAGGGTTGT	Amplifying partial sequence of allele 2

Table 4.1. Primers used in this study

APPENDIX B: Chapter 4 Figures

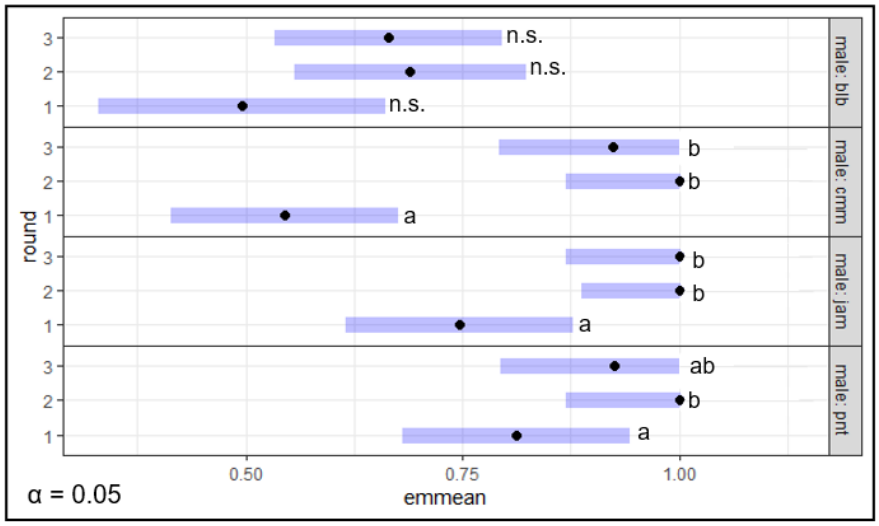


Figure 4.1. Measurements of pollen tube front for all pollen donors. *Blue bars depict the marginal pollen tube means (emmean) as a proportion of total style length at 48h post pollination. Female genotypes are depicted on the left vertical axis, and time-points or rounds are depicted on the right vertical axis. Pollinations were made at three discrete time points (rounds), separated by 30 days, with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse. Pollen donor species are represented by the following three letter species codes: S. bulbocastanum (blb), S. commersonii (cmm), S. jamesii (jam), and S. pinnatisectum (pnt).*

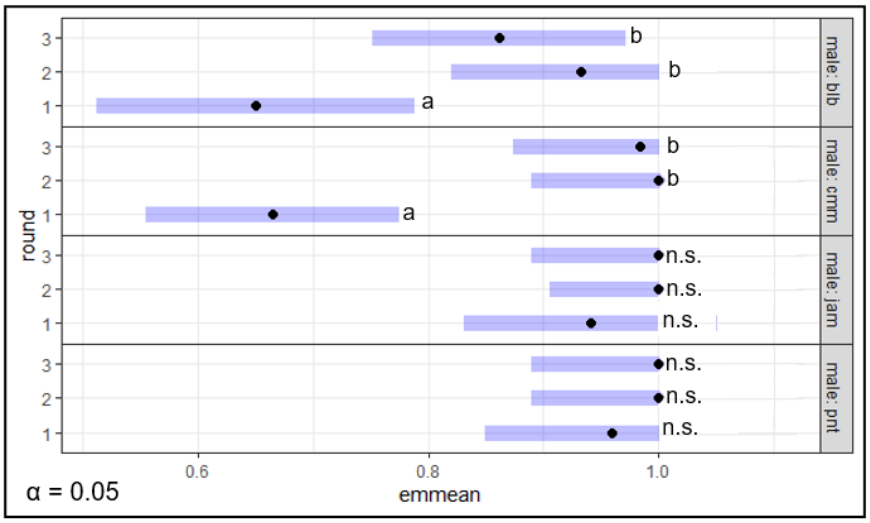


Figure 4.2. Measurements of the longest pollen tube for all pollen donors. *Blue bars depict the marginal pollen tube means (emmean) as a proportion of total style length at 48h post pollination. Female genotypes are depicted on the left vertical axis, and time-points or rounds are depicted on the right vertical axis. Pollinations were made at three discrete time points (rounds), separated by 30 days, with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse. Pollen donor species are represented by the following three letter species codes: S. bulbocastanum (blb), S. commersonii (cmm), S. jamesii (jam), and S. pinnatisectum (pnt).*

