VALIDATION OF A QTL IDENTIFIED FOR TOMATO SPOTTED WILT VIRUS RESISTANCE AND CANDIDATE GENE EXPLORATION IN *ARACHIS HYPOGAEA*

by

SYDNEY LEE WEBB

(Under the Direction of Peggy Ozias-Akins)

Genetic resistance to Tomato spotted wilt virus (TSWV) is important for yield protection in susceptible *Arachis hypogaea* (cultivated peanut). A single resistance source, PI 203396, has been used in breeding since the 1990s. Recently, SSD6 and its resistant progeny NC94022, were introduced as a second source of resistance. A quantitative trait locus (QTL) was previously identified on chromosome A01. Within this QTL, a resistance gene and an insertion region from NC94022 were hypothesized to improve disease resistance. In the current study, the impact of this QTL and insertion region is explored through eight populations developed from both known sources of resistance. Presence of the insertion significantly improved field resistance with no agronomic trade-offs. Expression of candidate genes within this QTL and a nearby region with a large deletion are explored under mechanical TSWV inoculation. A glutamate receptor present only in NC94022 is selected as a candidate for further exploration.

INDEX WORDS: *Arachis hypogaea*, cultivated peanut, tomato spotted wilt virus (TSWV), genetic resistance, quantitative trait locus (QTL), candidate gene

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B.S., North Carolina State University, 2021

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2024

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December 2024

DEDICATION

To my amazing parents for all your love and support over the years. Even from afar you never failed to take care of your little girl. I wouldn't be who I am or where I am without you. You let me learn to be myself but were always there when I needed to come home. There were plenty of hard times but we always got through them together. For the two of you I will forever be thankful.

To my family, on earth and in heaven, by blood and those I got to choose. Thank you for being who you are and for all the support you have given me during this time. I send my love to each one of you. To my grandparents, great-grandparents, aunts and uncles, thank you for teaching me how to be brave and to face each day with faithful strength. I know each of you was with me through this and I couldn't be more appreciative of everything you mean to me. You will be a part of me forever. And lastly, to Jade Elaine, my best friend and my sister. You've stuck by my side through everything and I can't imagine not having you on this adventure of life. Thank you for being you.

All my love and gratitude to each and every one of you. Here's to the next chapter!

ACKNOWLEDGEMENTS

First, a very big thank you to my advisory committee, your experience and love for peanuts showed me this work was so much more than just research but a commitment to the program and the producers. Dr. Peggy Ozias-Akins, thank you for taking me into your lab and allowing me to go on this great journey of grad school under your advisement. I have learned more than I ever imagined possible and I am grateful for all you have taught me. Thank you for guiding me through some challenging times and always being the support we needed to make it through each field season. I will forever remember what I have learned and the opportunities you have given me during this time. Thank you to my committee members, Dr. Corley Holbrook, Dr. Josh Clevenger, and Dr. Albert Culbreath. Your support and knowledge have helped me tremendously during my research and numerous presentations. You pushed me to learn new things and made sure I always understood the reason I was taking each step of the way.

To the POA peanut team, thank you for everything! You have not only become great friends but family. I can't imagine working with a better, more talented group of people. Stephanie, Brianna, Carlos, and Chinaza, thank you for being the first group I got to work with in the lab and for helping me find my way in Tifton. Gema, Prassana, Swikriti, Sameer, Raissa, Debbie, and Nayeli, thank you for being great additions to our team along the way. I look forward to seeing where each of you goes and cheering you on as you have done for me. To the rest of the peanut group and the POA lab, thank you for all your guidance and assistance during my time. I learned so much from each of you and am grateful for all of you. Dr. Joann Conner

and Dr. Sailaja Bhogireddy, thank you for all your help. I would not have been able to do nearly as much with transformation without your guidance.

To Dr. Juliet Chu, thank you for your experience and everything you did to guide this project and help me get started. You will always be a peanut member to me! To Dr. Baozhu Guo and Dr. Walid Korani, thank you for the work you did on the foundation of this project and along the way. To Jason Golden, Shannon Atkinson and the USDA peanut breeding team, thank you so much for all your help in and out of the field. I couldn't have done all this field work without your help and dedication each season.

To the UGA Tifton Campus, thank you for becoming a second home and for the wonderful memories. Mrs. Lesa, thank you for all your support and knowledge, you helped me in more ways than you will ever know and it is all much appreciated. Skylar, thank you for all the popcorn Thursdays and campus gatherings. And to all the PBGG Tifton students, thank you for being amazing friends and colleagues. I couldn't name you all without leaving someone out but please know that you each holds a place in my heart for your friendships that have helped me through these years.

Thank you to the University of Georgia and the Institute of Plant Breeding, Genetics and Genomics for this opportunity and support through the last three years. I could not have imagined a better graduate school experience and have greatly appreciated everything. Thank you to all the professors for guiding me and teaching me as not just a student but a colleague. All your hard work to get us prepared for the future was always appreciated.

Lastly and most importantly, I thank my family and God for getting me this far and supporting me through all the challenges of the last few years. I wouldn't be where I am without you and I will forever be grateful for this experience and all it has taught me.

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CHAPTER 1

Introduction

Cultivated peanut (*Arachis hypogaea* L.), also known as groundnut, is an important legume crop around the world. Over 30 million hectares of peanut were harvested worldwide in 2022, making it the ninth most produced row crop (FAO, 2024). China leads world peanut production followed by India, Nigeria, and the United States (U.S.), which produced six percent of total world production in 2023 (USDA, 2024c). Within the U.S., the state of Georgia produces over half the country's peanuts, exceeding 1.42 million metric tons (mt) and $783 million production value (USDA, 2024a, 2024b). World consumption of peanuts reached 42.5 million mt in 2018, making it the most consumed nut by over 40 million mt (WorldAtlas, 2018). The nutritional value of peanut is a key component to many people's daily diet. Peanut seed is 22-30% protein and 10-20% carbohydrate, with additional nutrition and health benefit coming from numerous vitamins (E, K, and B complex), minerals (Ca, P, Mg, Zn, and Fe), and fiber (Variath & Janila, 2017). Peanuts are also important for their oil content and as a source of nutritious animal feed (Pandey et al., 2012; Variath & Janila, 2017).

Arachis hypogaea L. belongs to the genus *Arachis* which originates from South America, specifically the regions around western Brazil, northeast Paraguay, and northern Argentina (Grabiele et al., 2012; Kochert et al., 1996). *Arachis* is composed mostly of diploid species (2n = 2x = 20), except for a few tetraploid species (2n = 4x = 40), including the domesticated *A. hypogaea* and the wild *A. monticola* (Bertioli et al., 2016; Kochert et al., 1996; Stalker, 2017). The genus is further divided into nine taxonomic sections based on morphology, geographic

center of origin, and cross compatibility with other groups in the genus (Simpson et al., 2001). Section *Arachis* is the largest and most advanced, containing the domesticated *A. hypogaea*. This allotetraploid species was produced from a cross between the diploid A-genome *A. duranensis* Krapovickas & W.C. Gregory and B-genome *A. ipaensis* Krapovickas & W.C. Gregory (Bertioli et al., 2016; Grabiele et al., 2012). These ancestors are estimated to have diverged from one another approximately 2.6 million years ago before undergoing a single hybridization event around 4,000 years ago. Hybridization was followed by a polyploidization event that doubled the chromosome number and produced the AABB genome of *A. hypogaea* (Bertioli et al., 2016; Kochert et al., 1996; Stalker, 2017). The species further underwent a genetic bottleneck period when limited gene flow occurred, resulting in a highly homogeneous population from which humans could select for domestication (Bertioli et al., 2016; Bertioli et al., 2019; Grabiele et al., 2012; Krapovickas & Gregory, 1994). Over time this bottleneck and human selection for specific quality and production traits constrained the genetic diversity in *A. hypogaea*.

Within *A. hypogaea*, two subspecies are distinguished by their morphological patterns. *A. hypogaea* subsp. *hypogaea* has a prostrate growth habit with no flowers on the mainstem and a longer growing season. *A. hypogaea* subsp. *fastigiata* has an upright growth habit with flowers occurring on the mainstem and a shorter growing season (Kochert et al., 1996; Krapovickas & Gregory, 1994). Within these subspecies are six botanical varieties - *hypogaea* and *hirsuta*, and *fastigiata*, *peruviana*, *aequatoriana,* and *vulgaris*, belonging to subspecies *hypogaea* and *fastigiata*, respectively (Krapovickas & Gregory, 1994). Along with these taxonomic classifications are four main market types of cultivated peanut (Stalker, 2017). Virginia type, concentrated in Virginia and the Carolinas, has a larger seed and is used for the in-shell and confectionary markets. Runner types are grown throughout the south and southeastern region,

including Georgia, and have smaller seeds than virginia types. These are commonly used in processing markets and account for more than 85% of the total U.S. peanut production (AmericanPeanutCouncil, n.d.). Valencia and spanish types are grown on small acreage in New Mexico, Texas and Oklahoma, and are used mainly for in-shell markets. (Stalker, 2017).

Literature Review

Peanut Breeding

Plant breeding is the process of developing improved varieties by making crosses and selecting lines with the most desirable traits. Peanut breeders have also recognized the importance of improving the narrow genetic base of peanut. Expanding on this foundation with new genetic combinations is essential for the conservation of such an important crop. The process of improving peanuts began with domestication of early *A. hypogaea* species when humans selected for plants with greater yield, larger seeds, and single chamber pods without an isthmus to separate multiple seeds (Kochert et al., 1996). Peanut has a geocarpic growth habit where flowers are pollinated above ground and pegs extend downwards so pods and seeds develop underground (Krapovickas & Gregory, 1994). Wild species have long, weak pegs that may easily break during harvest to leave pods in the ground and enhance survival. This results in yield loss under modern harvesting practices, so humans have selected for shorter, stronger pegs that can withstand harvest. (Kochert et al., 1996). Over time, selection for these traits and implementation of breeding programs have increased peanut yield and quality, resulting in a six-fold yield increase between 1909 and 2017 (Holbrook, 2019). Total pod yield and plant biomass are important agronomic traits for production. The spread and shape of above ground structures are also important for consideration with modern farm machinery. Peanuts are an indeterminate crop and will continuously produce new pods throughout the growing season, therefore time to maturity is an essential consideration for consistent quality at harvest time.

The process of domestication further decreased the already limited genetic diversity of *A. hypogaea*, specifically with regard to resistance to biotic and abiotic stresses. In peanuts there are many factors that may limit production of a crop. Abiotic stresses such as drought and heat or

soil fertility can hinder production in even the most disease-free environments (Holbrook & Stalker, 2003; Variath & Janila, 2017). A long list of biotic factors have significant effects on peanuts, but the most notable include peanut root-knot nematode (*Meloidogyne arenaria*); soilborne diseases such as stem rot (*Agroathelia rolfsii*), Sclerotinia blight (*Sclerotinia minor*), and Cylindrocladium black rot (*Cylindrocladium parasiticum*); foliar diseases such as early and late leaf spots (*Passalora arachidicola* and *Nothopassalora personata*, respectively); viruses such as tomato spotted wilt virus (TSWV); and aflatoxin, produced by *Aspergillus flavus* and *A. parasiticus* (Holbrook & Stalker, 2003; Kemerait et al., 2024). Resistance to many of these diseases has been explored and incorporated into cultivated peanut using the genetic variability available in *A. hypogaea* or through introgressions from wild diploid relatives (Holbrook et al., 2016; Stalker, 2017).

Agronomic and resistance traits in peanut are greatly influenced by genotype by environment (GxE) interactions, where a plant's genetic expression is impacted by its environment (Holbrook & Stalker, 2003; Variath & Janila, 2017; Weinig & Schmitt, 2004). Breeding practices must take this into consideration when producing a variety to be grown in multiple environments. This requires breeding lines to be evaluated in multiple locations over multiple years before being considered suitable for release as a new variety, which can increase the time and money spent by a program. Molecular breeding techniques can enhance traditional breeding practices to better understand traits and select appropriate genotypes while considering the GxE effect and reducing the time it takes to reach release.

Molecular Breeding

Marker-assisted selection (MAS) is an important practice in modern breeding where the presence or absence of a genetic marker can identify a plant's potential to show a specific phenotype (Collard et al., 2005). This tool requires not only polymorphisms between genotypes, but detailed maps to understand what genetic information is available. It can be especially useful within cultivated peanut to discover and apply any desirable variability that may benefit producers. Polymorphisms have been identified using various marker types over the years. Restriction fragment linkage polymorphism (RFLP) was the first system to show a sufficient number of polymorphisms in the *A. hypogaea* genome (Holbrook & Stalker, 2003). These were followed by randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and single nucleotide polymorphism (SNP) markers (Guo et al., 2013; Pandey et al., 2012). SSR and SNP markers are important in modern breeding to develop genetic maps and MAS for important traits. Sequencing tools such as the Affymetrix 'Axiom Arachis' SNP array and Khufu have been developed to identify SNPs associated with genetic regions of interest and applicable for MAS (Clevenger et al., 2017b; Clevenger et al., 2018; Korani et al., 2019; Korani et al., 2021; Pandey et al., 2017a).

Linkage maps are used to indicate the relative position of genetic markers within the genome and can be developed using the segregation of polymorphisms in a mapping population (Collard et al., 2005). These mapping populations should be segregating for one or more traits of interest if the linkage map is to be useful for identifying the genetic foundations of the given trait. F_2 populations derived by selfing F_1 hybrids, backcross (BC) populations derived from crossing an F_1 with one of its parents, and recombinant inbred line (RIL) populations derived from inbreeding single F_2 plants for several generations are common types of mapping

populations used in peanut studies (Collard et al., 2005). Early selfing or backcross generations can be used to produce linkage maps, though segregation may impact consistency until the lines are sufficiently inbred or homozygous. To limit the effect of segregation and the GxE interactions, RILs are often used because their high homozygosity allows the same population to be studied in multi-year and multi-environment tests with no genotype change (Khera et al., 2016; Weinig & Schmitt, 2004).

Given the complexity of *A. hypogaea'*s tetraploid genome, the first linkage maps were produced using diploid species followed by interspecific tetraploid populations. The first linkage map of *Arachis* was developed using RFLP markers from an F_2 population of *A. stenosperma* x *A. cardenasii*, two diploid A-genome species (Halward et al., 1993). A second map derived from an F_2 population of A-genome species was developed by Moretzsohn et al. (2005) using SSR markers to differentiate *A. duranensis* x *A. stenosperma*. Likewise, a B-genome map was developed from an F_2 population of *A. ipaensis* x *A. magna* using several marker types (Moretzsohn et al., 2009). Interspecific tetraploid maps have been generated using synthetic amphidiploids to cross with *A. hypogaea* varieties to map the tetraploid genome and expand genetic diversity (Burow et al., 2001; Foncéka et al., 2009). Foncéka et al. (2009) used an *A. duranensis* x *A. ipaensis* amphidiploid to represent the progenitor species of cultivated peanut in their mapping study. The first map of cultivated peanut was produced using SSR markers from a RIL population of cultivated x cultivated (Varshney et al., 2009). Since then, several cultivated maps have been produced, including high-density and consensus maps of multiple populations (Gautami et al., 2012; Hong et al., 2010; Qin et al., 2012; Zhou et al., 2014). Development of these maps, along with sequencing of the A- and B-genome diploid ancestors and *A. hypogaea*

cv. Tifrunner, have allowed for further understanding of the genetic foundation available in cultivated peanut (Bertioli et al., 2016; Bertioli et al., 2019).

Detailed linkage maps and segregating populations provide an important foundation for identifying the genetic control of important phenotypes, including resistance traits. Quantitative trait locus (QTL) mapping uses genotypic and phenotypic data to associate the underlying region controlling a quantitative trait (Collard et al., 2005). Association between markers on linkage groups and phenotypic data collected from parents and progeny of a population is used to determine the location of QTL(s) on the genetic map. Markers closely linked to the given QTL can then be used to select for the presence or absence of the QTL region and allow for identification of favorable individuals in the lab before going to the field. This reduces the time and money required to evaluate every individual in a population and focuses only on those with the greater potential for success. Because QTLs are a region rather than a specific point, sequences within the QTL can be more closely explored to identify potential candidate genes responsible for the trait of interest (Pandey et al., 2012).

Tomato Spotted Wilt Virus

Tomato spotted wilt virus (TSWV) belongs to the genus *Orthotospoviruses*, family *Tospoviridae*, and order *Bunyavirales* (CABI, 2020). Within the virus envelope are three single-stranded RNA molecules, each bound to a nucleocapsid protein. The medium RNA is unique to *Bunyaviridae* tospoviruses and produces a non-structural protein used to facilitate cell-to-cell movement of the virus through the plasmodesmata of the host plant. The small RNA produces a second non-structural protein that facilitates RNA silencing suppressor activity in the vector and host cells. Glycoproteins on the surface of the virus envelope are important for acquisition of the

virus in the vector and replicase proteins are produced to ensure replication in the plant host (Sherwood et al., 2009). This complex plant virus has been found to infect over 1,300 host plants and has been identified on every continent except Antarctica after being discovered in Australia in 1915 (CABI, 2020; Culbreath et al., 2003). Important host plants in the U.S. include tomato (*Lycopersicon esculentum*), pepper (*Capsicum annuum*), tobacco (*Nicotiana tabacum*), and peanut, along with other vegetable and horticultural crops (CABI, 2020; Culbreath et al., 1991; Gitaitis et al., 1998).

TSWV is naturally vectored by thrips, specifically *Frankliniella fusca* (tobacco thrips) and *F. occidentalis* (western flower thrips) in peanut (Riley et al., 2011; Srinivasan et al., 2018). Both the larval and adult stages of thrips feed on plant tissue, including leaves and flowers, but only adults that acquired the virus during the larval stage are able to transmit it (Riley et al., 2011; Sherwood et al., 2009). Transmission occurs during feeding when saliva of a viruliferous adult thrips is mixed with cellular contents and the virus is left behind in the host plant. The short pupal period between larva and adult is the only non-feeding stage, during which the thrips is in the soil. The feeding process itself can cause damage, leaving behind silvering streaks on leaves and stunting young plants (Srinivasan et al., 2018). Thrips populations typically peak one to two times during a peanut growing season, though warmer temperatures may lead to shorter life cycles and greater thrips populations in a field (Buechel, 2021; Srinivasan et al., 2018). When peak populations align with periods of young peanut plants it can be problematic for both plant development and increased disease transmission.

TSWV was first reported in the U.S. in 1971 when it entered into Texas; it was identified in all other peanut producing states by the late 1980s (Culbreath et al., 2003; Holbrook, 2019). The virus quickly became detrimental to peanut production, causing an average yield loss of

12% and over $40 million economic burden by 1997 (Srinivasan et al., 2017). The introduction of genetically resistant peanut varieties reduced loss to almost zero by 2011. Loss from TSWV has begun to rise again in recent years, with a 5% yield and over $35 million value loss in 2022 (Kemerait, 2024). Symptoms in peanut appear as concentric ringspots and chlorosis of leaves, stunting of above ground tissue, and misshapen or discolored pegs, pods, and kernels below ground (Culbreath et al., 2003; Srinivasan et al., 2017). Asymptomatic infections are possible but are not accounted for with visual observations, rather an enzyme-linked immunosorbent assay (ELISA) or similar test is required for confirmation of virus presence (Tillman & McKinney, 2018).

Field management practices to reduce TSWV have been highly encouraged since the virus peaked in peanut production around 1996 and the University of Georgia Tomato Spotted Wilt Risk Index for Peanuts was established (Brown et al., 2005). This risk index provides growers with information on the potential risk for TSWV in their fields and helps them determine the best management practices to reduce their losses. In 2003 the TSWV risk index was incorporated into the broader program Peanut Rx (https://peanutrx.org/) to provide farmers with information on TSWV and other important peanut diseases including leaf spots, stem rot, and nematodes (Kemerait et al., 2004). The risk index is updated yearly based on results from the previous growing season and the most recent research and extension information available. Selection of resistant cultivars is the top recommendation for limiting the effects of TSWV in the field, and the most recent resistance ratings for available cultivars is maintained in Peanut Rx. Cultural practices promoted by Peanut Rx include planting date, planting density and row pattern, application of in-furrow insecticides, tillage practices, and crop rotation (Kemerait et al., 2024). For TSWV, selecting a planting date to avoid peak thrips populations around young

seedlings, twin row patterns with higher plant populations, and in-furrow application of the insecticide Phorate to reduce thrips populations are the most important mitigation practices after variety selection (Brown et al., 2005; Culbreath et al., 2003; Kemerait et al., 2024).

Genetic resistance to TSWV

Genetic resistance to TSWV has been identified and successfully implemented using single resistance genes in both tomato (*Lycopersicon esculentum*) and pepper (*Capsicum annuum*). Multiple genes from the same family have been identified in tomato for TSWV resistance, including dominant alleles *Sw-1a* and *-1b, Sw-5a* through *-5e, Sw-6,* and *Sw-7,* and recessive alleles *sw-2, sw-3* and *sw-4* (Finlay, 1952; S. Qi et al., 2022; Stevens et al., 1991). *Sw-5b* contains nucleotide binding and leucine-rich repeat (NB-LRR) domains and confers resistance to a range of tospoviruses which has made it a top choice for incorporating genetic resistance in tomato (de Oliveira et al., 2018). *Sw-7* confers resistance to a wide range of TSWV isolates but has yet to be cloned and implemented in breeding as widely as *Sw-5* (S. Qi et al., 2022). A single-dominant resistance gene has also been reported in pepper, *Tsw*, though it confers resistance only against TSWV isolates (Boiteux & de Ávila, 1994; Sundaraj et al., 2014). Each of these genes results in a hypersensitive response to TSWV infection, i.e., localized cell death at the site of infection that can limit systemic spread in resistant plants. No hypersensitive response has been observed in peanut; therefore, it is believed that peanut's resistance to TSWV is more complex than a single resistance gene (Shrestha et al., 2013; Sundaraj et al., 2014; Tseng et al., 2016).

When TSWV first appeared in the U.S., Florunner (Norden et al., 1969) was the predominant runner peanut variety, having desirable agronomic traits and good yield but high

susceptibility to TSWV (Clevenger et al., 2017b; Culbreath et al., 1992). This susceptibility in a large portion of the peanut population led to the high peak of yield loss seen in the mid-1990s. Southern Runner (Gorbet et al., 1987) showed mild TSWV resistance but was not a predominant variety at the time (Culbreath et al., 1992; Sundaraj et al., 2014). Georgia Green (Branch, 1996) was released in 1995 and quickly became the predominant runner cultivar, controlling over 70% of the certified seed production by 2001 (Clevenger et al., 2017b; Sundaraj et al., 2014). This important turn of events was made possible by Georgia Green's TSWV resistance derived from its parent Southern Runner, a progeny of PI 203396 (Gorbet et al., 1987). Though it had only been previously evaluated for its resistance to late leaf spot, PI 203396 was determined to be the source of resistance in Georgia Green (Holbrook & Stalker, 2003).

PI 203396, *A. hypogaea* subsp. *hypogaea*, was collected in 1952 from a market in Puerto Alegre, Brazil (Isleib et al., 2001). Since its incorporation into breeding programs, this PI has contributed 1,022 additional markers to the runner germplasm, greatly expanding the genetic diversity of *A. hypogaea* cultivars (Clevenger et al., 2017b). It has become one of the most impactful introductions into modern peanut breeding, as Isleib et al. (2001) concluded it could have over $200 million annual impact for growers in the Southeast U.S. when grown under high disease pressure. Southern Runner and Georgia Green are considered first generation resistance from this PI; second generation resistance consists of varieties such as Georgia-06G, Georgia Greener, Georganic, and Tifguard (Sundaraj et al., 2014). Georgia-06G (Branch, 2007) is one of the most widely grown runner varieties in current peanut production, not only for its TSWV resistance but its desirable agronomic traits and consistently high yields in varying environments. Georgia-06G made up 72% of the certified seed produced in Georgia and Alabama in 2022 (Skaggs, 2024).

Continued collaboration between peanut breeders and pathologists identified a second source of TSWV resistance in NC94022, developed from a cross between PI 576638 (SSD6) x N91026E (Culbreath et al., 2005). NC94022's consistently high level of TSWV resistance is desirable for peanut production, but the agronomic traits derived from SSD6 make it undesirable for modern markets. SSD6 is a *hirsuta* landrace (*A. hypogaea* subsp. *hypogaea* var. *hirsuta* Kohler) collected from Guanajuato, Mexico (USDA, 1996). The prostrate growth habit, deeply constricted and beaked pods, and hairy stems allowed it to adapt over time to its local environment in Mexico, but these traits, especially the pod characteristics, make it undesirable for U.S. production (Barrientos-Priego, 1998).

With two known sources of genetic resistance in cultivated peanut, Shrestha et al. (2013) compared their mechanisms of resistance to further characterize the genetic materials available to breeders. Thrips mediated inoculation was used to provide the most realistic inoculation conditions in a controlled environment. Georgia Green, though considered to have mild resistance, represented the susceptible variety in this study. Georgia-06G, Georganic, Tifguard, and NC94022 represented resistance from the two available *hypogaea* sources. No significant difference was found between resistant genotypes for the TSWV symptoms observed, but there was a significantly higher level of virus copies found in NC94022 than the resistant plants derived from PI 203396. While all resistant lines screened in Shrestha et al. (2013) were deemed field resistant based on symptoms, viral load indicates that NC94022, and likely SSD6, have a different resistance mechanism than PI 203396. This not only means there is a chance to incorporate two different resistance mechanisms into breeding programs but to also combine resistances in a single variety.

QTLs for TSWV resistance

QTL analysis was used to evaluate the source of genetic resistance to TSWV in cultivated peanut with further aims to implement MAS and identify candidate genes underlying the resistance trait. Two RIL populations, the S- and T- populations, have been used to map QTLs linked to TSWV resistance from NC94022 and PI 203396, respectively. Qin et al. (2012) first developed these RIL populations: the S-population from a cross of TSWV resistant NC94022 with TSWV susceptible SunOleic 97R (Gorbet & Knauft, 2000) and the T-population from a cross of TSWV resistant Tifrunner (Holbrook & Culbreath, 2007) with TSWV susceptible GT-C20. Linkage maps using SSR markers were developed with 172 and 239 marker loci for the S- and T- populations, respectively (Qin et al., 2012). QTL analysis identified a major QTL on chromosome A01 of the S-population with 35.5% percent variation explained (PVE) and a QTL on LGJ15 of the T-population with 12.9% PVE (Qin et al., 2012). Continuing studies aimed to further develop the linkage maps of these populations and better resolve the QTL regions associated with TSWV resistance of each source.

