GENERATION OF *HT-B* AND *HT-B* PLUS *S-RNASE* KNOCKOUT LINES TO UNDERSTAND SELF-COMPATIBILITY IN DIPLOID POTATO

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Breeding, Genetics, and Biotechnology – Plant Biology – Master of Science

2021

ABSTRACT

GENERATION OF *HT-B* AND *HT-B* PLUS *S-RNASE* KNOCKOUT LINES TO UNDERSTAND SELF-COMPATIBILITY IN DIPLOID POTATO

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Domesticated potato (*Solanum tuberosum* L.) is the world's third most important food crop and is a food security crop according to the Food and Agriculture Organization of the United Nations. Currently, commercial potatoes are autotetraploid and mainly produced via asexual clonal propagation. The autotetraploid nature of most cultivated potatoes in combination with acute inbreeding depression when self-fertilized over multiple generations cause challenges in making advances with traditional breeding schemes. The benefits of moving potato to a diploid breeding model include a simplified breeding scheme, easier fixation of desirable alleles, and generation of inbred lines that may be used to generate F1 hybrids with heterotic potential. A major hinderance to self-compatibility originates from the gametophytic self-incompatibility (GSI) system in which the *S-RNase* and *HT-B* genes play a critical role. Utilizing CRISPR-Cas9 gene editing, *HT-B* and *HT-B* + *S-RNase* knockout (KO) lines were produced. *HT-B* KOs produced parthenocarpic fruit but remained self-incompatible. However, the *S-RNase* and *HT-B* double KOs were self-compatible. Self-compatibility was measured quantitatively using fruit set, fruit weight, and seed count. Fruit set varied across both self-incompatible and self-compatible lines, with no clear trend in statistical significance. Double KO lines consistently displayed higher fruit weight than incompatible lines. Seed count served as the best measure of self-compatibility, with *S-RNase* and *HT-B* double KO lines producing up to three times mean seed per fruit when compared to *S-RNase* only KOs from prior studies. The lines with the highest levels of self-compatibility will serve as useful additions in advancing potato breeding to the diploid level.

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Dedicated to my husband, Steven Morgan

ACKNOWLEDGMENTS

I want to thank my adviser Dr. David Douches for his guidance throughout my time at Michigan State University. To my mentor Dr. Felix Enciso Rodriguez, I offer my utmost gratitude for his excellent mentorship throughout my research and the confidence he helped to restore in myself. A special thank you to Dr. Daniel Zarka, who is a true guru when it comes to molecular techniques and helped me understand and work through the intricate details of my project. I am also highly appreciative of the members of my graduate guidance committee Drs. David Douches, C. Robin Buell, Guo-Qing Song, and Addie Thompson. I am thankful to C. Robin Buell for helping me find the right lab and her guidance on genomics, Guo-Qing Song for input on genome editing, and Addie Thompson for help with statistical analysis. I also want to thank Leo Pham from the Michigan State University Statistical Consulting Center with help on statistical analysis.

Thank you to my close friends in the lab Thilani Jayakody, Kate Shaw, Chen Zhang, William Behling, and Natalie Kaiser for their companionship and helpful support in completing my project. Thank you to the members of the potato breeding program Dr. Swathi Nadakuduti, Monica Hufnagel, Kelly Zarka, and the many others who provided intellectual resources for my research. To my husband, father, mother, sister, brother-in-law, and friends outside of lab I love you and thank you for supporting me so that I could make it to where I am today.

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POTATO BACKGROUND, BREEDING, AND GENE EDITING

I. Potato evolutionary origins, species, and consumption

The evolutionary origins of cultivated potato may be traced to a group of roughly 20 tuber bearing species in the *Solanum brevicaule* complex (a morphologically similar group of wild species) originating in the Andean highlands of South America (Spooner *et al.*, 2005). Incorporating both cultivated potato and its wild relatives, there are now over 1,000 species within the *Solanum* genus (Machida-Hirano, 2015). The most agriculturally relevant section of *Solanum* is *Petota* (subsection *Potatoe*) since it contains *S. tuberosum* in addition to all other tuber-bearing species (Gutaker *et al.*, 2019; Machida-Hirano, 2015). *S. tuberosum* Group Tuberosum, evolved from a hybridization between the *S. stenotonum* complex and an unknown species (Grun, 2009; Hosaka, 2003; Hosaka and Hanneman, 1998).

Domestication of potato species selected for traits that are still desirable in modern day potato lines, such as increased yield and reduction of bitter tasting glycoalkaloids (Hardigan *et al.*, 2017; Lightbourn and Veilleux, 2007; Spooner *et al.*, 2005). Above ground traits selected for domestication include high vigor and extensive segregation of flowering traits (Spooner *et al.*, 2005). Modern cultivated potatoes still retain a plethora of diversity from its wild progenitors, with ranges in peel and flesh color from yellow to purple and shape from cylindrical to spherical (Spooner *et al.*, 2012; Ovchinnikova *et al.*, 2011). The ploidy of cultivated potato (section *Petota)* is also highly diverse with diploid (2n = 2x = 24), triploid (2n = 3x = 36), tetraploid (2n = 4x = 48), and even pentaploid (2n = 5x = 60) cultivars (Spooner *et al.*, 2010).

Potato has been a staple food crop in South American countries for thousands of years and written accounts of the spread of potatoes to Europe date back as early as 1567 (Machida-Hirano, 2015; Ríos *et al.*, 2007; Spooner *et al.*, 2005). Currently, potatoes are consumed by billions of people and serve as an essential staple food in many countries with over 370 million metric tons produced yearly across the world as of 2019 (Figure 1.1) (FAOSTAT, 2021; Devaux *et al.*, 2014).

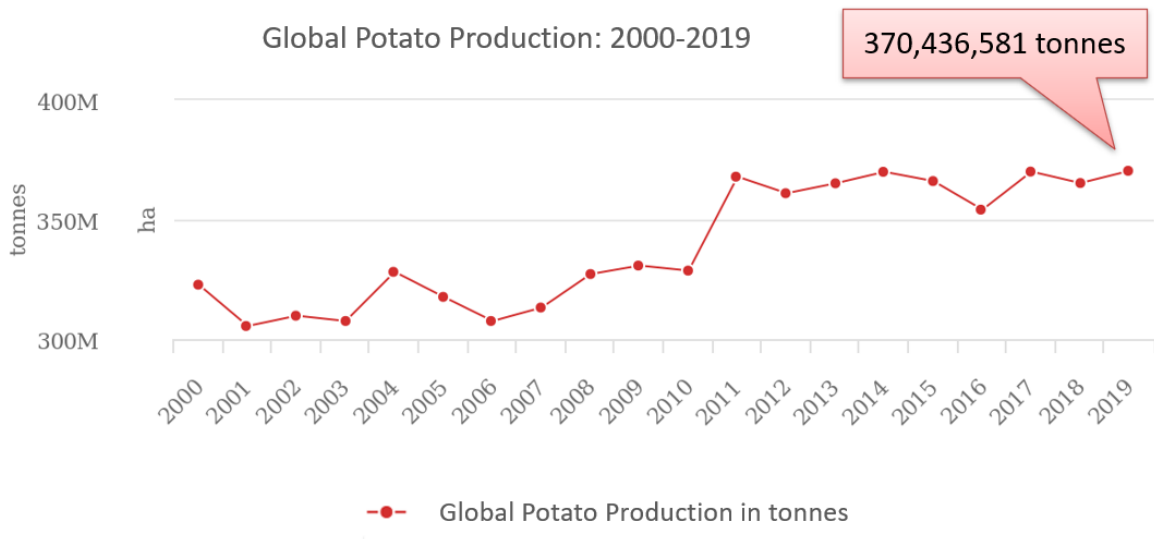

Figure 1.1 Global potato production in metric tonnes from 2000 – 2019.

Worldwide potato production has increased greatly from 2000 to 2011 but shows less growth from 2012 to 2019. Source: FAOSTAT (March 19, 2019).

This popularity in consumption comes from potatoes' many beneficial qualities including being an inexpensive yet highly nutritious food and relative ease of cultivation (Devaux *et al.*, 2014). Unprocessed and freshly cooked potato generally produce 11-12 mg of vitamin C / 100 g of tissue, have greater protein / g of tissue (dry weight) when compared with cereals, and lower fat content than legumes making it a relatively healthy food choice (Campos and Ortiz, 2019; Machida-Hirano *et al.*, 2015; Haase and Haverkort, 2006; Camire *et al.*, 2009). Potatoes also serve as an important source of carbohydrates in the human diet and are especially important as a source of fiber for

children (Kolasa, 1993; Camire *et al.*, 2009; Drewnowski and Rehm, 2013). Additionally, cultivated potato has a higher average yield per unit area than most crops, averaging at 40.6 metric tons per hectare in North America alone (Drewnowski and Rehm, 2013; Camire *et al.*, 2009).

i. Increase in human population and climate change in relation to potato

Anticipated global food consumption by humans by year 2050 is projected to increase by 100-110% due to an estimated rise in population to 9.6 billion by the year 2050 (Tilman *et al.*, 2011; Searchinger, 2013). The environmental impact of ensuring potato and other food stability crop production keeps pace with human population growth is substantial with 25% of greenhouse gas emissions and 70% of fresh water use attributed to agriculture (Searchinger, 2013). The resulting climatic changes from this expansion include global temperature increase, drought, and flooding from human expansion which will negatively affect many biological systems including food crops (Costinot *et al.*, 2016).

Potato production is highest in temperate regions and the predictive effects of climate change on potato production is highly dependent upon the region of growth (Hijmans, 2003; Haverkort, 1990). Generally, potatoes are sensitive to temperatures above 17°C and below 0°C which causes diminished tuber growth or damage to plant tissue, respectively (Stol *et al.*, 1991; Levy *et al.*, 1991). The expected increase in global temperature between 1.4°C and 5.8°C by 2100 (Smithson, 2002) is predicted to have relatively small effect at higher altitudes where either the location of potato production or the use of later-maturing cultivars may be implemented (Hijmans, 2003). However, in lower altitudes where altering planting time is not a viable option, climate change is predicted to significantly decrease potato production (Hijmans, 2003). To supply the rapidly expanding human population and account for areas negatively affected by climate change, utilizing

every possible genomic and agricultural resource to supply a growing population under environmental stress is imperative (Soyars *et al.*, 2018).

II. Potato breeding

When producing a new potato cultivar, there are dozens of traits to consider including flesh color, processing quality, disease resistance, drought tolerance, and predominantly yield (Slater *et al.*, 2014). Conventional breeding of tetraploid potatoes often relies on pedigree breeding and is inefficient, retaining less than 10% of the progeny in the first year due to high segregation of the aforementioned traits of interest in the F1 families (Haynes *et al.*, 2012; Slater *et al.*, 2016). Moving away from a tetraploid breeding and considering other breeding platforms is necessary to ensure potato production keeps up with global needs.

i. Pitfalls in tetraploid breeding

Within the U.S. the most widely produced potato varieties are Russet Burbank, Russet Norkotah, and Ranger Russet, all of which are tetraploid (2n = 4x = 48) (Bethke *et al.*, 2014, USDA 2021). Russet Burbank was initially released in the early 1900s and has been the dominant potato crop in the US ever since (Bethke *et al.*, 2014). Increase in yield for the Russet market class and other autotetraploid varieties, over the last century may be mainly attributed to improved agricultural techniques (e.g. improved fertilizer composition) as well as breeding (Tan *et al.*, 2016; Douches *et al.*, 1996). However, progress has been slow since providing ideal environmental conditions has limited potential for crop genetic improvement and the tetraploid breeding cycle takes nearly 10-12 years for new cultivar generation (Lindhout *et al.*, 2011).

The lengthy breeding progress of cultivated potato comes from its autotetraploid nature which makes it more difficult to predict and control allele frequencies when compared to other diploid crops such *Zea mays* (Hirsch *et al.*, 2016; Lindhout *et al.*, 2011). Additionally, the highly heterozygous genetic state of potato varieties makes it difficult to fix desirable alleles and the clonal vegetative propagation of potato is more time consuming and expensive when compared to true seed crops (Jansky *et al.*, 2016; Lindhout *et al.*, 2011; Manrique-Carpintero *et al.*, 2015). Tetraploid potato varieties are self-compatible, yet experience severe inbreeding depression when self-fertilized (Zhang *et al.*, 2019). Due to inbreeding depression, the hypothetical amount of generations it would take to generate an inbred tetraploid potato line using traditional breeding methods becomes unfeasible (Campos and Ortiz, 2019). This inhibition of the generation of inbred lines excludes the possibility of producing F1 hybrids with heterotic potential from inbred line crosses in tetraploid varieties.

ii. Diploid potato breeding benefits

As a solution to the pitfalls of tetraploid potato breeding, a self-compatible diploid potato breeding scheme offers a plausible alternative with a plethora of benefits. Diploid potato species have a simplified breeding scheme and new genetic diversity could be incorporated from wild diploid potato varieties such as disease resistance against potato pathogens such as late blight, blackleg, and soft rot (Jansky *et al.*, 2014; Bradshaw *et al.*, 2006). The biodiversity available within wild germplasm also offers resistances to abiotic factors such as heat and cold stress (Bradshaw *et al.*, 2006). The generation of inbred lines and a breeding system that results in F1 progeny with increased yield due to hybrid vigor as seen in other diploid crops such as *Z. mays* is another a major incentive to switch to a diploid breeding scheme (Hirsch *et al.*, 2016; Jansky *et al.*, 2016).