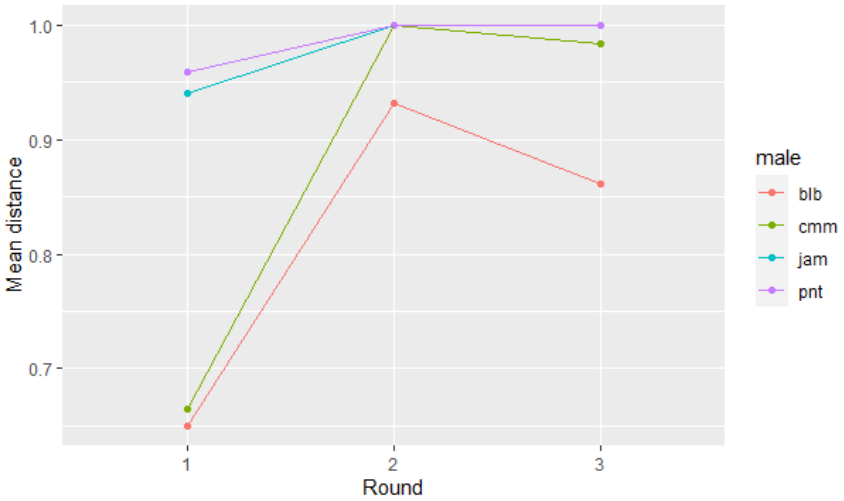
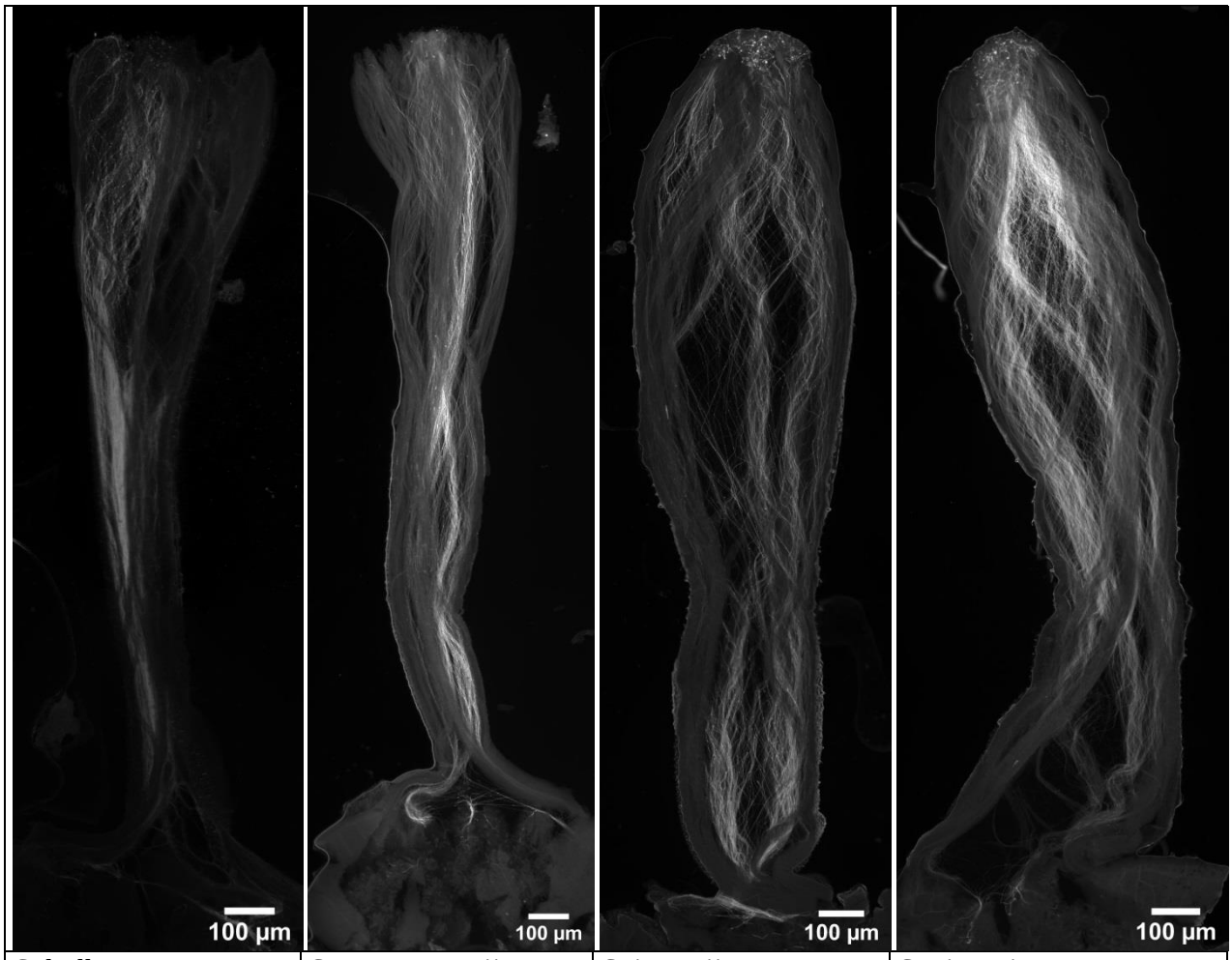


