

DISSERTATION

MAPPING RHIZOCTONIA ROOT AND CROWN ROT RESISTANCE FROM SUGAR BEET  
GERMPLASM FC709-2 USING NEW GENOMIC RESOURCES

Submitted by

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## ABSTRACT

### MAPPING RHIZOCTONIA ROOT AND CROWN ROT RESISTANCE FROM SUGAR BEET GERMPLASM FC709-2 USING NEW GENOMIC RESOURCES

Sugar beet (*Beta vulgaris* subsp. *Vulgaris*) provides about 35% of the refined sugar globally, and over half of the domestic production in the United States. Sugar beets are primarily grown in temperate climates from plantings in late spring and harvest in the fall. In the United States sugar beets are grown in four diverse regions: the upper Midwest (Minnesota and North Dakota), the far west (California, Idaho, Oregon, and Washington), the Great Plains (Colorado, Nebraska, Montana, and Wyoming), and the Great Lakes (Michigan).

Multiple pests and pathogens continue to threaten tonnage and recoverable sugar yields. These are controlled through planting genetically resistant cultivars, agronomic cultural practices and chemical applications throughout the growing season. With a shrinking set of chemical and cultural control options to manage these production threats, the need for continued improvement upon host plant resistance is important. Decades of global breeding efforts to improve disease tolerance in sugar beet has been effective, but molecular and genomic guided breeding and disease resistance characterization in sugar beet is only now emerging.

The most important root pathogen in sugar beet is Rhizoctonia Root and Crown Rot (RRCR) caused by the fungal pathogen *Rhizoctonia solani*. This disease is estimated to cause up to 50% localized losses, and regularly causes 57 million dollars in economic losses per year despite the use of tolerant varieties, chemical control, and cultural practices. Public sugar beet pre-breeding has developed hundreds of widely utilized lines with novel traits and combinations

of traits, including for RRCR resistance. One such line, FC709-2, displayed exceptional tolerance to *Rhizoctonia solani* released from the United States Department of Agriculture sugar beet breeding program in Fort Collins, Colorado. This germplasm line is base for many RRCR resistant cultivars used by growers around the world.

In this study, new germplasm, genetic, and genomic resources revolving around FC709-2 were developed. These resources include a new germplasm line derived from the purification of FC709-2. By using stricter selection pressure and single seed decent a more homogenous seed lot was created to be used by other breeding programs. A new reference genome created from a single highly RRCR resistant plant using the most recent sequencings and bioinformatic technologies will be used to discover genes that are responsible for a wide array of plant interactions. Last, novel QTLs associated with RRCR resistance were discovered using a biparental mapping population and bulk segregate analysis. Collectively, these results show that discovering novel RRCR resistance genes in a highly resistant germplasm line using a purpose-built reference genome is a streamlined and accurate method. With these new resources in place researchers around the world can use them to discover the genes responsible for RRCR resistance, create markers for more accurate selections, and follow the methods described to be implemented in other plant breeding programs.

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## DEDICATION

*For my parents, my wife Savannah, my future daughter, and for myself*

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# **Chapter 1: Introduction and Literature Review**

## **1.1 History of Sugar beet and its economic value**

Sugar beet (*Beta vulgaris* L.) is grown for the extractable sweetener sucrose, commonly known as table sugar. Sucrose is a disaccharide composed of glucose and fructose, produced and translocated in various plant tissue (Koch 2004, Li, Wu et al. 2017). In sugar beet, sucrose accumulates in the enlarged taproot, while the other major sucrose producer sugarcane accumulates sucrose in the stem. Sugar cane (*Saccharum officinarum* L.) and sugar beet are the number one and two most important sources of commercial sugar produced worldwide (Draycott 2008). Sugar cane is a tropical perennial grass and sugar beet is a biennial dicot grown in temperate climates. Sugar beet in a cropping system is grown as an annual for sugar production. With the advancements in breeding, sucrose from both species has increased over time to the extent that it is inexpensive and abundant for most of the world's population. However, this was not always the case throughout history.

Sugar cane has been cultivated longer than sugar beets. Crystallized sucrose production began in India over 2,300 years ago (Hill 1952). Improvements in sugar manufacturing occurred in Persia and in Eastern Mediterranean cultures, so that by 800 AD sucrose was widely available in the Arab world. Europeans had to import sugar, so it remained an expensive commodity. Europeans expanded cane cultivation to their tropical American colonies in the 1500s. By the 1800s, cane sugar held a central position in the world economy.(Scott and Jaggard 1993). The cultivation of sugar beet as a source of sugar in Europe can be traced French emperor Napoleon Bonaparte's restrictions on the import of British goods. This led to the British blockading France to deny access to its own ports. The blockade led to a drastic decrease in sugar, which prompted a

Napoleonic decree to increase beet sugar production. This increase in manufacturing elevated beet sugar science and innovation in the industry (Scott and Jaggard 1993).

Sugar beet was developed primarily from fodder beet, a common crop grown for animal consumption. Markgraf, in 1747, was the first to report the sweet substance isolated from sugar beet was the same as the substance from sugar cane. His student Franz Carl Achard in the 1780s was the one to identify, select, and propagate sweeter forms of the substance (table sugar). Achard's petition to develop a sugar factory in Silesia (now Poland) for the manufacturing of beet sugar from white fodder beet was granted in 1801. The first varieties of sweet fodder beets were high yielding, but mostly water weight, and sucrose percentages were low. Specific gravity was used to determine which beets had the highest sugar content. The now routine progeny test, widely used in breeding, was likely first applied to increase sucrose content in beets. In 1859, Volmorin used specific gravity to select mother roots for seed production, and tested progeny to select only those with high specific gravity to advance in the breeding program (Gayon and Zallen 1998). Over the next century sucrose levels have increased from 4% (fresh weight) to 15% or more (Wiley 1890, Archimowitsch 1956, Ware 2000). Today many sugar beet hybrids achieve 18% sucrose in optimal growing conditions.