Unfortunately, a major barrier to diploid potato breeding comes from the self-incompatible nature of the majority of wild and cultivated diploid species (Phumichai *et al.*, 2005; Jansky *et al.*, 2014). Incompatibility within *Solanum* is due to multiple contributing factors including the gametophytic self-incompatibility (GSI) system and in specific cases late-acting self-incompatibility (LSI) (Takayama and Isogai, 2005; Seavey and Bawa, 1986; Peterson *et al.*, 2016).

iii. Gametophytic Self-Incompatibility

GSI is governed by a multigene complex called the "*S*-Locus" which consists of a female determinant and a male determinant, both of which are linked transcriptional units and are inherited as a single segregating unit called a "S-haplotype" (Figure 1.2a) (Takayama and Isogai, 2005; Silva and Goring, 2007). Within GSI, whether or not a plant will be self-incompatible is determined by the gamete's own S-haplotype, unlike in sporophyte self-incompatibility (SSI) (as seen in the Brassicaceae family) which is determined by the diploid genome of the parent plant (Silva and Goring, 2007).

The sole female *S*-locus determinant is a glycoprotein ribonuclease named "S-RNase" and was initially identified to be exclusively produced in the pistil in tobacco (*Nicotiana alata*) in the late 1980s (Murfett *et al.*, 1994; Lee *et al.*, 1994; McClure *et al.*, 1989). Within the Solanaceae species, *S-RNase* has two highly variable regions (HVa and HVb) and five highly conserved regions (C1-C5) (McClure *et al.*, 1989; Mccubbin and Kao, 2000; Enciso-Rodriguez *et al.*, 2019). The highly variable regions are translated into a domain on the S-RNase protein which is exposed on the molecular surface and allows for interaction with the male determinant (Matton *et al.*, 1997; Matton *et al.*, 1999). Domain swapping simulations and S-RNase pollen rejection experiments support differences in these hypervariable regions and allow for the recognition of cross or self-

pollen which are integral to the specific self-pollen rejection reaction in the GSI system (Matton *et al.*, 1997; Matton *et al.*, 1999). Despite the highly variable regions, the main eight helices and seven *β*-strands comprising the S-RNase protein remain similar across species and likely contribute to the main function S-RNase plays as a ribonuclease (Matton *et al.*, 1997). In order to reject self-pollen, the S-RNase must retain its function as a ribonuclease and degrade the pollen RNA as shown by radioactive tracer experiments (Huang *et al.*, 1994; McClure *et al.*, 1999). When S-RNase recognizes RNA from the pollen which matches its own S-haplotype and degrades it this causes a cytotoxic effect upon the pollen tube ceasing its growth before it may reach the ovule (Takayama and Isogai, 2005). This failure of the pollen tube to reach the ovule is the defining factor in how GSI prevents fertilization.

Though S-RNase is the sole *S*-locus female determinant, there are other pistil factors that are related to self-incompatibility/pollen rejection in GSI (Goldraij *et al.*, 2006). In particular, asparagine rich HT proteins (HT-A and HT-B), 4936-factor, and a 120 kDa glycoprotein within Solanaceae are of interest and are implicated in self-incompatibility reactions (Goldraij *et al.*, 2006; Covey *et al.*, 2010; O'Brien *et al.*, 2002). Immunolocalization experiments within *Nicotiana* showed HT-B, 120 kDa glycoprotein, and factor 4936 are not required for uptake of S-RNase into the pollen tube but are involved in a hypothesized sequestering mechanism within the *Nicotiana alata* (Goldraij *et al.*, 2006). Within this sequestering mechanism, S-RNase is encapsulated in a vacuole membrane brought in through endocytosis from the extracellular membrane in the style into the pollen tube (Figure 1.2b and c) (Goldraij *et al.*, 2006). After endocytosis, the 120 kDa glycoprotein acts to label the compartmental membrane containing S-RNase and the HT-B/4936-factor are shown to be necessary for release of S-RNase from its vacuole and subsequent pollen rejection (Goldraij *et al.*, 2006). Further support for HT-B being necessary for the self-

incompatibility response comes from a study within self-incompatible *Solanum chacoense*, in which RNA interference (RNAi) was used to suppress the *Nicotiana* homologous genes (*ScHT-A* and *ScHT-B*) and produced self-compatible *S. chacoense* plants when *HT-B*, but not *HT-A* was targeted (O'Brien *et al.*, 2002). Though *HT-A* expression was not relevant to self-incompatible responses in *S. chacoense*, significant effects from *HT-A* were observed in wild tomato species (*Solanum* Section *Lycopersicum*) (Tovar-Méndez *et al.*, 2017; Covey *et al.*, 2010). Both *HT-A* and *HT-B* mapped to unilateral incompatibility/incongruity (UI) QTL, however the *HT-B* gene was found to be mutated and expressionless whereas the HT-A gene was intact, expressed, and contributed to UI (Tovar-Méndez *et al.*, 2017; Covey *et al.*, 2010). Though many major contributors to the female determinant SI response have been confirmed, the exact biochemical mechanism behind how HT proteins, 4936-factor, and a 120 kDa glycoprotein function remains unknown.

The male determinant within GSI comes from multiple *S-locus F-Box* (*SLF*) genes (Takayama and Isogai, 2005; Sassa *et al.*, 2007; Kubo *et al.*, 2010). SLF was initially difficult to isolate and characterize due to the repetitive sequences surrounding the *SLF* gene, however in a transformation experiment the *SLF* gene in *Petunia inflata*, *PiSL*, was confirmed to be the pollen self-incompatibility determinant (Entani *et al.*, 1999; Wang *et al.*, 2003; Sijacic *et al.*, 2004). After achieving gene identification and characterization of *SLF*, further studies showed there were other genes contributing to the male determinant self-incompatibility response and were named the *S-Locus F-Box Brothers* (*SFBB*) (Kubo *et al.*, 2010; Sassa *et al.*, 2007). Similar to the female contributors to the self-incompatibility response, the exact underlying chemical mechanism still leaves much to be discovered. However, it is generally well accepted that SLF acts in a self-

preservation mechanism, degrading S-RNase and preventing cytotoxicity within the pollen tube (Sijacic *et al.*, 2004; Kubo *et al.*, 2015).

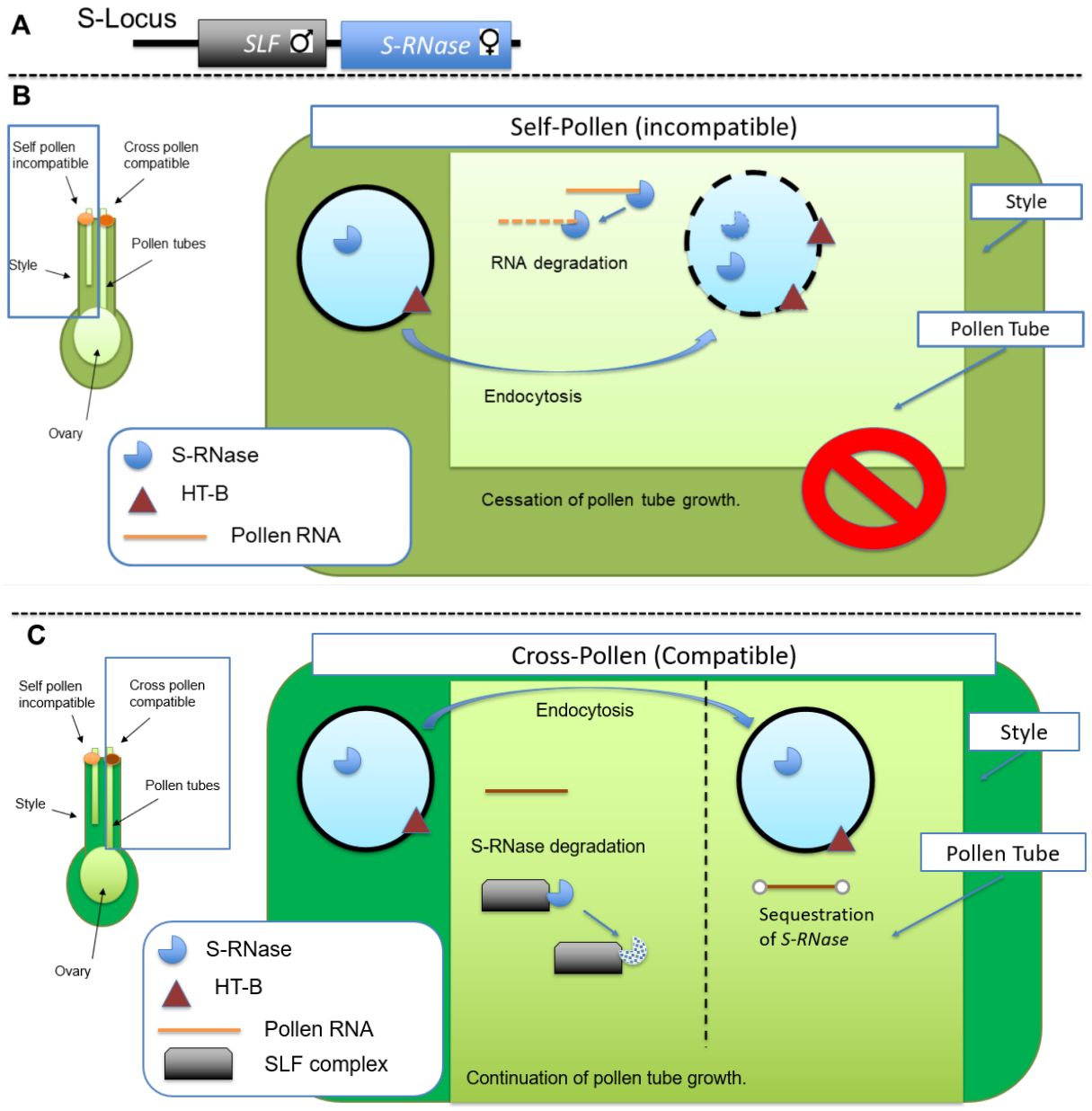

Figure 1.2 GSI models for incompatible and compatible reactions.

(A) The male determinant (SLF) and female determinant (S-RNase) on the S locus. (B) Example of self-incompatible pollen and no pollen tube growth. If the HT-B protein remains intact, the membrane encapsulating S-RNase will become unstable and release S-RNase which degrades S-RNase and causes cessation of pollen tube growth.

Figure 1.2 (cont'd)

(C) Example of self-compatible pollen tube growth. Prevention of contact between S-RNase and the pollen RNA. Left of the black dotted line shows prevention of RNA degradation by SLF acting as a detoxification molecule and degrading the S-RNase. Right of the black dotted line shows sequestration of S-RNase and therefore prevents contact between S-RNase and the pollen RNA, allowing for continuation of pollen tube growth. Models are derived from (Goldraij *etal.*, 2006; Takayama and Isogai, 2005).

When considering possible mechanisms behind the self-incompatibility response and relevant contributors, there are two main models that emerge: the S-RNase degradation model and the compartmentalization model (McClure *et al.*, 2011). In the S-RNase degradation model, SLF is the sole factor that degrades S-RNase and protects the pollen RNA (Figure 1.2b) (Huang *et al.*, 2008; Zhang *et al.*, 2009). In the compartmentalization model, SLF does not degrade all S-RNase, but rather the majority of S-RNase is kept sequestered away from the pollen RNA and does not encounter the pollen RNA (Figure 1.2c) (Goldraij *et al.*, 2006). It is important to note that these models are not necessarily mutually exclusive, it is quite possible that the majority of S-RNase are sequestered in vacuoles but some S-RNase proteins are still present in the cytoplasm of the pollen tube in lower quantities, or the opposite of this may be true (McClure *et al.*, 2011). The ambiguity surrounding GSI and other mechanisms related to self-incompatibility make this topic of research well worth additional study.

iv. Late Acting Self-Incompatibility

Though most species belonging to the Solanaceae, Rosaceae, and the Scrophulariaceae families adhere to the traditional GSI model and may attribute lack of fertility to cessation of pollen tube

growth, there are notable exceptions to this within angiosperms, and more specifically in the DRH population derived from a doubled monoploid, *S. tuberosum* Group Phureja DM 1-3 516 R44 (DM) and a hybrid S. *tuberosum* group Tuberosum RH89-039-16 (RH) (Seavey and Bawa, 1986; Peterson *et al.*, 2016; Duarte *et al.*, 2020). These "exceptions to the rule" display pollen tube growth from the stigma of the plant to the ovules, a clear discrepancy from the traditional GSI model, and instead must therefore adhere to some form of LSI (Seavey and Bawa, 1986; Peterson *et al.*, 2016). Currently, possible mechanisms behind LSI include pre-fertilization inhibition, ovarian inhibition within the ovule, and post-zygotic rejection of the embryo.

Pre-fertilization inhibition causes failure of fusion between the male and female gametes in producing a zygote and may be caused from low pollen viability characterized by low pollen production (Deng *et al.*, 2017). Another possible cause for pre-fertilization inhibition is lack of stigma receptivity in pollen capture and subsequent germination of the pollen tubes (Matton *et al.*, 1999; Seavey and Bawa, 1986).