The T-population was further mapped with 378 then 418 markers using the same RIL population (Pandey et al., 2017b; Pandey et al., 2014). Eleven QTLs were identified across multiple chromosomes for TSWV resistance, with resistance alleles coming from Tifrunner while susceptibility alleles were derived from GT-C20 (Pandey et al., 2017b). Agarwal et al. (2018) again mapped the population and identified a major QTL on chromosome B09 with 40.7% PVE, spanning a physical distance of 1.55 Mb and containing 114 gene annotations. This region was not mapped in the previous T-population studies and can be further explored for its effect on TSWV resistance from Tifrunner and PI 203396.

The S-population was further mapped with 206 then 248 marker loci using the original RIL population (Khera et al., 2016; Pandey et al., 2014). Agarwal et al. (2019) used a bin-mapping approach to further improve the S-population linkage map, producing a final map of 5,816 bins over 20 LGs and a total of 2,004 cM. NC94022 showed consistently low symptoms under TSWV pressure in each of the mentioned studies. QTL analysis identified major QTLs on chromosome A01 of the S-population, with resistance derived from NC94022; QTLs from each previous study were found in common locations on the updated maps (Agarwal et al., 2019; Khera et al., 2016; Qin et al., 2012). The greatest PVE for this QTL (36.51%) was identified in Agarwal et al. (2019). Two QTLs in this study overlapped within an 89.5 kb region on A01 containing 14 annotated genes. Kompetitive allele specific PCR (KASP) markers were designed using SNPs to distinguish allelic variation between resistant and susceptible lines within the population at this QTL (Agarwal et al., 2019). Khera et al. (2016) also identified QTLs in the S-population for both early and late leaf spot resistances, with one early leaf spot resistance QTL overlapping the major TSWV resistance QTL on A01.

Separate studies mapped TSWV resistance from Florida-EP™ '113', a TSWV resistant line derived from NC94022 x ANorden (Tseng et al., 2016; Zhao et al., 2018). An F_2 population of Florida-EP™ '113' x Georgia Valencia revealed a QTL on chromosome A01 for TSWV resistance derived from NC94022. This QTL was located between the major QTLs of Qin et al. (2012) and Khera et al. (2016) when comparing similar markers between all three studies (Tseng et al., 2016). This finding indicates that NC94022 and Florida-EP™ '113' may have different resistance QTLs even though they derive from the same source. More evaluation of both is needed. Zhao et al. (2018) refined the map developed in Tseng et al. (2016) and identified a 0.8 Mb interval on A01 containing nine gene models. TSWV resistance from Florida-EP™ '113'

was shown to have high heritability overall, but heritability was impacted by disease intensity in the field and the difference between visual symptom ratings versus immunostrip confirmation of virus presence (Tseng et al., 2018). Both NC94022 and Florida-EP™ '113' maintain high resistance to TSWV across all studies, confirming that NC94022, and likely its progenitor SSD6, is an important source of resistance for further exploration and implementation into breeding programs for improved peanut varieties.

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CHAPTER 2

VALIDATION AND INCORPORATION OF A QTL FOR RESISTANCE TO TOMATO SPOTTED WILT VIRUS IN AGRONOMICALLY IMPROVED *ARACHIS HYPOGAEA*

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To be submitted to *Crop Science*

Abstract

Arachis hypogaea (cultivated peanut) is an important crop that can be highly susceptible to Tomato Spotted Wilt Virus (TSWV). Genetic resistance is desired to prevent major yield losses. SSD6 and its progeny NC94022 have been introduced as resistance sources for breeding new varieties. Genetic evaluation of NC94022 previously identified a quantitative trait locus (QTL) for resistance on chromosome A01. An insertion region from NC94022 was identified from genome sequence and molecular markers were designed for marker-assisted selection. Eight populations derived from crosses of eight unique parents with a single recombinant inbred line (RIL) containing the insertion region were genotyped to identify individuals with and without the insertion. Field evaluation over three generations revealed a significant increase in TSWV resistance when the insertion region is present. All populations showed improved resistance compared with susceptible varieties and their unique parents but were not as resistant as NC94022 or the RIL parent. These results indicate the potential for improved TSWV resistance from SSD6 but show that additional resistance loci need to be explored. All lines were evaluated for agronomic traits and a subset were further genotyped for other traits of interest, including late leaf spot and nematode resistance and high oleic acid. Progenies were identified with genetic traits for diverse oleic acid profiles and multiple disease resistances, along with the field identified TSWV resistance. Further evaluation of these populations to select the best lines is necessary to provide producers with improved varieties and further understanding of genetic resistance.

Introduction

Cultivated peanut (*Arachis hypogaea* L.) is an important crop around the world for its nutritional and economic value. Over 50 million metric tons (mt) of peanuts were produced across Asia, Africa, and the Americas in 2023 (USDA, 2024c). The United States (U.S.) produces six percent of the world's peanuts, with the state of Georgia producing 55% of this, valued around $783 million (USDA, 2024a, 2024b). Eighty-five percent of the U.S. peanut market is runner-type peanuts followed distantly by virginia type at 10% of the market share (peanutsusa.com). Both market types are essential to the U.S. and world peanut markets, meeting different needs for manufacturing and consumer food products.

A. hypogaea is an allotetraploid species (2n = 4x = 40) of the genus *Arachis*. Cultivated peanut resulted from a hybridization of two wild diploid species (2n = 2x = 20), the A-genome *A. duranensis* Krapovickas & W.C. Gregory and B-genome *A. ipaensis* Krapovickas & W.C. Gregory (Bertioli et al., 2016; Grabiele et al., 2012). This event was followed by a spontaneous chromosome duplication and a period of genetic bottleneck that reduced available genetic diversity within the *A. hypogaea* species (Bertioli et al., 2019; Kochert et al., 1996; Stalker, 2017). Domestication introduced improved growth habit, yield, and harvestability compatible with modern farming practices (Kochert et al., 1996). Plant breeding has further improved these traits, with breeding programs increasing yields from 750 kg/ha to over 4,500 kg/ha between 1909 and 2017 (Holbrook, 2019). Breeding has also introduced resistance to diseases and abiotic stresses that may have been lost between *A. hypogaea* and its wild relatives or isolated landraces (Holbrook, 2019; Holbrook & Stalker, 2003; Pandey et al., 2012; Variath & Janila, 2017).

Tomato spotted wilt virus (TSWV) is one of the most destructive diseases for U.S. peanut production. In 2022, Georgia alone saw a five-percent yield loss to TSWV that cost producers

over $35 million (Kemerait, 2024). TSWV was first identified in U.S. peanuts in 1971 in Texas and is found in all peanut producing states today (Culbreath et al., 2003; Holbrook, 2019). This virus has been identified on all major peanut producing continents and is known to infect over 1,300 species besides peanut (CABI, 2020). In the U.S., TSWV is transmitted by two thrips species, *Frankliniella fusca* (tobacco thrips) and *F. occidentalis* (western flower thrips) (Riley et al., 2011; Srinivasan et al., 2018). Only adult thrips that acquired the virus as larva are able to transmit the virus to new plants (Riley et al., 2011; Sherwood et al., 2009). Thrips populations will peak one to two times during an average peanut growing season, but warmer temperatures can quicken the life cycle, resulting in greater thrips numbers during the season (Buechel, 2021; Srinivasan et al., 2018). If TSWV is present within these populations it can lead to greater transmission of the virus throughout a field and surrounding area. TSWV symptoms in peanuts present as chlorotic and eventually necrotic ringspots on the leaf surface and stunting of plant tissue above ground. Below ground symptoms may occur as misshapen or discolored pods and kernels (Culbreath et al., 2003). Asymptomatic infections may occur, with infected plants potentially being considered resistant when using visual assays only (Tillman & McKinney, 2018).

When TSWV first appeared in the southeastern U.S., Florunner (Norden et al., 1969) was the predominant runner variety in peanut production. Desired for its agronomic traits and yield, Florunner was also highly susceptible to TSWV, leading to over 12% yield loss by 1997 (Srinivasan et al., 2017). Resistance was found with the release of Southern Runner (Gorbet et al., 1987), followed by Georgia Green (Branch, 1996) which became the predominant runner variety by 1999 and helped save peanut production in the southeast (Clevenger et al., 2017b; Sundaraj et al., 2014). Georgia Green obtained its TSWV resistance from PI 203396, through its

moderately resistant parent Southern Runner (Gorbet et al., 1987). PI 203396 is considered one of the most impactful introductions to cultivated peanut, helping to expand the genetic diversity available in *A. hypogaea* and providing a significant economic benefit to producers encountering high disease pressure (Clevenger et al., 2017b; Isleib et al., 2001). Georgia Green is considered "first generation" resistance as newer varieties have been released with improved resistance, including the current most widely grown variety, Georgia-06G (Branch, 2007; Shrestha et al., 2013; Srinivasan et al., 2017; Sundaraj et al., 2014). Introduction of such resistance and improved management strategies reduced yield loss from TSWV to almost zero by 2010 (Srinivasan et al., 2017). Recent years, however, have seen an increase in TSWV pressure and loss, emphasizing the importance of having new resistant varieties available for grower selection (Kemerait, 2024).

Genetic resistance was also found in PI 576638 (also known as SSD6), an *A. hypogaea* subsp. *hypogaea* var. *hirsuta* landrace collected from Mexico (USDA, 1996). TSWV resistance from this PI was identified through its highly resistant offspring, NC94022 (F NC94022-1-2-1-1-b3-B; SSD6 x N91026E) (Culbreath et al., 2005). NC94022 has maintained significant TSWV resistance over many years of evaluation under high disease pressure (Culbreath et al., 2005; Li et al., 2012; Shrestha et al., 2013). The *hirsuta* background is undesirable in the current U.S. markets and production systems, but the high TSWV resistance makes this source an important donor for peanut breeding programs (Barrientos-Priego, 1998). Resistance mechanisms of NC94022 and PI 203396 are believed to be different, making the combination of these two sources of further interest to establish future varieties with enhanced resistance to TSWV (Shrestha et al., 2013).

Several studies have aimed to identify the genetic foundation of both sources by identifying quantitative trait loci (QTL) that may be linked to resistant phenotypes. Qin et al. (2012) developed two recombinant inbred line (RIL) populations: the T-population was a cross between PI 203396 resistant Tifrunner (Holbrook & Culbreath, 2007) and susceptible GT-C20; the S-population was a cross between NC94022 and susceptible SunOleic 97R (Gorbet & Knauft, 2000). Linkage map development and QTL analysis identified a major QTL on chromosome A01 of the S-population with 35.5% phenotypic variation explained (PVE) and a QTL with 12.9% PVE on linkage group 15 of the T-population (Qin et al., 2012). A second QTL from the T-population was identified on chromosome B09 with 40.7% PVE (Agarwal et al., 2018; Pandey et al., 2017b; Pandey et al., 2014). Several QTLs have consistently been identified on A01 using the S-population, with each subsequent publication showing increased QTL resolution (Agarwal et al., 2019; Khera et al., 2016; Qin et al., 2012). A major QTL with 36.51% PVE was identified on A01 mapped to an 89.5 Kb region containing 14 genes (Agarwal et al., 2019). Three SNPs identified within this QTL region were used to develop kompetitive allele specific PCR (KASP) markers that could differentiate between resistant and susceptible genotypes. Development and implementation of such markers associated with resistant phenotypes allows the application of marker assisted selection (MAS) that is aimed to reduce the time and cost required to evaluate and release a new variety to producers (Collard et al., 2005). Khera et al. (2016) identified two major QTLs for TSWV on A01 that overlapped with other QTLs for early or late leafspot resistance, exploring the opportunity for multiple disease resistance from these populations.

Using the Axiom_Arachis 58K and 48K SNP arrays to genotype the S-population RILs resulted in discovery of two new QTL between 12.04 to 12.34 Mb and 12.24 to 12.53 Mb,

respectively (Clevenger et al., 2018; Guo, 2021; Pandey et al., 2017a). Within the upper QTL lies an NBS-LRR (nucleotide binding site – leucine rich repeat) resistance gene and a 63 kb insertion was identified across across portions of both QTLs. The insertion was identified in NC94022 but not the susceptible SunOleic 97R or Tifrunner genotypes, making it of particular interest. Evaluating this QTL is the focus of the present study.

The objective of our study was to validate the effect of the TSWV-resistance QTL on chromosome A01 in a newly developed population derived from a cross between SSD6 and Tifrunner. This cross aimed to combine both sources of TSWV resistance into useful populations within our breeding program and variety pipeline. The second objective was to evaluate TSWV resistance derived from SSD6 and PI 203396 alone and in combination, using our populations as subjects. The final objective was to identify lines within these populations that may present multiple disease resistances with favorable agronomic traits using MAS and field evaluation.

Materials & Methods

Population development

The initial Peanut Genome Project strategic plan recognized a need for structured populations that were relevant to U.S. peanut production, and researchers moved forward with the development of 16 RIL populations (Holbrook et al., 2013). One of these, Tifrunner x SSD6, was the source of line F155 which was used to establish our populations. This RIL was selected for its consistent TSWV resistance and the presence of an insertion within the QTL region similar to NC94022. Eight populations were developed from RIL-F155 to incorporate improved TSWV resistance from SSD6 into advanced breeding lines and elite varieties. The eight unique parents of these populations were selected for their agronomic traits, including desirable growth habit and yield, TSWV resistance from PI 203396, and various disease resistances and oleic/linoleic acid contents (Table 2.1). Initial crosses were made in the greenhouse in 2020 and F_1 hybrids grown in 2021 to collect F_2 seed for further evaluation.

Genotyping

Mature seeds were randomly selected from the available F_2 seed for genotyping; a total of 2,407 seed representing 26 F_1 individuals across the eight populations were selected. Genomic DNA was extracted from a thin slice (3-5 mm diameter) of each seed following a high-throughput method from Xin et al. (2003) as detailed in Chu et al. (2011). SNP markers were previously identified within the A01 QTL region to differentiate between parental genotypes, Tifrunner or SSD6 (Chu, unpublished data). KASP assays were developed for three of these markers to identify the NBS-LRR resistance gene (R-gene) and insertion regions of interest (Table 2.2). Thermocycling and endpoint genotyping were performed on a Roche LightCycler® 480 II (Roche Applied Science, Indianapolis, IN) following a modified protocol described in

Chu et al. (2016). Each 5 µL reaction contained 2.5 µL KASP 2x genotyping mix, 0.07 µL primer mix, 1.93 µL water, and 0.5 µL 10X diluted DNA template. The primer mix (100 µM) contained 100 µM of each allele specific primer, 100 µM of common primer, and water to 100 µL. The thermal cycling program was completed as follows: activation at 95 C for 15 min, followed by 9 cycles of 94 C for 20 sec and 61 C for 1 min with the annealing temperature decreasing by 0.6 C per cycle, followed by 32 cycles of 94 C for 10 sec and 55 C for 1 min, and lastly 6 cycles of 94 C for 20 sec and 57 C for 1 min. Pre- or post-melt cycles occurred at 30 C for 1 sec and the plate was cooled to 25 C during reading. Oligos were 5'-labeled with FAM and HEX fluorophores to indicate separation of sample genotypes. If insufficient separation occurred after the first run, an additional three to nine cycles of 94 C for 20 sec and 57 C for 1 min were added as necessary to produce sufficient separation. Automated scatterplots identifying homozygous and heterozygous groups were analyzed for final genotype results.

Standard polymerase chain reaction (PCR) was used to confirm presence or absence of the insertion region in a subset of 261 individuals that were called as the susceptible allele or unknown at one or both insertion region KASP markers. Primers were designed to identify a 914 bp portion of the NC94022 specific insertion (Table 2.3). Tifrunner specific primers amplify a 699 bp region outside of the insertion (Table 2.3). Because of similarities within the insertion sequence and just outside, Tifrunner specific primers are present in all genotypes, i.e. lines with the insertion will amplify both sets of primers, while lines without the insertion will have only the Tifrunner specific amplicon (Figure 2.1). Genomic DNA was extracted from selected plants using a cetylrimethyl ammonium bromide (CTAB) method. Young leaflet tissue (70-100 mg) was frozen and pulverized using a vortex and 3-5 metal beads (4 mm diameter) in a 2mL microcentrifuge tube. Tissue was kept on liquid nitrogen and not allowed to thaw during

grinding. Extraction of DNA proceeded as follows: 500 µL of 2x CTAB (100 mM Tris-HCl Buffer [pH 8.0], 20 mM EDTA [pH 8.0], 1.4 M NaCl, 2% CTAB [hexadecyltrimethyl ammonium bromide], 1% PVP [MW 40,000], 0.2% 2-mercaptoethanol, and water to final volume) was added to ground tissue and mixed completely, followed by incubation at 65 C for 15 min. Chloroform: Iso-amyl alcohol (500 µL of 24:1) was added to each sample and inverted to mix, followed by centrifugation at 12,000 rpm for 5 min. The supernatant was transferred to a new microcentrifuge tube without disturbing the bottom layer and 400 µL Isopropanol was added followed by inversion to mix and centrifugation at 14,000 rpm for 5 min. The supernatant was discarded by decanting and the DNA pellet was washed with 500 µL of 70% ETOH. Dried pellets were resuspended in 250 µL of TE buffer (10 mM Tris, 1mM EDTA, pH to 8.0) with 1 mg/mL RNAse.

PCR was carried out using JumpStart Taq DNA polymerase (MilliporeSigma, Sigma-Aldrich, St. Lois, MO). Each 20 µL reaction contained 2 µL of 10X PCR buffer, 1.6 µL of 2.5 mM dNTPs, 1 µL each of 5 µM stock forward and reverse primers, 1.2 µL of 25 mM $MgCl_2$, 2 µL of 10% PVP, 0.2 µL BSA (10 µg/µL), 0.2 µL JumpStart *Taq* (2.5 U/µL), 9.8 µL HPCL grade water, and 1 µL 10X diluted DNA. The PCR reaction was initiated with activation at 94 C for 5 min, followed by 40 cycles of denaturation at 94 C for 30 seconds, annealing at 50 C for 30 seconds, and extension at 72 C for 1 min, and a final extension at 72 C for 7 min. Amplified products were visualized on a 1% agarose gel at 100V for 30 min; 1 µL of PCR product, 1 µL 10X loading dye, and 8 µL HPLC water was run for each sample.

Individual plant field evaluation

Individual seeds showing presence of the resistance allele at the R-gene were selected for further field evaluation. Genotyped seed was transplanted to the field on April 27, 2022, 13 days

after seeding in the greenhouse. Seedlings were planted at 1m spacing with 0.81-m between two rows of a bed and 0.96-m between neighboring beds. All field studies were done at the Gibbs Farm, Tifton, GA (31° 25' 51.816" N, 83° 35' 10.968" W). Early planting time and increased space between individual plants increases potential interactions with natural thrips populations for higher TSWV pressure. No chemical management was applied to the field at any time for thrips control. Fields were sprayed for leaf spot and white mold diseases, insecticides for worms and other non-thrips pests, and herbicide for weed management (Jason Golden, personal communication). Each plant was evaluated for TSWV resistance on August 10th at 118 days after greenhouse planting. TSWV was scored on a zero to five scale based on presence of TSWV symptoms and overall stunting (Table 2.4) (Li et al., 2012). Plants showing no symptoms were presumed to be resistant to TSWV as no additional testing was performed to confirm virus presence in the plant.

Plants with a score of zero to three were considered for advancement to the next generation; 565 plants were selected across all populations for harvest on September 6th. All pods were harvested from each selected plant.

Replicated plot field evaluation

Based on seed availability, 537 of the harvested lines were used for further studies in 2023. Five resistant and five susceptible lines, categorized based on 2022 results, from each population were advanced to the F_4 generation at the Illinois Crop Improvement Farm in Puerto Rico. All other lines were planted at the F_3 generation in an unreplicated trial in April 2023 to screen for resistance and agronomic traits. The $F_{3:4}$ families were planted in a separate field to further study their TSWV resistance in comparison with their parents and known resistant or susceptible lines. These 80 lines were planted on June 5, 2023, in a randomized block design with three

replications alongside TSWV resistant NC94022 and RIL-F155, seven of the eight unique parents (no seed was available for CS207), TSWV resistant York and Georgia-06G, and TSWV susceptible MarcI. Georgia-06G was used as a comparison for agronomic traits desired by growers. Each plot was 3-m long with 0.8-m between the two rows of each plot and 0.96-m between each bed. Seeds were directly planted in the field at a rate of 4 seed per 0.31m. Again, planting time and increased space were used to enhance TSWV pressure. Field management occurred as previously described.

Plots were visually rated for TSWV on October 3rd and 4th, at 120 and 121 days after planting. Percent of the plot canopy showing typical TSWV symptoms was represented by a one to ten scoring system, with 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10 equaling 0%, 1-10%, 11-20%, 21-30%, 31-40%, 41-50%, 51-60%, 61-70%, 71-80%, 81-90%, 91-100%, respectively (Culbreath et al., 1997; Tillman et al., 2007; Tseng et al., 2016). Each plot was evaluated for agronomic traits above and below ground, including canopy growth habit and distribution of pods around the taproot or along branches after inverting. All replicated F_4 generation plots were harvested on October 27th. Plots screened at the F_3 generation in a separate field were harvested on August 11th. Total weight of dried pods was collected for each plot. Pod and seed phenotypes were observed for the replicated plots only to confirm that progeny obtained the desirable traits of parental cultivars over the undesirable traits of SSD6 (Barrientos-Priego, 1998). Presented results from 2023 are for the replicated trail only.

Thirty-one of the 80 F_4 lines evaluated in 2023 were selected for further evaluation at the F_5 generation in 2024. These lines were again planted at the Gibbs farm in a different field than previous years. A randomized complete block design with three replications of each line was used, with Georgia-06G, TifNV-HG, and TifGP-2 as check lines for TSWV resistance and

agronomic traits. TifNV-HG and TifGP-2 are the unique parents for two of our populations (Table 2.6) All plots were planted on April 9th and final TSWV ratings were taken at 122 days after planting using the percent conversion scale described above. Plots were harvested on August 19th and total plot yield was collected as in previous generations.

Multiple trait genotyping

Resistance to multiple diseases is important for peanut producers. Each unique parental cultivar selected to develop our populations was chosen for its desirable agronomic traits and the presence of other important traits (Table 2.1). All 80 $F_{3:4}$ lines were screened with molecular markers linked to these additional traits. The high throughput DNA samples genotyped for F_2 selection in 2022 were again used here.

KASP markers (Table 2.5) were used for disease resistances following the same protocol described previously. Five markers were used across chromosomes A02 and A03 for late leaf spot (LLS) resistance (Lamon et al., 2021). Two markers on chromosome A09 were used to identify root-knot nematode resistance from an *A. cardenasii* introgression (Chu et al., 2016).

A HybProbe melting assay was used to screen for high oleic acid content based on the AhFAD2B mutation using the protocol and primers described in Chu et al. (2011). Each 2.5 µL reaction contained 0.6 µL of 5x Roche genotyping master mix, 0.18 µL of 25 µM MgCl2, 0.12 µL of 50 µM antisense primer, 0.3 µL of 50 µM sense primer, 0.3 µL each of 20 µM HybProbe-1 and -2 for the antisense and sense primers, respectively, and 1.51 µL water. Reactions were run on the Roche LightCycler® 480 II with each melting curve produced as follows: pre-incubation at 95 C for 10 min, followed by 55 cycles of amplification at 95 C, 57 C and 72 C for 10 sec each, followed by a melting curve analysis at 95 C for 1 min, 40 C for 2 min and an increase to 95 C at 0.11 C per sec with continuous fluorescence acquisition, and finally a cool down to 40 C.

Melting curves for oleic content were analyzed between 55 and 68 C. Peaks around 68 C indicate a high oleic allele, while peaks around 60 C indicate a normal oleic allele. Samples may also be heterozygous with peaks at both temperatures.

Statistical analysis

All statistical analyses were conducted in R-studio using analysis of variance (ANOVA) to determine statistical differences in TSWV ratings, post-harvest traits, and the effect of QTL presence on TSWV resistance. Tukey's honest significant difference (HSD) test was used to separate individuals or families for each trait. Correlation between TSWV ratings in each generation was compared using Pearson's correlation. A p-value threshold of 0.05 was used to determine significance for all analyses.

Results

Genotyping

Of the 2,407 F_2 seed genotyped, 1,691 individuals contained the favorable allele from SSD6 for the R-gene and were planted for evaluation in 2022. Only 1,651 plants survived transplanting to reach TSWV evaluation, of which 1,406 individuals contained the insertion region. The remaining 245 individuals with the R-gene only were grouped into three of the eight populations (Figure 2.2). All populations were used to evaluate TSWV resistance, but only the three with separate groupings of the QTL region were used to explore the effect of the insertion region on resistance. Eight lines lacking the insertion region were evaluated further as part of the 80 lines in the $F_{3:4}$ generation.

TSWV resistance

TSWV intensity was high during all three years of this study. During growth of the F_2 generation in 2022, TSWV symptoms were first observed in early June, around two months after transplanting into the field. Plants showing the earliest symptoms had greater stunting and higher disease ratings at the end of the season. All populations showed a distribution of individuals between resistant (0) and susceptible (4 or 5), with moderate TSWV resistance between scores of 1 and 2 on average (Figure 2.3). Lines harvested in 2022 scored only between 0 to 3, with lines having the highest resistance scores taking priority.

TSWV rating in the F_4 replicated plots in 2023 again ranged from resistant to highly susceptible, with average scores across the three replications ranging from 1.83 to 9.67 for all populations. There was a significant difference (p-value < 0.001) between lines from SSD6-derived populations and all check lines, including the seven unique parents (Figure 2.4). No lines were significantly more resistant than NC94022 and only two lines, 817 and 804, showed better

resistance across the three replications than RIL-F155 (Figure 2.5, Table 2.6). All lines were on average more resistant than susceptible check MarcI with a TSWV score of 10 (p-value < 0.001). Five lines had a single replication and three lines had two replications with susceptibility equivalent to all MarcI plots; all other plots have resistance scores less than 10 (Figure 2.5). The level of significance varies for each population in comparison to their unique parent but the average TSWV score for each population is better than that of its parent when available for testing (Figure 2.4). All populations have significantly better TSWV resistance than Georgia-06G (p-value < 0.001), with only 13 of the 80 lines being in a more susceptible significance group (Figures 2.4 and 2.5).