Figure 4.3. Estimated marginal means plotted against the different time-points *(rounds) used in this study for all pollen donors showing effect of plant age on interspecific pollen tube inhibition. The vertical axis depicts the estimated marginal means of the longest measured pollen tube as a proportion of the total length of the style. The horizontal axis plots the three pollination time-points (rounds) used in the study with the first time point being approximately six weeks after plants were transferred from tissue culture to the greenhouse with subsequent pollination rounds separated by 30 days. Pollen donor species are represented by the following three letter species codes: S. bulbocastanum (blb), S. commersonii (cmm), S. jamesii (jam), and S. pinnatisectum (pnt).*



S. bulbocastanum	*S. commersonii*	*S. jamesii*	*S. pinnatisectum*

Figure 4.4. Styles of *S. verrucosum* SV607845.02 pollinated with each of the four pollen donors. *Pollen tubes are visible at the base of the style and penetrating the ovary at 48h post pollination showing an absence of interspecific stylar barriers in these pollinations.*

>Solanum_verrucosum_S-RNase_SV607845.02_1
GGGGAAACTGGAAAATGGTTAAACCACAACTCACATCAGCTCTCTTCATTGTGCTTT
TTGCTCTTTCTCCCGCTTATGGGGATTTCGATTCCCTCCAACTGGTATTAACATGGCC
AGCATCATTTTGCCATGTTAATGATTGTGTGCGAATAGCTCCAAAAAACTTCACGAT
TCACGGGCTTTGGCCGAATAAAGAGGGAACGGTGCTGCAGAACTGCAAGCCAAAAC
CTAAGTATGTTAATTTCAAGGTAAGCAATAGCATTTTTTTAGAGCCCGCTTTTCCGCT
CAGTTCAATTTACTTGAAAGATTCTTTTCGAAATGCTTACAGGATAAGATGTTCAAC
GATCTTGACAAACACTGGATTCAGTTGAAGTTTGATGAAGATTATGGTGAAAAGGA
ACAACCTTTATGGCTCTATCAATATTTTAAGCATGGATCTTGTTGTCAGAAAATGTAC
AACCAAAACACGTATTTTAGTCTAGCCTTGCGCTTAAAAGACAGGTTTGATCTTCTG
AGAACTCTCCAAATACATCATATTTTTCCTGGATCAAGTTATACATTTAAGAAAATC
TTTGATGCCGTCAAGACAGCTACTCAAATGGATCCTGACCTTAAGTGTACTAAAGGA
GTACCGGAACTATATGAAATAGGCATATGTTTCACCCCAAATGCAGATGCTCTGATT
CCATGTCGTCAAAGTAATACATGCGATAGGACAGGAAAAATCTTTTTTCGCTGAACA
ACTTCACAT