Ovarian inhibition may be considered another form of pre-fertilization self-incompatibility though is often categorized separately (Seavey and Bawa, 1986). Ovarian inhibition was characterized as early as 1933 in *Hemerocallis thunbergia* and has since been seen in other species such as *Medicago sativa*, *Lilium candidum*, and *Lotus corniculatus* (Society and Club, 1933; Seavey and Bawa, 1986). This form of self-incompatibility involves a failure of the pollen tubes to penetrate into the ovules due to slowing the pollen tube growth or inhibiting access (Seavey and Bawa, 1986). The underlying genetic factors are under control of several loci and the exact genes and the biochemical mechanisms dictating this form of inhibition are unknown (Seavey and Bawa, 1986; Bubar *et al.*, 2011).

Post-zygotic rejection was first characterized in 1937 in *Gasteria verrucose* showing that when self-incompatible lines were crossed with pollen from the same plant or from a genetically variable pollen donor, both crosses produced lines with pollen tubes that grew equally well throughout the style (Sears, 1937). Interestingly, the basis of this sterility within this study was not attributed to inability of the embryo/endosperm to continue cell division, but rather abortion of the ovule (Sears, 1937). Support for this comes from observations of dividing endosperm in the nuclei but degradation of the ovules themselves when incompatible crosses were made (Sears, 1937; Seavey and Bawa, 1986). In more recent studies, a more exact mechanism behind post-zygotic rejection remains elusive on both a genetic and biochemical level (Lipow and Wyatt, 2000). This is due in part to the difficulty of phenotypically assessing post-zygotic rejection which may require the researcher to wait for fruit to mature to evaluate fertility and genetic references being limited to two or possibly three poorly defined loci related to post-zygotic rejection (Naaborgh *et al.*, 1992; Lipow and Wyatt, 2000). It has been suggested that post-zygotic LSI still involves factors that interact with the S-locus, but which factors and how remains undefined (Lipow and Wyatt, 2000). When considering what LSI mechanism is responsible behind pollen tube growth in incompatible crosses in DRH *S. tuberosum* lines, post-zygotic rejection seems the most likely due to confirmed pollen fertility and complete pollen tube growth observed from the stigma to the ovaries/ovules (Peterson *et al.*, 2016). However, since there are so many unknown factors surrounding this post-zygotic rejection and its association with the S-locus, no clear underlying mechanism is apparent.

III. Enhancements in potato breeding using modern techniques.

From 1920 to 1989, the national average potato (*S. tuberosum*) production within the USA has increased six-fold from 5.6 to 33.6 tonnes per hectare mainly due to enhancements of soil nutrients,

watering practices, pesticide application, and use of traditional breeding methods (Douches *et al.*, 1996). Though these traditional breeding methods and improvements in agricultural techniques have helped potato production come a long way from over a century ago, the current genetic gain of potato is slow compared to crops such as maize (*Z. mays*) and soybean (*Glycine max*) (Specht and Williams, 2015; Duvick, 1980; Douches *et al.*, 1996). Complimenting traditional breeding with modern genetic tools is now necessary if potato production is to keep up with other crops.

i. Genomic resources

Important milestones for genomic resources include the sequencing of reference genomes including the 844 Mb diploid genome of the doubled monoploid *S. tuberosum* group Phureja (DM1-3) and self-compatible *Solanum chacoense* M6 genome (Potato Genome Sequencing Consortium, 2011; Leisner *et al.*, 2018; Pham *et al.*, 2020). The *S. tuberosum* group Phureja DM1-3 and *S. tuberosum* group Tuberosum RH-89-039 genomes, annotation datasets, and phenotypic data from a diversity panel of 250 potato clones may be found on an online database Spud DB (http:// potato.plantbiology.msu.edu/) (Hirsch *et al.*, 2014). Genomic tools outside of Spud DB include multiple SNP arrays which were derived from tetraploid potatoes (Vos *et al.*, 2015; Schmitz Carley *et al.*, 2017). Genetic resources serve to accelerate potato breeding by allowing integration of approaches such as Marker Assisted Selection (MAS) and genomic selection. Additionally, cutting edge gene editing technology becomes more feasible when annotated reference genomes are available.

ii Gene editing in plants using CRISPR-Cas9

Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated protein 9 (Cas9) (CRISPR-Cas9) gene editing may be used for specific gene targeting in plants (Butler *et al.*, 2017; Cao *et al.*, 2016). CRISPR-Cas9 targets genes precisely using a RNA–guided DNA endonuclease Cas9 to generate a double stranded break (DSB) within the targeted gene (Yin *et al.*, 2017; Soyars *et al.*, 2018; Jiang and Doudna, 2017). Following a DSB, the subsequent error prone repair mechanism Non-Homologous End Joining (NHEJ) within the transformed cell may cause gene knockout (KO) (Butler *et al.*, 2017; Jiang and Doudna, 2017; Yin *et al.*, 2017). Alternatively, homology directed repair (HDR) allows for error free repair or if a donor template flanked by homology arms are provided, insertion of a foreign gene, precise gene modification, or deletion (Jiang and Doudna, 2017).

Though certainly a powerful tool, CRISPR has limitations. Depending on what associated Cas protein is being used (Cas9, Cas10, or Cas12, among others) a Protospacer Adjacent Motif is necessary for the nuclease to cut the correct sequence (Swarts and Jinek, 2018; Jiang and Doudna, 2017). For example, Cas9 requires a 5'-NGG-3' PAM sequence and Cas12a requires a 5'-TTTN-3' PAM sequence (N = A,T,G, or C ; R = A or G ; H = A, C, or T) (Jiang and Doudna, 2017; Paul and Montoya, 2020). Currently, there is progress in generating CRIPSR-Cas9 variants that allow for "near-PAMless" CRISPR systems that are able to recognize 5'-NRNH-3' and NGN PAMs which allows for more possible gene targets (Walton *et al.*, 2020; Miller *et al.*, 2020; Zhang *et al.*, 2021). However, these advances in engineering Cas9 variants are in its infancy and will need time to integrate into mainstream gene editing.

An additional limitation of CRIPSR-Cas9 comes from the possibility of off targeting, which may cause unexpected and/or unwanted changes in the targeted genome (Chen *et al.*, 2019; Zhang *et*

al., 2015; Hsu *et al.*, 2016). To combat unwanted targeting there are multiple strategies to decrease off-targeting in CRIPSR-Cas9 systems. Strategies include truncating the gRNA which may decrease off targeting by 5000-fold in a wild type Cas9 system or using the shortened gRNA in concert with an altered nickase form of Cas9 paired with two sgRNAs, each of which only cleaves one strand (Zhang *et al.*, 2015; Fu *et al.*, 2014).

A social rather than scientific issue with CRISPR-Cas9 editing is legislation restricting the production and release of gene edited/genetically modified organisms. Laws vary across the world with the European Union (EU) defining GM crops as any organism which has its genetic material altered in a way that does not occur naturally whereas restriction within the USA is less strict, only considering transgenic plants as GM material (Kim and Kim, 2016; Wolter and Puchta, 2017). Therefore, a crop such as the *polyphenol oxidase* (*PPO*) KO mushrooms, which contain small deletions (1–14 bp) in the *PPO* gene but no foreign DNA, would be considered outside of GMO regulation in the USA, but would be regulated in some European countries (Kim and Kim, 2016). Despite any challenges or limitations, CRISPR-Cas9 has already proven to be a useful tool for gene editing in a wide variety of crops. In the citrus fruit tree, Duncan grapefruit (*Citrus paradisi*), Cas9 KO of the *CsLOB1* gene resulted in resistance to *Xanthomonas citri*, a proteobacteria known to cause citrus canker (Jia *et al.*, 2017). Disease resistance was also achieved in rice (*Oryza sativa*) against another proteobacteria, *Xanthomonas oryzae,* by knocking out the *OsSWEET13* gene (Zhou *et al.*, 2015). Aside from direct application, CRISPR-Cas9 editing is extremely useful for functional analysis of genes, as may be seen in the *slmapk3* gene KO in tomato which firmly established *slmapk3* as a gene conferring drought tolerance (Wang *et al.*, 2017). For improving potato crops in particular, CRISPR-Cas9 editing has produced useful edits in both tetraploid and diploid potatoes (Dahan-Meir *et al.*, 2018; Enciso-Rodriguez *et al.*, 2019; Ye *et al.*, 2018). In

tetraploid potatoes, the *StGBSS* starch synthase gene was targeted and gene edits were achieved in all four alleles in 2% of the regenerated lines, resulting in an increased amylopectin/amylose ratio which grants a more "waxy" potato that is more likely to retain its shape after being cooked (Andersson *et al.*, 2017). In diploid potato, self-compatible potatoes have been generated via gene KO of the *S-RNase* gene (Enciso-Rodriguez *et al.*, 2019; Ye *et al.*, 2018). These examples show just a glimpse of the multiple applications of CRISPR-Cas9 and the many potential benefits which may be gained from gene editing.

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Chapter 2

HT-B AND S-RNASE CRISPR-CAS9 DOUBLE KNOCKOUTS SHOW ENHANCED SELF-FERTILITY INDIPLOID SOLANUM TUBEROSUM

[*To be submitted to the Plant Biotechnology Journal*]

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FE-R, NM-C and DD conceptualized the study. FE-R designed the CRISPR-Cas9 constructs. FE-R, SL, DZ, DD, SN and CRB contributed to the experimental design. SL and FE-R performed sequencing of KO lines. SL performed chloroplast counting, stylar squash analysis, self-

compatibility assessments, statistical design, and statistical analysis. SL and FE-R wrote the manuscript and performed laboratory experiments. The Manuscript was approved of by all authors.

Acknowledgements:

Funding for this study came partially from Michigan State University Project GREEEN award (GR19-015) to DD. We would like to thank Dr. Addie Thompson from the Department of Plant, Soil, and Microbial Sciences and Leo Pham from the Michigan State University Statistical Consulting Center for help with statistical analysis.

I. Summary

The gametophytic self-incompatibility (GSI) System in diploid commercial potato (*Solanum tuberosum*) poses a substantial hybridization barrier in potato breeding by hindering the generation of inbred lines. One solution is gene editing to generate self-compatible diploid potatoes which will allow for the generation of elite inbred lines with fixed favorable alleles and heterotic potential. The *S-RNase* and *HT* genes have been demonstrated to be contributing factors in GSI in the Solanaceae family and self-compatible *S. tuberosum* lines have been generated by knocking out S-RNase with CRISPR-Cas9 gene editing. This study employed CRISPR-Cas9 to knockout *HT-B* either individually or in concert with *S-RNase* in the diploid self-incompatible S. tuberosum line DRH-195. Using seed count from self-pollinated fruit as the defining characteristic of self-compatibility, *HT-B*-only knockouts produced little or no seed. In contrast, double knockout lines of *HT-B* and *S-RNase* displayed levels of seed count that were up to three times higher than targeting *S-RNase* alone, indicating a potential synergistic effect between *HT-B* and *S-RNase* in self-compatibility in diploid potato. Contradictory to the traditional GSI model, self-incompatible

lines displayed pollen tube growth reaching the ovary, but ovules fail to develop into seeds indicating late-acting self-incompatibility in DRH-195. Lines generated from this study will serve as a valuable resource for diploid potato breeding.

II. Introduction

Potato (*Solanum tuberosum* L.) represents one of the most economically important species within the Solanaceae family along with tomato, eggplant, pepper, tobacco and petunia (Wu and Tanksley, 2010; Olmstead et al., 2008). Potato is the world's third most important food crop (Devaux et al., 2014) contributing to global food security with an annual production of over 370 million metric tons (FAOSTAT, 2021). Commercial potato varieties are heterozygous, autotetraploid, and experience acute inbreeding depression when self-pollinated over multiple generations which complicates traditional breeding schemes (Potato Genome Sequencing Consortium, 2011; Zhang et al., 2019). Shifting potato production from tetraploid clonal propagation to diploid self-compatible reproduction has several advantages including a shorter breeding cycle, introgression of advantageous alleles from wild diploid potato relatives, and the generation of inbred lines (Lightbourn and Veilleux 2007; Jansky et al 2016.) The generation of inbred lines is of particular interest as this is the most direct approach for yield increase via heterosis, increasing genetic gain, and fixing favorable allelic combinations as seen in grain crops such as maize (*Zea mays* L.) (Hosaka and Hanneman, 1998; Li et al., 2018, Jansky et al 2016). However, the self-incompatible nature of most diploid potatoes is a significant barrier in the generation of inbred diploid potato lines.