TSWV ratings in 2024 showed similar trends to 2023, with average scores of the three replications ranging from 1.67 to 8.67. There was again a significant difference between lines from the SSD6-derived populations and check lines used in 2024 (p-value < 0.001), including two parental lines and Georgia-06G (Figure 2.6). Lines 817 and 804 again showed high resistance, along with lines 819 and 1469, which showed higher resistance in 2024 than in previous years (Figure 2.6, Table 2.6). Unlike previous years, there was not a significant difference between the overall populations and the checks (p-value = 0.099) (Figure 2.7).

There was not a significant correlation between TSWV scores in 2022 and 2023 for lines evaluated in both generations (p-value = 0.22) (Figure 2.8). There was a weak positive correlation between the two years (R = 0.14). Correlation between lines evaluated in both 2023 and 2024 was significant, however. A very strong, significant positive correlation was seen between these two studies (p-value < 0.001, R = 0.78), even with different planting times and environments (Figure 2.9).

Only three populations allowed for evaluation of lines with and without the insertion region. There was a significant difference between TSWV resistance of lines with the insertion region and those without; p-value less than 0.001 in all years (Figure 2.10). When the insertion region is present, the average TSWV score is 5.6 compared to 8.46 when the insertion region is absent, based on 2023 ratings (Figure 2.10B). These averages are lower in 2024, 3.33 and 6.4 with and without the insertion, respectively (Figure 2.10C). These populations contain resistance from both SSD6 and PI 203396 genetic sources. When the insertion region is present, lines from this combination are significantly more resistant than PI 203396 sources alone (p-value < 0.001) (Figure 2.11). Lines without the insertion region are comparable to their unique parents and susceptible MarcI (Figure 2.11). Again, no lines are consistently more resistant than NC94022, even with the insertion region and combined resistance sources. Two lines, 817 and 804, show the most similar resistance to NC94022 (Figure 2.5). Consistency of the resistance in these lines was seen in 2024 (Figure 2.6) but further observations of these lines and others with resistance should be explored.

Agronomic traits

All lines from the eight populations were phenotypically similar to their unique parents in pod and seed characteristics, including shape, size, and color. No numerical data was collected for these traits. Georgia-06 showed symptoms of TSWV on the seed coat, including dark red discolored patches (Figure 2.12). Only one experimental line showed these symptoms under the same TSWV pressure and environmental conditions. TSWV is not seed transmitted but symptoms may appear on some seed coats (Srinivasan et al., 2017).

Total yield was collected as pounds per acre (LB/Acre) under high TSWV pressure for each plot in 2023 and 2024. All but five lines produced significantly higher yields than Georgia-

06G in 2023 (p-value < 0.001) (Figure 2.13). There was a significant difference between each RIL-derived population and Georgia-06G (p-value < 0.001) except for population C2924, which did however yield numerically higher than Georgia-06G on average (Figure 2.14). Susceptible MarcI yielded higher than Georgia-06G in 2023. Only seven of the eight populations were evaluated in 2024 and there was not a significant difference between their yields and those of the check lines (p-value = 0.17) (Figure 2.15). There was however a numerical difference in the median values for each population, with the three replications of Georgia-06G averaging higher than all but one line, 922 (Figure 2.15 and 2.16). Georgia-06G yielded over nine times greater in 2024 as it did in 2023 (Figure 2.14 and 2.16). All lines, including the checks, yielded higher in 2024 than in 2023, though the negative correlation between years was not significant (p-value = 0.18; R = - 0.23) (Figure 2.17).

When comparing yield to TSWV ratings, there was a significant but weak negative correlation between TSWV score and total plot yield in 2023 (p-value = 0.008; R = - 0.27) (Figure 2.18A). This correlation was not significant in 2024 with weaker negative correlation between yield and TSWV than 2023 (p-value = 0.3; R = - 0.18) (Figure 2.18B).

Multiple trait MAS

Of the 80 F_4 lines screened with the LLS, nematode, and high oleic acid markers, 21 showed the favorable allele for one additional trait and 14 for a combination of two traits (Table 2.7). No lines had markers for all three traits. LLS and nematode resistances were screened with multiple markers so any line containing the resistance allele for at least one of these markers was considered positive for the trait. Seventeen of the lines positive for non-TSWV traits also had high TSWV resistance during the first two seasons of evaluation (Table 2.7).

Discussion

Impact of TSWV was high across Georgia peanut production regions in 2022 and 2023 (Kemerait, 2024) and our research plots were no exception to this trend. Given this status, the continued exploration of genetic resistance is essential for producers to combat yield loss to TSWV. Numerous years of use in peanut production have shown the significant effect of resistance from PI 203396, but losses to TSWV remain high (Clevenger et al., 2017b; Isleib et al., 2001; Srinivasan et al., 2017). The introduction of SSD6 has shown potential to provide further resistance to TSWV, with its resistance likely coming from a QTL on chromosome A01 (Agarwal et al., 2019; Guo, 2021; Khera et al., 2016; Qin et al., 2012). An insertion region within this QTL, identified from resistant parent NC94022 of the S-population, is believed to be the important factor in this resistance. Marker assisted selection was used in our study to identify individual lines from eight populations with the presence or absence of this insertion region, along with an NBS-LRR resistance gene found within a close neighboring QTL (Guo, 2021). Identifying lines with improved TSWV resistance using MAS could have significant impact on the time and expenses associated with releasing resistant peanut varieties as seen with the release of TifNV-high O/L with nematode resistance and high oleic acid (Collard et al., 2005; Holbrook et al., 2017). Our populations here are a combination of resistance from both SSD6 and PI 203396 to not only identify the effects of the A01 QTL on resistance but explore a combination of resistances.

Evaluation of individual plants in 2022 provided preliminary data on the resistance present in these populations. While TSWV ranged from high resistance to high susceptibility, presence of the insertion region significantly increased resistance overall. Plants with both the R-gene and insertion region showed a significantly lower TSWV score than those with the R-gene only,

indicating the importance of the insertion region for resistance. In 2023 and 2024 replicated trials, the presence of the insertion region again showed a significant decrease in TSWV rating, further confirming the importance of the insertion and validating the usefulness of the SNP markers used to identify this region. The QTL region explored here is more narrow than that identified on A01 in the previous reports of Qin et al. (2012), Khera et al. (2016), and Agarwal et al. (2019), matching most closely to that presented by Guo (2021). All reports showed that presence of the major QTL on A01 is related to improved TSWV resistance derived from NC94022. Agarwal et al. (2019) developed KASP markers that separated the resistant from susceptible lines and the markers used in this study are a further exploration of that work.

The goal of MAS is to limit the plants evaluated in the field by using genetics to establish a starting population. Markers used here were successful in identifying the insertion region, and TSWV ratings indicate that absence of this region accompanies increased susceptibility. While there is a significant difference between the rating of lines with and without the insertion region, some lines with the insertion region still showed a potential to be susceptible to TSWV. Therefore, the markers identifying the insertion region may be used to eliminate a number of susceptible lines, but those with the region should still be phenotyped to confirm field resistance. It also emphasizes the need to screen over multiple years and under multiple environmental conditions, as some individuals with the insertion region were considered resistant in 2022 but were deemed susceptible under replicated plot conditions in 2023. This may in part be due to the varied planting times, April vs June in 2022 and 2023, respectively, and different environmental conditions each year resulting in different disease pressure (Riley et al., 2011; Srinivasan et al., 2018). April planting in 2024 showed consistent results for TSWV pressure and ratings as in

2023, indicating that even under different environments and planting times, the lines in our populations have potential to show improved and consistent resistance.

Another explanation is that of asymptomatic infections or missed infection using individual plants (Tillman & McKinney, 2018). Plants showing no symptoms were presumed resistant and lack of replication in 2022 likely resulted in false negatives in some cases. Introduction of plot replication in 2023 and 2024 provided a more reliable representation of the resistance in these populations. High resistance was maintained in replicated plots but expectations of resistance or susceptibility based on individual plant observations were inconsistent.

Resistance from SSD6 is observed when focusing on the A01 QTL region, as described above. Effect of combining resistance from PI 203396 and SSD6 was observed when comparing our eight populations to lines from only PI 203396, including the unique parents and Georgia-06G. When looking at lines with and without the insertion region, those without have TSWV scores most similar to the control lines derived from PI 203396. Some lines with the insertion region are similar to these controls, but most show a greater resistance. In comparison to NC94022 or RIL-F155, both derived directly from SSD6, lines with the insertion region and PI 203396 in their background are significantly more susceptible on average. Lines 817 and 804 are the only two with resistance in the same significance group as RIL-F155, making them important lines to focus on in future studies. Repeated evaluation of these lines in 2024 indicates they can maintain their high TSWV resistance across planting times and environments.

Our results agree with previous reports that NC94022 shows high resistance to TSWV under field observation (Baldessari, 2008; Culbreath et al., 2005; Shrestha et al., 2013). This consistent resistance of NC94022 indicates the potential that even greater resistance from SSD6 is still available. Our populations were selected with a specific focus on the A01 QTL using the

insertion region without consideration for other areas of possible importance. Tseng et al. (2016) and Zhao et al. (2018) each reported a second possible QTL derived from NC94022 for TSWV resistance using Florida-EP™ '113', showing there may be other regions involved in TSWV resistance from SSD6. Genomic evaluations using the Khufu sequencing pipeline (Korani et al., 2021) are being done to compare SNPS across the sequence of SSD6 with that of Tifrunner, NC94022, our RIL parent, and the populations developed here to identify further areas that might indicate important locations for resistance (data not shown). Pangenome evaluations are also of use for whole genome comparison and can be implemented for both the insertion region and other areas of chromosome A01 or beyond (Sameer Pokhrel, personal communication).

Agronomic traits and multiple disease resistance were important points of exploration in this study. Observation of individual plants and small plots showed that all lines produced would meet grower expectations for pod and seed characteristics. SSD6 shows the typical hirsuta characteristics of deeply constricted and beaked pods with purple seeds (Barrientos-Priego, 1998). The lines produced here lack these characteristics, matching more closely to the desired varieties and breeding lines used as unique parents. Above ground traits were similar to those of Georgia-06G for all lines, with slight variations of mainstem height, spread, and leaf color in the field. Yields of all but five experimental lines were greater than that of Georgia-06G under the given conditions in 2023. This establishes a good foundation for the success of these lines with both TSWV resistance and quality production. It is important though to further test these lines, as Georgia-06G was not as resistant or productive as expected under these small plot, high TSWV conditions in 2023 (Branch, 2007; Skaggs, 2024; Sundaraj et al., 2014). Low production by Georgia-06G in this test may in part be due to the late planting, lower seed density, and no TSWV management practices used in this study, all opposite of what growers and variety trials

implement to obtain high yields and productive plots. Results from 2024 show a slight increase in the average resistance and much improved yields of Georgia-06G across the three replications compared to 2023. Improved production under similar disease conditions in 2024 versus 2023 emphasizes the need for multiple year and environment evaluations as a single year may result in less than expected results. This second year of studies shows that our breeding lines may not produce as high as Georgia-06G as seen in 2023, but they are still competitive under high disease pressure.

Marker assisted selection to identify lines with potential multiple disease resistances and varying levels of oleic acid contents demonstrates the value of these populations for further cultivar development. Lines from our populations were identified to have resistance alleles to late leaf spot and root-knot nematodes, both significant pests in Georgia peanut production (Kemerait, 2024; Kemerait et al., 2004). Multiple disease resistance is an important production factor in environments affected by various diseases simultaneously. Oleic acid content is important for consumer products, especially confectionary products that may require longer shelf life with maintained quality. Providing producers with varying oleic acid profiles allows them to broaden the products they provide buyers and consumers. Multiple disease resistance and varying oleic acid contents combined with quality TSWV resistance and acceptable agronomic traits enhances the usefulness and profitability of these populations for producers in the future.

Table 2.1 Unique parents for development of eight RIL-derived populations

Population ID	Unique Parent	Market type	MAS traits	Cultivar release
C2920	TifNV-HG (C1805-617-2 x Georgia-06G)	Runner	Nematode resistance High oleic	(Holbrook et al., 2023)
C2921	TifJumbo (C1805-2-9-16 x Bailey Hi O/L)	Virginia	Nematode resistance Late leaf spot resistance * High Oleic	(Holbrook et al., 2024)
C2923	CS196 (Georgia-13M x C2593-F2-34)	Runner	Late leaf spot resistance High oleic	Breeding line **
C2924	CS207 (TifNV High O/L x C259-F2-293)	Runner	Nematode resistance Late leaf spot resistance High oleic	Breeding line **
C2925	TifNV-High O/L (Tifguard x Florida-07)	Runner	Nematode resistance High oleic	(Holbrook et al., 2017)
C2926	Tif GP-2 (C-99R x COAN)	Runner	N/A	(Holbrook et al., 2012)
C2927	Georgia-18RU (GA 052530 x GA 032913)	Runner	N/A	(Branch, 2019)
C2928	Georgia-16HO (Georgia-07W x Florida-07)	Runner	High oleic	(Branch, 2017)

* Marker indicated resistance only, not verified in field
** Holbrook, USDA Crop Genetics and Breeding Research

Table 2.2 KASP markers for TSWV marker-assisted selection

Marker ID	Marker position *	QTL region	Allele specific primer [resistant / susceptible allele]	Common primer
TSWV_18	12,060,020	R-gene	GGTCTCGAAAGTTTATAGGGCA **[G/A]**	GGTGTGAGATTATATCTAATAGTAC GAG
TSWV_22	12,333,618	Insertion	GGTTTTTATCGGTTCACTATGGGTT TGAC **[T/C]**	GTCCGCTAATTAGACCGCACAAG
TSWV_10	12,366,499	Insertion	AAAGACTATTCTCCAGTTCCCAG **[T/G]**	GGAATGTTTGGGAGGACAAG

*positions on arahy.Tifrunner.gnm2 from https://www.peanutbase.org/genome/ and Bertioli et al., 2019

Table 2.3 PCR primers to identify presence of insertion region within A01 QTL

Primer	Identifies	Sequence (5' – 3')	Expected band (bp)
Common	NA	GGGAGAACCAATCCCTTGA	NA
NC94022 specific	Insertion present	GCCGCCATTATTTATCGTTT	914
Tifrunner specific	No Insertion	GGAAGAATAACATTCATGTCCAAA	699

Table 2.4 Single plant TSWV rating system (Li et al., 2012)

TSWV score	Phenotype description
0	No symptoms No stunting
1	Minimal symptoms No stunting
2	Moderate symptoms Mild stunting
3	Moderate symptoms Noticeable stunting
4	High symptoms High stunting
5	Severe symptoms Severe stunting

Table 2.5 KASP markers used for late leaf spot and nematode resistance traits

Resistance	Location (chromosome_bp)	Allele specific primer [resistant / susceptible]	Common primer
Late Leaf Spot	A02_910314	AAAATTAGCAACGGCCAAAA**[T/C]**	TGACGTACGTAGAGATCAAATGG
	A02_2618876	GGTGGTGATGGTAGGGAAG**[A/T]**	ACCGTTTGGTTGAGCAGATT
	A02_85484881	GCACAAAACAATGTGCCTGTA**[T/A]**	AAGTGGATGCATTGGTGGTT
	A02_80149907	ACGTGCTTGTCCTCTAAGG**[T/C]**	GAATAGGACAAAAATGCAATGTG
	A03_134516425	TTTCGGTGTCATCCCCA**[G/C]**	CAGCTATTATATGCTTCATTCATTG
Nematode	Rma_A09_5946954	GAAGGTCGGAGTCAACGGATTTATC CCTTTTCCCTCTCTCTTT**[T/C]**	CAGCAGCAGCTTTCCTTTCT
	Rma_A09_37106181	**[A/G]** **	**

** proprietary primer sequences from LGC Biosearch Technologies (Middlesex, UK)

Table 2.6 Line IDs for F_4 lines evaluated in replicated field studies

Population	C2920	C2921	C2923	C2924	C2925	C2926	C2927	C2928
Unique parent	TifNV-HG	TifJumbo	CS196	CS207 *	TifNV-High O/L	Tif GP-2	Georgia-18RU	Georgia-16HO
Line IDs in population	10	124	475	738	798	828	891	1079
	29	153	483	757	803	829	915	1084
	45	154	506	761	804	843	920	1088
	46	163	514	767	805	847	922	1139
	58	269	542	768	809	848	925	1216
	69	277	603	776	810	868	928	1259
	84	282	609	787	811	869	949	1292
	86	312	616	790	815	879	987	1348
	89	344	683	793	817	880	998	1445
	95	377	722	795	819	881	1063	1469

* CS207 not included as a unique parent check in 2023 replicated field study

Table 2.7 Multiple trait MAS for lines evaluated at F_4 generation. Ten lines screened for each population.

Population	Unique parent	Late Leaf Spot	Nematode	High Oleic Acid	Late leaf spot + Nematode	Late leaf spot + High Oleic Acid	Nematode + High Oleic Acid	+ TSWV resistance
C2920	TifNV-HG	--	1	4	--	--	1	3
C2921	TifJumbo	2	2	2	1	0	0	3
C2923	CS196	2	--	1	--	5	--	4
C2924	CS207	0	0	1	2	1	1	3
C2925	TifNV-HighO/L	--	2	0	--	--	3	3
C2926	Tif GP-2	--	--	--	--	--	--	--
C2927	Georgia-18RU	--	--	--	--	--	--	--
C2928	Georgia-16HO	--	--	4	--	--	0	1
Total		4	5	12	3	6	5	17

-- phenotype is not present in population based on unique parent genotype; traits listed for each parent in Table 2.1

Figure 2.1 Distribution of PCR primers across the A01 insertion region to identify NC94022 specific insertion. Black bar represents portion of chromosome A01. A) Tifrunner genotype, location of where insertion would be located is indicated by gray bar. B) NC94022 genotype, 63 kb insertion represented by gray bar. Yellow bar shows location of common primer in both genotypes; blue bar shows location of Tifrunner specific primer; green bar shows location of NC94022 specific primer; primer sequences identified in Table 2.3. * = Tifrunner amplicon, 699 bp. ** = NC94022 insertion amplicon, 914 bp.

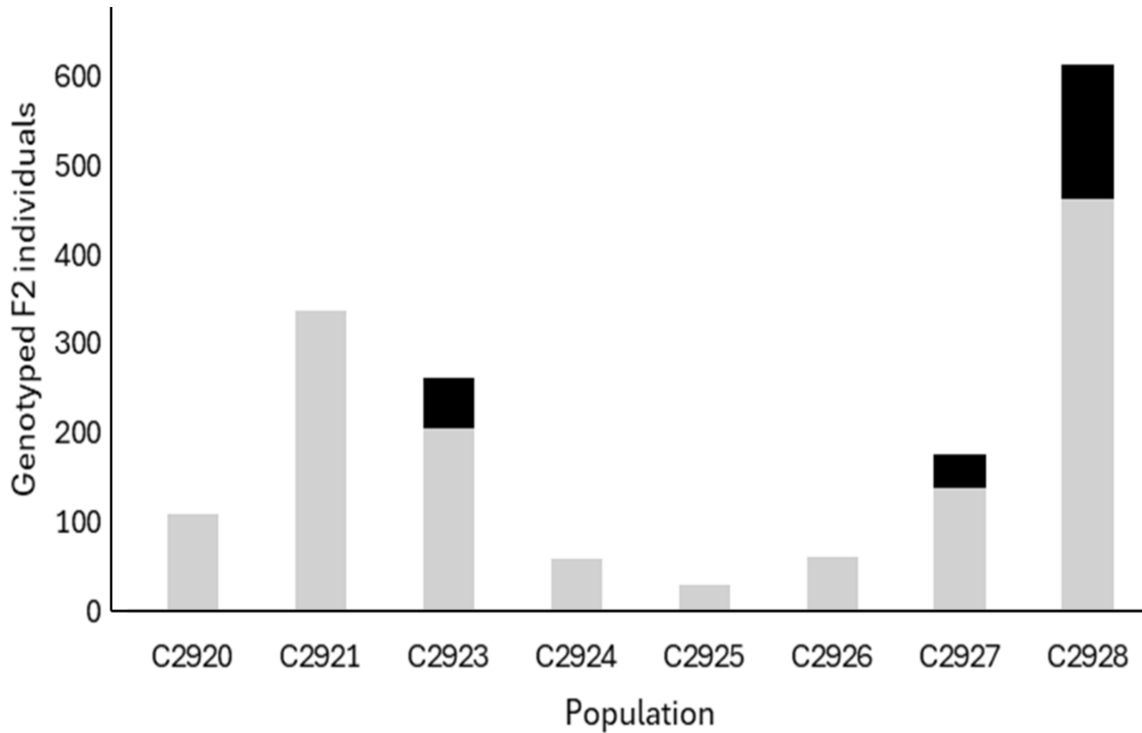

Figure 2.2 Distribution of genotypes at F_2 generation across eight populations. Gray bar shows individuals containing the R-gene and insertion region based on KASP and PCR genotyping. Black bars show individuals without the insertion region, R-gene only.

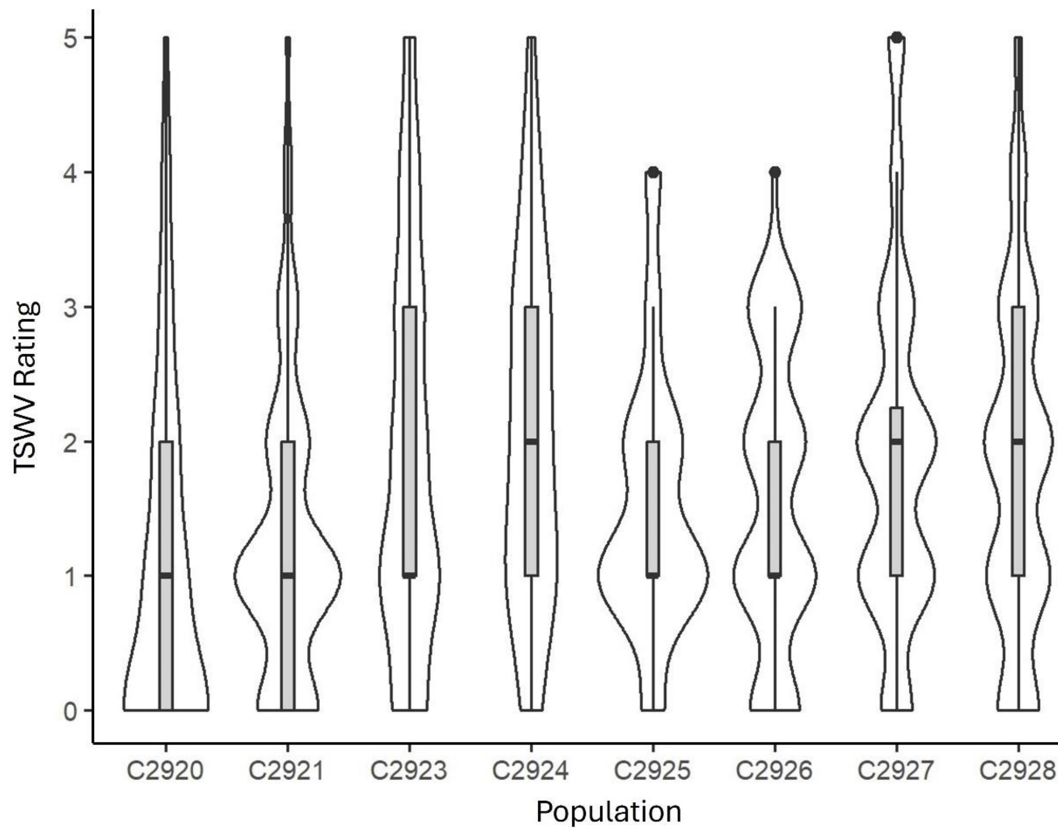

Figure 2.3 Violin plots showing distribution of TSWV ratings of F_2 individual plants during 2022 field evaluation. Ratings for all individuals across eight populations. Boxplot inside violin plot shows distribution of data points. Black horizontal line indicates median and gray boxes represent data within the interquartile range. Black dots represent outliers beyond the upper extreme quartile.