>Solanum_verrucosum_S-RNase_SV607845.02_2
ACGATTCACGGGCTTTGGCCGGATAAAGAGGGAACGGTGCTGCAGAACTGCAAGCC
AAAACCTAAGTATGTTAATTTCAAGGATAAGATGTTCAACGATCTTGACAAACACTG
GATTCAGTTGAAGTTTGATGAAGATTATGGTGAAAAGGAACAACCTTTATGGCTCTA
TCAATATT

Figure 4.5. Partial sequences of the two *S-RNase* alleles identified in *S. verrucosum*. *Allele SV607845.02_1 was captured using the SP3 primers designed by Ye et al. (2018). The SV607845.02_2 allele was capture using primers designed from conserved S-RNase regions.*

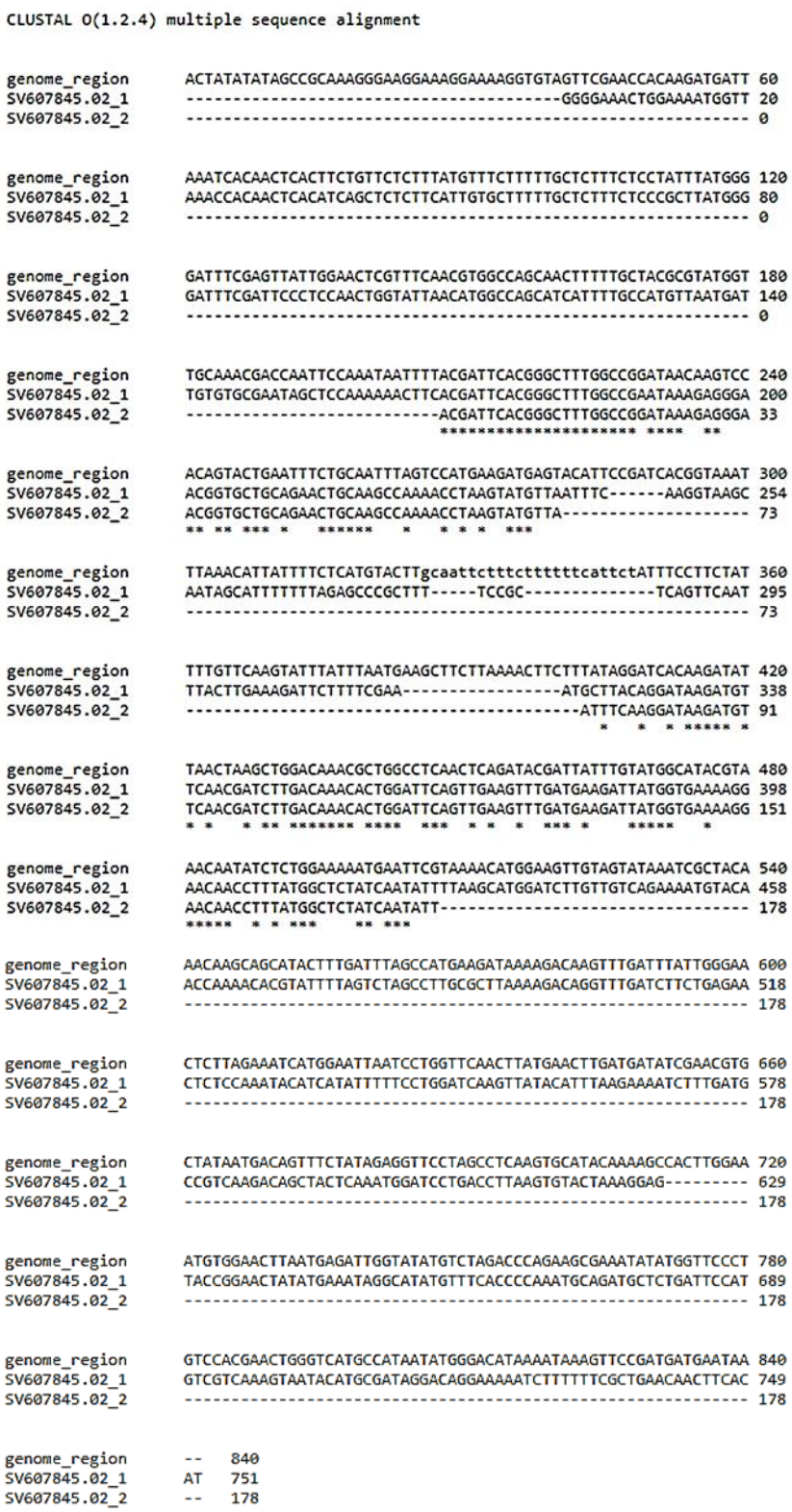