Self-pollen rejection in potatoes is usually controlled by the gametophytic self-incompatibility system (GSI), which is present mainly in the Solanaceae, Rosaceae, and the Scrophulariaceae

families (Boivin *et al*., 2014). The GSI model asserts that factors from both the female determinant (S-RNase in the style) and male determinant (S-locus F-Box (*SLFs*) proteins from the pollen) contribute to self-incompatibility (SI) (Kubo *et al.*, 2010; McClure, 2006; McClure *et al.*, 1990; McClure *et al.*, 1989; Sijacic *et al.*, 2004). In cross pollinated plants, the SLF proteins prevent S-RNase-mediated pollen RNA degradation via a proteosome system, acting as a detoxification mechanism (Kubo *et al.*, 2010; McClure *et al.*, 2011; Takayama and Isogai, 2005). Detoxification will not occur if the S-haplotype between the pollen and the style match, as a consequence self-pollinated plants with matching S-haplotypes are not fertilized (McClure *et al.*, 1990; McClure *et al.*, 2011, McClure *et al.*, 1989; Takayama and Isogai, 2005). Currently, diploid breeding programs use native genetic sources to remove SI barriers. The dominant *Self incompatibility inhibitor* locus (*Sli*) from self-compatible *S. chacoense* lines has been well documented (Clot *et al.*, 2020; Hosaka and Hanneman, 1998; Jansky *et al.*, 2016; Jansky *et al.*, 2014). M6, an inbred *S. chacoense* line, has been a common source of *Sli* in diploid potato breeding (Clot *et al.*, 2020; Jansky *et al.*, 2014). In some instances, the introgression of the *Sli* into cultivated diploid materials has allowed breeders to overcome SI and move potato breeding towards the generation of inbred lines (Alsahlany *et al.*, 2021; Kaiser *et al*., 2021). However, introgression of *Sli* from M6 into other germplasm is time-consuming and could lead to linkage drag and fixation of undesirable traits such as high tuber glycoalkaloid content and other inferior tuber traits (Jansky *et al.*, 2014). Furthermore, other undefined factors interact with the expression of *Sli*-based self-compatibility (SC) during inbreeding, adding confounding effects to obtain self-compatible plants through traditional breeding (Kaiser *et al*., 2021).

To avoid the introgression of undesirable genes through conventional breeding, targeted mutagenesis using Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-

associated protein 9 (Cas9) (CRISPR-Cas9) gene editing may be used for gene targeting to alter specific traits (Butler *et al.*, 2017; Cao *et al.*, 2016). CRISPR-Cas9 has already proven to be a viable option for generating self-compatible plants, as was demonstrated with the knockout of *S-RNase* (Enciso-Rodriguez, *et al.*, 2019; Ye *et al.*, 2018). In the *S-RNase* KO study reported by Enciso *et al* (2019), plasticity in both gene edited and non-edited lines was observed in SI and fertility (fruit count and seed count).

Though *S-RNase* is a major modulator of SI in potato, there are other potential contributing factors such as HT proteins previously reported in the Solanaceae family (Goldraij *et al.*, 2006; O'Brien *et al.*, 2002; Tovar-Méndez *et al.*, 2017). In a hypothesized sequestering model, S-RNase and HT-B proteins undergo endocytosis from the extracellular matrix (ECM), encapsulating S-RNase in a vacuolar compartment (Goldraij *et al.*, 2006, McClure *et al.*, 2011). Immunolocalization experiments suggest that HT-B is present on the membrane containing S-RNase and is degraded in self-compatible pollen tubes, yet remains functional in incompatible pollen tubes (Goldraij *et al*., 2006, McClure *et al.*, 2011). Degradation of HT-B is associated with stability of the vacuolar membrane containing the S-RNase, and only occurs if the *S*-haplotypes between the style and the pollen are dissimilar (Goldraij et al., 2006, McClure *et al.*, 2011). Alternatively, if the *S*-haplotypes are identical, HT-B will remain intact, yet the vacuolar membrane gets degraded which leads to release of the S-RNase and inhibition of fertilization (Goldraij et al., 2006, McClure *et al.*, 2011). This sequestering model is based on studies in tobacco (*Nicotiana alata)* and a wild potato relative (*S. chacoense*) where both species displayed SC when the *HT-B* gene was suppressed via RNA interference (RNAi) (Mcclure *et al.*, 1999; O'Brien *et al.*, 2002). Another *HT* gene, *HT-A*, was suppressed in *S. chacoense* but did not result in SC (O'Brien *et al.*, 2002). *HT-B* and *HT-A* were shown to contribute to SI and interspecific unilateral incompatibility (UI) in *Solanum* Section

Lycopersicum; due to the lack of significant interaction in *S. chacoense, HT-A* was not included in this study (Covey *et al.*, 2010; Tovar-Méndez *et al.*, 2017).

These genetic factors, in concert with environmental parameters and ontogeny, influence the SI response, which can show plasticity in fertility metrics such as fruit set (Enciso-Rodriguez, *et al.*, 2019; Mena-Ali and Stephenson, 2007; Stone *et al.*, 2006; Webb and Williams, 1988). Generally, lower fruit set is observed at higher temperatures and the SI response breaks down as the plant matures (Enciso-Rodriguez *et al.*, 2019; Mena-Ali and Stephenson, 2007; Stone *et al.*, 2006; Travers *et al.*, 2004; Webb and Williams, 1988). This study seeks to understand the role of *HT-B* and its possible interactions with *S-RNase* in *S. tuberosum* by targeting *HT-B* for KO either individually or in addition to *S-RNase* using CRISPR-Cas9-mediated gene editing while also considering environmental factors and plant age. In addition to enhanced understanding of the SI response in *S. tuberosum,* self-compatible lines resulting from gene edits may be used as a resource for self-compatibility in reinventing potato as a diploid crop.

III. Results

i. Detection of the HT-B gene in potato

Primers were designed from conserved *HT-B* regions of multiple *Solanum* spp. and used to perform Rapid amplification of cDNA ends (RACE) to amplify the *HT-B* open reading frame (ORF) from DRH-195. The DRH-195 *HT-B* gene is 401 bp and contains two exons and one intron (Figure 2.1a). The predicted protein encodes a 101 amino acid peptide and has an Asn/Asp rich motif toward the C-terminal region. The predicted HT-B potato protein is highly similar (79%, sequence ID: BAC00942.1) to a reported HT protein from *S. peruvianum*, a wild tomato relative. However, the potato HT predicted peptide lacks the first four aa (MAFN). A similar trend is observed in HT

aa sequences reported for most wild relatives of potato (*S. chacoense, S. bulbocastanum* and *S. pinnatisectum*) where only partial cDNA sequences are available, lacking up to 17 amino acids from the start codon relative to HT-B peptide sequences from the tomato clade. BLASTn analysis using the potato reference genome sequence (DM v6.1) (Pham *et al.*, 2020) revealed the *HT-B* gene was on chromosome 12, close to the annotated *HT-A* gene for this species.

ii. Targeted mutagenesis of HT-B and S-RNase using CRISPR-Cas9

Three separate constructs 121, 123, and 124 were used within this study. *HT-B* KOs were produced with two separate constructs each containing a unique gRNA (sgRNA-HTB1 in 121 or sgRNA-HTB2 in 123) which targeted 44 bp or 77 bp downstream of the start codon in exon 1 of the *HT-B* gene, respectively (Figure 2.1a). The sgRNA targeting S-RNase is sgRNA1 and is described in Enciso et al. (2019). CRISPR-Cas9 cassettes harboring sgRNA-HTB1, sgRNA-HTB2 and sgRNA-HTB1 + sgRNA1 were transfected along with double stranded oligo deoxynucleotide (dsODNs) into diploid DRH-195 potato protoplasts using PEG-mediated transformation to assess if the constructs were functional. Unlike negative controls (just dsODNs), each sgRNA combination was able to generate double strand breaks (DSBs) and incorporate dsODNs at each *HT-B* and *S-RNase* targeted region in either direction 5’ to 3’ or 3’ to 5’.

After confirming edits in protoplasts, over 1000 DRH-195 leaf explants were used for *Agrobacterium*-mediated transformation between all constructs. The percentage of shoots recovered were 67%, 55%, and 42% from explants on selective media in the 121, 123, and 124 constructs respectively (Table S1). Shoots were screened using a combination of PCR gel electrophoresis and restriction enzyme digest assays for *bona fide* gene edits; 24 candidate lines were identified after screening over 300 lines using PCR amplification and restriction enzyme

digest tests. From the 24 candidate lines, 12 were confirmed to have gene edits using Sanger Sequencing and were included with a non-edited and *S-RNase* KO line for downstream analyses.

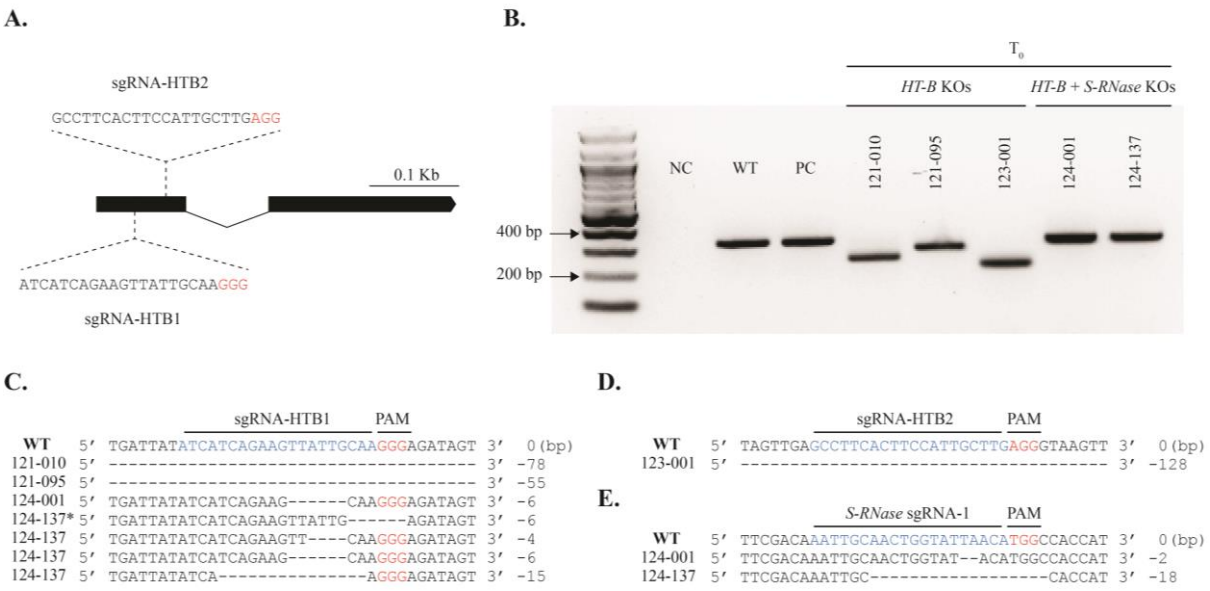

Figure 2.1 CRISPR-Cas9 gene-based mutagenesis of *HT* genes.

(A) The gene architecture of *HT-B* and location of two gRNAs belonging to separate constructs designed to target conserved regions on exon 1 between the DM and RH alleles (B) *HT-B* edits compared to the WT and PC which have no *HT-B* edits. Homozygous deletions of > 30bp were observed in 121_010, 121_095, and 123_001, one small < 10bp homozygous deletion (124_001), and one chimeric line (124_137) with multiple chimeric alleles (124_137*). Small deletions in the PC, 124_001, and 124_137 lines are not noticeably different from the WT or PC which have no *HT-B* gene edits. (C) DNA sequences of representative *HT-B* deletion

Figure 2.1 (cont'd)

with a large deletion in the *HT-B* gene resulting from sgRNA-HTB2 targeting. (E) Representative *S-RNase* edits. All lines are homozygous except for the PC line which has heterozygous deletions and the chimeric 124_137. (c,d,e) Deletions are represented by dashes.

iii. CRISPR-Cas9 editing events result in HT-B and HT-B + S-RNase gene KO

CRISPR-Cas9 editing produced *HT-B* and/or *S-RNase* KOs by disrupting the gene(s) targeted (Figure 2.1b-e). Lines targeted with the sgRNA-HTB1 are labeled as "121" and the line targeted with sgRNA-HTB2 is labeled as "123" (Figure 2.1b, c). Lines with successful edits in both the *HT-B* and *S-RNase* genes have the initial digits "124". The wild type DRH-195 and self-compatible positive control *S-RNase* KO (DRH-195.158) are labeled as "WT" and "PC", respectively. Gene KO in the PC was described previously in Enciso-Rodriguez, *et al.* (2019). Representative samples from *HT-B* and *HT-B + S-RNase* KO lines are shown in Figure 2.1b-e, sequences from all 14 lines are shown in Figure S2.1. All edits of *HT-B* and *S-RNase* in T_0 lines occurred near the Protospacer Adjacent Motif (PAM) sequence in exon 1 of each gene (Figure 2.1). Most edits for both *HT-B* and *S-RNase* were homozygous, while non-homozygous *HT-B* mutations were chimeric (three differing edits) in the 121_008, 121_020, and 124_137 lines. Frequencies of alleles for all chimeras may be found in Supporting Table S2.2. Regarding edits in the *HT-B* gene, a 1 bp insertion was recovered in the 121_005 line with the other mutations ranging from a small 1 bp deletion (121_008_C4) to several large > 50 bp deletions (121_010, 121_062, 121_095, and 123_001) (Figure S2.1). The largest deletion observed was 128 bp (Figure 2.1d). Most lines with small *HT-B* deletions produced a frameshift mutation resulting in a premature stop codon or disrupted the reading frame likely resulting in a dysfunctional protein. The only exceptions were two *HT-B*

alleles with deletions that did not exhibit a frame shift mutation in the chimeric lines 121_020_C3 and 124_137_C4 (Figure 2.S1). All *S-RNase* edits produced frameshift mutations which altered the predicted amino acid sequence.

iv. Ploidy determination and pollen viability

Endoreduplication commonly occurs in regenerated potatoes (Karp *et al.*, 1984; Owen *et al.*, 1988). Tetraploids are generally self-compatible due to the heteroallelic pollen effect and would act as a confounding factor within this study (Campos and Ortiz, 2019; McClure *et al.*, 2011; Nettancourt, 1977). Therefore, to further characterize the events and avoid the confounding effect of tetraploid potatoes, the number of chloroplasts in guard cells in the T_0 lines were counted to determine ploidy (Karp *et al.*, 1984). Tetraploid potatoes have an expected an average of 12-14 chloroplasts per guard cell whereas diploids have an average of 6-8 chloroplasts per guard cell; any line with an average > 8 were removed from this study. One quarter of lines exhibited chromosome doubling and were excluded from this study. The 14 lines included in this study had an average of 6-8 chloroplasts per guard cell indicating diploidy (Figure S2.2). To ensure any variation in fertility was not due to male sterility, pollen from each gene edited line were stained with acetocarmine; all lines produced viable pollen and exhibited pollen tube growth (Figure S2.3).

v. Stylar squash analysis shows complete pollen tube growth in all lines

Three self-pollinated styles for each of the 14 selected lines were viewed under a 4′,6-diamidino-2-phenylindole (DAPI) fluorescent microscope using an aniline stain. As observed for the WT and PC, all 14 lines showed pollen tube growth from the stigma through the style down to the ovaries (Figure 2.2a). The one exception was 121_008 which lacked any pollen tube growth. The lack of

pollen tube growth and self-incompatibility in the WT line has previously been observed in other angiosperm species (Seavey and Bawa, 1986). This pollen-stylar interaction observed may be due to Late-Acting Self-Incompatibility (LSI).