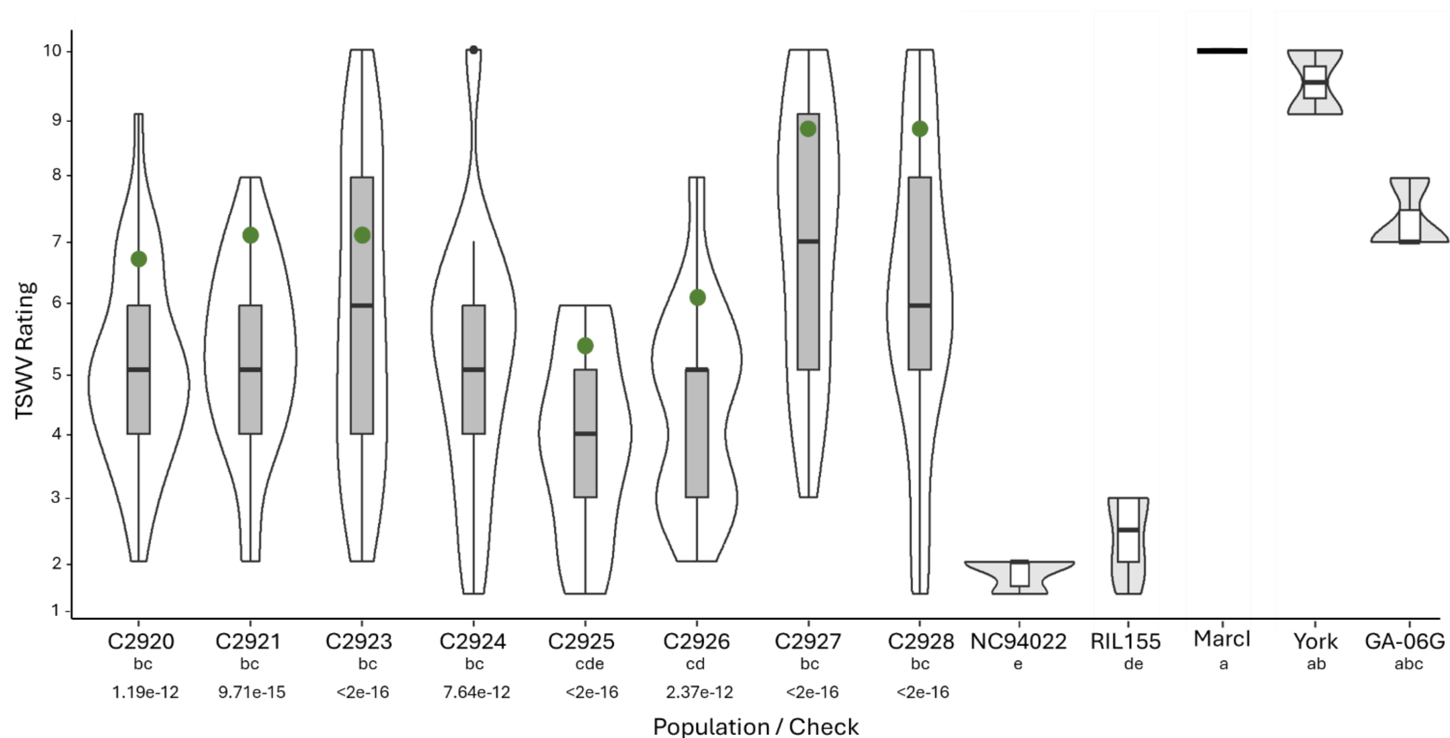

Figure 2.4 Violin plots showing distribution of TSWV ratings of replicated plots during 2023 field evaluation. Ratings for 80 $F_{3:4}$ families across eight populations (white violin plot) and check lines (gray violin plots). Significant difference between average values of eight populations and check lines, p-value < 0.001. Significance groups identified under population name. Green dots represent TSWV rating for unique parent of given population, when available. p-values for difference between unique parent and progeny lines indicated under population name. Box plots within violin plots show distribution of data points. Black horizontal line indicates median in all boxes. Gray and white boxes represent data within the interquartile range of populations and checks, respectively. Black dot on C2924 represent an outlier beyond the upper extreme quartile.

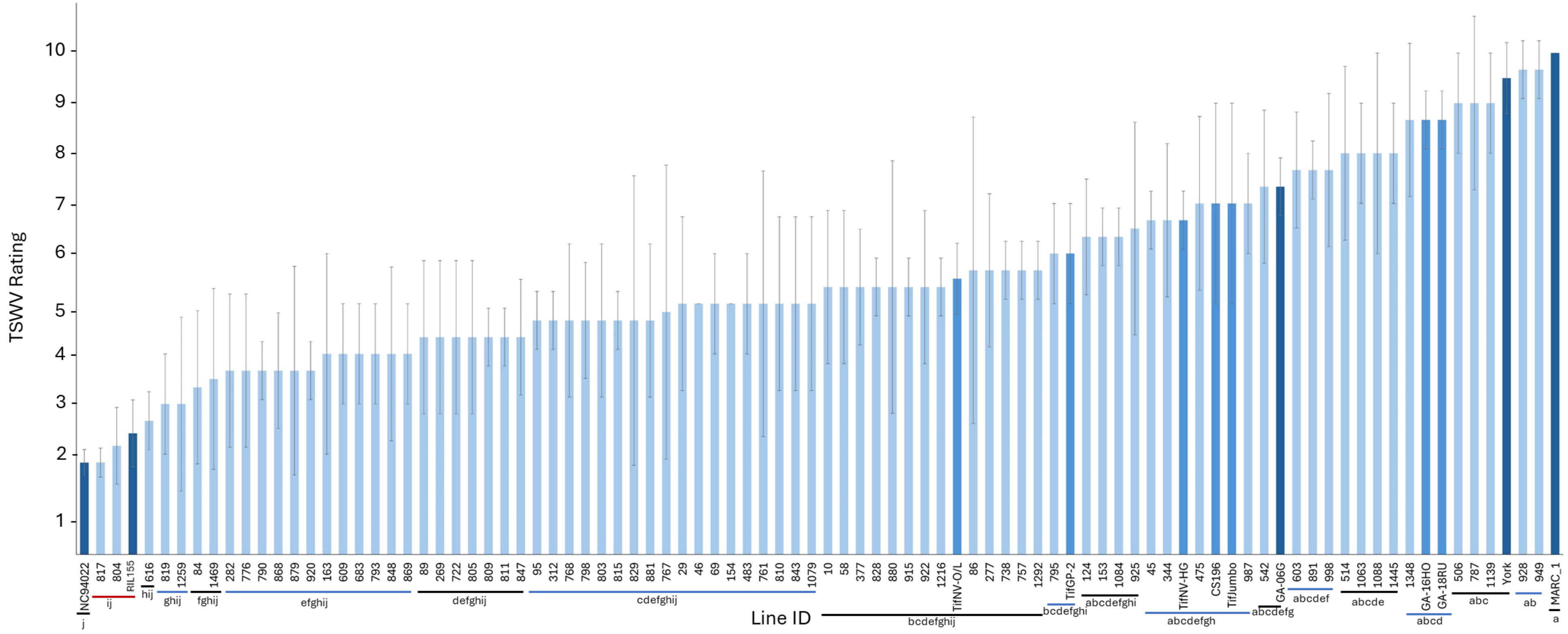

Figure 2.5 TSWV rating of individual F_4 lines and checks during 2023 field evaluation. Average of all replicated plots with error bar showing distribution of rating. Dark blue bars represent check lines. Medium blue bars represent unique parents of populations. Light blue bars represent the 80 F_4 lines. Significant difference across all lines, p-value < 0.001 Significance groups indicated under Line IDs; alternating black and blue lines indicate separation of 19 significant groups from A to J based on Tukey-HSD for ANOVA. Red line indicates Line IDs in closest significant group to RIL-F155 and NC94022. Table 2.6 provides breakdown of lines into eight populations with parents.

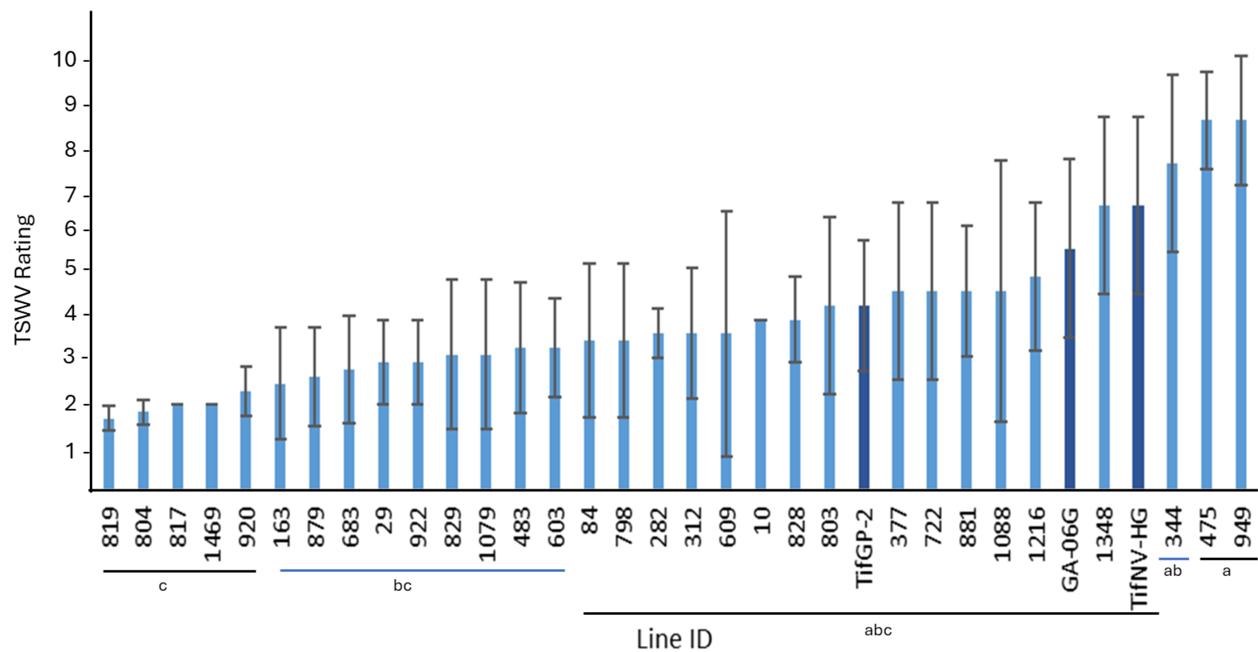

Figure 2.6 TSWV rating of individual F_5 lines and checks during 2024 field evaluation. Average of all replicated plots with error bar showing distribution of rating. Dark blue bars represent check lines. Light blue bars represent the 31 F_5 lines. Significant difference across all lines, p-value < 0.001 . Significance groups indicated under Line IDs; alternating black and blue lines indicate separation of five significant groups from A to C based on Tukey-HSD for ANOVA.

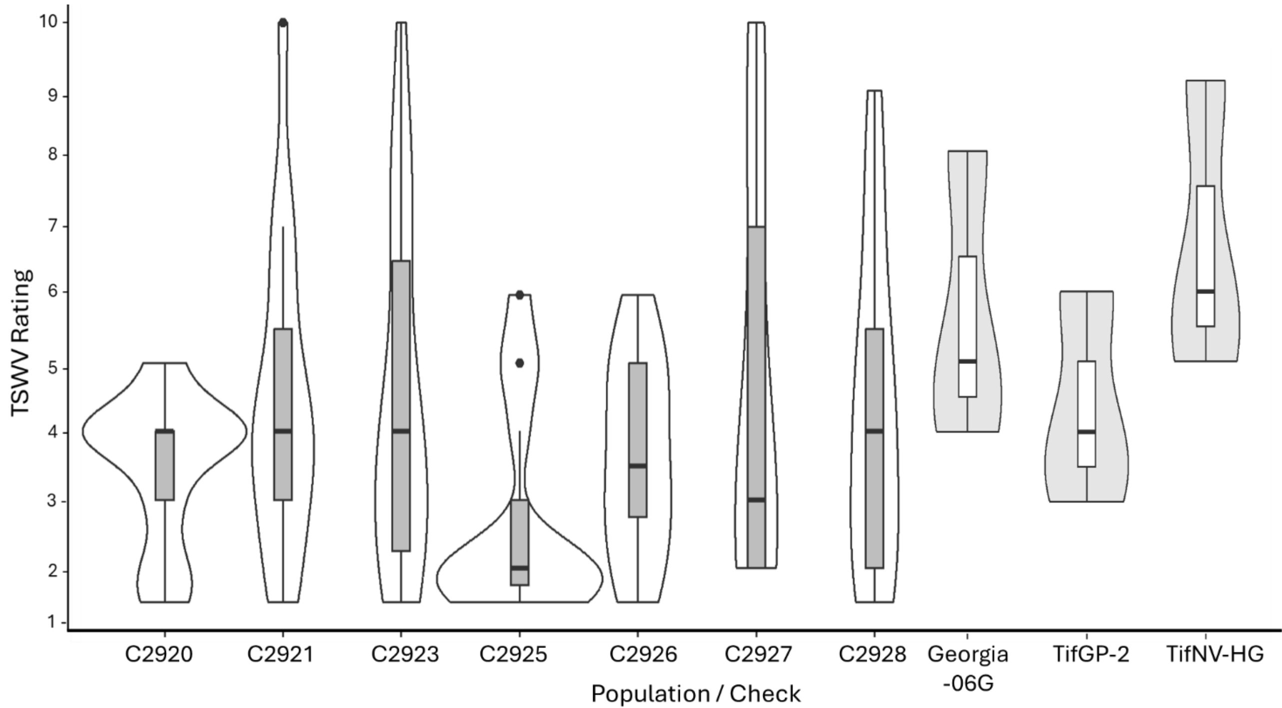

Figure 2.7 Violin plots showing distribution of TSWV ratings of replicated plots during 2024 field evaluation. Ratings for 31 $F_{4:5}$ families across seven populations (white violin plot) and check lines (gray violin plots). No significant difference between the seven populations and checks, p-value = 0.099. Box plots within violin plots show distribution of data points. Black horizontal line indicates median in all boxes. Gray and white boxes represent data within the interquartile range of populations and checks, respectively. Black dots on C2921 and C2925 represent outliers beyond the upper extreme quartile.

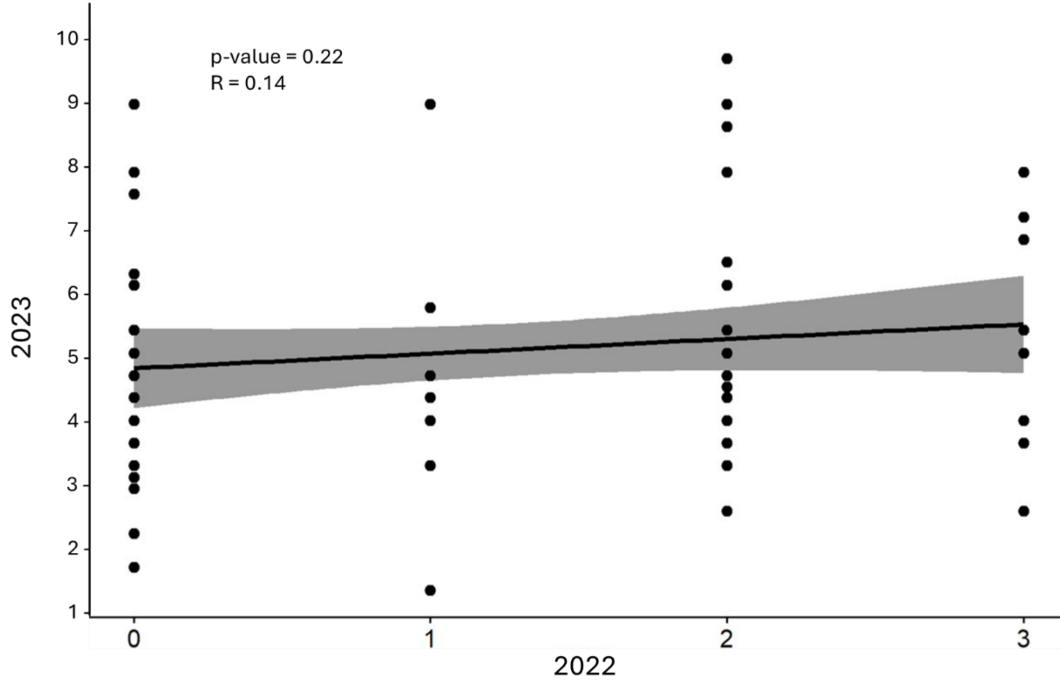

Figure 2.8 Pearson's correlation of TSWV ratings between individual plants and replicated plots in 2022 and 2023. Lines evaluated at the F_2 and F_4 generation in 2022 and 2023, respectively. Average TSWV rating of replicated plots used to determine 2023 value for correlation with individual value in 2022. p-value = 0.22, R = 0.14

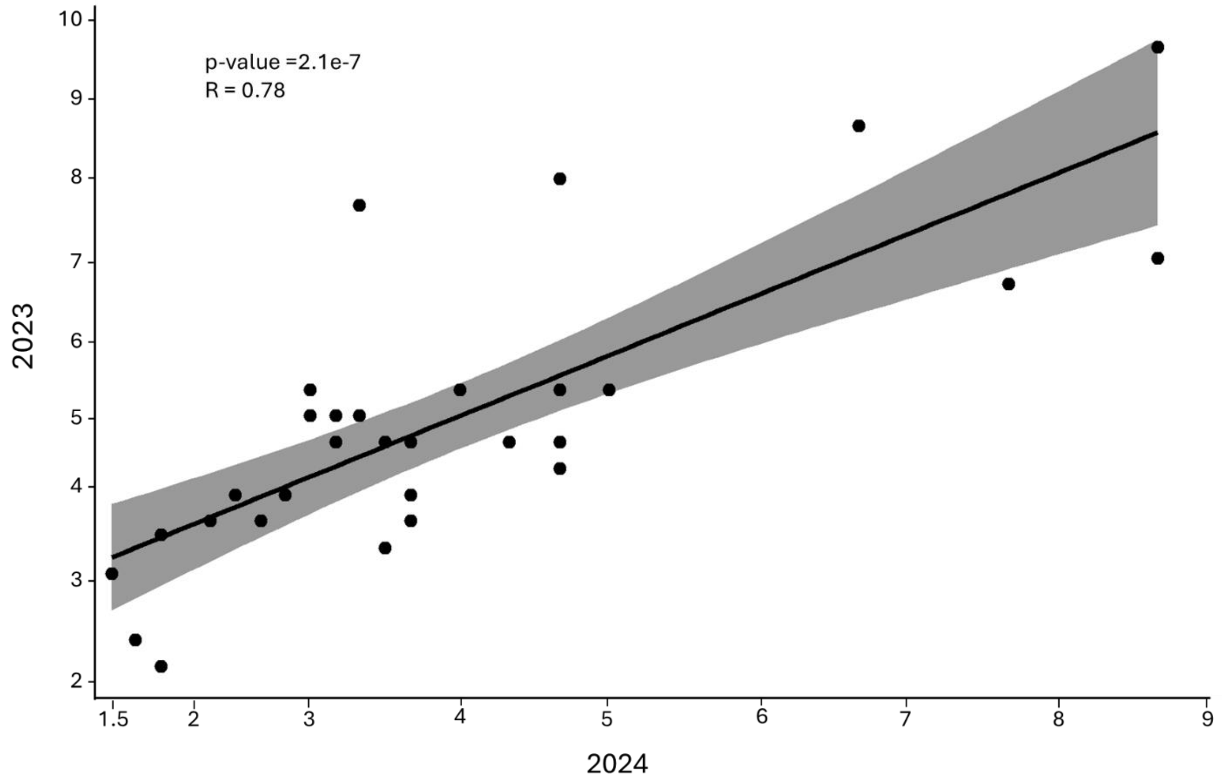

Figure 2.9 Pearson's correlation of TSWV ratings of replicated plots for lines evaluated in 2023 and 2024. Lines evaluated in the F_3 and F_4 generations in 2023 and 2024, respectively. Average TSWV rating of three replicated plots used for both years. p-value < 0.001. R = 0.78.

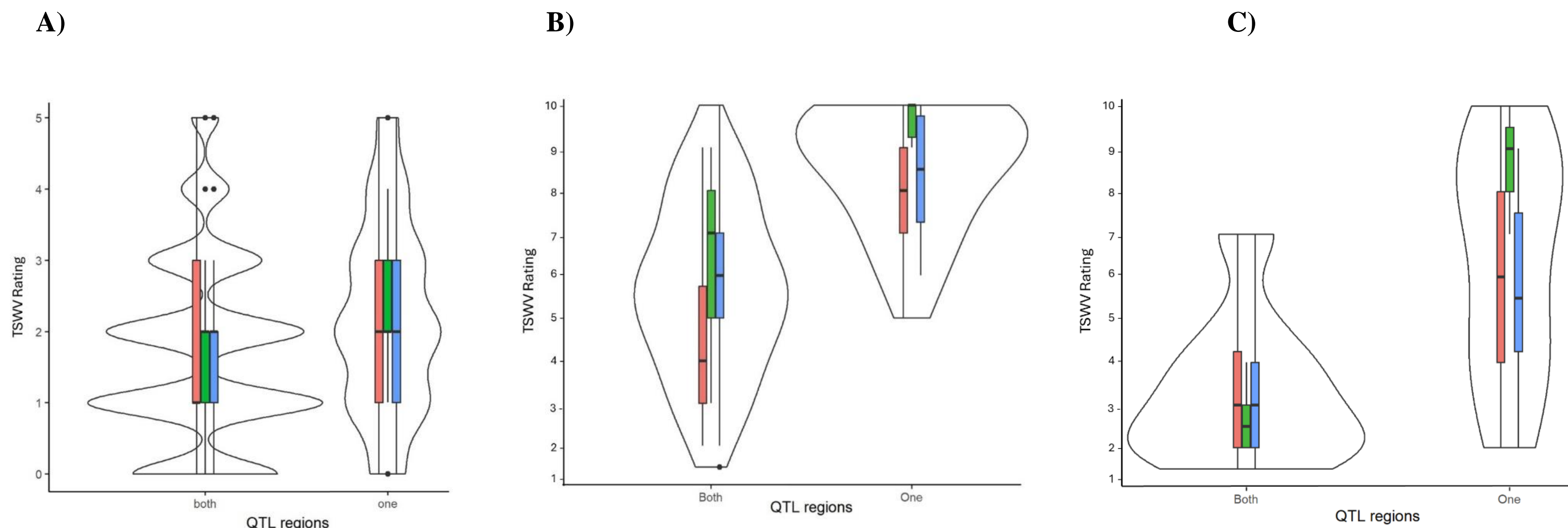

Figure 2.10 QTL effect on TSWV resistance. Comparison of TSWV ratings in lines with and without the insertion region, across three populations with both genotypes. A) ratings from 2022 field evaluation. p-value < 0.001. B) ratings from 2023 field evaluation. p-value < 0.001. C) ratings from 2024 field evaluation. p-value < 0.001. Red, green, and blue plots represent three populations containing both genotype groups.

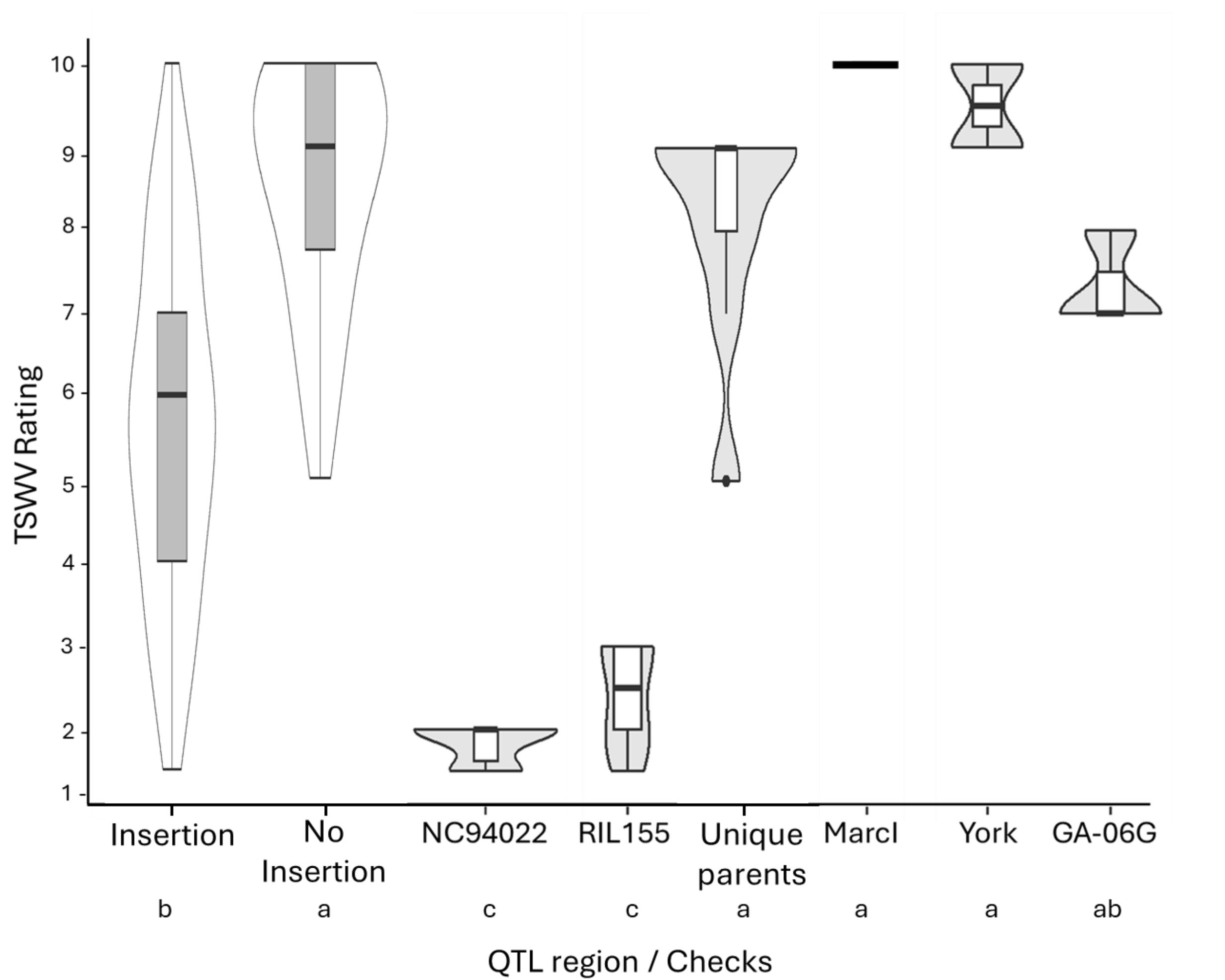

Figure 2.11 Distribution of TSWV ratings with and without the insertion region of the A01 QTL. Comparison of F_4 replicated lines from three populations representing both genotypes with check lines. Significant groups based on ANOVA Tukey test identified under x-axis name. p-value < 0.001.

Figure 2.12 TSWV symptoms on seed. Normal Georgia-06G seed coat on left, symptomatic seed coat on right. Quarter used for size reference.