Figure 4.6. The two *S-RNase* alleles of *S. verrucosum* identified in this study *aligned to the genome region of the publicly available assembly GCA_900185275.1 available through NCBI.*

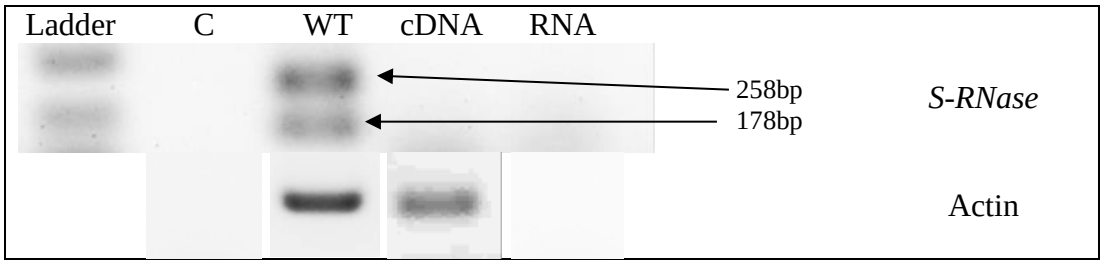


Figure 4.7. *S-RNase* expression in *S. verrucosum* SV607845.02. *No S-RNase expression was observed after RT-PCR. Total RNA was isolated from 100 styles collected from newly opened flowers within 24 hours after anthesis. Samples were DNase treated before using RT-PCR to assess expression of S-RNase*