By the mid-1800s, sugar factories began opening around the world. The first U.S. sugar factory opened in 1841, in Northampton, Massachusetts, but closed shortly after its first season. This was attributed to low yield of beets due to disease pressures, unsuitable growing conditions, improper cultivar availability, and naive agronomic and sugar manufacturing practices. The first profitable factory opened in Alvarado, California in 1870. Many factories were built across America prior to World War One, but most closed by the 1960s as improvements in germplasm, agronomy, and processing generated surplus sugar. Globally, a majority of the sugar beet

production takes place across Northern Europe and North America. Beets of all crop types are grown on every continent except Antarctica. Sugar beets need to be processed in order to extract sucrose, so factories need to be built in relatively close proximity to the production areas. This reduces transportation cost from the large amounts of biomass being transported. Factories generally service more than 6000 hectares of beets during a processing campaign lasting around 4-6 months. Sugar beet is a technical crop to grow, and agronomic practices developed from physiological considerations have been key in increasing productivity (Scott & Jaggard, 1993). Plant populations have increased over time as more attention has been paid to early-season growth and survival of seedlings. Yield of sugar beets has doubled over the past 50 years (Scott and Jaggard 1993, Hoffmann and Märlander 2005, Panella, Campbell et al. 2016) although percent sucrose has increased marginally during this time.

In the United States, sugar beets contribute 50-60% of sucrose delivered. Worldwide beets are 19-26% of total sucrose production. The predominant use of sucrose is for human consumption with 61% of the U.S. deliveries in 2016 being used in soft drinks, candy, and baked goods such as cookies, cakes, and breakfast cereals (Table 16a in USDA Economic Research Service, 2016). Less than 2% of 2016 U.S. sucrose deliveries were for non-food uses (Table 16a in USDA Economic Research Service, 2016). Fatty acid derivatives of sucrose can be polymerized for use in plastics, inks, and coatings. This is why the breeding target for beets has been for higher sugar yielding varieties.

Additional uses for sugar beets are still being discovered today. One use that has been recently discovered is using beets as a form of biofuels (Panella 2010, Eggleston and Lima 2015). Sucrose to ethanol via fermentation with yeast is a well-known technique that has been used for hundreds of years. Corn grain ethanol fermentation has been the primary source for biofuel energy

production. Energy beets represent a new class of beet crop type (McGrath, Townsend et al. 2016). Germplasm resources for energy beet typically come from existing sugar beet hybrids. In addition to being a source for ethanol, biogas can also be utilized using energy beet lines.

Breeding and cultivation of beets for new uses will likely continue to employ breeding methods used in sugar beets (Panella 2010, McGrath, Townsend et al. 2016). Historic uses for beets beyond sucrose production includes ancient vegetables, animal fodder, and co-products recovered during the sucrose extraction process (pectin, betaine, emulsifiers etc.) (Cholewa, Guimaraes-Ferreira and Zanchi 2014, Eggleston and Lima 2015, McGrath, Townsend et al. 2016, Ralla, Salminen et al. 2017). Beet pulp (the insoluble root tissue left after sucrose extraction) and molasses (the liquid remaining after sucrose refining) are used as animal feed or as feedstocks for the manufacturing of pharmaceuticals. Other opportunities for beets are the production of inulins, fructans, and novel monomers, such as xylose and arabinose, for polymer feedstocks (Werpy and Petersen 2004, Gurel, Gurel and Lemaux 2008). As the target products of sugar beet continue to evolve over time, so will breeders' techniques and knowledge to obtain those products.

## **1.2 Physiology of Sugar Beet**

Sugar beet (*Beta. vulgaris* spp. *vulgaris* L.) is a core eudicot clade in the order of Caryophyllales, family Amaranthaceae (formerly Chenopodiaceae), subfamily Betoideae. Plants in the order are often found in stressful environments and are thought to have diverged from the two core eudicot clades (Astrid and rosid) by 100 million years ago (Group 1998). Evidence suggests the Beta species had differentiated from its closest relatives around six million years ago, the *Patellifolia* species (Romeiras, Vieira et al. 2016). Sea beet, also known as wild beet (*Beta vulgaris* spp. *maritima*), is commonly known as the wild ancestor of cultivated types of beets (Stevanato, Trebbi and Saccomani 2017). Sea beets are often found within a few meters of the sea

growing on the beach and coastal areas. Seed dispersal is facilitated by the seed floating on sea water and transported by ocean currents (Werpy and Petersen 2004, Fievet, Touzet et al. 2007). Sea beets are indigenous to the Mediterranean coasts ranging from Morocco to Lebanon to Spain and north along the Atlantic coast to Scandinavia. In their original state, *B. vulgaris* types and subspecies are outcrossing, wind-pollinated, self-incompatible, and cross compatible.

Cultivated beets can be classified by their use as a crop (sugar, fodder, leaf/chard, table beet/beetroot) (Lange, Holtgräwe et al. 2008). In Ancient times beets were likely collected and then cultivated for their leaves for both food and medicinal uses (Stevanato, Trebbi and Saccomani 2017). The leafiness of the biennial varieties was likely favored over the annual types, but this is uncertain as the origination of cultivated types is unknown. The first known account of the swollen root characteristic is also unclear, but it is possible that it originated in Persia (Zossimovich 1940). By the eighteenth century, swollen roots were widely used for food and fodder. The first dedicated sugar beet cultivars were available by the 1860s (Biancardi et al., 2019).

The shape and morphology of enlarged and single unbranched root crop taproots are completely different from those of the wild type varieties. Wild type roots are thin and highly branched. Leaf beet roots are often thicker but retain the highly branched nature of the wild types. Leaf beets are selected for their foliage and petiole color (ie. rainbow chard). Color in beet consists of alkaloid betalain pigments that serve similar functions as anthocyanins in most angiosperms (Grotewold 2006, Brockington, Walker et al. 2011, Lopez-Nieves, Yang et al. 2018). Hypocotyl color has often been used in sugar beet breeding as a physical marker to assure hybridity, since red hypocotyl color is dominant to green color (Panella, Lewellen and Hanson 2008). Sugar beet roots have been selected to be uncolored because of impurities that can form and hinder the sucrose production in processing. These impurities have been somewhat responsive to selection in the

breeding process (Hoffmann and Märländer 2005). Impurities are only a concern in sugar beets and not the other beet crop types.