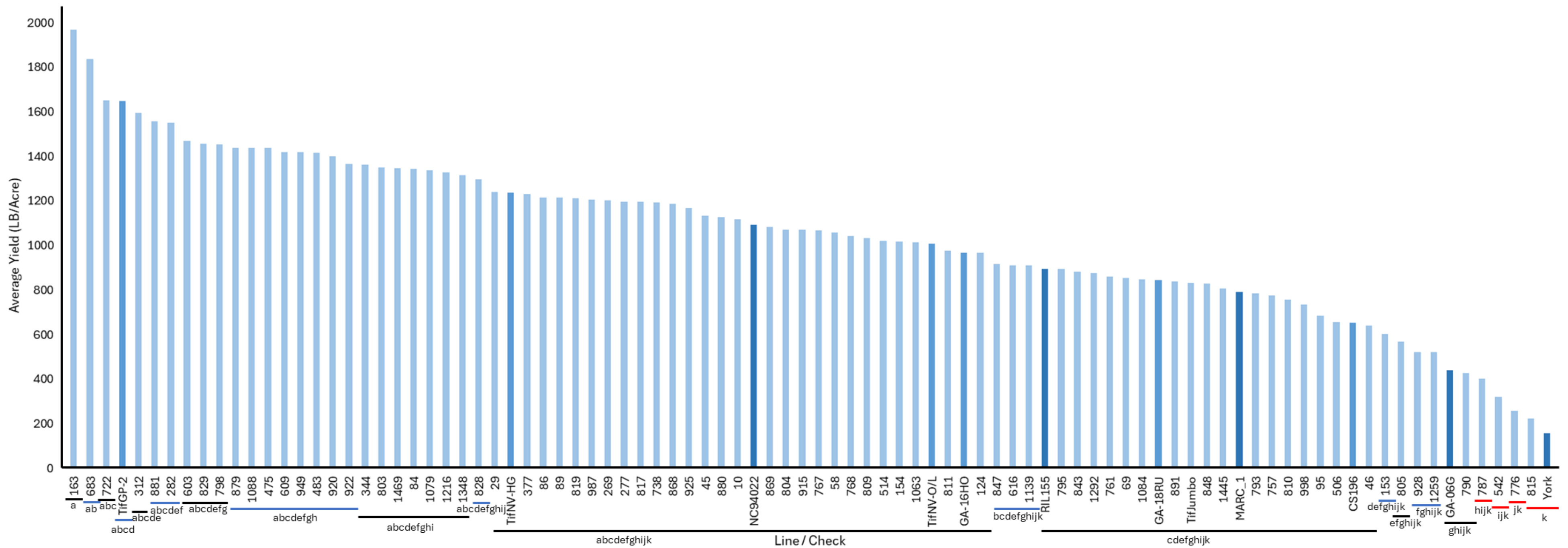

Figure 2.13 Total plot yield of individual F_4 lines and checks during 2023 field evaluation. Average of all replicated plots. Dark blue bars show check lines. Medium blue bars represent unique parents of eight populations. Light blue bars represent the 80 F4 lines. Significant difference across all lines, p-value < 0.001. Significance groups indicated under Line ID's; alternating black and blue bars below indicate separation of 21 significant groups from A to K based on Tukey-HSD for ANOVA. Red line indicates Line IDs in lower significant groups than Georgia-06G. Table 2.6 provides breakdown of lines into eight populations with unique parent.

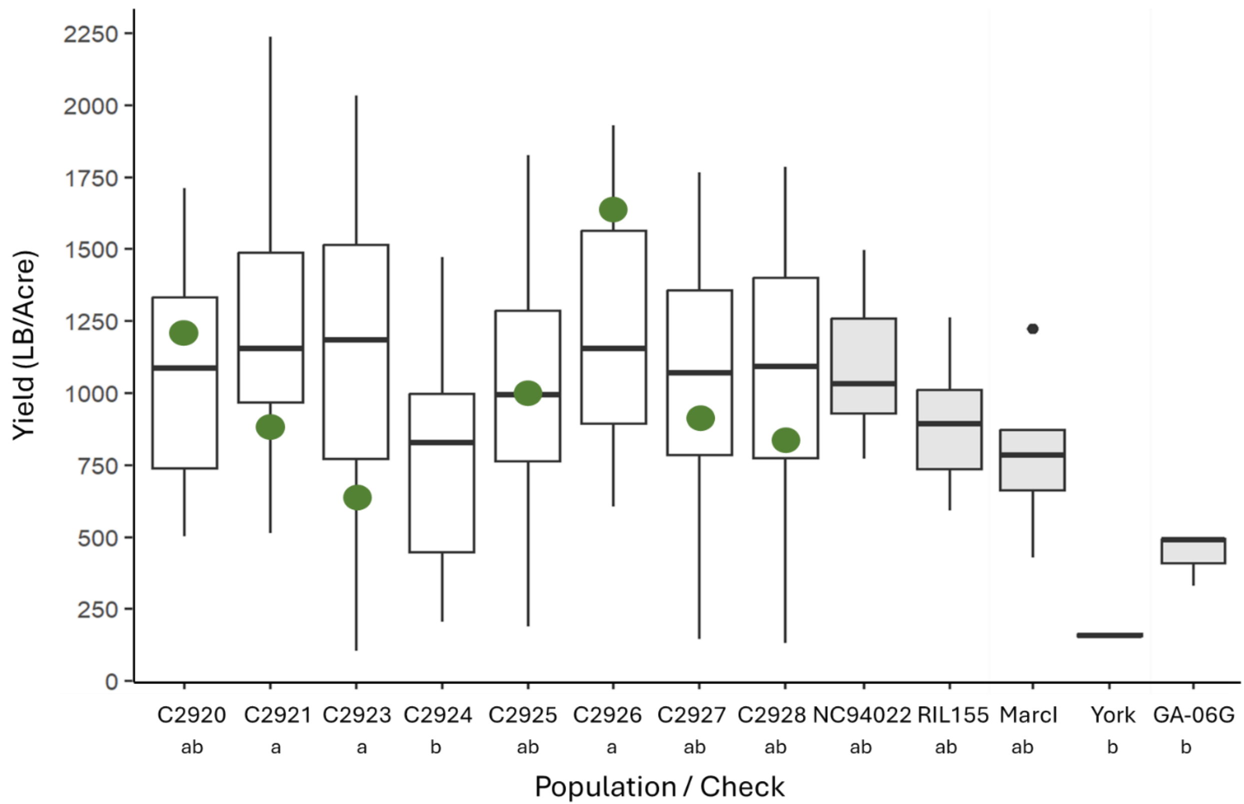

Figure 2.14 Distribution of total plot yield of replicated plots during 2023 field evaluation. All pods harvested for 80 F_4 plots across eight populations (white plots) and check lines (gray plots). Green dots represent yield for unique parent of given population, when available. Black horizontal line indicates median and boxes represent data within the interquartile range. Black dot in MarcI represents an outlier beyond the upper extreme quartile. Significant difference between eight populations and check lines, $p\text{-value} < 0.001$; significant groups identified by letters under x-axis

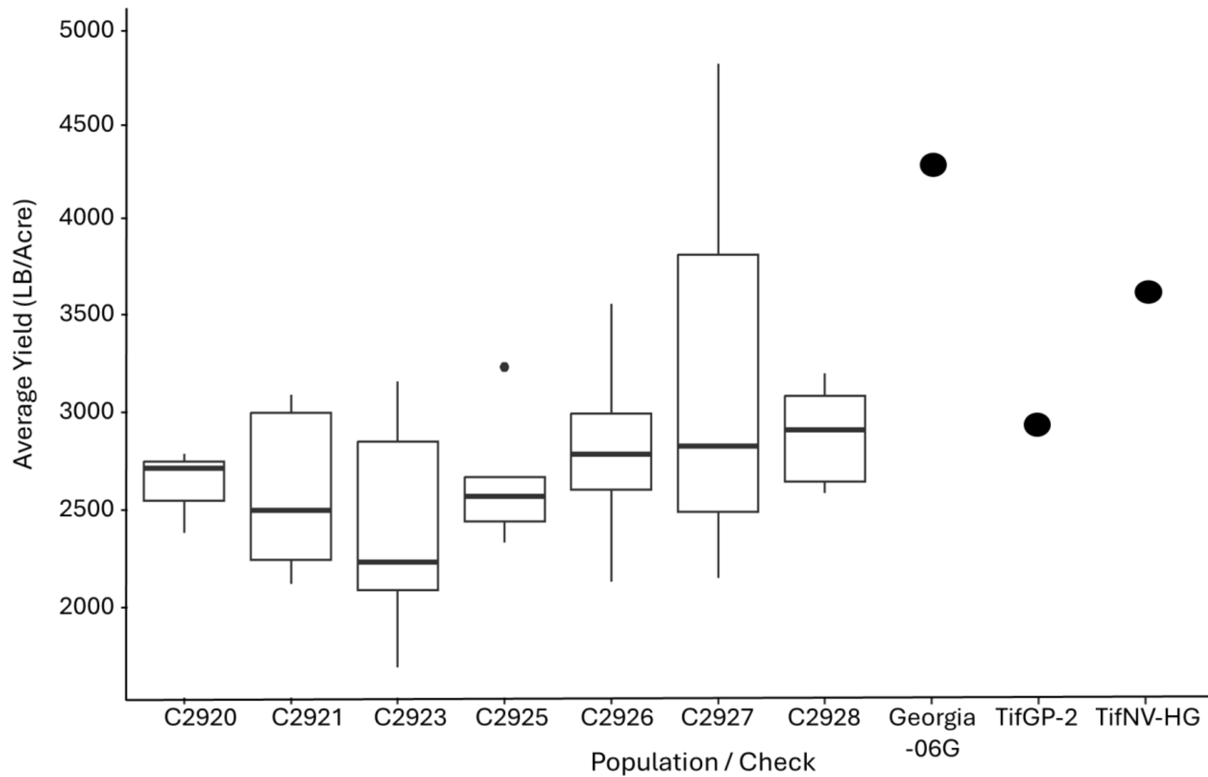

Figure 2.15 Distribution of average total plot yield of replicated plots during 2024 field evaluation. All pods harvested for 31 F_5 plots across seven populations (white plots) and check lines (black dots; only one value available for each). Black horizontal line indicates median and boxes represent data within the interquartile range. Black dot in C2925 represents an outlier beyond the upper extreme quartile. No significant difference between the populations and checks, p-value = 0.17.

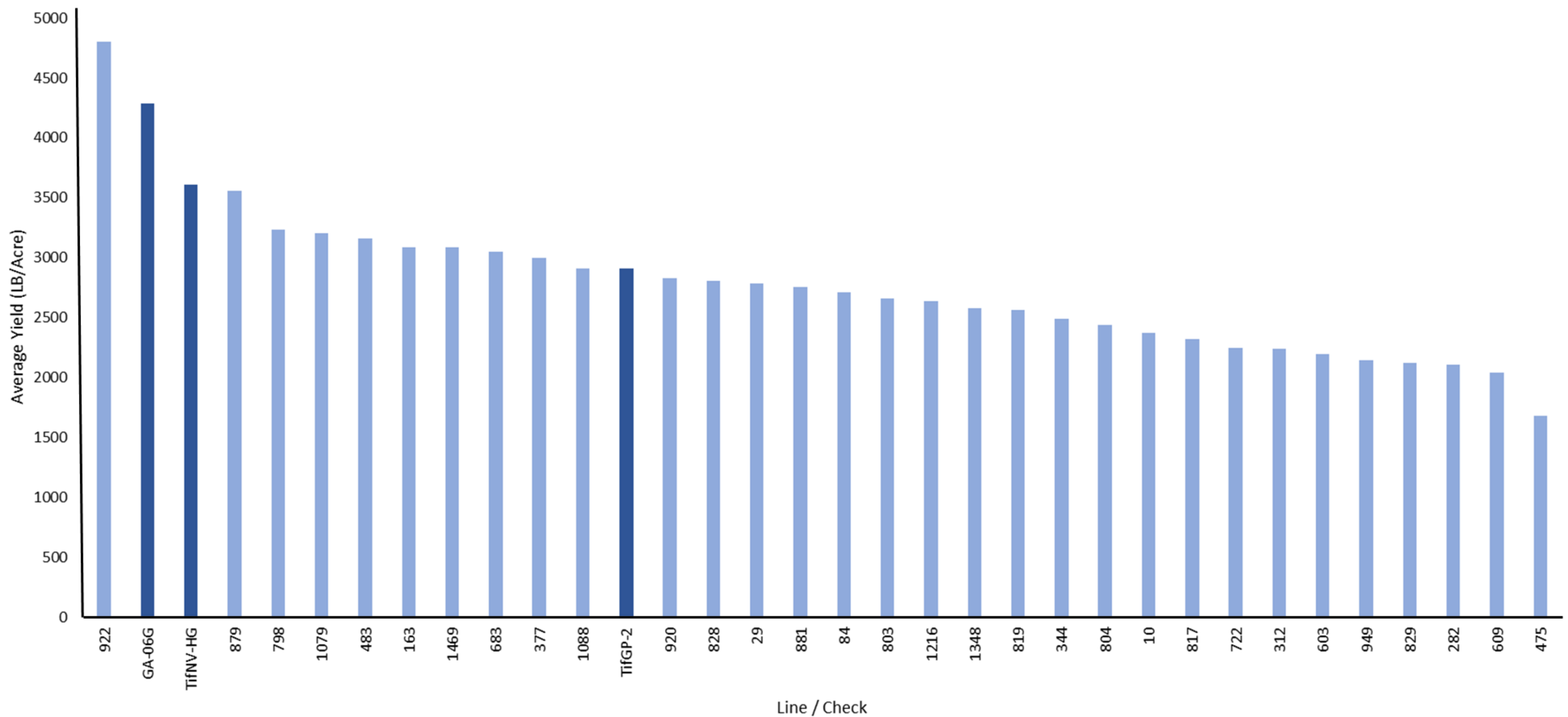

Figure 2.16 Total plot yield of individual F5 lines and checks during 2024 field evaluation. Average of all replicated plots. Dark blue bars show check lines. Light blue bars represent the 31 F5 lines. No significant difference across the lines, only numerical difference in average yields across the three replications. Table 2.6 provides breakdown of lines into populations.

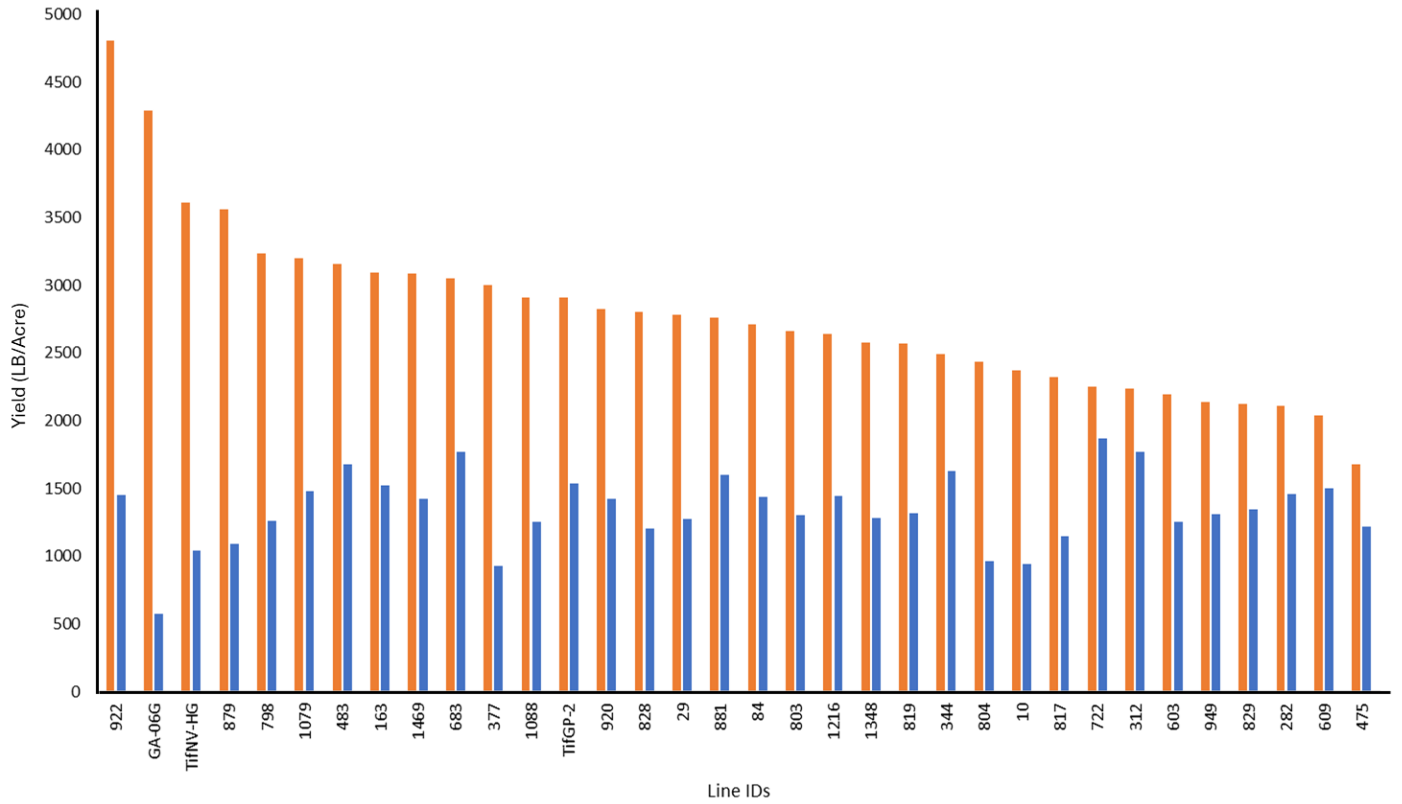

Figure 2.17 Yield comparison between lines grown in 2023 and 2024 replicated plots. Each bar represents average yield across three replications. Orange bars show yield in 2024, blue bars show yield in 2023. Pearson's correlation between years: p-value = 0.18, R = - 0.23.

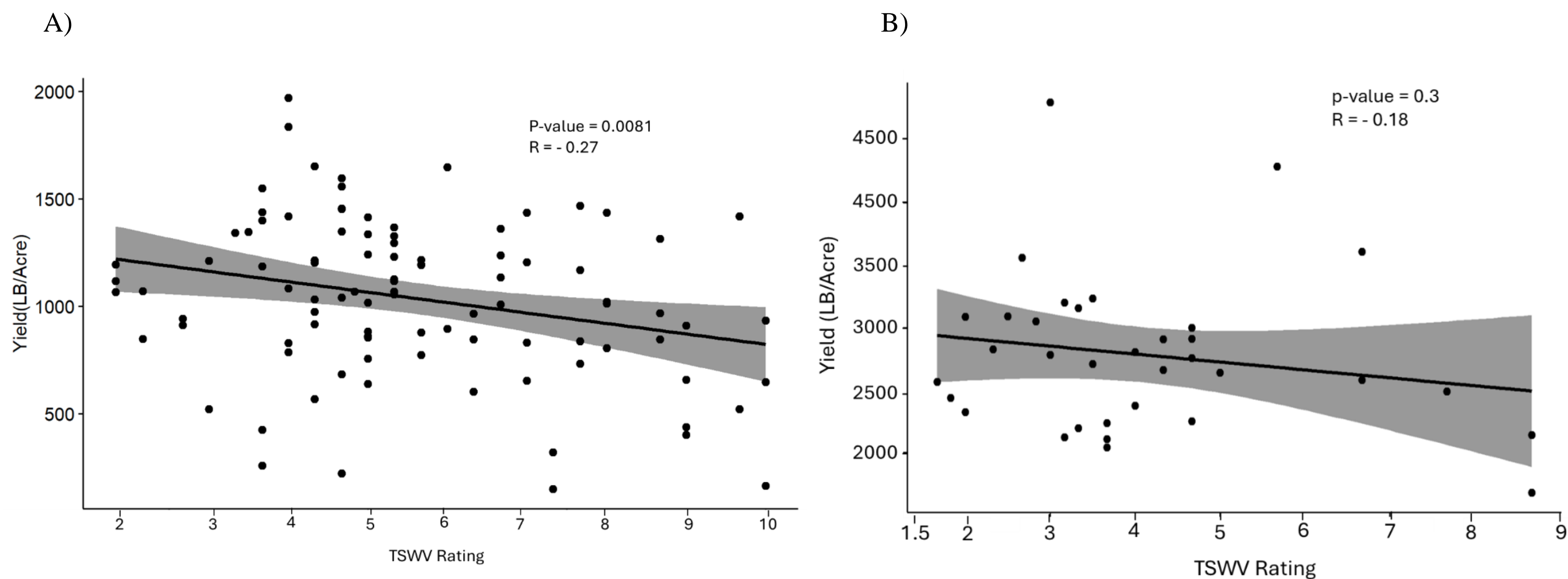

Figure 2.18 Pearson's correlation of TSWV rating and total plot yield for replicated plot trials. Average values for three replications indicated by points. A) 2023 yield, p-value = 0.008, R = - 0.27. B) 2024 yield, p-value = 0.3 R = - 0.1

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CHAPTER 3

IDENTIFICATION OF CANDIDATE GENES FOR TSWV RESISTANCE FROM *ARACHIS HYPOGAEA* 'NC94022'

Introduction

Arachis hypogaea (cultivated peanut) is an economically important crop, providing a farm gate value over $790 million to the state of Georgia and $1.6 billion to the United States (U.S.) in 2022 (Georgia, 2024; USDA, 2024b). Peanut producers face above and below ground challenges when trying to produce the best crop each season. Tomato spotted wilt virus (TSWV) is a significant problem in U.S. peanut production, causing over $35 million value loss for Georgia farmers in 2022 (Kemerait, 2024). Profit loss is the result of increased costs for disease management and reduced yield at the end of the season. PeanutRx provides producers with strategies to limit loss from TSWV including information on how and when to plant, insecticides available to manage thrips populations that vector the disease, crop rotation to interrupt pest life cycles, and most importantly, selection of genetically resistant varieties (Kemerait et al., 2024). Genetic resistance allows the plant an opportunity to defend itself when exposed to TSWV infection in the field.

Natural resistance to TSWV was identified in *A. hypogaea* in the mid-1990s after the new variety Georgia Green (Branch, 1996) was identified as resistant compared with susceptible varieties being grown at the time. Georgia Green is derived from Southern Runner, a progeny of PI 203396 (Gorbet et al., 1987). PI 203396 was shown to be resistant to TSWV and has since

been incorporated into numerous released varieties to have over $200 million in annual impact for growers under high disease pressure (Isleib et al., 2001). This PI has contributed significant genetic and molecular variation to the limited diversity of the *A. hypogaea* runner-type breeding programs (Clevenger et al., 2017b). PI 576638, also known as SSD6, was identified as a second source of genetic resistance to TSWV. This source is being incorporated into breeding programs and evaluated for the mechanism of resistance through its highly resistant progeny NC94022 (Culbreath et al., 2005).

Genetic resistance from PI 203396 and SSD6 has been explored using the T- and S-populations, respectively (Qin et al., 2012). No single resistance gene has been identified in cultivated peanuts for TSWV. Resistance genes have been identified for TSWV in other susceptible species, including tomato and pepper (*Lycopersicon esculentum* and *Capsicum annuum*, respectively) (Boiteux & de Ávila, 1994; de Oliveira et al., 2018; Finlay, 1952; S. Qi et al., 2022; Stevens et al., 1991; Sundaraj et al., 2014). These NBS-LRR (nucleotide binding site – leucine rich repeat) genes are linked to a hypersensitive response to TSWV infection in these crops. No hypersensitive response has been identified in peanut, making its resistance appear to be more complex than a single gene (Shrestha et al., 2013; Sundaraj et al., 2014; Tseng et al., 2016). Identification of specific candidate genes is important for understanding the genetic mechanisms of resistance in peanut as has been done in tomato and pepper.

Resistance from SSD6 is the focus of this study. Two neighboring quantitative trait loci (QTL) were identified on chromosome A01 using the S-population, produced from a cross between NC94022 and TSWV susceptible SunOleic 97R (Agarwal et al., 2019; Guo, 2021; Khera et al., 2016; Qin et al., 2012). Within these QTLs are two specific regions of interest, an NBS-LRR resistance gene (R-gene) and a 63 kb insertion that is present in NC94022 but not

SunOleic 97R (Table 3.1) (Guo, 2021). Further analysis of the S-population identified a new 500 kb region of interest on chromosome A01 located upstream of the previously identified QTL (Table 3.1) (J. Clevenger, unpublished). Genomic tools can help narrow down these large regions to identify candidate genes for TSWV resistance. Similar work has previously been done to narrow QTL regions to identify candidate genes for TSWV and other diseases in peanut. Agarwal et al. (2018) and (2019) identified SNPs within and around candidate genes in QTLs for TSWV resistance in the T- and S-populations, respectively. This study focuses on a more narrow region of the QTL identified in Agarwal et al. (2019) for the S-population. Guo (2021) first presented on the R-gene and insertion regions specifically explored here. Candidate gene regions have also been identified for early and late leaf spot, stem rot, bacterial wilt, and nematode resistances, among others (Clevenger et al., 2017a; Han et al., 2018; Luo et al., 2020; F. Qi et al., 2022; Zhang et al., 2019). Several of these studies have identified regions for resistance to both TSWV and leaf spot in a single population, emphasizing the idea of multiple disease resistance (Agarwal et al., 2018; Zhang et al., 2019).

Once candidate genes are identified it is important to explore their expression patterns and empirically test their function. Gene expression may be constitutive or inducible, when genes respond to a stimulus. For viral infection, mechanical inoculation can be used to examine expression of candidate genes under various inoculation states. This method is explored here based on previous examples of mechanical inoculation in peanut (Mandal et al., 2001; Mandal et al., 2002; Shrestha et al., 2015). Inoculation of peanut can be challenging and inconsistent, and assessing the effects of specific candidate genes for resistance can be overshadowed if multiple genes are involved. Transformation of susceptible varieties with the candidate gene is an alternative to eliminate the confounding factors of other resistance mechanisms. Rather than

using *A. hypogaea* as the transformation recipient, *Nicotiana tabacum* (tobacco) can be used as a model species for easy *Agrobacterium*-mediated transformation and mechanical inoculation. *N. tabacum* is highly susceptible to TSWV (Culbreath et al., 1991), and the thin leaves of tobacco compared to the thick cuticle of peanut makes mechanical inoculation more successful. Spassova et al. (2001) transformed tobacco lines with the *Sw-5a* and *-5b* candidate genes from tomato and found them to play a role in TSWV resistance. Our study will use a similar approach to test candidate genes for resistance from *A. hypogaea*.

The objective of this study is to explore the identified regions on chromosome A01 for specific candidate genes related to disease resistance. Expression patterns of selected genes with and without TSWV inoculation will classify candidate gene(s) as constitutively expressed or inducible. The expression patterns will inform further experiments on their overexpression in *N. tabacum* after *Agrobacterium*-mediated transformation. This study will provide preliminary data for prioritizing candidate genes from SSD6 that may be responsible for TSWV resistance and ultimately improve the accuracy of marker-assisted selection for this trait in peanut breeding programs.