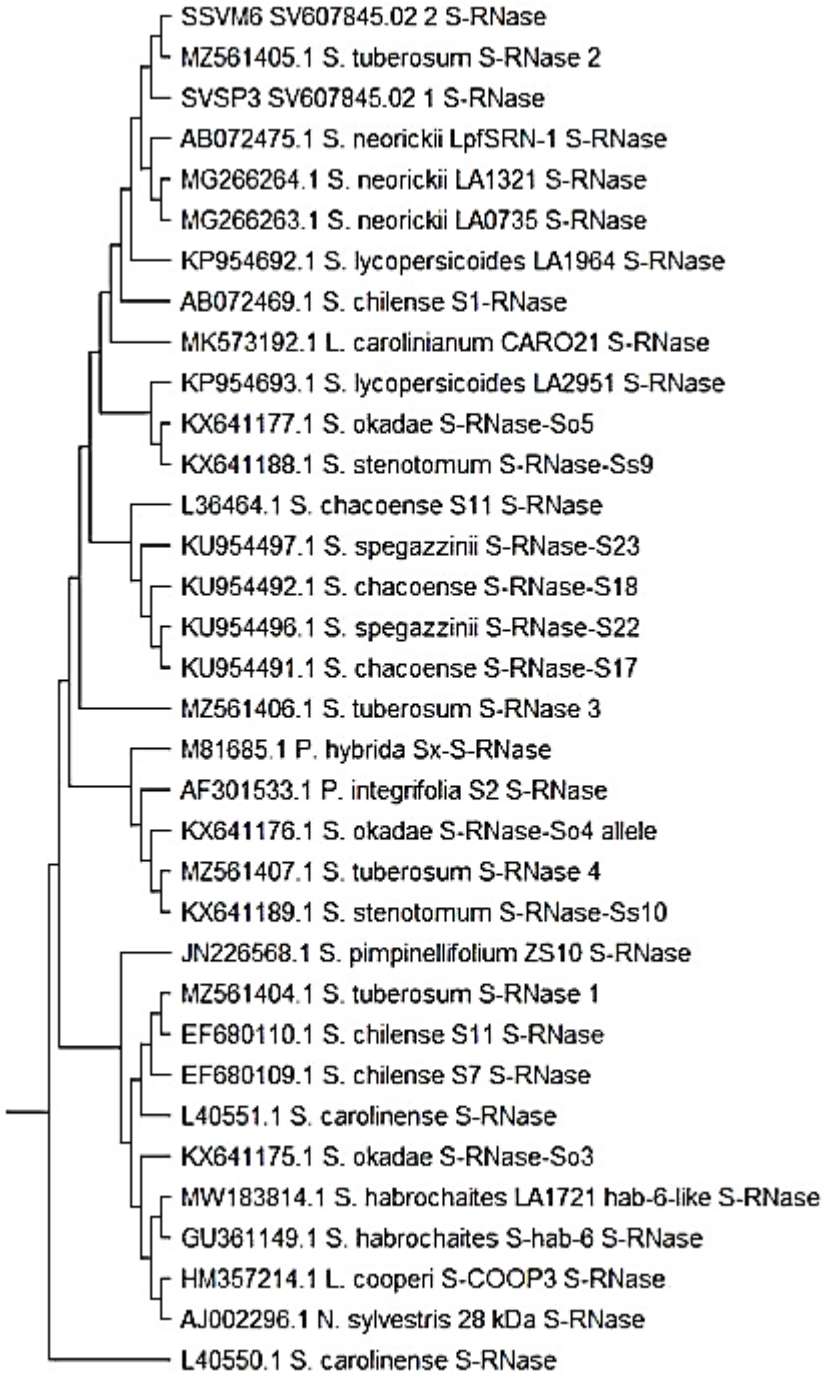


Figure 4.8. UPGMA phylogenetic tree showing the similarity of both SV607845.02 *S-RNase* alleles to the S-RNase 2 from *S. tuberosum* and their relation to other known *S-RNase* alleles within Solanaceae. *Sequences are publicly available and were retrieved from NCBI, alignment and phylogenetic analysis was created using MEGA version 11.0.9.*

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The past decade has seen a renewed interest in diploid potato breeding as current and past breeding efforts at the tetraploid level have been plagued by lengthy breeding cycles, excessive heterozygosity, and inbreeding depression which impedes the fixation of desirable alleles (Lindhout et al., 2011; Stokstad, 2019). Genetic improvement of potato at the diploid level would accelerate the generation of new varieties, and simplify efforts to incorporate and stack valuable agronomic and abiotic resistance traits into existing germplasm (Jansky et al., 2016; Kaiser et al., 2021). The use of diploid germplasm also simplifies the introgression of traits from wild species that have been major sources for disease resistance and abiotic stress tolerance.

Because previous attempts to introgress wild species traits have focused on incorporating traits into tetraploid potato germplasm; the movement towards a diploid breeding system has reignited interest in understanding how to best access the genetically isolated 1EBN wild species without complex ploidy manipulation, somatic fusion, or embryo rescue that have previously been required (Carputo et al., 1997; Ramon and Hanneman, 2002; Polzerová et al., 2011; Bethke et al., 2017). Unfortunately, due to prezygotic stylar barriers and differences in effective ploidy between 1EBN species and cultivated germplasm, accessing the traits from these species is extraordinarily difficult (Novy and Hanneman, 1991; Jansky and Hamernik, 2009; Städler et al., 2021). By better characterizing the prezygotic barriers between 1EBN species and cultivated diploids, further research can better understand the significant postzygotic barriers that inhibit the use of these valuable species.