The sugar beet taproot, the harvested product, consists of water (~75%), soluble solids (~20%; ~75% as sucrose), and insoluble solids (~5%) (Hoffmann and Märländer 2005, Hoffmann 2010). These values can range by genotype and growing environment. Sucrose content and total dry matter are highly correlated (Hoffmann and Märländer 2005, McGrath and Trebbi 2007). Sucrose biosynthesis occurs in sugar beet as it does in other plants and the means and mechanism of its accumulation are becoming clearer (Winter and Huber 2000, Lunn and MacRae 2003, Etxeberria, Pozueta-Romero and Gonzalez 2012), including through identification of the transporters that load it into the phloem (Nieberl, Ehrl et al. 2017) and unload it into the vacuoles of the taproots (Jung, Ludewig et al. 2015). Sugar beet leaves play an important role in sucrose production. The ratio of biomass between leaves and roots is under genetic control, and can be selected for higher root-to-leaf ratios resulting in increased sucrose yield (Snyder and Carlson 1978, Snyder 1985).

Sucrose is accumulated in vacuoles of the root parenchyma cells. Sucrose is typically concentrated within the innermost 5-6 of 12-15 concentric cortical rings at the point of maximum root width. Supernumerary cambial rings, which are unique to beets, begin developing within a few weeks after germination and continue to increase in width throughout the growing season (Kenter, Hoffmann and Märländer 2006). These ring features are key in beet's ability to store a large number of high-value compounds. (Describe development with Hayward 1938). The pulp remaining after sucrose extraction is mostly plant cell-wall material. Generally, pulp is dried for sale as animal and pet feed. Sugar beet cell walls are unique in that they have low levels of xyloglucan and high levels of pectin, are rich in neutral sugar side chains, and have highly

acetylated pectic homogalacturonan (McGrath, Townsend et al. 2016). These properties of the beet taproot provide for a rich environment for pathogens to infect and proliferate.

Disease management is critical for sugar beets. Beet pathogens affect all crop types to varying degrees (Harveson, Hanson and Hein 2009). This includes various fungi, bacteria, viruses, nematodes, and insects. There is a continuing need for high levels of genetic resistance or tolerance to many of these biotic stressors (Biancardi 2005, Biancardi, McGrath et al. 2010, Biancardi, Panella and Lewellen 2012, Biancardi and Tamada 2016). A majority of public breeding efforts go towards introgressing disease and stress tolerance into germplasm releases (Doney 1995, Panella, Campbell et al. 2016). Diseases and pests affect beets in all stages of its lifecycle (Harveson, Hanson and Hein 2009), and genetic tolerance or resistance exists for most. The most common seedling diseases include Pythium, Aphanomyces, and Rhizoctonia damping off, some of which can be controlled with chemical seed treatments and genetic resistance. Additional genetic resistance is continuously sought after in breeding programs for long term control. The major fungal root diseases include Rhizoctonia, Aphanomyces, and Fusarium, which is primarily controlled using genetics. Rhizomania “crazy root,” caused by Beet Necrotic Yellow Vein virus (BNYVV), which is transmitted by the soil organism *Polymyxa betae*, is one of the most destructive beet diseases in the world (Biancardi and Tamada 2016). At least two genes (*Rz1* and *Rz2*) are being deployed either individually or in combination with one another in an effort to genetically control for the virus. There have been resistance-breaking strains of the virus and new sources of resistance are continuously being searched for. Another major worldwide sugar beet disease is Cercospora leaf spot (*Cercospora beticola*), which has the potential to defoliate the crop and cause economic yield losses. Other yield impacting diseases include curly top (Geminiviridae,

Curtovirus) and powdery mildew (*Erysiphe betae*). Sugar beet cyst nematodes (*Heterodera schachtii*) is another major emerging sugar beet pest worldwide.

Recent achievements in beet breeding relate to the identification of new sources of resistance and introgression of these into sugar beet germplasm (Doney 1995, Biancardi, McGrath et al. 2010, Panella, Campbell et al. 2016). *Beta maritima* will continue to be an important genetic source of disease resistance for sugar beets. Due to their outcrossing nature, disease resistance genes in beets tend to act in a dominant or additive nature. Only a few of these resistances have been well characterized and will be the focus of many breeding programs moving forward.

### **1.3 Crop Wild Relatives**

Sugar beets primary gene pool is in the Section Beta, whereas the Sections *Corollinae* and *Nanae* and the genus *Patellifolia* are considered the secondary and tertiary gene pools (Harlan and de Wet 1971, Kadereit, Hohmann and Kadereit 2006, Biancardi, Panella and Lewellen 2012). Wild species outside of the primary gene pool such as *Patellifolia* (*P. patellaris*, *P. procumbens*, and *P. webbiana*), carry important disease resistance traits. However, these traits cannot be introgressed because of a lack of chromosome pairing resulting in the inability to have homologous recombination (Tränkner, Lemnian et al. 2016). A chromosome translocation from *P. procumbens* was effective at transferring sugar beet cyst nematode resistance traits, but with it came a significant yield drag (Savitsky 1975). These varieties were only used during times of heavy infestations (Panella and Lewellen 2007). In the following studies it is clear that this gene (*HsIpro-1*) is not sufficient for full protection against sugar beet cyst nematode (McGrath, Townsend et al. 2016). Other disease resistant traits that *Patellifolia* harbors include powdery mildew, Cercospora Leaf Spot, and Beet Curly Top virus (Curtis 1968, Biancardi 2005). The taxon remains an attractive and rich genetic resource for sugar beet improvement.

The more useful taxon has been the Sea Beet (*Beta maritima*), providing many novel resistance genes (Frese, Desprez et al. 2000, Panella and Lewellen 2007, McGrath, Panella and Frese 2011, Biancardi, Panella and Lewellen 2012). The first documented transfer of these disease resistant genes from sea beet to sugar beet was achieved by Munerati, using sea beet growing in Italy's Po Delta as a source of resistance to *Cercospora* leaf spot (Munerati, Mezzadrolì and Zapparoli 1913, Biancardi, Panella and Lewellen 2012). In recent years, sea beet has been mined for resistance to rhizomania. The widely used single dominant gene for resistance (*Rz1*), discovered in commercial germplasm, has been overcome by the virus. Other genetic resources were then screened, especially sea beet, and the discovery of another resistance gene (*Rz2*) was found. This gene had a greater effect than that of *Rz1*. Most commercial hybrids used today are carrying either *Rz1* or *Rz2*, or both (Biancardi, Lewellen et al. 2002, Biancardi, Panella and Lewellen 2012).