Materials & Methods

Gene identification

Three regions on chromosome A01 were explored for candidate genes (Table 3.1). Version two of the Tifrunner genome was obtained from PeanutBase along with gene annotations (Bertioli et al., 2019; Dash et al., 2016). Hifiasm was used to develop genome sequences of NC94022 and SSD6 (Josh Clevenger, unpublished).

An NBS-LRR resistance gene (R-gene) was previously identified as Arahy.1PK53M (Guo, 2021). The annotated gene sequence was obtained from Tifrunner and aligned to the QTL region of NC94022 for comparison. A 125 kb sequence containing the 63 kb insertion region on NC94022 was extracted and run through FGENESH (Solovyev et al., 2006) using the *A. duranensis* prediction-based model to identify coding sequences (CDS) of potential genes within the region. A second location upstream of these two regions was identified between 10.85 and 11.33 Mb of Tifrunner as another potential region for resistance. GEvo via CoGe (Lyons & Freeling, 2008) was used to compare Tifrunner to NC94022 to identify significant regions of difference between the genomes. A 34 kb sequence flanking and spanning a deletion in NC94022 was extracted and genes within were identified using FGENESH as described above. Moving forward, this region is denoted as the "deletion" region for identification purposes. Coding sequences of each gene were used to search for similarity with the Tifrunner.gnm2 version 1 genome sequence using the BLAST tool on PeanutBase. Resulting gene sequences from Tifrunner were aligned with predicted sequences from NC94022 using GeneiousPrime (http://www.geneious.com) to identify differences in the sequences from each source.

Pangenome alignment of Tifrunner, SSD6, and NC94022 was done to further investigate all regions of interest and to confirm any differences between Tifrunner and NC94022 that were

identified using other sources (Sameer Pokhrel, unpublished). Tifrunner was used to represent the sequence of SunOleic 97R in this study.

Primer design for gene expression

Primers were designed to differentiate between gene sequences in NC94022 and Tifrunner. Three glutamate receptor genes from the insertion region and the R-gene were assessed in experiment one; two genes from the deletion region were additionally evaluated in experiment two. Primers were designed using predicted cDNA sequences and the IDT PrimerQuest™ Tool (https://www.idtdna.com/pages/tools/primerquest) (Table 3.2). When possible, primers were designed to identify only sequences from NC94022 or Tifrunner, although some genes did not show sufficient polymorphism to allow differential primer design.

Presence of TSWV virus in all plants was determined using primers from Jain et al. (1998), with 5' ATGTCTAAGGTTAAGCTC 3' and 5' TTAAGCAAGTTCTGTGAG 3' as the forward and reverse primers, respectively. These primers identify the nucleocapsid protein (N-gene) of the virus. The resulting amplicon is 800 bp in length.

Gene expression under mechanical inoculation

Gene expression was assayed using tissues from mechanical inoculation of NC94022 and SunOleic 97R, parents of the S-population. TSWV-infected leaf tissue was collected from peanut plants at the Gibbs farm, Tifton, GA in August 2021 and stored at -80 C until needed for inoculation. TSWV susceptible *N. tabacum* var. K326 (provided by J. Michael Moore, UGA-CAES) was used as a positive inoculation control. Two inoculation experiments were conducted; Table 3.3 details the differences between them. Experiment one was completed in September 2022 and experiment two in March 2023. Growth chamber conditions were set to 27 C with a

16-hour light, 8-hour dark cycle. Plants were watered regularly during each experiment to maintain plant health.

The mechanical inoculation process was modified from Mandal et al. (2001). TSWV-infected tissue (2.5 g) was finely ground using liquid nitrogen and added to 50 mL potassium phosphate buffer (0.027 M of K_2HPO_4, 0.0235 M KH_2PO_4, and water), with 50 μL 2-Mercaptoethanol (0.1%). Celite 545 (0.5 g) was added to the ice-cold solution after mixing. Leaflets to be inoculated were dusted with carborundum and a cotton swab soaked in inoculum was rubbed across the surface from base to tip twice. An 18G needle and syringe was used to prick and deposit droplets of inoculum into the base of each inoculated leaf. Excess inoculum was rinsed with water after three minutes. A mock inoculation was completed following the same process without the addition of the TSWV infected tissue. Uninoculated plants were left untreated. Two leaves were inoculated on each plant and tagged with string for future identification. All plants were monitored for TSWV symptoms and physical damage from the inoculation process.

Tissue samples were collected for RNA extraction pre- and post-inoculation (Table 3.3). One leaflet was collected at each time point. Early time points were collected from an inoculated leaf while later time points were collected from newly formed leaves to test systemic spread of the virus. One inoculated leaf was left untouched for continued virus presence after early sample collection. Each leaflet was flash frozen in a 2 mL microcentrifuge tube with liquid nitrogen and stored at -80 C until ready for processing. RNA extraction was completed for all samples at the same time using the Qiagen RNeasy plant mini kit (Qiagen, Germantown, MD). RNA concentration and purity of each sample was determined using a Synergy microplate reader (Agilent, Santa Clara, CA) and a 1.2% formaldehyde agarose gel. Bands on all gels were

visualized using an Azure 200 gel imager with the ethidium bromide setting (Azure biosystems, Dublin, CA).

cDNA was produced from each RNA sample using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA). A 1:5 cDNA dilution in nuclease-free water was used for downstream reactions. Each 20 µL PCR reaction to observe expression contained 10 µL of 2X GoTaq Green master mix, 2 µL each of 5 µM stock forward and reverse primers, 1 µL of 1:5 cDNA dilution, and nuclease-free water. PCR protocols were as follows: initiation at 95 C for 2 min, followed by 35 cycles of denaturation at 95 C for 30 sec, annealing at 60 C for 30 sec and extension at 72 C for 30 sec, and a final extension at 72 C for 5 min with an indefinite hold at 4 C. Annealing temperatures and extension times were modified as needed for each primer pair based on melting temperature and amplicon length (Table 3.2). PCR protocols to identify TSWV in samples were run separately with an initial denaturation at 95 C for 15 min, followed by 35 cycles of 95 C for 1 min, 52 C for 45 sec and 72 C for 1 min, and a final extension at 72 C for 10 min with an indefinite hold at 4 C (Jain et al., 1998). All PCR products were run on a 1% agarose gel at 100V for 30 min; 1 uL sample and 9 uL of water were added to each well. Bands were observed as described previously.

Candidate gene selection and cloning

Based on gene expression results and evaluation of TSWV resistance in the field (Chapter 2), a glutamate receptor within the insertion region of NC94022 was selected as the candidate gene for further evaluation. Genomic DNA (gDNA) and cDNA were used as templates for plasmid development. Primers were designed to amplify the gene from the start to stop codons with restriction enzymes added to the ends to match cut sites made in the vector (Table 3.4). *Sal*I and *Kpn*I were added to the forward and reverse primers, respectively, with a six base

pair leader sequence on the 5' end of each primer. The binary expression vector pBINplus35S was used for overexpression of the candidate gene (Dubey et al., 2017; van Engelen et al., 1995). This vector contains kanamycin (Kan) resistance for bacteria and plant selection, with a CaMV (Cauliflower mosaic virus) 35S promotor and a NOS (nopaline synthase) terminator to regulate expression of the inserted gene. Genomic DNA was extracted from NC94022 plants grown in the greenhouse using the DNeasy Plant Mini kit (Qiagen, Germantown, MD). Based on RT-PCR results, cDNA was selected from two NC94022 inoculation samples for amplification of the target gene sequence. Each 25 µL amplification reaction contained 5 µL of 5X GXL buffer, 2 uL dNTPs, 0.5 µL each of forward and reverse primer at 0.25 µM final concentration, 1.5 µL DNA template, 0.5 µL primeSTAR GXL DNA polymerase (Takara Bio, USA), and HPLC water. Quantity of the final cDNA reaction was increased to 100 uL and gDNA to 50 uL to ensure sufficient product after purification. To amplify sufficient product, the following cycling conditions were implemented: activation of 98 C for 1.5 min, followed by five cycles of 98 C for 10 sec, 55 C for 15 sec and extension at 68 C for the given time, followed by 25 cycles of 98 C for 10 sec, 60 C for 15 sec and 68 C for the given extension time, and a final extension at 68 C for 5 min. Genomic DNA samples were allowed to extend for 7.5 min and cDNA samples were extended for only 2.5 min, based on their expected lengths of 7.4 and 2.7 kb, respectively. PCR products were purified using the QIAquick PCR purification kit with spin columns. The total volume of purified PCR products was digested with *Sal*I and *Kpn*I using 1 µL each of high-fidelity enzymes (NewEngland Biolabs, Ipswich, MA), 6 µL cutSmart 10X buffer and water to a total volume of 60 µL. Reactions were allowed to run at 37C for 1.5 hours.

E. coli cells containing empty pBINplus35S plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen, Germantown, MD). Vector DNA was digested with *Sal*I and *Kpn*I

following the same protocol described above with the addition of 2 µL QuickCIP (NewEngland Biolabs, Ipswich, MA) to dephosphorylate the cut ends. Digested insert and vector products were purified separately using the Qiagen PCR purification kit. T4 ligase (Promega, Madison, WI) was used to ligate the inserts to vector in a 3:1 molar ratio. Each 10 µL reaction contained 1 µL 10X ligase buffer, 1 µL T4 ligase, 3:1 insert to vector, and nuclease free water to total. An aliquot of each reaction was run on a gel to confirm ligation by visualizing bands of greater sizes than individual components.

Ligated products were transformed into NEB 5-alpha competent cells (New England Biolabs, Ipswich, MA) as follows: 2 µL of ligation product was mixed into 25 µL of thawed competent cells and placed on ice for 30 min, the cell mixture was heat shocked at 42 C for exactly 30 sec and placed on ice for 5 min, 250 µL of room temperature SOC media was added and the solution was placed at 37 C for 60 min under constant shaking. A vector only control was used to check quality of the digestion and ligation process. Transformed cells were grown overnight on LB+Kan (50 µg/mL) agarose plates; 75 µL of cell solution was spread onto one plate and the remaining onto a second plate. Plates were removed after 12 to 16 hours and colony growth observed for each transformation.

Twenty-four colonies from each transformation were screened with PCR. Selected colonies were isolated in a 96-well plate in 50 µL LB+Kan media. Three vector only colonies were selected as negative controls. Colonies were grown overnight at 37 C under constant shaking. Two microliters of each sample were added separately to 50 µL of ½ TE buffer (10 mM Tris, 1mM EDTA, pH to 8.0; 1:1 ratio TE to HPLC water for ½ TE buffer) and heated to 95 C for 10 min. This stock lysate was maintained at 4 C until all downstream studies were completed. PCR was carried out using JumpStart Taq DNA polymerase (MilliporeSigma,

Sigma-Aldrich, Darmstadt, Germany) and primer pairs annealing within the insert and vector sequences (Table 3.5)**.** Primers to identify the NOS terminator sequence of the plasmid were used as a positive control. Each 10 µL reaction contained 1 µL of 10X PCR buffer, 0.8 µL of 2.5 mM dNTPs, 0.5 µL each of 5 µM stock forward and reverse primers, 0.6 µL of 25 mM $MgCl_2$, 1 µL of 10% PVP, 0.1 µL BSA (10 µg/µL), 0.1 µL JumpStart *Taq* (2.5 U/µL), 4.9 µL HPCL grade water, and 1 µL stock bacterial lysate. The PCR reaction was initiated with activation at 94 C for 5 min, followed by 40 cycles of denaturation at 94 C for 30 seconds, annealing at 50 C for 30 seconds and extension at 72 C for 1 min, and a final extension at 72 C for 7 min. Amplified products were separated on a 1% agarose gel at 100V for 30 min; 1 µL of PCR product, 1 µL 10X loading dye, and 8 µL HPLC water was run for each sample. Bands were visualized as described previously.

Colonies positive for an insertion were grown overnight on LB+Kan plates at 37 C. Plasmid DNA was extracted using the Qiagen miniprep kit and double digested using *Sal*I and *Kpn*I as previously described. A single enzyme digest using *Eco*RI was done following the same method. Products were run on a 1% agarose gel as above and only the cDNA samples were selected for further processing. Whole plasmid sequencing (Eurofins, Louisville, KY) was conducted to confirm the inserted cDNA sequence.

Results

Gene identification

Alignment of the R-gene, Arahy.1PK53M, from Tifrunner.gnm2 to NC94022 identified no sequence differences between the two genotypes when using FGENESH prediction for NC94022 CDS in the region. Pangenome alignment identified a 1.7 kb insertion in NC94022 compared to Tifrunner in the region of the R-gene, around 12.061 Mb and 12.181 Mb of Tifrunner and NC94022, respectively (Figure 3.1a). This was not identified when gene expression primers were designed as Pangenome evaluation was completed after expression was determined. Location of primers on the 5' end of the gene would not have identified this difference on the 3' end of the gene. This insertion extends the last exon of the gene in NC94022 (Figure 3.1b), though field results do not indicate that presence of the R-gene alone is significant for resistance (Chapter 2).

FGENESH prediction of the larger 63 kb insertion region from NC94022 (Table 3.1) identified 12 predicted gene models (Figure 3.2a). Eight models were full genes with the other four models being only two short exons. A glutamate receptor and kinetochore protein were identified from these models using BLAST. Each gene is duplicated four times in the same glutamate receptor + kinetochore protein order.

BLAST results indicate the glutamate receptor is not a 100% match to any other glutamate receptor in the Tifrunner or NC94022 genome. On chromosome A01, the highest similarities were found with Arahy.NV2IFB (99.1% identity with 36% query coverage) and Arahy.5N45JG (74.7% identity with 38% query coverage) which are located upstream and immediately downstream of the insertion region, respectively (Figure 3.2, Table 3.6). Both genes were among the gene predictions from the NC94022 sequence and were identical to Tifrunner.

The insertion glutamate receptor had high CDS and protein sequence differences when aligned with both Tifrunner genes and any other glutamate receptors in the region, including Arahy.VZ8YXD on the 5' end of the insertion; these differences are represented by the high percent identity but short query coverages in Table 3.6.

The kinetochore protein had the highest BLAST identity to Arahy.H2X3VG on chromosome A10 (90.2% identity with 83% query coverage) and Arahy.0E6AR9 (90.4% identity with 83% query coverage) on B10. Neither CDS had consistent sequence similarity when aligned to the predicted gene. No glutamate receptors were identified around Arahy.H2X3VG and the few glutamate receptors within 1 kb on either side of Arahy.0E6AR9 were significantly different from those found in the insertion region. Comparison of the four glutamate receptor + kinetochore protein duplications identified no sequence differences between any of the coding regions or their upstream promotor regions, evaluated up to 5 kb from the start codon of each glutamate receptor. Similarities between the glutamate receptor in each duplication are indicated in Table 3.6. Only three single nucleotide differences were identified between the duplications, with all SNPs occurring in intron or non-coding areas. Pangenome alignment confirmed the insertion region (Figure 3.2b). SSD6 contains the same region as NC94022 but is half the size with only two glutamate receptor/kinetochore protein duplications compared to four. Coding sequences for both genes are the same in SSD6 as in NC94022.

GEvo analysis of Tifrunner and NC94022 identified a deletion within the second target region around 11 Mb of chromosome A01, upstream of the insertion region (Table 3.1). FGENESH of this region in NC94022 identified two genes, a pleiotropic drug resistance gene (Arahy.IK6XD2) and a RNA-binding protein (Arahy.R6E3N5) (Figure 3.3a). The deleted portion was within the 3'untranslated regions (UTRs) of IK6XD2, removing the last 13 of the 20

UTRs predicted in the Tifrunner.gnm2 version 1 gene annotations. The coding region of this gene was unchanged between NC94022 and Tifrunner. An insertion of six base pairs was identified in exon 13 of R6E3N5 between Tifrunner and NC94022. No other differences in this gene were found between genotypes. Pangenome alignment confirmed these differences and identified that the same were also present in SSD6 as NC94022 (Figure 3.3b and c).

Inoculation and gene expression

Mechanical inoculation resulted in few infections and symptomatic plants in this experiment. No inoculated NC94022 showed symptoms in either experiment (Figure 3.4a). Only one inoculated SunOleic 97R plant showed symptoms in experiment two; all other plants showed no symptoms (Figure 3.4b and c). All inoculated tobacco plants showed symptoms and stunting compared to uninoculated plants (Figure 3.4d). PCR screening of the TSWV N-gene confirmed presence of TSWV in symptomatic tobacco and peanut plants only, i.e. there were no asymptomatic plants (Figure 3.5).

Gene expression results indicate that all genes tested are constitutively expressed (Figure 3.6, 3.7 and 3.8). Time point after inoculation and inoculation method did not alter expression. The R-gene and glutamate receptors Arahy.5N45JG and Arahy.NV2IFB are expressed in both genotypes as expected based on primer design and sequence comparison (Figure 3.6 and 3.7). The glutamate receptor within the insertion region was only expressed in NC94022 as predicted based on sequencing. Genes within the “deletion” region, Arahy.IK6XD2 and Arahy.R6E3N5, were also expressed in both genotypes (Figure 3.8).

Candidate gene selection and cloning

Based on sequence comparisons and gene expression results, four potential candidate genes were identified across the two regions on chromosome A01 for TSWV resistance: the R-gene,

insertion region glutamate receptor unique to NC94022 and both genes in the deletion region. Based on results from field studies (Chapter 2) the insertion region provided a significantly greater resistance than the R-gene alone. Genes within the deletion region have not been explored under field evaluation. Therefore, the glutamate receptor in the insertion region was selected for further cloning and testing.

PCR screening of the transformed and isolated colonies showed bands for a single colony in each gDNA and cDNA sample (Figure 3.9). The identified gDNA colony showed two bands rather than the expected one and was therefore not explored further. Two cDNA colonies, further identified as c56 and c63 based on their cDNA sample source, have the expected single band around 1 kb (Figure 3.9b). Single enzyme digestion of cDNA colonies with *Eco*RI showed multiple bands for both samples, with c56 having the expected four bands and c63 having only three (Figure 3.10). Double digestion with *Sal*I and *Kp*nI shows two bands for both samples, confirming presence of the vector and an insert (Figure 3.10).

Whole plasmid sequencing of both clones confirmed the correct insert sequences (Figure 3.11). Sequencing indicated that the cDNA insert of c63 is smaller than c56, explaining the differences seen by digestion (Figure 3.10). Alignment of plasmid sequences with the expected insert gene sequence shows that c63 is missing exon 2 of the gene (Figure 3.12). The protein sequence of the shorter c63 insert sequence shows an early stop codon compared to the full sequence of c56. cDNA sequence for final c56 and c63 plasmids are included in Table 3.7.

Discussion

A diverse set of candidate genes was identified within the two regions of interest on chromosome A01 of NC94022. Members of three and two different gene families were identified in the QTL and deletion regions, respectively. Arahy.1PK53M, a NBS-LRR gene, was previously identified in the QTL region (Guo, 2021) and was further investigated here. R-genes are commonly involved in disease resistance as they can detect pathogen associated avirulence proteins and initiate the plant's specific response pathways (Belkhadir et al., 2004; DeYoung & Innes, 2006). TSWV resistance genes in tomato, including *Sw-5a* and *-5b*, belong to the NBS-LRR domain and have been successfully implemented into breeding for resistance (de Oliveira et al., 2018; S. Qi et al., 2022; Spassova et al., 2001). These R-genes initiate a hypersensitive response to TSWV infection in tomato which has not previously been identified in peanut. Still, the function of R-genes in peanut's resistance is plausible.

Within the insertion region of the QTL were multiple glutamate receptors and a kinetochore protein. Arahy.5N45JG, Arahy.NV2IFB, and Arahy.VZ8YXD were identified outside the insertion region in all genotypes and have similar sequences to the glutamate receptor identified within the 63 kb insertion sequence (Table 3.6). Glutamate receptors are membrane bound channels that transport molecules across cell membranes and can play an important role in signaling pathways, including defense signaling in response to biotic and abiotic stressors (Ahmed et al., 2023; Davenport, 2002; Simon et al., 2023). It is reasonable to believe that the unique glutamate receptor genes within the insertion region present only in NC94022 and not SunOleic 97R could be involved in TSWV resistance from this genotype. Field data shows the significance of this region compared to the R-gene to improve TSWV resistance. The kinetochore protein also identified in the insertion region showed similarity to proteins on

chromosomes B10 and A10, with B10 having neighboring glutamate receptors. These proteins are involved in chromosomal alignment and separation during cell division (Yu et al., 2000) but are not as likely to be associated with disease resistance as other candidates identified.

CoGe and pangenome alignments identified a deleted region between Tifrunner and NC94022 upstream of the previously identified QTL region. FGENESH prediction of the sequence around this deletion identified two candidate genes with alterations between NC94022 and Tifrunner. The identified deletion was located at the 3'end of Arahy.IK6XD2, a pleiotropic drug resistance gene. This gene contains a p-loop nucleotide binding protein fold with ATPase activity. Pleotropic drug resistance genes belong to the ATP-binding cassette transporter family, which help transport molecules across the cell membranes and can be expressed in response to biotic stresses (Crouzet et al., 2006; Nuruzzaman et al., 2014). There were no differences between the CDS of this gene in NC94022 and Tifrunner, but the identified deletion removed several of the 3' UTRs in NC94022. UTRs are involved in the stability and regulation of transcribed mRNA sequences and can be involved in important protein-protein interactions (Mayr, 2019). Modification or deletion of this region could alter the function or regulation of this gene in NC94022 compared to Tifrunner or SunOleic 97R.

Arahy.R6E3N5, a RNA-binding protein, was identified on the 3' end of the deleted region. This gene is involved in RNA processing and post-transcriptional gene regulation. It may also be involved in defense if able to bind to viral RNA particles, such as the RNA of TSWV (Huh & Paek, 2013; Woloshen et al., 2011). A six base pair insertion was found in exon 13 of this gene in NC94022 compared to Tifrunner, resulting in an insertion of two amino acids in the protein chain. This insertion may result in a functional change of the protein but further evaluation is necessary to confirm.

Primers designed to identify each of these genes were not able to differentiate successfully between the two genotypes, except for the insertion glutamate receptor genes that are not present in Tifrunner. Each gene showed base pair level differences in the sequence, but suitable primers could not be designed for these minor differences and show amplification when tested in genomic DNA. Nevertheless, these primers were useful for examining expression of the genes to identify constitutive expression versus expression only under inoculation. As all target sequences were amplified with RT-PCR, it was concluded that all candidate genes are constitutively expressed in both NC94022 and SunOleic 97R. The insertion glutamate receptor is the only exception to this as it was only identified in NC94022 and amplified in this genotype only as expected. Expression occurred at all time points tested. Only one SunOleic 97R plant showed symptoms and infection with TSWV inoculation. Comparison of this plant to others that were inoculated but not infected shows that expression of these genes are not dependent on infection with TSWV.

Lack of infection does not appear to impact our gene expression study but improvement in the infection rate may aid future studies of this kind. Mechanical inoculation is difficult in peanuts due to the thick cuticle layer of the leaves. Comparatively, the thin leaves of tobacco allow it to be highly susceptible to mechanical inoculation. All inoculated tobacco in our study quickly showed typical TSWV symptoms and PCR confirmed infection by presence of viral RNA. There are many factors that influence the success of mechanical inoculations and each study may produce different results even with minimal changes to the protocol (Mandal et al., 2001). Still, mechanical inoculation is best for consistency and controllability when identifying response to inoculation by a genotype. Use of thrips-mediated transmission may be more replicable of field transmission and the resulting resistance mechanisms, but there is greater risk

of inconsistency therefore the mechanical rub and prick method was chosen for this study (Mandal et al., 2001; Shrestha et al., 2015).

The glutamate receptor identified within the insertion region of NC94022 was selected as the final candidate gene for this study. This gene sequence was amplified from two cDNA samples from inoculation experiment two and inserted into a binary plasmid vector pBINplus35S for overexpression of the gene. Overexpression with the 35S promotor is predicted to mimic the duplications of this gene in the insertion region. These duplications are predicted to amplify the effect of this gene. Increased copy number of resistance genes has been found to improve resistance to soybean cyst nematode in soybean. The *Rhg1* locus contains 3 genes and increased copy number of this locus results in increased resistance to SCN, with varying resistance seen with varying copy number (Cook et al., 2012; Shaibu et al., 2020). Overexpression of this candidate gene in susceptible tobacco will provide further information on its effect on resistance and perhaps the role of duplication. Pangenome analysis showed a difference between the number of duplications in the insertion between SSD6 and NC94022, with two and four duplications, respectively. Quantitative PCR between these two genotypes could further indicate the importance of duplications of the candidate gene for resistance.

Sequencing of the two final plasmids c56 and c63 showed different cDNA and therefore mRNA sequences produced from the insertion glutamate receptor genes. The insertion sequence of c63 appears to lack exon 2 compared to c56, implying different transcription patterns of this gene. This was not apparent until the final plasmids were sequenced but may be important to explore in terms of resistance in the future. The sequence of c63 appears to have an early stop codon that would shorten the protein and potentially alter the function compared to c56. For

further transformation steps only c56 will be initially explored, but it is important to consider the different transcripts from this gene if it is shown to be involved in resistance.

Future steps in this study include transformation of TSWV susceptible *N. tabacum* var. K326 with plasmid c56 containing the full candidate gene. The same mechanical inoculation procedure used here will be used on homozygous T1 transformants with and without the candidate gene to screen for its effect on TSWV resistance. This will allow us to further narrow the source of genetic resistance from NC94022 and SSD6, which will provide a more specific target region for marker assisted selection in the breeding program.

Table 3.1 Sequence regions and genome coordinates on chromosome A01 for candidate gene exploration and FGENESH gene prediction.