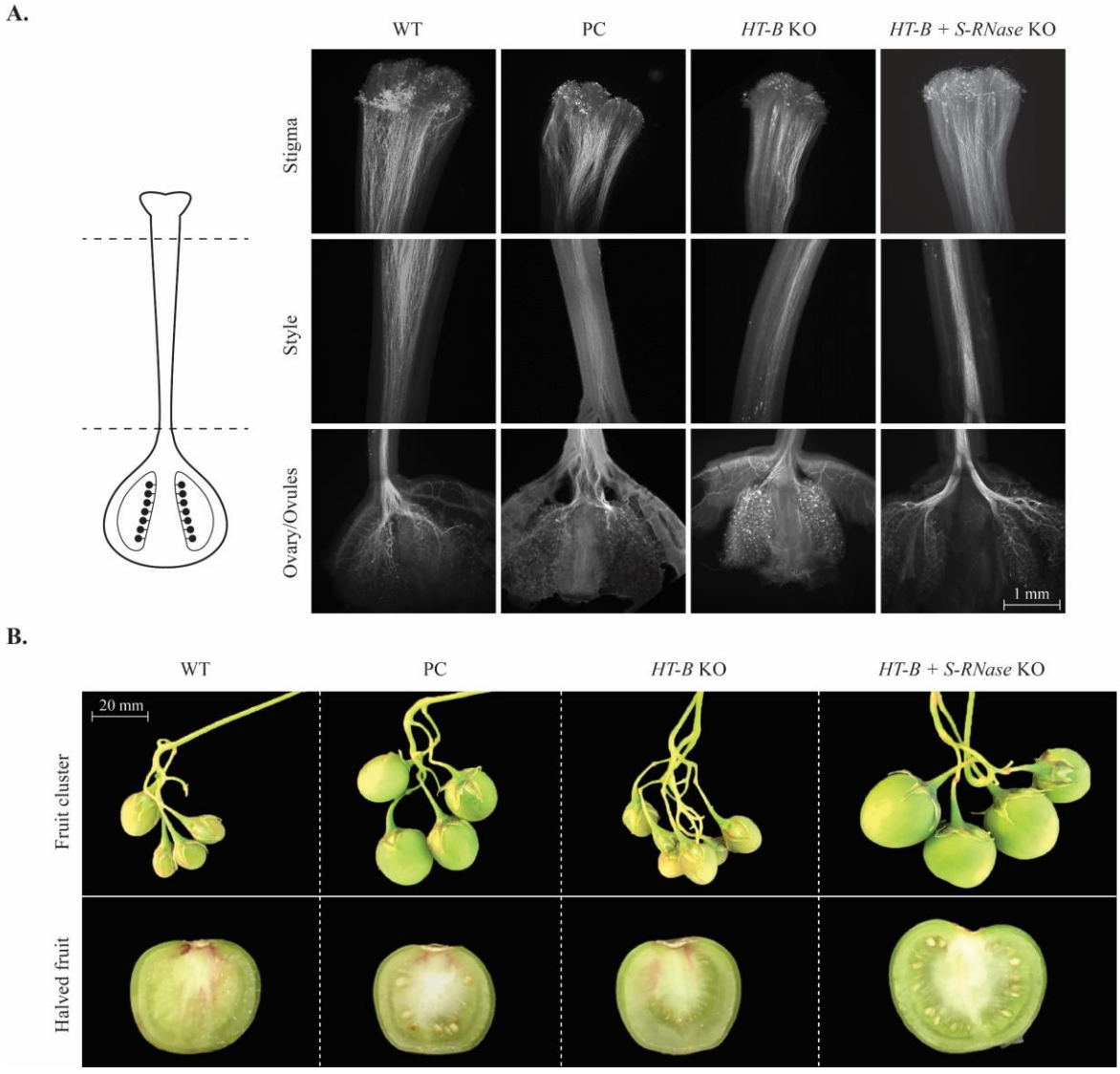

Figure 2.2 Qualitative assessment of pollen tube growth, fruit size, and seed count.

(A) Pollen tube growth stained with aniline blue. Successful pollen tube growth from the stigma to the ovary in the WT, PC, *HT-B* KO, *HT-B* + *S-RNase* KO lines. Samples above are representative of fourteen lines and 42 styles viewed. (B) Fruit size comparison is to scale; halved fruit are enlarged and not to scale. Fruit shown is representative of the most common

Figure 2.2 (cont'd)

phenotype observed for the WT, PC, *HT-B* KO, *HT-B* + *S-RNase* KO lines. The *HT-B* and the *HT-B* + *S-RNase* samples are from the 121_020 and 124_137 lines, respectively. Parthenocarpic fruit shown for WT and *HT-B* KO lines. Seed was produced in the PC (*S-RNase* KO) and in the *HT-B* and *S-RNase* KO lines. Parthenocarpic fruit is noticeably smaller than seed producing

vi. Double KO lines display self-compatibility and enhanced self-fertility

Quantitative analysis of self-fertility was performed using fruit set, fruit weight, and seed count analysis. Fruit set occurred in all lines including the self-incompatible WT, indicating that fruit set is not necessarily indicative of SC in this study (Figure 2.3a). For example, out of all 14 lines, 121_005 had the second highest probability of setting fruit (64.0% +/- 6% SE) and yet only had a mean seed per fruit of 0.56 +/- 0.10 SE (Figure 2.3a and b). As seen in Figure 2.3c, *HT-B* + *S-RNase* KOs and the PC had significantly greater fruit weight than the WT and *HT-B* KO lines ($P < 0.05$, Tukey's test) that was noticeable in the fruits (Figure 2.2b). Interestingly, there was a significant increase in fruit weight between plants pollinated at one month and two and a half months ($p < 0.05$, ANOVA), but no significant relationship between plant age and seed count was observed ($p > 0.05$, ANOVA). The WT and *HT-B* KO lines exhibited less than three seeds per fruit, the PC produced a moderate seed count (38.9 +/- 2.8 SE), and the *HT-B* + *S-RNase* KOs ranged from 14 – 128 seeds (Figure 2.3c). The *HT-B* + *S-RNase* KO line (124_005) with the lowest seed count (13.9 +/- 1.1 SE) also had severe floral mutations (Figure S2.4). The 124_001 and 124_137 lines (*HT-B* and *S-RNase* KOs) had up to three times greater seed count than the PC (*S-R-Nase* KO).

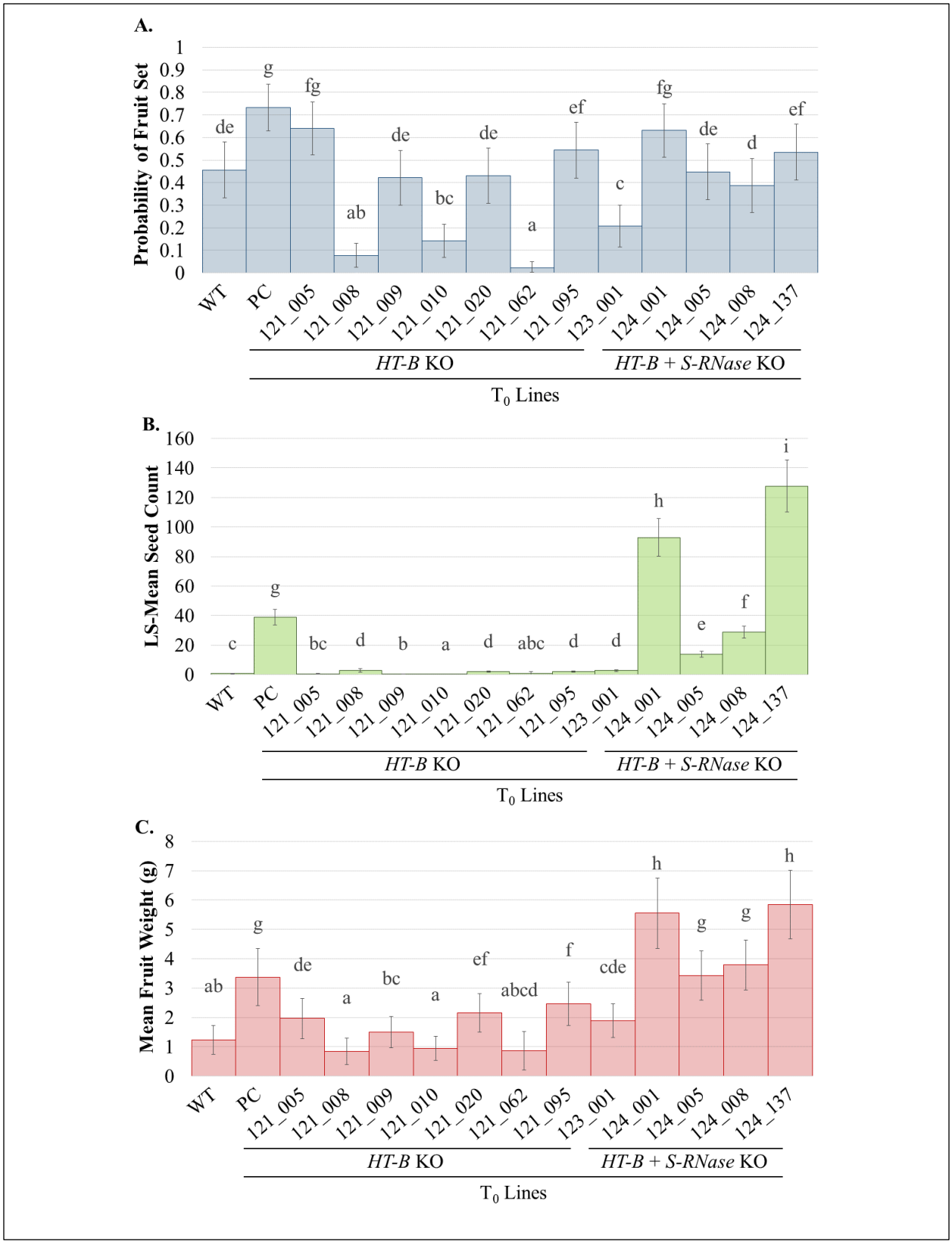
A.
Probability of Fruit Set
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
de
g
fg
ab
de
bc
de
a
ef
c
fg
de
d
ef
WT
PC
121_005
121_008
121_009
121_010
121_020
121_062
121_095
123_001
124_001
124_005
124_008
124_137
HT-B KO
HT-B + S-RNase KO
T0 Lines
B.
LS-Mean Seed Count
160
140
120
100
80
60
40
20
0
c
g
bc
d
b
a
d
abc
d
d
h
e
f
i
WT
PC
121_005
121_008
121_009
121_010
121_020
121_062
121_095
123_001
124_001
124_005
124_008
124_137
HT-B KO
HT-B + S-RNase KO
T0 Lines
C.
Mean Fruit Weight (g)
8
7
6
5
4
3
2
1
0
ab
g
de
a
bc
a
ef
abcd
f
cde
h
g
g
h
WT
PC
121_005
121_008
121_009
121_010
121_020
121_062
121_095
123_001
124_001
124_005
124_008
124_137
HT-B KO
HT-B + S-RNase KO
T0 Lines

Figure 2.3 (cont'd)

Figure 2.3 Quantitative analysis of self-fertility.
(A) Significant differences in fruit set ($p < 0.05$, Tukey's test) were observed between different lines, but there are no consistent trends in fruit set when comparing *HT-B*, *HT-B* + *S-RNase*, PC, and the WT. (B) Fruit weight is significantly higher ($p < 0.05$, Tukey's test) in self-compatible fruits with *HT-B* + *S-RNase* KO knockout lines together with PC compared to the WT or *HT-B* only knockouts. (C) Double knockout lines 124_001 and 124_137 have significantly higher seeds ($p < 0.05$, Tukey's test), than the PC. The WT and *HT-B* KOs had significant lower seed count ($p < 0.05$) with < 5 seeds per fruit on average. (A/B/C/D) Lines with the same letter are not significantly different from one another.