The United States Department of Agriculture Agricultural Research Service (USDA-ARS) National Plant Germplasm System contains about 2700 holdings from seeds collected in the wild, materials, and germplasm releases. This material can be accessed through the Germplasm Resources Information Network (<https://npgsweb.ars-grin.gov>). The seed stored for distribution are maintained at the Western Regional Plant Introduction Station in Pullman, Washington. There they test viability of stored seed and distribute seed that is viable. If quantities are insufficient, they increase seed quantities for accessions that have poor viability or the quantity has fallen below critical levels, identify gaps in the collection, and arrange for seed transfers and collection trips to fill in the gaps of poorly represented geographic regions. In Europe the well-maintained collection is the Dutch-German *Beta* collection, located in the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany, which has more than 200 accessions. Materials are shared

between the U.S. and European germplasm centers with the goal of defining core germplasm sets. Germplasm characterization is facilitated by the germplasm centers in collaboration with beading programs around the world. The genetic diversity is high within *B. vulgaris* (Li, Lühmann et al. 2011, Andrello, Henry et al. 2016). Allelic diversity in wild species may be as high as three times of that found in cultivated species (Mita, Dani et al. 1991, Richards, Brownson et al. 2004, Saccomani, Stevanato et al. 2009). Utilizing this diversity will result in the enhancement of sugar beet germplasm. Going back to crop wild relatives with cutting edge sequencing technology and breeding techniques will allow breeders to bring forward enhanced traits into elite sugar beet germplasm varieties.

### **1.3 Sugar Beet Breeding**

Sugar beets grown commercially in the U.S. and Europe today are hybrids. These hybrids are produced using a complex cytoplasmic male sterility (CMS) system with at least two recessive alleles (Mikami, Yamamoto et al. 2011). These cultivars are also monogerm (Savitsky 1950), which involves another monogenic-recessive allele. Monogerm varieties eliminate the laborious work of thinning stands to economic population densities. Typically, new traits are difficult to introgress into CMS seed parents. Pollen parents are generally multigerm, open-pollinated, and mass-selected for disease resistance. This is because it is easier to fix the generally dominant or co-dominant resistance alleles in pollinators versus seed parents. Commercial hybrids are often heterozygous for disease resistance alleles, which can reduce efficacy of resistance, but this is still unclear due to the lack of molecular identification of most resistance genes. Outcrossing is the primary method of pollinating, but inbreeding can be utilized. A gametophytic self-incompatibility system prevents self-pollination but allows for cross pollination (LUNDQVIST, ØSTERBYE et al. 1973, LARSEN 1977). Historically, sugar beet inbreds have not been used as parents in hybrids.

Throughout sugar beet breeding history there have been many different innovations that have helped modernize the crop.

At the very dawn of sugar beet breeding most if not all cultivars were open-pollinated populations that had been mass-selected for increased root weight and increased proportion of sucrose in the root. One of the earliest hurdles for breeders was whether to select for increased sucrose content (highest possible sugar per root) or to target higher root yield. This led to the two broad groups of cultivated beets: the E-types (harvest tonnage) and the Z-types (high-sucrose types). Sucrose yield is determined by the harvest weight multiplied by the proportion of sucrose in the beets, minus losses due to storage and processing (McGrath and Panella 2018). Sucrose content in the root is expressed as a percentage of root fresh weight. Over time breeders have increased sucrose content from around 6% to around 18% in modern day hybrids. Sucrose concentration is quantitatively controlled with high heritability. Five quantitative trait loci (QTL) associated with sucrose content were detected using a mapping population, but only two influenced root yield, which is generally considered a non-additive trait (Schneider et al., 2002). Strong negative correlation between sucrose content and root yield has been identified (Pritchard 1916, Coons 1936). The most promising route for higher absolute sugar yield is likely to increase total dry mass, also known as biomass.

The two major breeding innovations in beets were discovered and developed in the decades after 1940. Both are essential in creating modern sugar beet varieties. First was the discovery and deployment of monogerm seed. This trait is controlled by a single recessive gene (*m*) that determines one seed per seed ball (Savitsky 1950). Beet flowers are perfect and monoecious, lack petals, and are allogamous. Pollen from flowers is primarily wind dispersed with some insect pollination. Beet seed is considered a fruit (botanically an utricle), with one true seed per fruit.

The wild-type state for beet seed is the multigerm seed ball that is a result from the fusion of multiple flowers. Flowers are born in axils on indeterminate inflorescences (racemes). When the flowers senesce, the seed becomes corky and woody. This is thought to help the seed be dispersed via ocean currents around the Mediterranean. Monogerm seed was the key in reducing the amount of labor that went into growing sugar beet. Having a single plant come from a single seed allowed for proper planting populations as well as allowing each root to grow to economic size.

The second major achievement in sugar beet breeding was the deployment of CMS for hybrid seed production. This system is dependent on the development of fertility-restoring genotypes. Restorers are conditioned by two recessive nuclear genetic loci (x and z) that prevent pollen fertility in CMS mitochondrial genotypes but restore fertility in normal cytoplasmic genetic backgrounds. These lines are often referred as either “maintainer lines” or “O-types” in breeding programs (Owen 1945, Powling and Ellis 1983, Kitazaki, Arakawa et al. 2015). Although this has been a major achievement in breeding it has come with heavy selection pressure in the restorer lines resulting in a genetic bottleneck (Taguchi, Hiyama et al. 2014). Hybrids show considerable heterosis, particularly in root yield (Schwegler, Gowda et al. 2014, Ćurčić, Taški-Ajduković and Nagl 2017). Hybrids are almost solely grown throughout the world, and this is why the development of CMS is considered the greatest achievement in sugar beet breeding.

Hybrids have been developed using many different crossing schemes. Hybrids were originally developed using three- and four-way prenatal crosses in the 1960s and 1970s. These were triploid hybrids, from which one of the parents is tetraploid, which showed better performance than early diploids. Over time diploids advanced into more elite varieties and are used in two-way crosses creating the diploid hybrids used today. Breeding at the diploid level

gives better control in creating unique niche varieties that perform particularly well for certain traits.