Sequence region	Location on Tifrunner *	Location on NC94022 **
R-gene	12.061 – 12.069 Mb	12.182 – 12.189 Mb
Insertion region	12.283 – 12.350 Mb	12.409 – 12.530 Mb 63 kb insertion region within
Deletion region	10.85 – 11.33 Mb Focus region = 11.085 – 11.110 Mb	11.210 – 11.250 Mb

* Tifrunner.gnm2 from PeanutBase
** NC94022 Hifi assembly 2 from Josh Clevenger, HudsonAlpha Institute for Biotechnology, Huntsville, Alabama

Table 3.2 Primer sequences designed to identify candidate gene cDNA from gene expression study

Candidate gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)	Exons identified	Target genotype
R-gene	AATCTGCTACTCCCGGACGA	GACAACTTCGACAGCATCAGAG	547	Exon 2 – Exon 6	Tifrunner, NC94022
Insertion glutamate receptor	GCCTTCCTCACACCATTTAC	CCATCCTTCAATGCCTTCTC	446	Exon 3 – Exon 5	NC94022
Arahy.5N45JG	GCCCTTAAGCCTGATGATAC	TGTGAAGGCCAGAAGAATTAG	697	Exon 2	Tifrunner, NC94022
Arahy.NV2IFB	GAATTGGAGTGCCACTTAGG	CCATCCTTCAAAGCCTTCTC	691	Exon 2 – Exon 5	Tifrunner, NC94022
Arahy.IK6XD2	CCACCAACATCTCACGTTAT	CTCTCCTAGCAAGCTCTAGT	887	5' UTR – 3' UTR #2 (across all exons)	Tifrunner, NC94022
Arahy.R6E3N5	CGGTCCATGAGCCAAATTA	ATCCTCCCTACGATGATGAG	449	Exon 2 – Exon 3	Tifrunner, NC94022

Table 3.3 Mechanical inoculation experimental design

Method	Experiment 1	Experiment 2
Number of plants	5 inoculated 5 mock-inoculated 2 uninoculated	4 inoculated 4 mock-inoculated 2 un-inoculated
Replications	Randomized in one tray for each treatment	2 replications
Inoculation	3-4 quadrifoliate stage 2 weeks post planting	Same as experiment 1
Sample collection	Pre-inoculation Post inoculation – 12 hr, 24 hr, 48 hr, 48 hr, 7 weeks (inoculated only) Inoculated tissue before 48 hr New tissue collected after 48 hr	Pre-inoculation Post-inoculation – 12 hr, 24 hr, 7 days, 14 days, 30 day (inoculated only) Inoculated tissue before 48 hr New tissue collected after 48 hr

Table 3.4 Primers designed to amplify insertions for candidate gene cDNA cloning

Primer	Leader sequence	Restriction site	Sequence specific primer
Forward	TAAGCA	GTCGAC (*Sal*I)	ATGATCAAAGTTTGGGTTCTTG
Reverse	TGCTTA	GGTACC (*Kpn*I)	TTACTTTGGGCAGTAATAATCT

Table 3.5 Primer sequence for PCR screen of transformed colonies

Test	Forward (5'-3')	Reverse (5'-3')
Positive control (Nos terminator)	TCCTGTTGCCGGTCTTGCGA	TGACACCGCGCGCGATAATTT
Presence of insert	GCCTTCCTCACACCATTTAC	TGACACCGCGCGCGATAATTT

Table 3.6 Percent identity of glutamate receptor duplications in the insertion region to surrounding glutamate receptors based on BLAST results. GR # indicates the order of the duplicated glutamate receptors, as seen in Figure 3.2. Arahy.(gene name) indicates gene hits from BLAST to Tifrunner genome.

	GR 1	**GR 2**	**GR 3**	**GR 4**	**Arahy. 5N45JG**	**Arahy. VZ8YXD**	**Arahy. NV2IFB**
GR 1	100%	100%	100%	100%	99.1% * 36% **	90.9% * 8% **	99.1% * 36% **
GR 2		100%	100%	100%	99.1% * 36% **	90.9% * 8% **	99.1% * 36% **
GR 3			100%	100%	99.1% * 36% **	90.9% * 8% **	99.1% * 36% **
GR 4				100%	99.1% * 36% **	90.9% * 8% **	99.1% * 36% **

* percent identity: percent of nucleotides that are the same between the sequences
** query coverage: alignment length of BLAST result relative to query sequence

Table 3.7 cDNA sequence of c56 and c63 plasmid inserts for candidate gene

c56

ATGATCAAAGTTTGGGTTCTTGTCATTGTGATGTTCTATAATGGTTTTCCATCAAAAG
GGACAAGCAATTGTAATAATGTTACAAGGCCAAGTACAGTTAATGTTGGAGCAATTT
TATCTTTTAATTCAACCATTGGAGGAGTGGCTAAAATTGCAATACAAGCAGCAGTAG
ATGATATAAATTCCAATGCAACCATTCTCAATGGAACTAAGTTTAATATCTCAATGC
AGGACACAAAATTCTCCCCCGGATTTCTAGGAATTATTGACTCATTAATATTGATGG
AGAAAGGCACTGTGGCAATAATTGGTCCACAGTACTCAGAAATGGCACATGTAATC
TCACACATTGCAAATGAGATGCAAGTACCTCTCTTATCATTTGCAGCAACAGATCCT
ACACTCACTTCTCTCCAATTCCCATATTTTGTTAGAACAACACAGAGTGATCTTTATC
AAATGTCTGCAGTGGCAGATATTGTTGATCACTTCCAATGGAGAGATGTGATTGCAA
TCTTCATTGATGATGATCATGGAAGAAATGGGGTTGCTGCATTAGGTGATAAGCTTG
CCGAAAAACGTTGCAAGATATCATATAAAGTACCCTTTAGGCCTAATAATAATAACA
ACATTAGTGAAGAAGAAATAAACAATGCATTATTCAAGATAGCTTTGATGGAATCA
AGGGTAATAGTTGTTCATATAGTAGCAGATTTAGGGTTAAAAGTTCTTAAGGTTGCT
CAATCACTTAGCATGATGGGGAGTGGTTATGTGTGGATAGCCACTGATTGGCTTTCC
ACTGTGTTAGATTCAAACCCTTCATTGTCCACAAGTTCAGCCATGAATGACATCCAA
GGTGTTATTACCTTGCGCATGTATACACCTGATTCAGAATTGAAGAGAAAATTTGTG
TCTAGGTGGAATAATAACCTAACCCTAAAAATGAATCATCAAGAGGGTCCTTTTGGA
TTGAACACCTTTGGTTTATATGCTTATGACACTGTTTGGGTCATAGCTTATGCTCTTG
ATGCTTTGTTTTCTGGGGGAAATAATAATAATAATAATAACAACAATCATAATA
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c63
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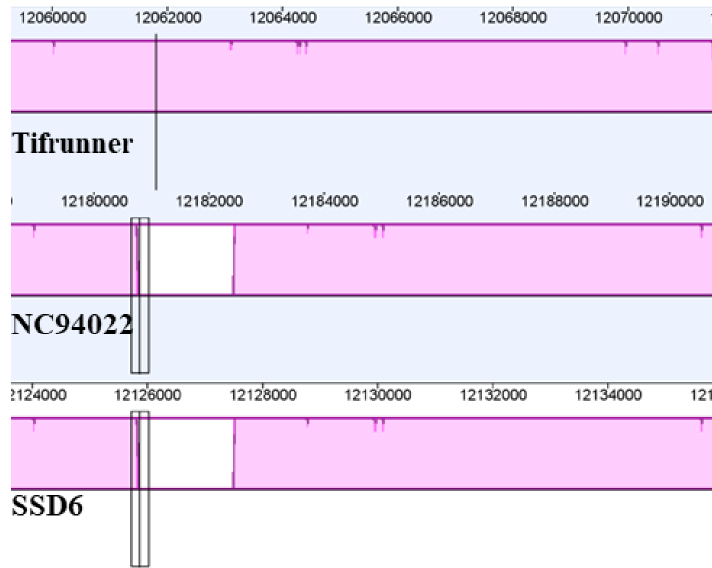
C

A)



B)

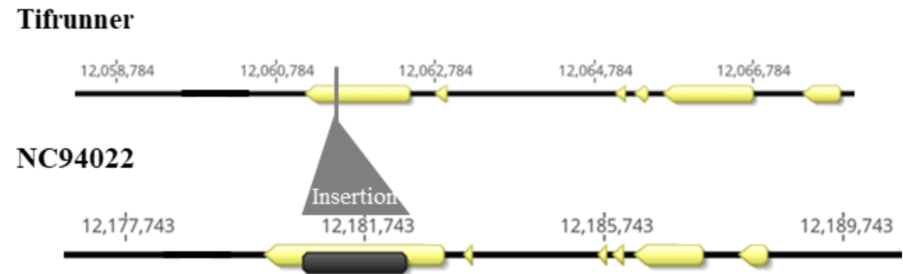
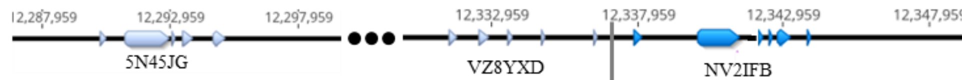


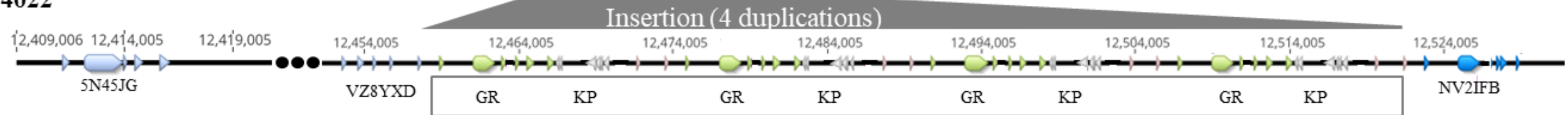
Figure 3.1 Alignment of Tifrunner and NC94022 R-gene region on chromosome A01. A) Pangenome alignment showing insertion in gene region. White block represents inserted sequence present in NC94022 and SSD6 that is not present in Tifrunner. B) Gene alignment in Tifrunner and NC94022; exons represented by yellow arrows. Gray line and box represent location of insertion on Tifrunner and NC94022, respectively. Insertion corresponds to white block in NC94022 sequence in part A. Size difference observed in extended exon six of NC94022 with insertion than Tifrunner without this sequence.

A)

Tifrunner



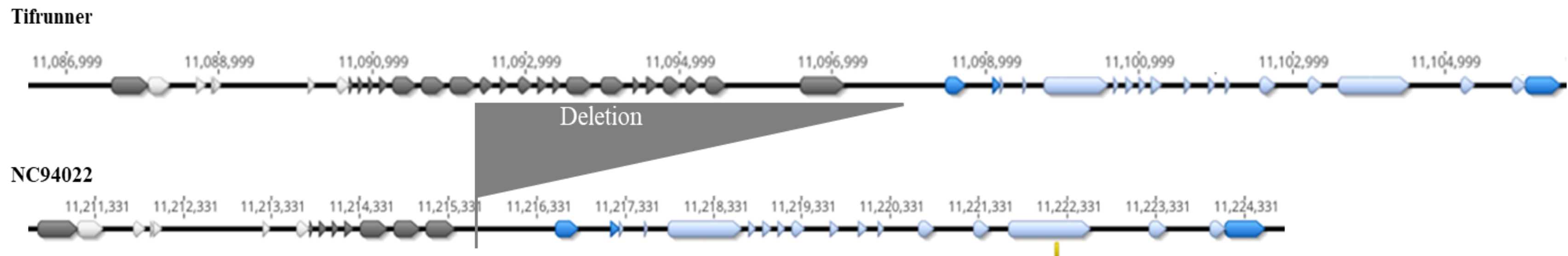
NC94022



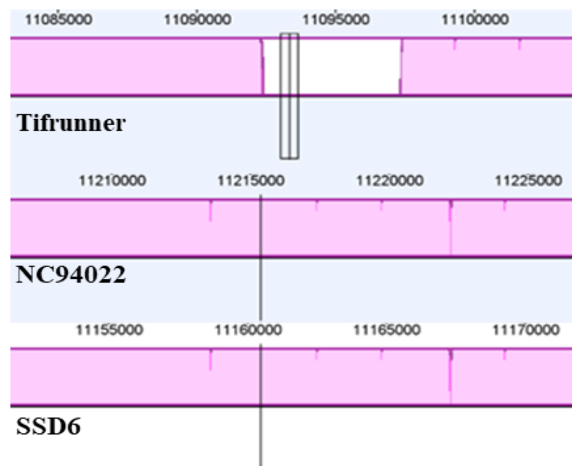
B)

Figure 3.2 Alignment of Tifrunner and NC94022 insertion region on chromosome A01. A) Gene alignment in Tifrunner and NC94022 with inclusion of unique insertion in NC94022. Blue arrows represent glutamate receptors outside insertion region; gene name listed with respective CDS. Insertion region genes represented by green (glutamate receptor) and light gray arrows (kinetochore protein). Glutamate receptors and kinetochore protein duplications in insertion represented by GR and KP, respectively. B) Pangenome alignment showing insertion region in NC94022 compared to Tifrunner. White blocks represent inserted sequence in NC94022 and SSD6 not present in Tifrunner. Insertion in NC94022 aligns with denoted insertion region in part A.

A)



B)



C)

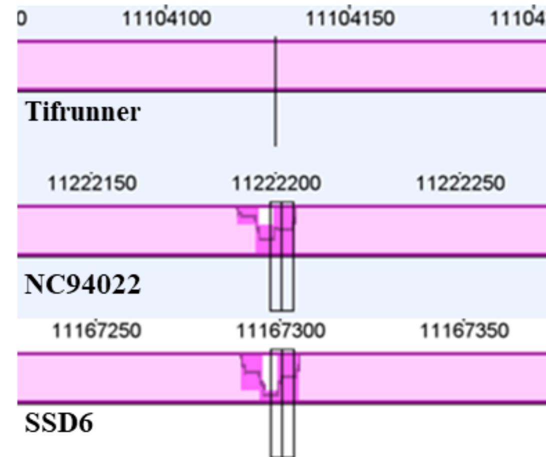


Figure 3.3 Alignment of Tifrunner and NC94022 "deletion" region on chromosome A01. A) Gene alignment in Tifrunner and NC94022 with inclusion of deletion between NC94022 and Tifrunner in the 3'UTRs of Arahy.IK6XD2 (gray arrows) and a six base pair insertion in exon 13 (yellow line) of Arahy.R6E3N5 (blue arrows). B) Pangenome alignment showing deletion region in NC94022 compared to Tifrunner. White box represents region present in Tifrunner that is absent in NC94022 and SSD6.
C) Pangenome alignment showing the six base pair insertion in NC94022 compared to Tifrunner. Lined box indicates sequence present in NC94022 and SSD6 that is absent in Tifrunner.

A)

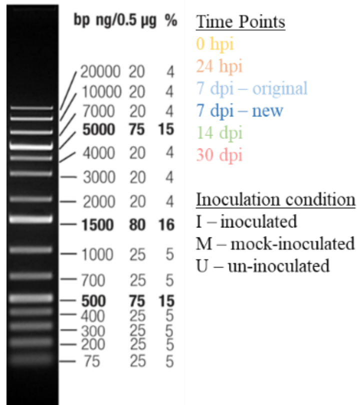
B)

C)

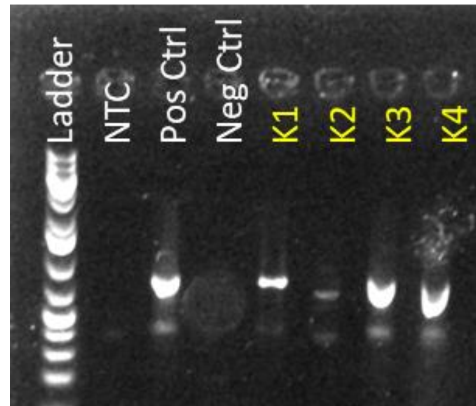
D)

Figure 3.4 Post-inoculation phenotypes of peanut and tobacco plants. Plants from experiment two replication 1 used as representative samples. All images were taken 30 days after inoculation. A) NC94022 peanut plants, left to right - inoculated x 2, mock-inoculated x 2, un-inoculated. B) SunOleic 97R peanut plants, left to right – inoculated x2, mock-inoculated x2, un-inoculated. Star identifies symptomatic plant. C) Symptomatic SunOleic 97R, symptomatic leaf circled. D) K326 tobacco plants, left to right – un-inoculated, inoculated x2.

A)



B)



C)

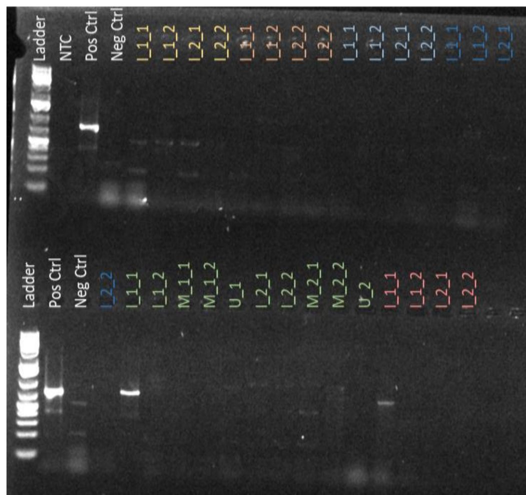


Figure 3.5 PCR confirmation of TSWV inoculation. Presence of TSWV N-gene identified by band size ~800 bp. Samples from study 2 used as examples. NTC = no template control
A) Key for gel details. B) Inoculated tobacco samples. C) Peanut samples, SunOleic 97R only. No symptomatic NC94022 plants.

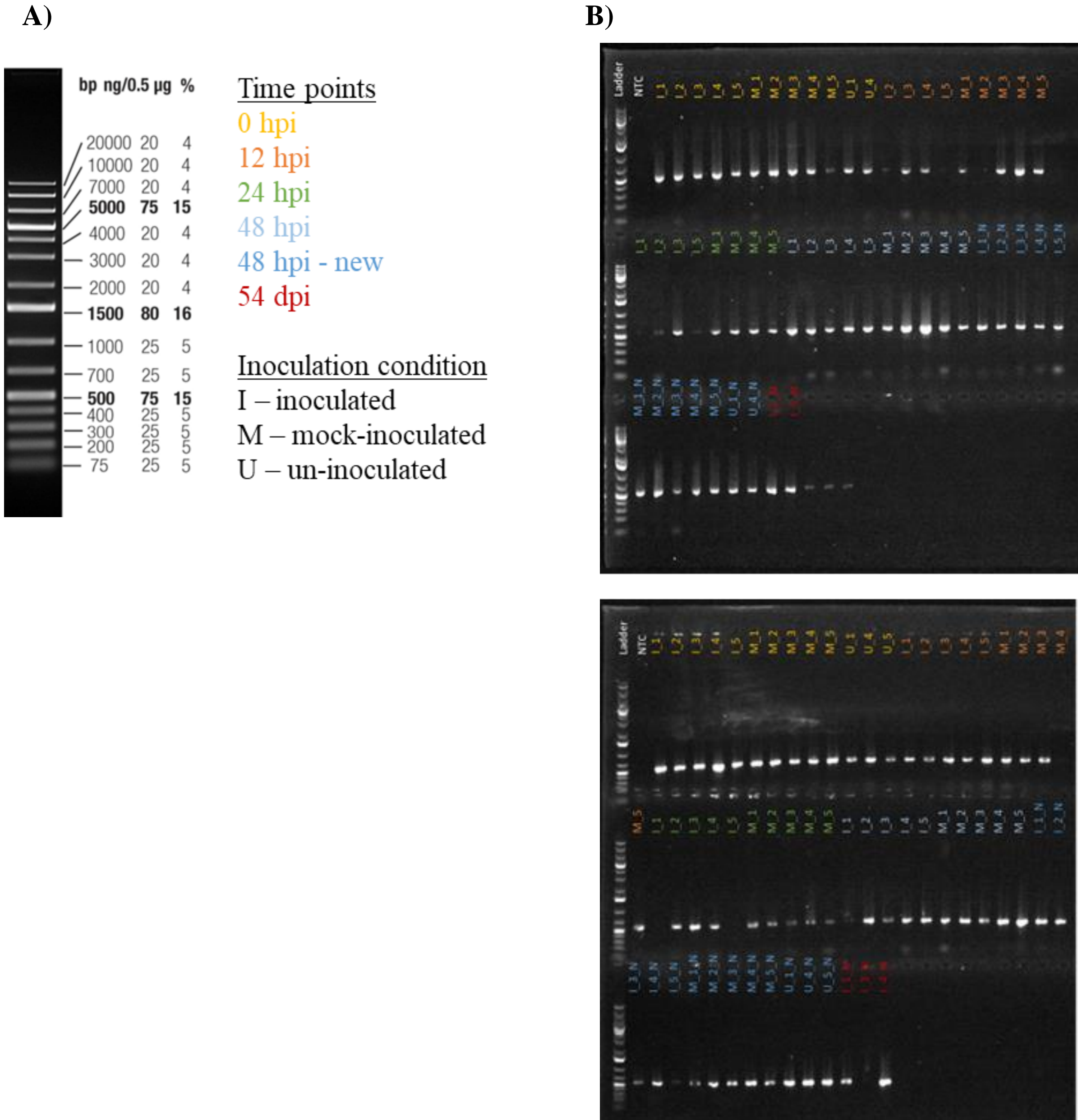


Figure 3.6 Gene expression of R-gene from QTL region on chromosome A01. A) Key showing gel details for samples from inoculation experiment 1. B) Expression results for the R-gene in NC94022 and SunOleic 97R on the top and bottom, respectively. Expected band size ~550 bp. Primer sequences for R-gene included in Table 3.2. NTC = no template control

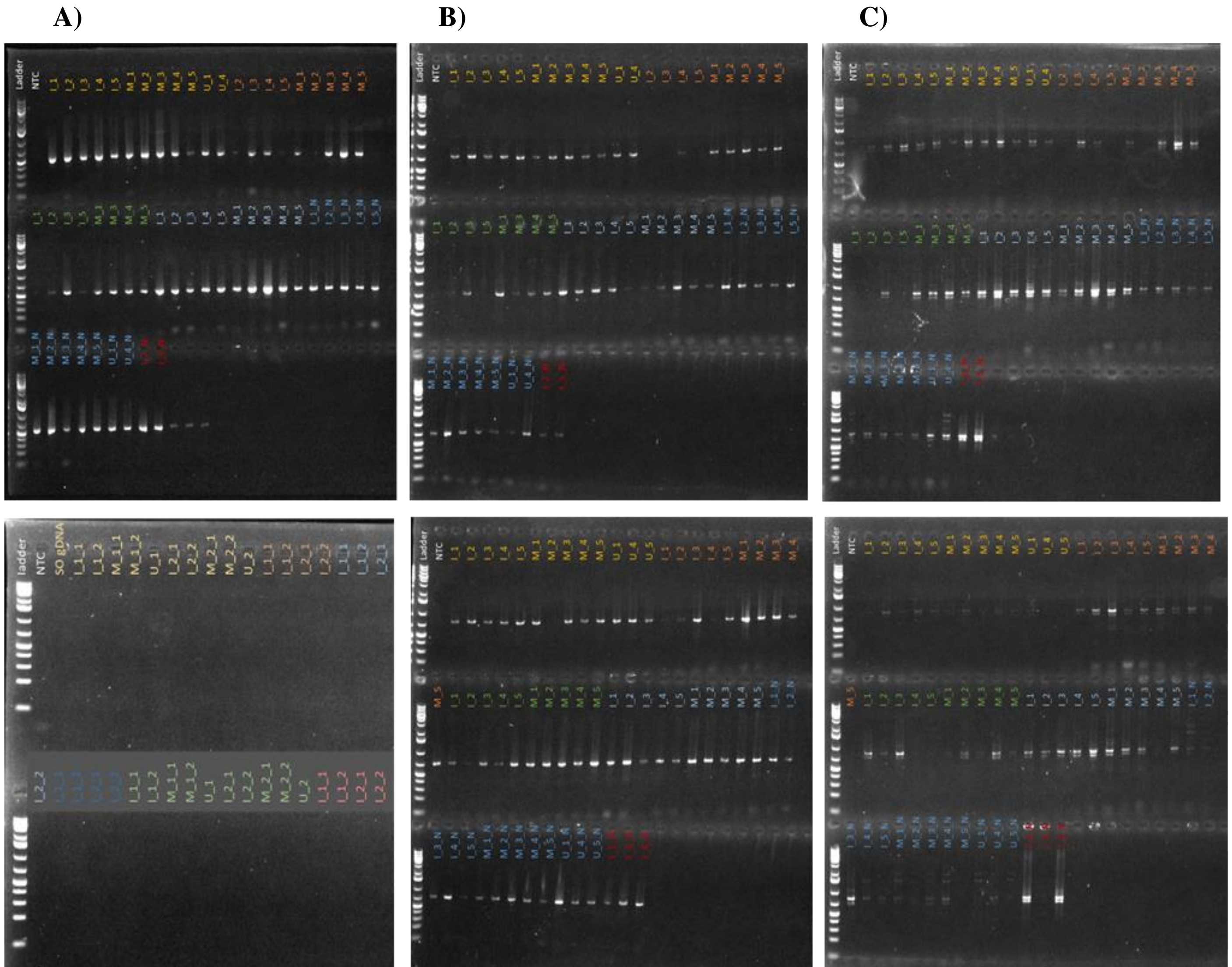


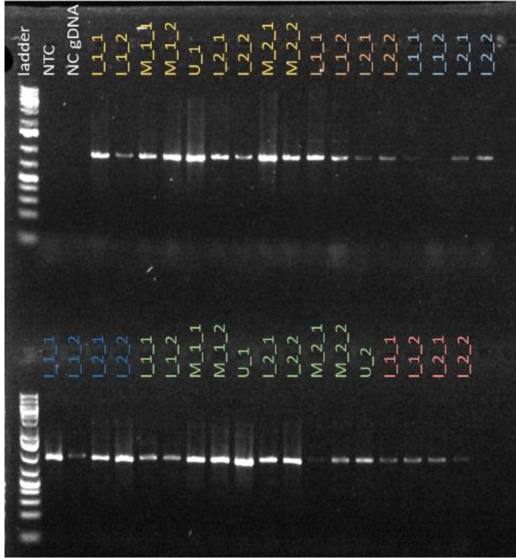
Figure 3.7 Gene expression of candidate genes from insertion region on chromosome A01. Expression results for NC94022 and SunOleic 97R cDNA samples on top and bottom row, respectively. Primer sequences for all genes included in Table 3.2; gene names as indicated in table. Key showing gel details for samples from experiment 2 shown in Figure 3.5A. Key showing gel details for samples from experiment 1 shown in Figure 3.6A. NTC = no template control

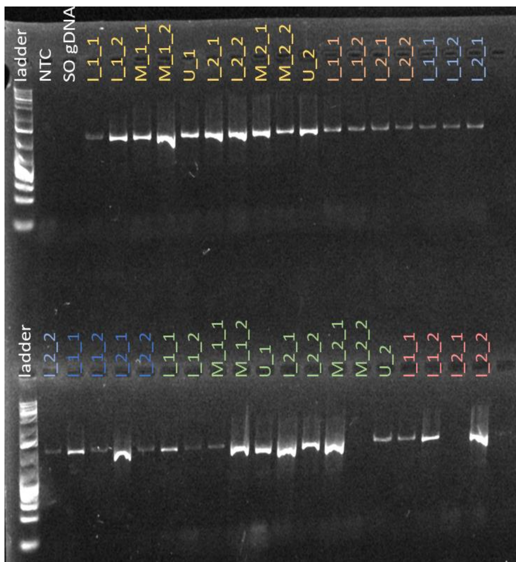
A) Insertion glutamate receptor: samples from experiment 2, expected band size ~450 bp. Expression observed in NC94022 only as gene is not present in SunOleic 97R.