IV. Discussion

Converting potato breeding from a tetraploid clonally propagated platform to a self-compatible diploid breeding system has far reaching advantages from increased genetic gain potential as well as sexual reproduction that will lower the cost of storage by using true seed instead of culture or tuber seeds (Howard, 1975; Jansky *et al.*, 2016). In this study, double KO lines with higher levels of self-fertility than previous *S-RNase* KO lines were generated (Enciso-Rodriguez, *et al.*, 2019). Prior studies have described self-compatibility in diploid potato as a qualitative measurement defined in terms of presence, absence, or displaying pseudo self-compatibility (Ye *et al.*, 2018; Enciso-Rodriguez *et al.*, 2019). While a qualitative viewpoint may be sufficient depending on the application, it may not accurately describe variation in self-compatibility and fertility often observed in *Solanum* species (Cipar *et al.*, 1964). Rather than qualitatively describing self-

(in)compatibility, this study quantitatively defines fertility by correlating the fruit weight, and seed count that may be anticipated from *S-RNase, HT-B,* and *S-RNase* + *HT-B* KO(s).

Using fruit set and weight as metrics of self-fertility, this study shows all self-pollinated lines produced fruit to varying degrees. DRH lines have shown plasticity in fruit set in previous studies (Peterson *et al.*, 2016; Enciso-Rodriguez *et al.*, 2019) and variation may occur due to environmental effects such as temperature, photoperiod, and humidity as reported in other members of the *Solanum* genus such as *S. carolinense* and *S. peruvianum* (Mena-Ali and Stephenson, 2007; Stone *et al.*, 2006; Webb and Williams, 1988). Plant age is another possible confounding factor when quantifying SI, as SI breaks down in older plants of *S. tuberosum* and other Solanum species (Eijlander *et al.*, 1997; Stephenson *et al.*, 2003; Travers *et al.*, 2004). To account for the effect of plant age, plant fertility was recorded at one month and two and a half months after the plants were transferred from tissue culture. The lines with the highest fruit weight (PC, 124_001, 124_005, 124_008, and 124_137) also had the highest seed count out of all 14 lines (Figure 2.3b, c) indicating larger fruits contain more seeds. Greater fruit weight observed mature versus young plants but lack of significant difference between seed count given differences in time supports that more mature plants give rise to larger fruits, but do not have increased self-fertility.

Due to the plasticity in fruit set and weight, seed count provides the most appropriate representation of self-compatibility/fertility within DRH-195. All double *HT-B* + *S-RNase* KO lines and the PC were self-compatible but ranged from low to high levels of self-fertility. The majority of fruit produced in the WT and *HT-B* KO lines was parthenocarpic, except for four lines (121_008, 121_020, 121_095, and 123_001) which had low seed count (less than three) per fruit. The 121_008, 121_020, 121_095, and 123_001 lines consistently produced between one and three seeds per fruit. The 124_001 and 124_137 lines displayed enhanced seed production indicating a

synergistic effect of *S-RNase* and *HT-B* in overcoming GSI. The self-compatibility reaction observed in *HT-B* + *S-RNase* KOs and the PC is likely due gene edits in *S-RNase* preventing any functional S-RNase protein from degrading pollen RNA. However, the biochemical mechanism underlying increased fertility/enhanced seed production is unknown. Immunolocalization has shown HT-B proteins are located on membrane vacuoles containing S-RNase and higher levels of HT-B protein are found in incompatible versus compatible pollinations, supporting that HT-B plays a role in sequestration/release of S-RNase, but prediction of how a knockout of both *S-RNase* and *HT-B* results in enhanced compatibility is hypothetical (Goldraij *et al.*, 2006; McClure, 2006; McClure *et al.*, 2011). Possible hypotheses include another unknown interaction outside of the *S*-locus with HT-B and an undefined factor which enhances seed set, or the removal of HT-B allows for enhanced, but incomplete sequestration of S-RNase. Enhanced seed set compared to the PC was not observed in 124_005 or 124_008. The low seed production in 124_005 is likely due to the severe floral mutation of petals, anther, and style fusion which inhibited pollination. These floral mutations were observed in (80%-100%) of flowers pollinated in the 121_008, 121_062 and 124_005 lines and likely contributed to 121_008 and 121_062 showing the lowest fruit set out of all the lines ($p < 0.05$, Tukey's test). The PC showed a higher mean seed count per fruit compared to 124_008 ($p < 0.05$, Tukey's test) which may be due to modifying loci other than *S-RNase* or *HT-B* or somaclonal variation resulting from potato regeneration and Cas9 activity (Figure 2.3c) (Goldraij *et al.*, 2006; McClure *et al.*, 1999; O'Brien *et al.*, 2002).

Self-incompatible plants displayed pollen tube growth from the stigma to the ovaries, a clear discrepancy from the traditional GSI model (Figure 2.2a). Though the GSI model may remain representative of other species within the Solanaceae, Rosaceae, and Scrophulariaceae families, our results support the hypothesis that *S. tuberosum* DRH-195 line has a different reaction to self-

pollination (McClure *et al.*, 2011; Sassa *et al.*, 2007; Thompson and Kirch, 1992). This incongruity is not entirely unexpected considering prior studies in the DRH potato population and other angiosperm species originally hypothesized to have GSI which also show complete pollen tube growth in self-incompatible lines (Peterson *et al*, 2016; Gibbs, 2014; Seavey and Bawa, 1986). Complete pollen tube growth, but lack of seed production support the source of SI is derived from LSI (Gibbs, 2014; Seavey and Bawa, 1986). The LSI in DRH-195 may be due to failure to penetrate the ovules, arrested pollen tube growth within the micropyle, syngamy failure, or a post-zygotic SI mechanism (Gibbs, 2014; Seavey and Bawa, 1986).

In this study, CRISPR-Cas9 was utilized to generate KOs in *HT-B* or *HT-B* + *S-RNase* within the diploid potato line DRH195. The resulting levels of compatibility were quantitatively characterized by comparing fruit set, fruit weight, and seed count with seed count being the essential defining factor in self-compatibility. The *HT-B* + *S-RNase* double KOs 124_001 and 124_137 showed seed count levels that were over two and three times greater than the PC which was the characterized as the most self-fertile *S-RNase* KO from a previous study (Enciso-Rodriguez, *et al.*, 2019). The 124_001 and 124_137 lines will provide a valuable contribution to developing self-compatible diploid potato lines in commercial cultivars and provide genetic resources to further understand SI/SC in diploid potato.

V. Materials and Methods

i. Plant material

The SI diploid potato line DRH-195 (2n=2x=24) described by Enciso-Rodriguez *et al*, (2019) was selected for this study. DRH-195 derived *S-RNase* KO line (DRH-195.158) from a previous study was used as a SC positive control (PC) line (Enciso-Rodriguez, *et al.*, 2019). WT and KO lines

were propagated *in vitro* on Murashige and Skoog (MS) medium (MS basal salts plus vitamins, 3% sucrose, 0.7% plant agar, pH 5.8) (Murashige and Skoog, 1962). *In vitro* plants were maintained in growth chambers with 16-h-light/8-h-dark photoperiod at 22°C and average light intensity of 200 μmoles m-2s-1.

ii. Primer design, amplification, and sequencing of HT-B

DNA was isolated from young leaves of DRH-195 using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and used for PCR with a Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, United States). *HT-B* primers were designed from conserved regions of reported *HT-B* gene/cDNA sequences retrieved from the National Center for Biotechnology Information nucleotide database (Table S2.3) and amplified using the following thermocycler conditions: one cycle of initial denaturation for 4 min at 94°C, followed by 34 cycles for 15 s at 30°C, 45 s at 56°C and 45 s at 72°C and a final extension of 5 min at 72°C. Amplicons were gel-purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Purified amplicons were A tailed, 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). Finally, 40 colonies were Sanger sequenced and aligned using Clustal Omega (Sievers et al., 2011).

iii. Single guide RNA (sgRNA) selection, assembly, and validation

Single guide RNAs were designed from the *HT-B* ORF, using the CRISPR RGEN tools (Park et al., 2015) and assembled using the Golden Gate cloning method in a T-DNA binary vector (pHSE401) carrying the Cas9, U6 promoter and scaffold guide RNA as described by Xing et al.,

(2014). Two separate constructs were generated to target different *HT-B* gene regions containing the sgRNA-HTB1 and sgRNA-HTB2, respectively. Additionally, a two-gRNA construct was assembled using the previously reported sgRNA-1 to the *S-RNase* gene (Enciso-Rodriguez *et al.*, 2019) and the sgRNA-HTB1. Finally, each assembled construct was transformed into *Agrobacterium tumefaciens* strain GV3101 pMP90 as described by Enciso-Rodriguez *et al.* (2019). On-target activity of the reagents was validated using DRH-195 protoplasts by tagging a dsODN into the CRISPR-Cas9 reagents induced double-strand breaks in the *HT-B* and *S-RNase* genes as described by Nadakuduti et al. (2019). PCRs amplifications were performed to detect the presence of dsODN at the target site on both genes.

iv. HT-B/S-RNase knockout and transformation events screening

DRH-195 leaf segments from 4-week-old tissue culture plants were used for *Agrobacterium*-mediated transformation as described by Enciso-Rodriguez *et al*. (2019). DNA from leaf tissue of plantlets from rooted transformation events (T_0) was isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and PCR-screens were performed using GoTaq Green Master Mix (Promega, Madison, Wisconsin) and the following thermocycler conditions: one cycle of initial denaturation for 5 min at 95°C, followed by 35 cycles for 15 s at 95°C, 45 s at 56°C and 45 s at 72°C and a final extension of 5 min at 72°C. Amplicons were viewed on a 2% agarose gel stained with SYBR-safe (Thermo Fisher Scientific, USA) and lines with noticeably large deletions were selected for this study (Thermo Fisher Scientific, USA). Lines WT-like size were subjected to a restriction enzyme (RE) digestion using HpyCH4V (sgRNAHTB1) and *SmlI* (sgRNAHTB2) (New England Biolabs, USA). RE digest screening could not be performed on *S-RNase* target regions due to lack of cut sites near the PAM region. Candidate lines were identified and underwent

additional PCR using Q5® High-Fidelity DNA polymerase (New England Biolabs, USA) and subsequent Sanger Sequencing and analyzed as described previously. From the initial candidate lines, 14 WT, PC, and selected T_0 lines were transferred from subculture into 10 cm diameter pots, then were transferred 3 weeks later to 7.6L plastic pots with a peat and perlite growth medium mixture (Bacto professional plating mix, Huston, Texas. Plants were grown in a greenhouse with light intensity of 250 µmoles m—2s—1, 16/8-h light/dark photoperiod and a temperature of 25° C, through the months of late September – December in East Lansing, MI. Plants were fertilized biweekly with Peters Professional 20:20:20 fertilizer (The Scotts, Co., Marysville, OH, United States) and watered regularly.

v. Self-pollination, pollen viability, and ploidy determination

Flowers were self-pollinated using mature pollen from at least four flowers from the same plant. T_0 prevent possible cross pollination all flowers pollinated in this study were covered with a fine mesh bag before anthesis, contained in a separate section of the greenhouse, and self-pollinated within a day of anthesis. Pollen viability was determined via germination and acetocarmine staining. Briefly, pollen was collected in 1.5mL Eppendorf tube and mixed via gentle pipetting with 50uL of germination solution [22.5 mg Sucrose + 12.5 mg Boric Acid + 18.4 mg $CaCl_2$ + 29.5 mg $Ca(NO_3)_2$ + 123 mg $MgSO_4$ / 250 mL distilled water] and then placed on a glass slide. After 24 hours, 20uL of acetocarmine was added to the slide and pollen tube growth was verified under 10X magnification with a brightfield microscope (Figure S2.3). To determine ploidy of all lines used in this study, chloroplasts were counted in 10 guard cells as described in Ordonez (2014) and lines with > 8 average chloroplasts were removed from the study (Figure S2.2).

vi. Aniline blue pollen tube stain and visualization

Ten flowers for each WT, PC, T_0 KO lines were self-pollinated for stylar squash analysis. Within 48 hrs post-pollination a minimum of six carpels were immediately submerged in 750 uL of a 3:1 100% ethanol:glacial acetic acid fixation solution and incubated for at least 24 hrs in the dark at room temperature. The fixation solution was removed, and carpels were then submerged in 750 uL of 6 M NaOH solution and left overnight at room temperature. The softening solution was removed and washed three times with distilled H_2O. The H_2O was removed and then 1 mL of 0.1% aniline staining solution [100mg aniline blue + 760 mg K_3PO_4 + 100 mL distilled H_2O] was added. Tubes were wrapped in tin foil and incubated while shaking gently for 1 hour. Carpels were gently squashed on a glass slide with a cover slip and viewed with Nikon Imaging Software (NIS) under 4X magnification with DAPI fluorescence microscopy filter.

vii. *Self-Compatibility Assessment and statistical analysis*

Fruit set, fruit weight, and seed count served as quantitative measures for comparing self-fertility in the WT, PC, and 14 T_0 KO lines. The 14 lines were clonally propagated four times in culture to give a total of 56 plants which were organized into a Randomized Complete Block Design (RCBD). From each of the 56 plants, 15 flowers were self-pollinated. To account for possible changes in self-compatibility due to plant age, pollinations were repeated at one month-old, and two-months-old, respectively. At each time point, 840 flowers were pollinated giving 1,680 total flowers pollinated within this study. Fruit set, fruit weight, and seed count data were recorded for each plant at each stage (Supplemental Data). ANOVA (alpha = 0.05) and Tukey pairwise comparison (alpha = 0.05) tests were performed in R using the glmmTMB (Brooks *et al.*, 2017), lme4 (Bates *et al.*, 2015), eemeans (Length, 2019), and multcompView (Graves *et al.*, 2015)

packages. Within this study, SC plants are defined as having both fruit set and at least one seed consistently observed in the fruit. In contrast, self-fertility is defined as having fruit set and consistently producing more than one seed per fruit. Seed count was used to define self-fertility as low, moderate, and high by having 4-20, 21-50, or > 50 seeds per fruit on average, respectively.