Seed production fields are very different from production fields. These fields are isolated from other seed production fields by 2-4 km. This prevents cross-pollination between non-target cultivars. Although as many precautions are taken as possible, cross-pollination happens and results in some off-types in production fields (large-leaf, red types, annuality, ect.). Large scale seed increases come from fields where rows of pollinators are planted next to rows of CMS seed parents. One generation, from seed-to-seed, takes around 11 months. This process can be shortened using greenhouse conditions. Breeder seed is typically produced on a smaller scale where single plants are placed in cages for self-pollinating. When seed is harvested it needs to go through processing to remove immature seed and the corky fruit tissue surrounding the seed. Seed will then be sized and polished before being primed and pelletized, to improve emergence and stand establishment (Paparella, Araújo et al. 2015). Cultivar approval in the U.S. comes from the factory districts, and in Europe it comes from national or regional agencies.

Beets are long-day plants that typically flower in the spring and early summer. Biennial types require a vernalization period that can be completed at almost every stage of growth. Temperatures of 4-6C for 10-16 weeks are sufficient for vernalization. Beets may de-vernalize if temperatures become too warm for too long. This phenomenon is not very well understood, and it is typically an issue in greenhouse seed production. The vernalization period to induce bolting and flowering is governed largely by the bolting gene locus, B (Pin, Benlloch et al. 2010, Pin, Zhang et al. 2012). Annual beet has the fully dominant allele B, while biennial beets are homozygous recessive (bb) at the bolting locus. Most flowering pathway genes are largely homologous to other flowering plants (Jung and Müller 2009, Blümel, Dally and Jung 2015).

Sugar beets are a complex and sometimes difficult crop to work with. The biennial nature of the crop results in slow turnaround for seed production, thus slowing the breeding process. Creating different populations (bi-parental, RILs, MAGIC, etc..) takes much longer than annual crops. Modernizing breeding programs using next-generation sequencing, marker-assisted selection, and genomic selection would allow for more accurate selection and make the breeding process more efficient.

#### **1.4 Modern Breeding for Disease Resistance**

An underlying goal in plant breeding is to provide growers with the best possible set of traits in their selected crop. Achieving this complete “genetic package” is done through many rounds of selecting those individuals and populations with desirable and heritable traits. With next generation sequencing and bioinformatics, we can now begin to discover more accurate markers and understand the genetic underpinning of desired traits. Phenotyping cannot be forgotten in this equation either. Without an accurate phenotype, association between a marker and the phenotype it is selecting for may not be very accurate. High throughput phenotyping is advancing at a rapid pace allowing for more plants to be phenotyped at a time as well as increasing the accuracy of phenotypes using multispectral cameras (Zhang, Li et al. 2017). With the combination of both higher throughput genotyping and phenotyping achieving a complete “genetic package” is becoming a greater possibility.

Few molecular markers for sugar beet traits are used today, at least in public sector pre-breeding programs. One of the most common ones used for disease resistance are the rhizomania genes Rz1 and Rz2 (Norouzi, Sabzehzari and Zeinali 2015). In Japan, markers have been developed for resistance to both *Aphanomyces* root rot and *Cercospora* leaf spot (Taguchi, Okazaki et al. 2010, Taguchi, Kubo et al. 2011). Two very important non disease resistance

markers are the CMS seed parents and their maintainers, Rf1 and Rf2 (Hagihara, Itchoda et al. 2005, Matsuhira, Kagami et al. 2012, Honma, Taguchi et al. 2014). Other diseases have had some potential markers like sugar beet cyst nematode (Kumar, Harloff et al. 2021) and Fusarium (De Lucchi, Stevanato et al. 2017) but they still need to be optimized. Ideally, markers would be located within the causal gene. Recombination between a marker and the gene it is identifying can cause the marker to predict the wrong phenotype. With DNA sequencing technologies becoming more advanced and cheaper the ability to find markers within genes is now the gold standard.

*Beta vulgaris* has nine chromosomes in its haploid state. Its genome size ranges from 714 to 758 Mb per haploid genome (Arumuganathan and Earle 1991). Highly repetitive DNA sequences make up roughly 60% of the beet genome (Dohm, Minoche et al. 2014). This includes ribosomal DNA repeats, multiple families of short repeat units, and various classes of transposable elements (Heitkam, Holtgraewe et al. 2014, Schwichtenberg, Wenke et al. 2016, Zakrzewski, Schmidt et al. 2017). Each chromosome has the repeat-sequence distribution pattern suggesting sugar beet is fully diploidized, with little or no duplication of its primary chromosome set (Halldén, Ahrén et al. 1998).

The German Agricultural Biotechnology Initiative (GABI) published the first chromosomally anchored reference sugar beet genome of the doubled haploid line ‘KWS2320’ (RefBeet) (Dohm, Lange et al. 2009, Dohm, Lange et al. 2012, Dohm, Minoche et al. 2014). RefBeet has been updated multiple times and continues to be a valuable source regarding gene content and context. Another valuable reference genome that has been completed in recent years was developed using USDA-ARS germplasm release ‘EL10’ (McGrath, Drou et al. 2013, McGrath, Townsend et al. 2016). Again, with sequencing getting less and less expensive, there will be a growing number of genome assemblies produced for different sugar beet accessions. The

importance of these additional genome assemblies for different sugar beet accessions is multi-fold, including capturing gene presence/absence variation, mapping traits of interest in specific backgrounds to avoid reference genome bias, and to capture as many different gene and allele combinations as possible.

### ***1.5 Rhizoctonia solani***

The form genus *Rhizoctonia* is a filamentous fungus that does not produce asexual spores and share a number of common features in their anamorphic states. This species complex is generally a soil-borne fungi, mostly associated with roots and usually pathogens, but some have been reported as saprophytic and symbiotic. This genus can be found worldwide in both agricultural and forest soils that can cause economic damage through foliar and root rot diseases in major crops. The genus concept of *Rhizoctonia* was established in 1815 by De Candolle and reviewed again in 1970 by Parmeter and Whitney. Features that are always present in this complex are brown pigmented hyphae, constrictions at branch points forming right angles and absence of mitospores.

*Rhizoctonia solani* (teleomorph = *Thanatephorus cucumeris* Frank (Donk)) is widely considered the most studied species within the genus. The fungus is most often recovered from soils around the world and is considered as an extremely destructive plant pathogen. It has a wide host range and can cause different symptoms depending on the host species. Symptoms on various crops include seedling damping off, black lesions on roots and seeds, stem rot, and rot of plant parts in contact with the soil. *R. solani* can also cause foliar lesions due to the germination of basidiospores on the leaf surface (García, Onco and Susan 2006).