B) Arahy.5N45JG glutamate receptor: samples from experiment 1, expected band size ~700 bp.

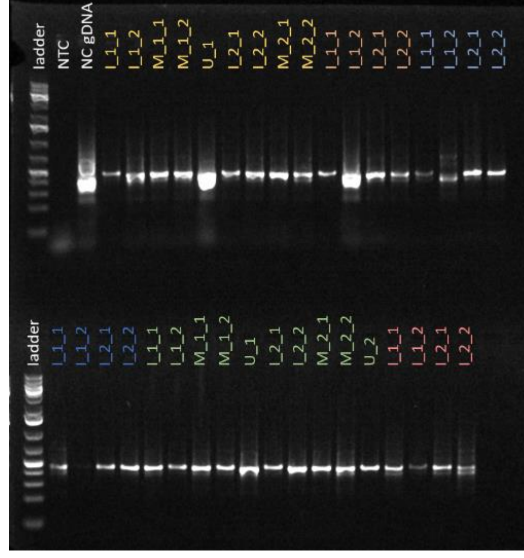
C) Arahy.NV2IFB glutamate receptor: samples from experiment 1, expected band size ~ 700 bp.

A)





B)



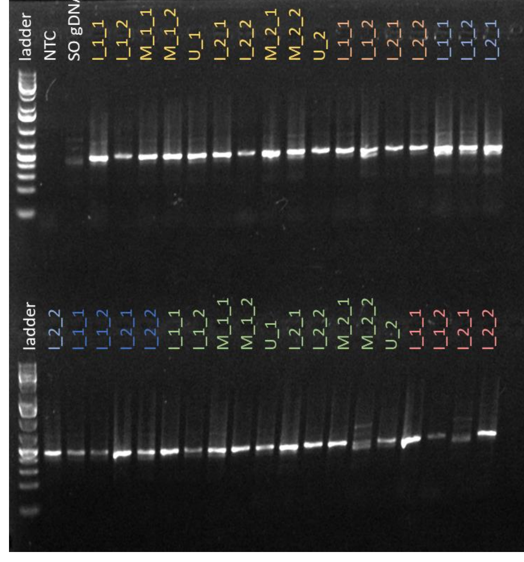
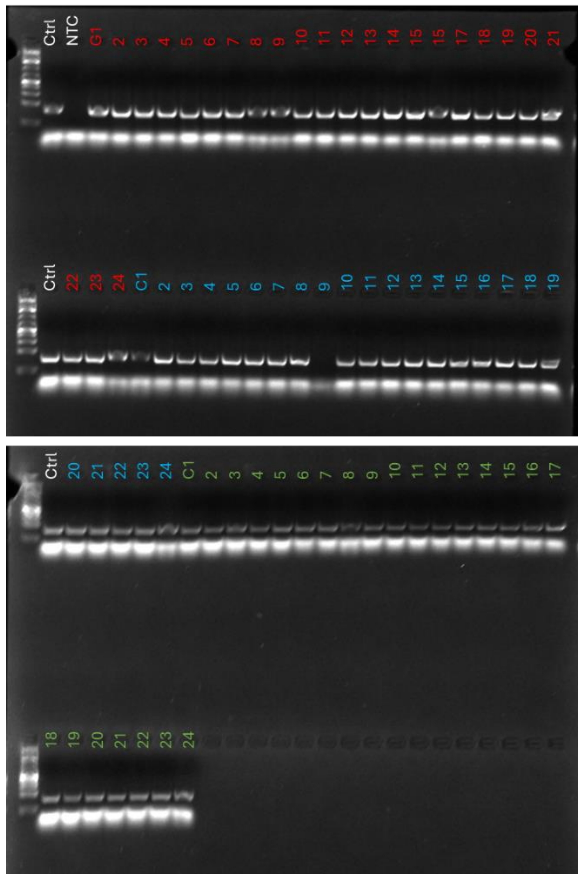


Figure 3.8 Gene expression of candidate genes from deletion region on chromosome A01. Expression results for NC94022 and SunOleic 97R cDNA samples on top and bottom row, respectively. All samples obtained from experiment 2; key showing gel details for samples from experiment 2 in Figure 3.5A. NTC = no template control.
A) Arahy.IK6XD2: expected band size ~ 890 bp. B) Arahy. R6E3N4: expected band size ~ 450 bp

A) **B)**



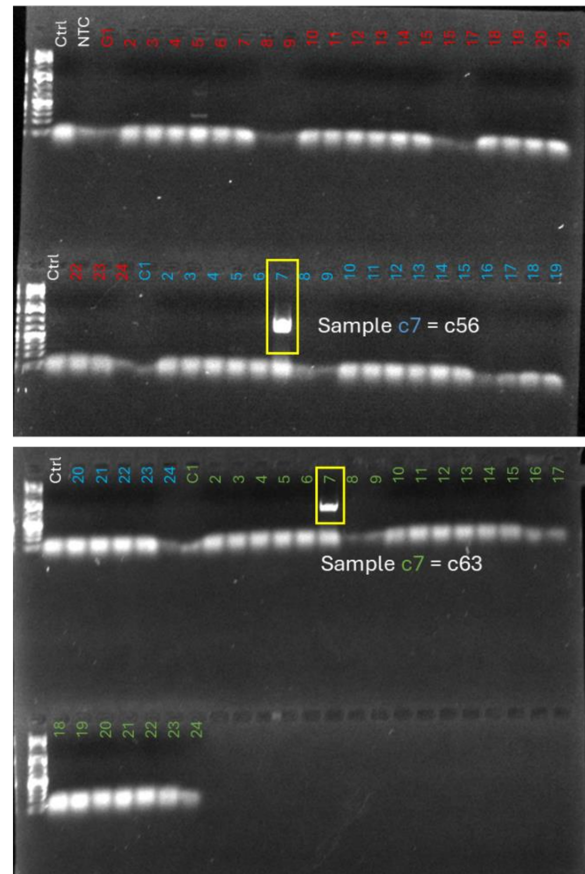


Figure 3.9 PCR screen of transformed colonies with ligated plasmids. Two gels for each for both A and B with the same samples across both primers. Primer sequences as given in Table 3.5. gDNA samples = red, cDNA samples = blue and green for the two cDNA samples, 56 and 63 respectively, used to amplify the glutamate receptor. Control = pBINPLUS35S colony with no insert. NTC = no template control.

A) Positive control amplifying within the Nos terminator of pBINPLUS35S plasmid; expected band size ~196 bp. B) PCR to identify presence of insert in plasmid; forward primer within cDNA insert (location identified in Figure 3.11) and reverse primer within NOS terminator. Expected band size ~1.2 kb if insert is present. Two samples with insert identified in yellow boxes; c7 blue renamed as c56 and c7 green renamed as c63 based on original cDNA source.

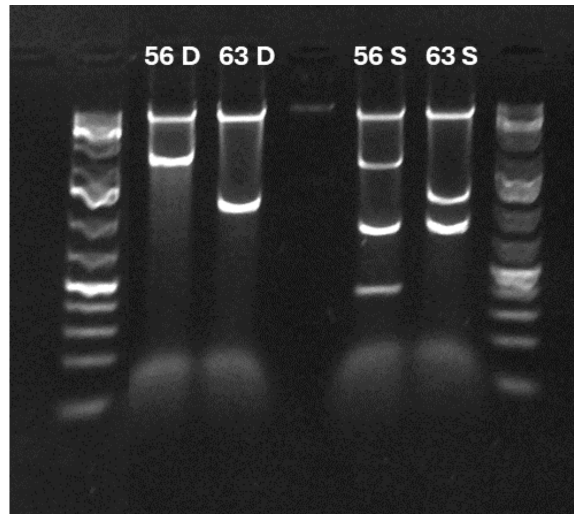
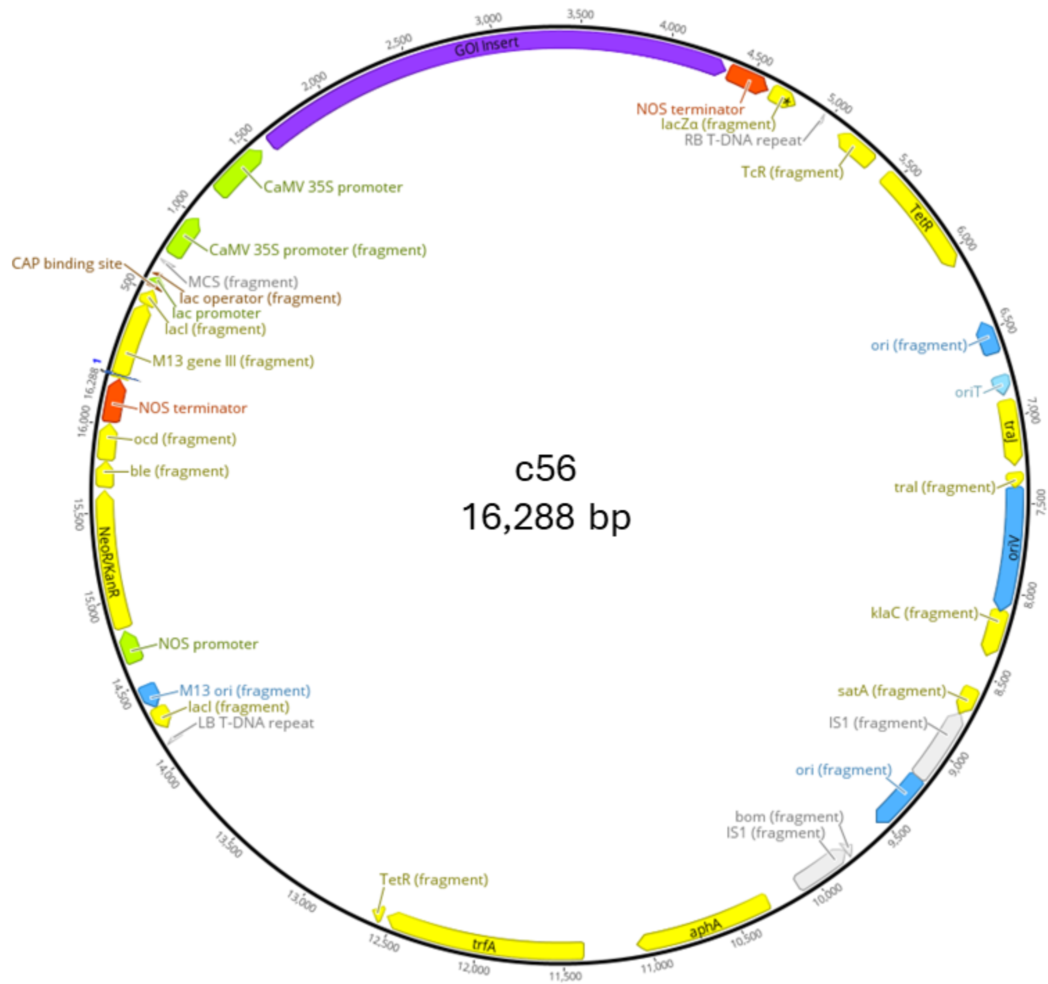


Figure 3.10 Enzyme digestion of positive cDNA clones to confirm insert presence. 56 and 63 indicate plasmid names c56 and c63, respectively (reference Figure 3.9). D = double digest with *Sal*I and *Kpn*I; expected band sizes of ~12.3 kb (pBINPLUS35S vector) & ~2.5 kb (gene insert). c56 meets expected, c63 does not (lower band for insert). S = single digest with *Eco*RI; expected band sizes of ~12.3 kb (pBINPLUS35S vector), ~2.5 kb, ~400 bp, & ~1kb. c56 meets expected, c63 does not (only 3 bands).

A)



B)

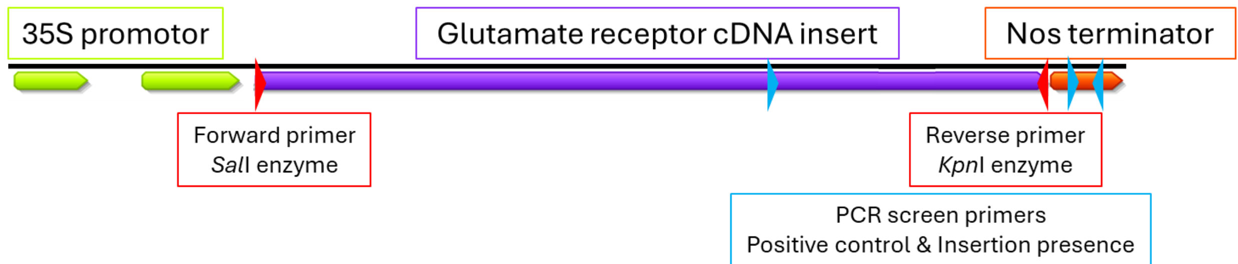
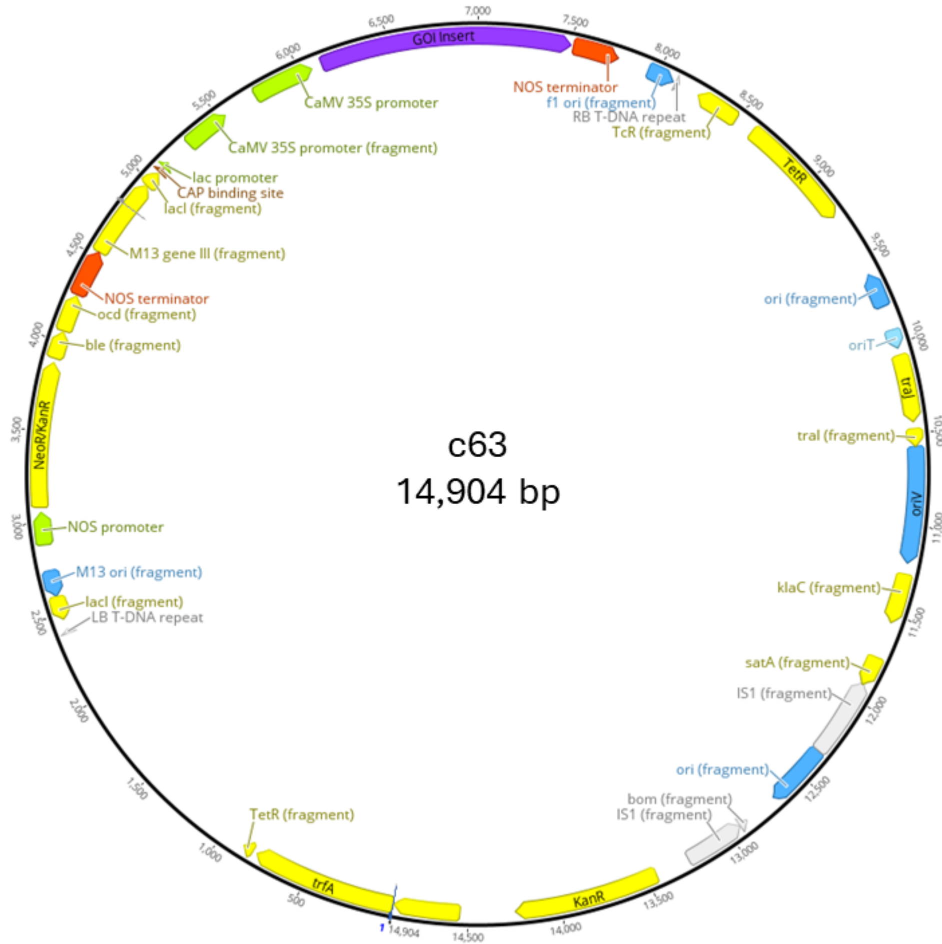


Figure 3.11 A Sequenced plasmids selected for further evaluation of candidate genes – plasmid c56. Insert of candidate gene represented in purple. c56 plasmid with full gene cDNA sequence insert. A) Full plasmid. B) Zoom of 35S promotor – insert – Nos terminator with restriction sites and PCR primers for screening plasmids identified. Primer sequences in Tables 3.4 and 3.5.

A)



B)

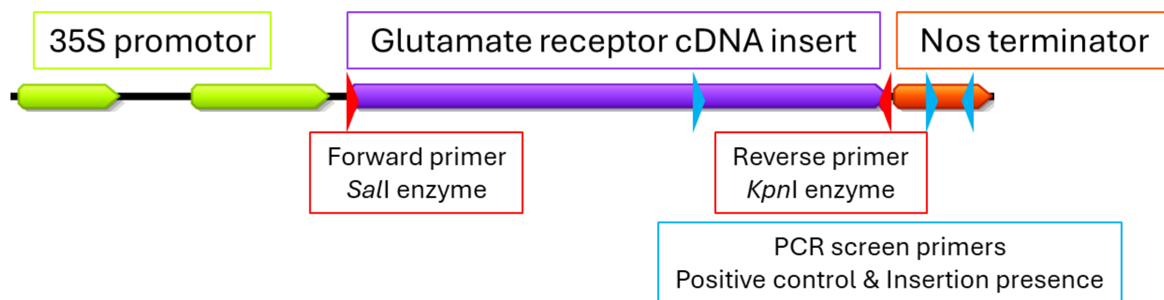


Figure 3.11 B Sequenced plasmids selected for further evaluation of candidate genes – plasmid c63. Insert of candidate gene represented in purple. C63 plasmid with partial gene cDNA sequence insert. A) Full plasmid. B) Zoom of 35S promotor – insert – Nos terminator with restriction sites and PCR primers for screening plasmids identified. Primer sequences in Tables 3.4 and 3.5.

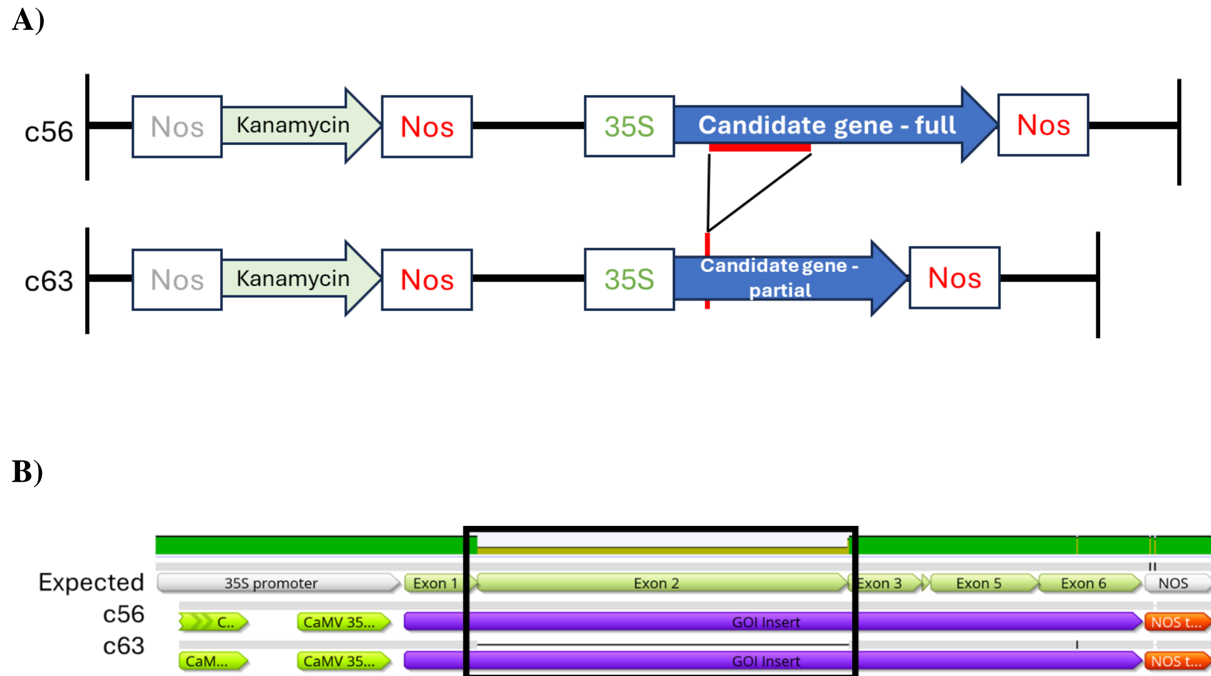


Figure 3.12 Comparison of c56 and c63 gene inserts in final plasmid sequences. A) Representation between left and right borders of plasmid, kanamycin resistance gene and insertion of glutamate receptor cDNAs. Missing sequence in c63 identified by red bar compared to c56. B) Exon comparison of c56, c63 and expected sequence based predicted cDNA sequence from FGENESH analysis and WPS sequencing of plasmids. Black box represents the missing section of c63 compared to c56.

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CHAPTER 4

CONCLUSIONS

Tomato spotted wilt virus is a significant problem in peanut production across much of the United States. When infection occurs in susceptible varieties it can result in yield and profit loss for producers. Genetic resistance is the most impactful way to combat disease in the field. Selection of the right varieties is the first step for the farmer, but providing these resistant varieties is the role of breeders and pathologists. It is therefore important for researchers to understand the foundation of the resistance they are developing. TSWV resistance from PI 203396 was identified in the mid-1990's and has had significant impact on breeding programs and the overall peanut industry. The goal of this project was to improve our knowledge of the genetic basis of disease resistance in an underutilized source of resistance to TSWV in *Arachis hypogaea,* SSD6 and NC94022*,* and incorporate it into the breeding program for future use.

Published studies reported a QTL for TSWV resistance on chromosome A01 of *A. hypogaea* that derives from NC94022, a highly resistant progeny of SSD6 (PI 576638). A resistance gene and an insertion region from NC94022 were found within a narrowed version of this QTL. Marker assisted selection was used to select lines from eight unique populations that contained this QTL. These populations were developed from a recombinant inbred line (SSD6 x Tifrunner) and eight unique parents to combine the two known sources of TSWV resistance with good agronomic traits. Individual F_2 plants with the R-gene and with or without the insertion region were evaluated in the field for their TSWV resistance. This first field study indicated that

when the insertion region is present there is a significantly lower TSWV rating based on symptoms and stunting of infected plants.

The most resistant and agronomically favorable individuals were harvested for further evaluation at the $F_{3:4}$ generation. These lines were planted in replicated plots alongside their parents and checks, NC94022, Georgia-06G, and TSWV-susceptible MARCI. A select set of lines were again evaluated at the $F_{4:5}$ generation alongside Georgia-06G and two parent lines in a new field than the previous years. TSWV resistance was evaluated based on symptom coverage of the canopy, and presence of the insertion region again significantly improved TSWV resistance. Improved resistance was observed in comparison to lines without the insertion region, but also lines with resistance from PI 203396 alone. Our populations were not as resistant as NC94022 but this opens the door to find other regions of interest for TSWV resistance from this source.

Alongside these field studies, lab evaluations were conducted to explore the source of resistance from NC94022 at the gene level. Two regions of the A01 QTL were examined for structural variations between NC94022 and Tifrunner using whole genome sequences. Pangenome alignments and gene prediction models were used to clarify the regions of interest and identify candidate genes for resistance. Four genes were selected as candidate genes, including the R-gene (Arahy.1PK53M) and unique glutamate receptor gene duplicated within the insertion region of NC94022, a pleiotropic drug resistance gene (Arahy.IK6XD2) and a RNA-binding protein (Arahy.R6E3N5) from the upstream deletion region. The sequence of each gene selected showed dissimilarities between NC94022 and Tifrunner. PCR primers for all of these genes were developed to detect gene expression in NC94022 and SunOleic 97R plants with and without mechanical inoculation. Expression results determined that neither inoculation status nor

time after inoculation impacted expression of the candidate genes. Based on field results and sequence comparisons, the unique glutamate receptor genes from the insertion region of NC94022, where the duplications are 100% identical, was selected as the final candidate gene. The cDNA sequence of this gene was inserted into a binary expression vector, pBINplus35S, for overexpression of the gene. The developed plasmid will be used to transform susceptible *N. tabacum* lines to test the glutamate receptor's effect on TSWV resistance.

The purpose of these projects was to provide a deeper understanding of the genetic resistance to TSWV provided by SSD6. PI 203396 has provided growers with significant resistance for many years, but TSWV is still a problem in many fields. Having a second source of resistance available to act alone or in combination with widely deployed resistance could greatly benefit peanut producers. The lines developed in this project will continue to be evaluated in the breeding program for their TSWV resistance and agronomic traits, but also for other traits they may possess. Several lines were identified to contain markers for nematode and late leaf spot resistance, as well as the high oleic acid trait. Field studies are necessary to confirm the presence of selected traits, but providing producers with multiple disease resistance is an important benefit in many field conditions. Future farmers will not only be able to access multiple disease resistance, but breeders will be able to more easily incorporate TSWV resistance using MAS once a more precise region for resistance is known. The markers used here to identify the QTL on A01 did select for lines with improved resistance, but more resistance can still be obtained from NC94022 and/or SSD6. Pangenome alignments and KHUFU sequencing will be used to explore other regions of interest for their involvement in resistance. New KASP markers can be designed to test these regions against the already collected phenotypes. Quantitative PCR will also be used to explore the differences between NC94022 and SSD6 in the

number of duplications present in the insertion region to determine if this plays a role in the different resistance levels seen.

In the end, this research will be of great benefit not only for peanut research but for producers and consumers. Peanut is an extremely important crop around the world and understanding the foundation of resistance to TSWV is an important piece to preserving this essential crop.