S-RNase and HT in interspecific crosses

The relationships of interspecific compatibility and incompatibility in *Solanum* section *Petota* are complex and intricate. Inquiry into these relationships has elucidated the pleiotropic and redundant function of *S-RNase* and *HT* which tandemly and independently mediate both interspecific and intraspecific pollen rejection (Baek et al., 2015; Tovar-Méndez et al., 2014, 2017). The outcome of interspecific pollinations is specific to the individual species involved and direction of the cross due to the direct and indirect involvement of multiple factors such as *HT* and *S-RNase,* 120 kDa, Cullin1 (*CUL1*), and farnesyl pyrophosphate synthase (*FPS2*) (Bedinger et al., 2011; Li and Chetelat, 2015; McCormick, 2018; Tovar-Méndez et al., 2017).

The findings presented here are consistent with previous work conducted in *Solanum* section *Lycopersicon* showing that *S-RNase* plays a central role in interspecific pollen rejection (Baek et al., 2015; Tovar-Méndez et al., 2014). Statistical analyses also demonstrated that *HT-B* alone is not a significant factor in these pollinations. These results do not contradict the findings from Tovar-Méndez et al (2014, 2017) where *HT* proteins played a significant role in interspecific pollen rejection. Since *HT-A* remained functional in all of the female genotypes used, these findings demonstrate the overlap in gene function between *HT-A* and *HT-B,* the greater importance of *HT-A*, or that *HT* is not a significant factor in these combinations implicating other mechanisms (Baek et al, 2015; Qin and Chetelat, 2021).

Therefore, there is a need to generate gene knock-outs of *HT-A* to further elucidate the function of *HT* in these pollinations as *HT-A* alone may mediate the *S-RNase* independent rejection of interspecific pollen. Future assessment of the role of *HT-A* in interspecific pollen rejection will inform our understanding of interspecific pollination barriers. Efforts are being made to make these gene edits and further assessment will be made after the edits are confirmed.

Sli and Solanum chacoense

This study draws the clear conclusions that *Sli* is not a significant factor in interspecific pollen rejection in these pollinations. The presence or absence of *Sli* did not change the outcome of any of the pollinations made, which is inconsistent with the previous findings of Sanetomo et al. (2014) despite the use of the same *S. pinnatisectum* PI (273232). These inconsistencies can be attributed to unknown factors in *S. chacoense* besides *Sli*. The pollen donors used in the assessments made by Sanetomo et al. (2014) came from interspecific crosses between *S. chacoense* and *S. phureja*, while the pollen donors used in these assessments were of one species, *S. chacoense*. The difference between pollen donors is the likely source of inconsistencies; the introduction of unknown genetic variables from *S. phureja* represents a confounding variable in the studies carried out by Sanetomo et al. (2014).

Although *Sli* played no significant role in these pollinations, it is possible that *S. chacoense* as a pollen donor is better able to bypass stylar barriers in EBN1 species such as *S. pinnatisectum* compared to *S. megistacrolobum*, *S. demissum*, and *S. phureja* (*S. tuberosum* Group *Phureja*) as reported by Sanetomo et al. (2014). Consequently, *S. chacoense* may be a valuable resource in accessing these EBN1 species regardless of *Sli* status. The limitations and possibilities associated with *Sli* with be more apparent when the genetic mechanisms behind *Sli* can be clearly identified. Further testing of a diverse panel of *S. chacoense* pollen donors would also clarify if pollen from these species is accepted to a greater degree in interspecific pollinations.

SC and interspecific compatibility in Solanum verrucosum

Currently, bridge crossing with *S. verrucosum* is among the most reliable and feasible methods to access EBN1 species (Jansky, and Hamernik; 2009). Unlike the methods proposed by

Sanetomo et al. (2014), Ramon and Hanneman (2002), Polzerová et al. (2011), and Carputo et al. (1997), the use of *S. verrucosum* bridge crosses is more accessible for most plant breeders as it does not require ploidy manipulation, interploidy crosses, or the use of advanced techniques such as somatic fusion or embryo rescue (Jansky and Hamernik, 2009). However, although the use of *S. verrucosum* as a bridge to access sexually isolated 1EBN species is has been demonstrated previously, our understanding of this method is poor (Jansky and Hamernik, 2009; Yermishin et al., 2014). Technically if the EBN hypothesis is correct, no hybrids should result from interspecific crosses between *S. verrucosum* and any 1EBN species (Johnston et al., 1980).