Classification of the different *Rhizoctonia* taxa began with looking at which plants it had infected but is now done through anastomosis groups. When teleomorphic stages are available the

basidia and basidiospores have also been used for taxonomic classification, but not all strains can be cultured with this stage present. With the advancement in molecular biology and next generation sequencing researchers are using these new tools to further differentiate species within the complex.

#### Infection process

The order of events that happen during the infection process of *R. solani*, include adhesion, penetration, colonization, and host reaction (Anderson 1982). When *Rhizoctonia* hyphae contact the external surface of a compatible host, there is a recognition of plant exudates that induces profuse hyphal branching and formation of infection structures. Infection structures that are formed during this process allow the fungus to penetrate the external plant tissue. These structures called infection pegs penetrate the cuticle and epidermal cell wall layer (Keijer 1996). This is when the host and pathogen exchange enzymes and exudates attempting to control one another. *Rhizoctonia* excretes a multitude of extracellular enzymes including pectinase, pectin lyase, cellulase, phosphatase, and pectin methylesterase (Bateman and Basham 1976). After penetration, colonization of plant tissue is accomplished by the production of hydrolytic enzymes that can degrade several cell walls beyond the advancing hyphae. This results in large areas of dead plant cells from the initial penetration and later the colonization of internal tissue. Most symptoms on hosts are large black lesions located on roots reducing nutrient and water uptake to the plant resulting in death or reduced viability.

#### **1.6 *Rhizoctonia solani* in Sugar Beets**

*Rhizoctonia solani* in sugar beets causes the disease *Rhizoctonia* Root and Crown rot disease (RRCR). It is a worldwide disease that causes significant economic loss in multiple crops (Ithurrart, Büttner and Petersen 2004). The fungus can attack sugar beets primarily in two different

manors: one during the seedling stage causing damping off and two infecting at later stages causing crown and root rot. Throughout this dissertation we will be primarily focused on the mature tissue infections.

*Rhizoctonia solani* strains in the soil form a species complex described in previous sections and the two primary anastomosis groups that infect sugar beets are AG-2-2- IIIB and AG-4. AG-1 and AG-5 have been shown to be slightly infections on sugar beets as well (Windels and Nabben 1989, Rush, Carling et al. 1994, Nelson, Helms et al. 1996, Carling, Kuninaga and Brainard 2002, Desouza, Bolton and Khan 2010). *Rhizoctonia* crown and root rot on sugar beet results from initial infection from sclerotia or mycelia (often on previous years plant debris), which can survive for many years in the soil (Cubeta and Vilgalys 1997). Typical RRCR symptoms on sugar beet are large black lesions on the taproot that can envelope or hollow out the entire beet if the infection is severe enough. Above ground symptoms are severe wilting, collapse, and yellowing of leaves (Windels and Nabben 1989, Herr 1996). Yield loss caused by *Rhizoctonia* infection varies from field to field but can reach up to 60% yield reduction (Buhre, Kluth et al. 2009). Adding infected beets into storage piles can also cause further yield reduction by causing the infection to spread to other uninfected beets. To reduce the inoculum load from year to year, growers use crops in their rotation that do not harbor the fungus. To reduce the infection in an established production field growers, apply the fungicide azoxystrobin to reduce early infection and improve stand counts, however this does not completely eliminate infection later in the season (Noor 2013). Resistant cultivars in addition to timely fungicide applications are the preferred means to control RRCR infection (Panella and Lewellen 2007, Nagendran, Hammerschmidt and Mitchell McGrath 2009).

Some unique obstacles when breeding for RRCR resistance in sugar beets is how the fungus is propagated. *Rhizoctonia solani* does not produce any asexual spores and only on rare

occasions produce sexual spores (Cubeta and Vilgalys 1997). This lack of spore production makes it difficult to precisely control the disease pressure applied to nurseries when screening germplasm for resistance. To evaluate germplasm for disease severity in the field, breeders typically rely on visual evaluation of an uprooted plant and giving it a score. This evaluation can be done within a greenhouse setting as well. With only using visual scale there can be a bias in the ratings leading to less accurate selection within breeding programs. This can slow progress by inadvertently selecting for unwanted traits or missing potential advantageous traits.

Next generation sequencing has been an extraordinary tool for the advancement of plant breeding over the past two decades. Sugar beet breeding programs have been slowly integrating this technology and some discoveries have been made regarding RRCR resistance. Currently two sugar beet genomes have been published (EL10.2 and RefBeet) and they have been instrumental in implementing marker-associated breeding (Dohm, Minoche et al. 2014, Funk, Galewski and McGrath 2018, McGrath, Funk et al. 2023). With these resources, researchers can get closer to identifying resistance genes for particular diseases. For *Rhizoctonia*, there have been a few genes of interest that have been associated with RRCR resistance. Holmquist et al. (2021) discovered in their transcriptome analysis that three genes encoding for major latex proteins are elevated in a partly resistant sugar beet genotype (Holmquist, Dörfors et al. 2021). Earlier some QTLs were described for RRCR resistance on chromosomes 4, 5 and 7 (Lein, Sagstetter et al. 2008). *Rhizoctonia solani* resistance has also been discovered on table beet (*Beta vulgaris* L. ssp. *vulgaris*). A QTL was discovered on chromosome 2 of table beet through mapping of F<sub>2:3</sub> populations of a resistant table beet by a susceptible sugar beet. A new reference genome for a *Rhizoctonia*-susceptible table beet inbred W357A was used for the mapping of the newly discovered QTL (Wigg, Brainard et al. 2023). Despite these discoveries, there remains a need to

map causal resistance genes and develop direct markers to increased selection accuracy in commercial breeding programs and enhance new trait discovery efforts in pre-breeding programs. Additionally, directly mapping the causal resistance genes underlying RRCR resistance in sugar beet will enable a deeper understanding of the molecular plant-pathogen interaction, which will inform more advanced approaches for improving resistance (ex. transgenesis or genome editing) and controlling losses to this pathogen.

### **1.7 Marker assisted plant breeding**

Plant breeding is the art and science of changing plant traits in order to produce desired characteristics. This can be accomplished in a multitude of ways that began with simply selecting plants with desirable traits and propagating them. Over time, new techniques and technologies have been developed and deployed making plant breeding much more effective and efficient.