APPENDICES

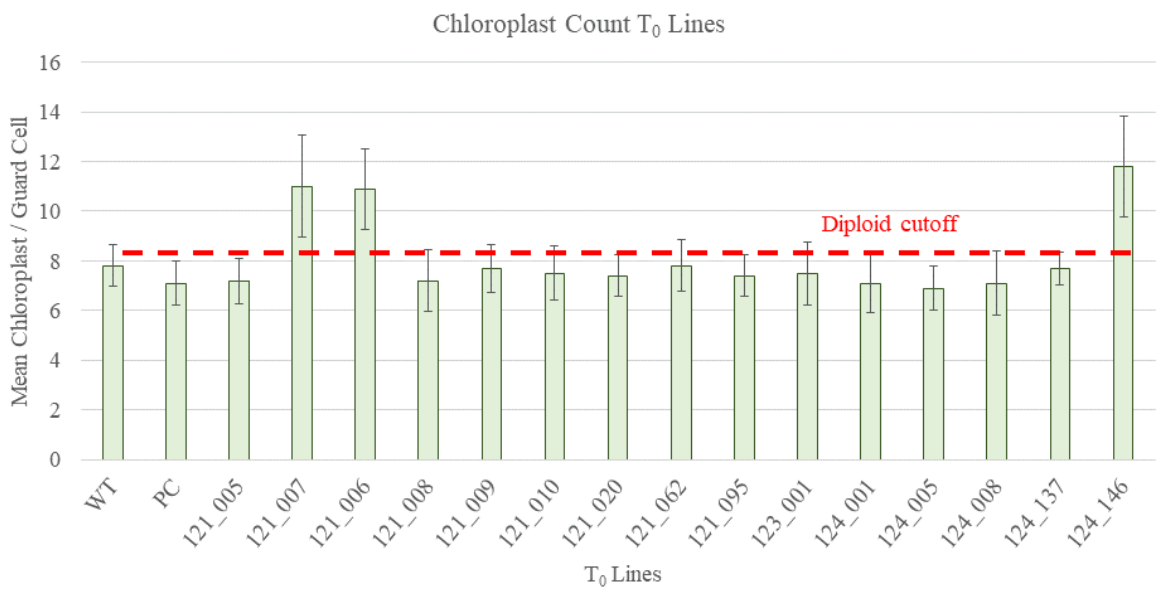

Figure S2.1 Ploidy determination for T_0 lines.

An average of 6-8 indicates a diploid *S. tuberosum* line. Lines selected for fertility assessment fall below the average cutoff of eight (dashed red line) chloroplasts per guard cell and are diploid. Error bars represent the standard deviation. T_0 lines selected for study in addition to 121_007, 121_006, and 124_146 lines which all had average chloroplast per guard cell count greater than eight and were removed from the study.

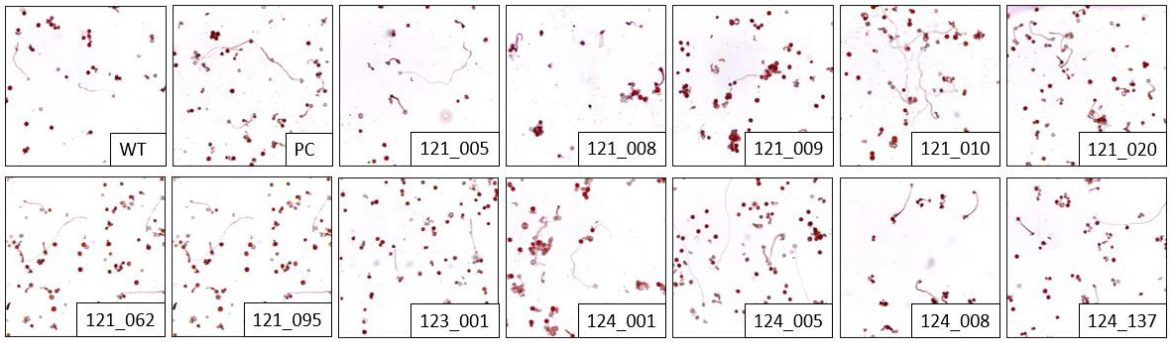

Figure S2.2 Pollen staining and germination for the T_0 generation.

All lines used in this study stained red when exposed to acetocarmine and generated pollen tube formation, indicating fertile pollen. Differences in self-compatibility are not due to infertile pollen, but rather a stylar or other non-male determinant.

Line	Edit		sgRNA-HTB1 PAM
WT		5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAGTTATTGCAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGG 3'
121_005	+1	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAGTTATTGACAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAG 3'
121_008_C1	-6	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAG------CAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGG 3'
121_008_C2	-5	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAGT-----CAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGG 3'
121_008_C3	-15	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAGTTATTG----------AGCCTTCACTTCCATTGCTTGAGG 3'
121_008_C4	-1	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAGTTATT CAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGG 3'
121_009	-15	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAGTTATTG----------AGCCTTCACTTCCATTGCTTGAGG 3'
121_010	-78	5'	ATGGCAAATATT----------TTGCTTGAGG 3'
121_020_C1	-6	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAG------CAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGG 3'
121_020_C2	-39	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCA----------TTGCTTGAGG 3'
121_062	-55	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTC----------ATTGCTTGAGG 3'
121_095	-55	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGG----------TCCATTGCTTGAGG 3'
124_001	-6	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAG------CAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGG 3'
124_005	-7	5'	ATGACAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAA-------CAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGG 3'
124_008	-15	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAGTTATTG----------AGCCTTCACTTCCATTGCTTGAGG 3'
124_137_C1	-6	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAGTTATTG-------AGATAGTTGAGCCTTCACTTCCATTGCTTGAGG 3'
124_137_C2	-4	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAGTT----CAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGG 3'
124_137_C3	-6	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCATCAGAAG------CAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGG 3'
124_137_C4	-15	5'	ATGGCAAATATTTTGCTTATATTTTCTTTGGTTCTTATGATTATATCA----------AGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGG 3'

			sgRNA-HTB2 PAM
WT		5'	TATATCATCAGAAGTTATTGCAAGGGAGATAGTTGAGCCTTCACTTCCATTGCTTGAGGGTAAGTTGTTTTTTAATTGTAGTTTTGCTATATTTAATTTT 3'
123_001	-128	5'	---------- 3'

Line	Edit		sgRNA-1 PAM
WT		5'	ATGGGGATTTCGACAAATTGCAACTGGTATTAACATGGCCACCATCATTT 3'
P.C.	-1	5'	ATGCGGATTTCGACAAATTGCAACTGGTATT-ACATGGCCACCATCATTT 3'
124_001	-2	5'	ATGCGGATTTCGACAAATTGCAACTGGTAT--ACATGGCCACCATCATTT 3'
124_005	-5	5'	ATGCGGATTTCGACAAATTGCAACTGG-----ACATGGCCACCATCATTT 3'
124_008	-2	5'	ATGCGGATTTCGACAAATTGCAACTGGTAT--ACATGGCCACCATCATTT 3'
124_137	-18	5'	ATGCGGATTTCGACAAATTGC----------CACCATCATTT 3'

Figure S2.3 All edited sequences for 121, 123, and 124 lines.

sgRNAs are in blue, PAM sequences are in red, and dashed Lines indicate deleted regions. 121_008, 121_020, and 124_137 are chimeric. All other lines have homozygous deletions between the different alleles.

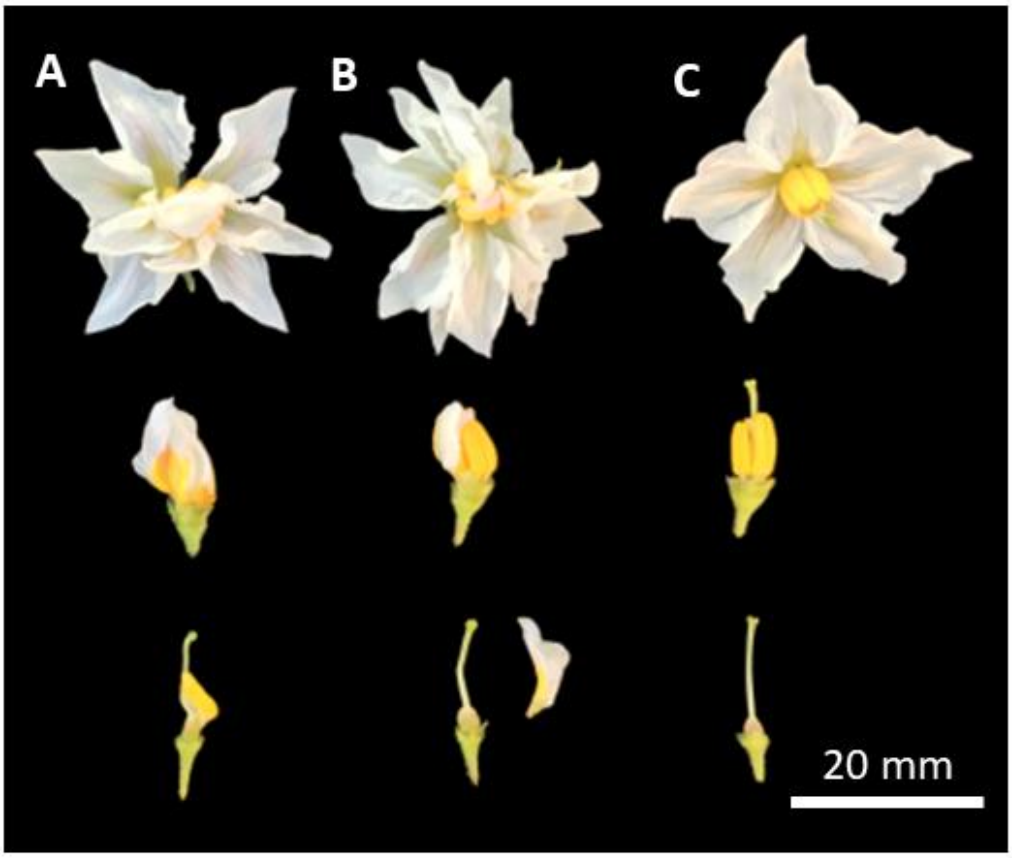

Figure S2.4 Floral mutations.

(A) Severe mutations defined as fusion of petals, anthers, and the style. Flower example from 124_005. (B) Moderate mutation defined as fusion of petals and anthers, but no fusion in the style. Flower example from 121_062. (C) No mutation, flower example from WT.

Table S2.1 Transformation efficiency.

Construct Code	sgRNA	Initial Explants	T_0 Lines	Transformation Efficiency (%) *
121	sgRNA-HTB1	220	148	67
123	sgRNA-HTB2	600	330	55
124	sgRNA-HTB1 + sgRNA-RNase**	600	250	42

**Percentage of recovered sprouts from selective regeneration media*

***Edits in either HT-B or S-RNase*

Table S2.2 Frequencies of chimeric allleles.

Line and chimera (C) #	Gene Edit	Sequence Edit*	Percentage (%)**
121_008_C1	-6	TCATCAGAAG - - - - - - CAAGGGAGATAGTTGAG	43%
121_008_C2	-5	TCATCAGAAGT - - - - - CAAGGGAGATAGTTGAG	25%
121_008_C3	-15	TCATCAGAAGTTATTG - - - - - - - - - - - - - - - - - AG	19%
121_008_C4	-1	TCATCAGAAGTTATT-CAAGGGAGATAGTTGAG	13%
121_020_C1	-6	TCATCAGAAG - - - - - - CAAGGGAGATAGTTGAG	62%
121_020_C2	-39	TCATCA -	38%
124_008_C1	no edit	TCATCAGAAGTTATTGCAAGGGAGATAGTTGAG	43%
124_008_C2	-15	TCATCAGAAGTTATTG - - - - - - - - - - - - - - - - - AG	57%
124_137_C1	-6	TCATCAGAAGTTATTG - - - - - - - AGATAGTTGAG	4%
124_137_C2	-4	TCATCAGAAGTT - - - -CAAGGGAGATAGTTGAG	18%
124_137_C3	-6	TCATCAGAAG- - - - - -- CAAGGGAGATAGTTGAG	9%
124_137_C4	-15	TCA - - - - - - - - - - - - - - - -AGGGAGATAGTTGAG	4%
124_137_C5	no edit	TCATCAGAAGTTATTGCAAGGGAGATAGTTGAG	65%

**Showing regions upstream of the sgRNA and downstream of the PAM. Deletions represented by a dash.*

***Percentage of alleles may vary depending on cell samples taken.*

PAM is in red.