The ability to produce hybrids between *S. verrucosum* and 1EBN species highlights our limited understanding of effectively ploidy and endosperm balance number. Recent research by Stadler et al. (2021) and others has demonstrated the complex dosage-sensitive nature of endosperm development and failure (Baek et al., 2016; Roth et al 2017; Coughlan et al., 2020). The use of discrete categories, such as endosperm balance numbers, is inconsistent with our current understanding of effective ploidy. The multigenic mechanisms that underpin effective ploidy are complex and genome wide (Ehlenfeldt and Hanneman, 1988; Lafon-Placette, and Köhler, 2016; Stadler et al., 2021). Additionally, individual species of the same EBN are not always closely related (Pritchard, 2005). Therefore, caution should be applied when using EBN to predict the outcome of interspecific crosses as it represents an outdated understanding of effective ploidy with demonstrable flaws.

The ability of *S. verrucosum* to form interspecific hybrids with species of differing EBN is likely partially rooted in its lack of a functional S-RNase (Eijlander, 1998). The absence of functional S-RNase allows interspecific pollen tube growth in what would otherwise be incompatible pollinations. While it is known that there is no detectable S-RNase at the protein

level (Eijlander, 1998), the use of primers targeted to conserved regions of *S-RNase* was able to locate *S-RNase* in the genome. These alleles for *S-RNase* in *S. verrucosum* are likely non-functional or inhibited in some way as no expression was ever observed. Due to the absence of *S-RNase*, and possibly other factors, prezygotic stylar barriers are not an important barrier in interspecific pollinations when *S. verrucosum* is the female parent. Non-functional genes for *HT* and other factors may further allow interspecific pollen to penetrate the style. Further analysis into the promotor regions of *S-RNase* and into other factors such as *HT* in *S. verrucosum* will clarify the causative genetic mechanisms allowing *S. verrucosum* to act as a receptive parent to self and interspecific pollen.

Although more resource and time efficient than other techniques, demonstrations of the *S. verrucosum* bridging technique by Jansky and Hamernik (2009) and Yermishin et al. (2014) however have shown a low efficiency. Only a few seeds are produced after a large number of pollinations, and often hybrid progeny lack sufficient fertility for further use (Hermsen and Ramanna, 1976; Jansky and Hamernik, 2009; Yermishin et al., 2014). The use of mentor pollen (Yermishin et al., 2014) and careful or no emasculation (Bamberg, 2020) may increase seed set, but only to a marginal degree. The use of *S. verrucosum* bridge crosses remain a viable option despite its low efficiency rate, as it is less labor and resource intensive compared to other methods used (Jansky and Hamernik, 2009). Nevertheless, improving the efficiency of this method is necessary for it to gain greater use. Therefore, further inquiry and manipulation of the genetic mechanisms underlying effective ploidy and endosperm balance number are likely to result in the largest increases in efficiency.

Plant ontogeny

Intra- and inter-specific compatibility is heavily influenced by environmental and ontogenic factors such as temperature, humidity, flower age, and plant age (Bienz, 1958; Henderson and LeClerg, 1943; Travers et al., 2004; Liao et al., 2016; Webb and Williams, 1988). Although variable GSI responses due to plant and floral age have been demonstrated in *S. carolinense* and *N. alata*, until now these findings have not been observed in interspecific pollinations (Travers et al., 2004; Liao et al., 2016). Results from statistical analysis indicate that plant age does have a significant impact on interspecific pollen rejection, consistent with the findings published by Travers et al. (2004) and Liao et al. (2016). There is still a lot to learn about plant ontogeny and the degradation of interspecific and GSI barriers with age. Since all of the plants in this study were planted at the same time it is unknown whether the age of the pollen donor or the female parent are more or less responsible for the break-down in prezygotic barriers. Further experiments that focus on the age of the pollen donor and female parent separately will aid in the identification of the causative mechanisms and the overall implications of plant age in breeding programs.

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