Prior to the genomic revolution, conventional breeding primarily relied on phenotypic selection of superior individuals among segregating progenies resulting from hybridization. This is how plant breeding, particularly in the case of sugar beet breeding, took place for decades until modern molecular techniques were developed. Many obstacles were difficult to overcome including genotypic and environmental variation when only looking at phenotypic data. One substantial issue with conventional phenotypic-based breeding, particularly in crops with long generation times or complex breeding schemes, is the amount of time and effort it takes to release a new cultivar. Depending on the species, this can take anywhere from 6 to 10 years, and if selection was not completely accurate it may take longer or fail all together.

Marker-assisted selection (MAS) involves selection of plants carrying genomic regions that are associated with a particular trait or traits of interest. In ideal scenarios, the molecular marker may even be within (or in complete linkage with) the causal genes, making the selection

extremely accurate. MAS works for both traits governed by single major genes as well as quantitative trait loci (QTLs). The benefits of using markers linked to genes in breeding programs is reducing the time between new cultivars by increasing the accuracy at which breeders can select their material. This would be a more phenotypic based program to a more genotypic program.

Marker-assisted evaluation can be used in multiple different manners within a breeding program not involving advancements of desired traits. This can begin with a pre-breeding type of program. Identifying correct cultivars and assessing their purity is essential when beginning to use germplasm in a breeding program. Having markers that can be used to confirm the true identity of individual plants can make a program more efficient. An example is in hybrid rice, where simple sequence repeat (SSR) and sequence-tagged microsatellite site (STS) markers are used to confirm purity rather than the standard “grow-out tests” that involve an entire growing season to assess morphological and floral characteristics (Yashitola, Thirumurugan et al. 2002). Another pre-breeding method that uses MAS is assessing the genetic diversity of their parental selections. Breeding programs depend on having a high level of genetic diversity for achieving progress from selection. Molecular markers have been a tool for characterizing genetic resources and providing breeders insight for selecting parents to be integrated into their program. An example of this is the comparison of marker haplotypes that enabled different sources of resistance to Fusarium head blight to be predicted and deployed worldwide (McCartney, Somers et al. 2004, Singh, Anderson et al. 2019).

Sugar beet breeding programs have drastically improved the speed and accuracy of their programs when implementing MAS. Artificial inoculation of pathogens in field and greenhouse trials have long been the protocol to evaluate and select germplasm to be advanced (Scholten, Panella et al. 2001). This has been very productive over the decades of selection, however disease

escapes or plants who may not have been infected and were deemed resistant, can be selected. These false selections genetically contaminate seed lots and can reduce advancement in germplasm production. Molecular markers eliminate disease escapes by accurately selecting those plants that contain the disease resistance traits. MAS can also reduce the amount of time and labor that goes into sugar beet breeding. Making selections at the seedling stage and culling those individuals that do not possess the disease traits drastically reduces the number of resources needed. With the advancement of next generation sequencing and bioinformatics, molecular marker discovery will become much easier to implement into breeding programs. Molecular markers do exist however they are proprietary to the seed companies and not available to the public. The leader in sugar beet breeding, KWS, has patented disease resistance regions and genes for Cercospora Leaf spot (TÖRJÉK 2020), Rhizomania (TORJEK 2020, MÜNNEKHOFF 2021, TORJEK 2022) and Sugar Beet Cyst Nematode (TÖRJÉK 2019). Public molecular markers will not only allow for the seed companies to check their germplasm for the resistance traits but also discover if the new public germplasm has novel sources of resistance.

Compared to other major commodity crops, sugar beet is a relatively new crop, particularly in light of its relatively recent domestication. The difficult reproductive biology in beet has largely been overcome by breeders through decades genetic studies, culminating in a deep understanding of the most efficient breeding schemes enabled by molecular markers. For most major sugar beet diseases, many sugar beet pre-breeding lines have been discovered or developed that have various disease resistance traits still many of the underlying molecular mechanisms have yet to be described. Through next generation sequencing, improved bioinformatic techniques, and improved phenotyping, the genes responsible for disease resistance can be uncovered. Ultimately, molecular

markers for these genes will allow for faster and more accurate integration into elite varieties for producers to grow in their fields.

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# **Chapter 2: Development of sugar beet pre-breeding lines FC709-4 and FC709-5 with improved resistance to Rhizoctonia Root and Crown Rot**

## **Introduction**

Like many root and tuber crops, sugar beet (*Beta vulgaris* subsp. *vulgaris*) can be infected by multiple pathogens that cause root and crown damage. *Rhizoctonia solani* is a ubiquitous, soil-borne necrotrophic fungal pathogen that continues to threaten sugar beet crop yields and quality globally, which causes the disease Rhizoctonia Root and Crown Rot (RRCR). As there are limited chemical options to control RRCR throughout the growing season, improvement of host plant resistance remains a primary objective of sugar beet breeding and pre-breeding programs. For decades, United States Department of Agriculture – Agricultural Research Service (USDA-ARS) sugar beet programs have focused on identifying novel resistance from germplasm collections like the USDA National Plant Germplasm System (NPGS), introgressing these traits into sugar beet crop type backgrounds, and releasing pre-breeding germplasm with improved resistance. Here we report the continuation of this mission through the development of new germplasm lines FC709-4 and FC709-5 with improved RRCR resistance derived from FC709-2. We envision these new germplasm will be useful as more uniformly resistant check lines for disease trials, as parents for breeding programs and mapping population development, and used to study the molecular mechanisms of underlying RRCR resistance.

Sugar beet (*Beta vulgaris* L.) is a relatively young crop in its domestication history. Sugar beet was developed from fodder beet in Eastern Europe in the 1700s. Fodder beets had high yields,

but most of that was water weight and low sucrose content. Over the next 100 years, through selecting high sucrose lines, improvement of sugar beet cultivars with high yield was achieved. It wasn't until the mid-20<sup>th</sup> century that breeders began to understand and implement genetic selection to improve sugar beet cultivars. One of the problems with early sugar beet varieties is that the seed was multi-germ resulting in multiple plants per seed ball. This would lead to labor intensive thinning of the crop. Discovering cytoplasmic male sterility (CMS) system allowed for the selection of monogerm cultivars which is how today's hybrids are still being produced (Owen 1945, Savitsky 1950, Mikami, Yamamoto et al. 2011). Modern research in *Beta* genetics and genomics is used to explore the applied uses of new and traditional, biochemical, and molecular techniques. These techniques are implemented to investigate the genetic relationships between cultivated and wild sugar beet lines. These tools also allow for investigation to determine genetic control of pathogenicity in important sugar beet pathogens, of resistant in sugar beet, and of the interactions between pathogens and sugar beet.