Table S2.3 Primers utlized.

Label	Sequence	Description
HTB-F	5' - CAACAAACTCATATAAAATGGC - 3'	*HT-B forward* primer
HTB-R	5' - TTATCTTTGCTTGGGCAAGGGC - 3'	*HT-B* reverse primer
S-RNase-F	5' - ATGTTTAAATCACTGCTTACATCAAC - 3'	*S-RNase* forward primer
S-RNase-R	5' - TCAGGGACGGAAAAATATTTTCCCTG - 3'	*S-RNase* reverse primer

APPENDIX B: Additional experiments: HT-A gene targeting

For the sake of brevity and due to timing of the experiments, certain aspects relevant to this experiment were left out of the main manuscript and supporting materials submitted to the Plant Biotechnology Journal. Within this section, the results concerning the *HT-A* gene and the T1 generation which were omitted from manuscript submission are covered.

As was mentioned in Chapter 1, *HT-A* was not shown to be necessary for SI reactions in *S. chacoense*, but was relevant to the UI response in *S. lycopersicum* (O'Brien *et al.*, 2002; Covey *et al.*, 2010). Therefore, *HT-A* is not necessary to complete the study but nonetheless would have served as a good compliment and should be considered for future experiments. When initially conceptualizing the study described in Chapter 2, *HT-A* was also initially considered for gene KO. The *HT-A* gene was identified in potato similarly to the *HT-B* gene. *HT-A* primers (Table S2.4) were designed from conserved *HT-A* regions of multiple *Solanum* spp. and used to perform Rapid amplification of cDNA ends (RACE) to amplify the *HT-A* open reading frame (ORF) within DRH-195.

Table S2.4 Primers used for *HT-A* amplification.

Label	Sequence	Description
HTA-F	5' - ATGGCATTCAAGGCAAATATCTT - 3'	*HT-A forward* primer
HTA-R	5' - CTAACAACAACAAATGGCTTTACAAA -3'	*HT-A forward* primer

Once isolated, *HT-A* was also included as a target gene within two different constructs. Both constructs, unfortunately, failed to generate KO lines. The first construct had a sgRNA-HTA1 targeting 661 bp downstream of the *HT-A* start codon in a CRISPR-Cas12a cassette, which generates DSB similarly to Cas9, but generally has lower efficiency in generating DSBs (Paul and Montoya, 2020). The Cas12a system was used to house sgRNA-HTA1 instead of a Cas9 system

because no suitable 5'-NGG-3' PAM site is located near a possible sgRNA sequence that was also unique from the *HT-B* gene. Cas12 requires a 5'-TTTN-3' PAM recognition site and was initially seen as a plausible alternative (Paul and Montoya, 2020). However, Cas12 has a lower success rate for inducing double stranded breaks than Cas9 and requires temperatures over 28°C in order to function with high activity (Malzahn *et al.*, 2019; Ming *et al.*, 2020; Paul and Montoya, 2020). The explants used for this study were not originally exposed to temperatures over 28°C. The failure of Cas12a to generate DSB due to temperature sensitivity has inhibited gene editing in plants in the past, and is likely the cause of the failure of the sgRNA-HTA1 containing Cas12 construct as well (Malzahn *et al.*, 2019).

Sprouts from explants were recovered from selective media which indicates they likely contain the Cas12a construct which contains a resistance gene. Exposing these potential gene edited events to increased heat as described in by Malzahn *et al.*, (2019) may yet produce gene edits and would be an interesting future experiment.

The *HT-A* gene was also targeted with the same construct that houses the sgRNA-HTB1 included in the main manuscript. The sgRNA-HTB1 targets the *HT-B* and *HT-A* genes 44 bp and 53 bp (respectively) downstream of the start codon. The sgRNA-HTB1 targets a region between the HT-A and HT-B genes which is conserved except for a single mismatched base pair (Figure S2.5). Initially, this mismatch was hypothesized to not pose a problem to Cas9 editing, since previous studies had reported that Cas9 could tolerate a single mismatch and still produce double stranded breaks and gene knockout lines (Feng *et al.*, 2014; Xing *et al.*, 2014; Hsu *et al.*, 2016). However, after performing PCR and gel visualization screens for large deletions on 140 plants, restriction enzyme digest assays, and sanger sequencing on randomly selected lines no edits were recovered within the *HT-A* gene. This same construct did produce edits in the *HT-B* gene, as is shown by the

multiple 121 lines with large deletions in the *HT-B* gene. These results indicate that failure to produce a double stranded break and *HT-A* KO was due to the mismatch rather than transformation failure or error in the Cas9 protein.

Figure S2.5 *HT-A* and *HT-B* target region comparison.

The sequences between *HT-A* and *HT-B* are for mostly conserved. However, mismatches are noticeable near the start codon of *HT-B* (ATG of the top sequence), immediately after the PAM sequence, and within the gRNA as indicated by the black arrow and red "G" nucleotide which is different from the sgRNA-HTB1 gRNA which has an "A" nucleotide.

APPENDIX C: Additional experiments: T1 fertility assessment

The T1 generational plants were also excluded from the initial Plant Biotechnology Journal submission due to time constraint. However, the methodology for T1 germination of select lines from the T0 generation is described here.

Roughly 20 T_1 seeds from select T_0 lines (as seen in Figure S2.6) *HT-B* KO, *HT-B* + *S-RNase* KO, PC, and WT samples were extracted from the T_0 fruit, exposed to 1500 ppm gibberellic acid overnight, and then placed directly into soil. Growth conditions thereafter were the same as described for the T_0 generation, except for higher temperatures due to the growth of the plants stretching through late February to April. All lines had seeds which germinated. T_1 selected lines were confirmed to be diploid in the same manner as described for the T_0 generation (Figure S2.6). A single germinated sample was kept from each selected line and observed for fruit set and seed count. Results regarding fertility were similar to the T_0 generation, showing self-incompatibility/low fertility in WT and *HT-B* KO lines, but self-compatibility/high fertility in the *S-RNase* + *HT-B* KOs. Interestingly, the WT and *HT-B* lines showed no fruit set, unlike in the T_0 generation. This could be due to the same plasticity mentioned previously or a decrease in fertility due to inbreeding depression (De Jong and Rowe, 1971; Travers *et al.*, 2004; Stone *et al.*, 2006; Webb and Williams, 1988) The fruit set and seed production observed in the double KO lines indicates the transmission of self-compatibility/self-fertility is stable within the T_1 generation.

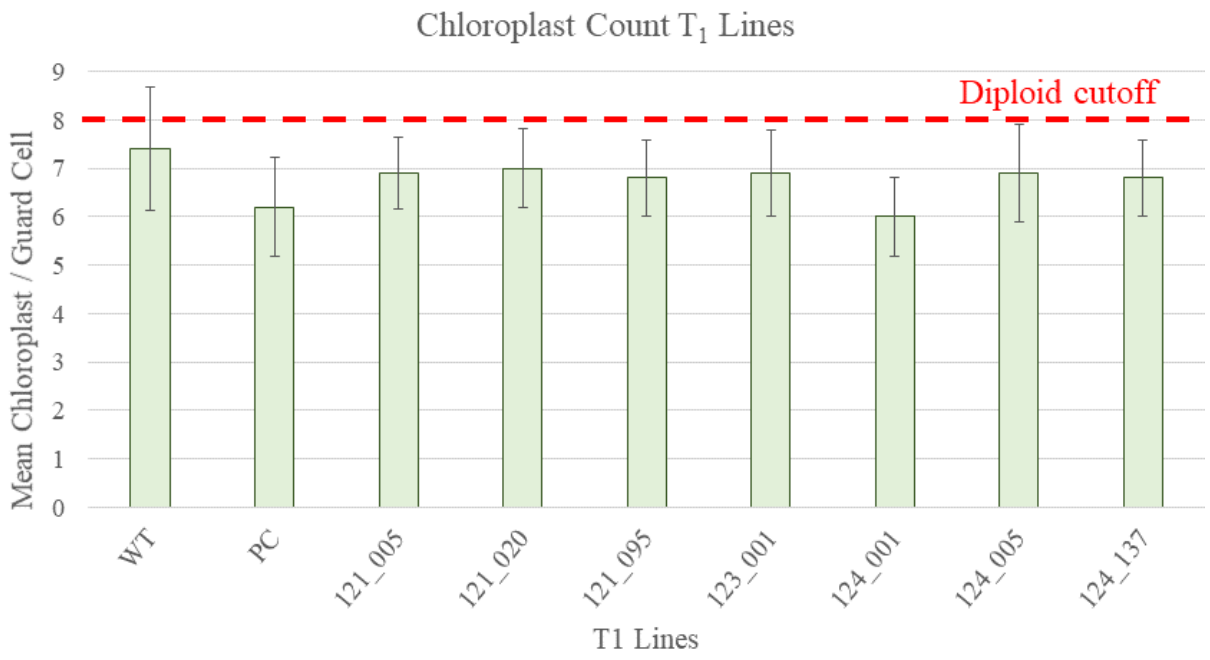

Figure S2.6 Ploidy determination for T_1 lines.

An average of 6-8 indicates a diploid *S. tuberosum* line. All T_1 lines selected for fertility assessment fall below the average cutoff of eight (dashed red line) chloroplasts per guard cell and are diploid. Error bars represent the standard deviation.

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Chapter 3

CONCLUSIONS AND FUTURE DIRECTIONS

I. Global potato production

Potato production is worldwide with weight production by percentage rated per continent as Asia (42.8%), Europe (38.1%), the Americas (12.8%), Africa (5.8%), and Oceania (0.5%) as of 2019 (FAOSTAT, 2021). Potatoes are a vital crop for both developed and developing countries with the top four producers being China, India, Russia, and the United States of America (FAOSTAT, 2021). Considering the agricultural significance of potato as a food security crop, it is of upmost importance to use every tool available to ensure production of this vital crop including identifying genes related to developing self-compatible lines (Devaux et al., 2014). The polyploid nature and inbreeding depression seen in commercial potato poses a substantial barrier to facilitating potato production but may be remedied by turning to diploid potato breeding as an alternative method (Potato Genome Sequencing Consortium, 2011; Jansky *et al.*, 2016). Successful gene edits in potatoes (e.g. overcoming self-incompatibility via targeted mutagenesis) demonstrate that CRISPR-Cas9 is a plausible approach to generating self-compatible lines (Enciso-Rodriguez, *et al.*, 2019; Ye *et al.*, 2018).

II. Contribution of *HT-B* + *S-RNase* KO lines to diploid potato breeding

The study described in Chapter 2 serves as additional support to genome editing and the possible benefits provided in relation to developing self-compatible cultivars for diploid potato breeding. The generation of double KO *HT-B* + *S-RNase* lines from idea conception to confirmation of self-compatibility and fertility assessments took roughly two years. This is significantly less time to

generate self-compatible plants in comparison to traditional breeding schemes seeking to incorporate self-compatibility which may take six or more generational cycles to achieve a similar effect (Alsahlany *et al.*, 2021). In addition to taking less time to produce a self-compatible line, the fertility of the top double KO lines produced are robust with an expected fruit set probability of over 50% for self-pollinated flowers and anticipated seed count of over 90 per fruit (Figure 2.3a and c). The self-compatible and highly self-fertile nature of the double KOs is also stable in the next generation, considering the T1 lines also had fruit set and seed produced (data not shown). In particular, the 124_001 line produced will provide the best candidate for future breeding programs as the deletions within this are homozygous and the line is robustly self-compatible (Figure 2.1c). Future directions for the 124_001 line would involve crossing to diploid lines that generally display more commercially desirable traits from the, MSU diploid breeding program (pers. Comm. D. Douches). The reason for this being that though the 124_001 has the benefit of being self-compatible the tubers are oblong, pink skinned, and would not be desirable as a chipping or table stock potato. The breeding scheme will include an initial cross between 124_001 X select cultivated dihaploids, backcrossing of the progeny, and then a recurrent selection breeding scheme selecting for progeny containing the KOs.

Another future study would be to consider as a compliment to the *HT-B* and *S-RNase* knockout experiment is to target *HT-A* in *S. tuberosum* to elucidate what (if any) role *HT-A* plays in the self-incompatibility response in diploid potato. Observing if *HT-A* plays a role in unilateral self-incompatibility in potato (similar to tomato) would be of interest as well (Covey *et al.*, 2010). 124_001 has a high level of SC and may also be crossed to *Sli*-based SC lines. This population would help potato breeders to understand the relationship between *Sli, S-RNase* KO, and *HT-B* KO in contributing to SC in diploid potato.

In closing, there are still many interesting aspects regarding self-compatibility to explore in diploid potato. Understanding the role of *HT-A*, the precise biochemical mechanisms behind self-incompatibility, and why some potato species display LSI instead of GSI still remains to be discovered. Elucidating these aspects is well worth while to enhance potato breeding progress as a diploid crop.

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