The USDA-ARS Sugar Beet Genetics Lab in Fort Collins, Colorado combines traditional methods of crop improvement with the use of new molecular biology techniques to understand the genetics of sugar beet and its wild relatives. The program began in the 1950s, with the ongoing primary mission of developing disease-resistant sugar beet germplasm. Since its inception, the Fort Collins group has largely focused on improvement of *Rhizoctonia* Root and Crown resistance, but also included secondary breeding targets of improved resistance to other diseases like sugar beet cyst nematode (*Heterodera schachtii*), fusarium yellow (*Fusarium oxysporum*), Rhizomania (Beet necrotic yellow vein virus), *Cercospora* Leaf Spot (*Cercospora beticola*), and the curly top (Beet curly top virus). Public sugar beet breeding is one of few remaining crops in the United States where all public sector breeders are USDA-ARS scientists (Panella, Campbell et al. 2016). Public

sugar beet pre-breeding programs heavily rely on utilizing the Beta gene bank, particularly Plant Introductions (PI, or PIs herein) accessions from the USDA-ARS National Plant Germplasm Systems (NPGS), to identify wild germplasm with potentially novel forms of disease or pest resistance. The USDA-ARS breeding community, in collaboration with a network of university scientists nationally, have been systematically screening the USDA-NPGS *Beta* collection for disease/pest resistance, with these activities organized largely through the Sugar Beet Crop Germplasm Committee. Lines with promising resistance from the gene bank are incorporated into the pre-breeding programs, and result in the release of pre-breeding lines with improved resistance. These publicly supported USDA-ARS pre-breeding lines have been critical sources of resistance for industrial breeding programs. Hybrid varieties grown commercially are entirely produced by industrial breeding companies, and all acres are planted to Roundup-Ready varieties (and are hence transgenic).

*Rhizoctonia solani* is a fungal species complex that causes a wide variety of symptoms and diseases (e.g., root, crown, and stem rot; damping-off; and wilting) in number economically important crops (Anderson 1982, Ogoshi 1987, Gonzalez, Pujol et al. 2011). The fungus can survive in soil or plant residues as mycelium or sclerotia for multiple years, making disease management difficult (Windels and Nabben 1989). *Rhizoctonia* can infect sugar beets throughout their life cycle. *Rhizoctonia* Root and Crown Rot (RRCR) is the disease when *Rhizoctonia solani* infests mature plants. Tissue in the crown and taproot are penetrated by the fungus that then secretes cell wall degrading enzymes killing adjacent cells. With the abundance of nutrients the fungus can colonize both living and dead host tissues, followed by sclerotia formation as a long-term survival structure (Mukhopadhyay 1987). RRCR crown symptoms include black lesions on the oldest petioles of the crown that can continue to rot the top of the taproot causing yellowing of

older leaves. Root symptoms include dry rot cankers on the surface of the root that can continue inward as the severity of the infection increases. Severe infections result in completely wilted leaves, deformed taproots, and plant death.

Genetic resistance to *R. solani* has been suggested to be polygenic (Hecker and Ruppel 1975). For breeding, mass or recurrent selection has been used, as well as visual evaluation in the field, after artificial inoculation is applied for adequate disease pressure. This is primarily how germplasm has been developed over several decades. Some of the first germplasm lines to be released primarily for their *Rhizoctonia* resistance qualities came from the USDA-ARS Sugar Beet Genetics Lab in Fort Collins, Colorado (Panella, Campbell et al. 2016). The first release for primarily *Rhizoctonia* resistance came in 1968 (FC701) (Hecker and Gaskill 1972) and subsequent releases over time have been the major sources of resistance used by private seed companies (personal communication). One such germplasm, “FC709-2” has been the resistant check in the *Rhizoctonia* Root and Crown Rot (RRCR) trials for the past 25 years (Panella 1999). Though this germplasm line has been the most resistant to RRCR, the genetic underpinnings of resistance in this background are largely unexplored.

The first QTL to be reported for RRCR resistance was by Lien et al. (2008). Using a traditional bi-parental population by crossing a highly resistant seed parent by a susceptible pollinator they developed segregating F<sub>2</sub> population. From this population individuals were selfed to create 95 F<sub>2:3</sub> families. The highly resistant seed parent was originally derived from FC702 a USDA Fort Collins line that is the original parent for FC709-2. This study developed a linkage map comprised of 38 expressed sequence tags (ESTs) with high similarity to genes that are involved in resistant reactions of plants (R-ESTs) and 25 bacterial artificial chromosomes (BACs) containing nucleotide binding site (NBS)-motifs typical for disease resistant genes (Lien et al.,

2008). Using this linkage map, the authors identified QTLs on chromosomes 4, 5, and 7 using a F<sub>2:3</sub> population developed from a cross between resistant and susceptible sugar beet parents.

Later, in a similar study, Wigg et al (2022) used F<sub>2:3</sub> populations this time crossing a resistant sugar beet line (FC709-2) by a susceptible table beet line (W364B) and a resistant table beet line (W364B) by a susceptible sugar beet line (FC901). Utilizing genotype-by-sequencing on segregating F<sub>2</sub> families they were able to identify a new RRCR associated QTLs on chromosome 2 accounting for 30% of the phenotypic variation. The QTLs on chromosome 2 had an LOD score of 5.32 for the interval of 56,099,742 to 62,113,791bp and a LOD score of 6.31 for the interval 7,511,726 to 33,304,080 bp.

The Fort Collins program released sugar beet germplasm line FC709-2 in 1999 as a multigerm (MM), non O-type, pseudo self-fertile pre-breeding line to serve as a source of RRCR resistance for commercial breeders. FC709-2 is the result of four cycles of selection for improved RRCR resistance from its parental line FC709 (Hecker and Ruppel, 1988). The first cycle of selection for FC709-2 was mass selection for resistance to RRCR in a nursery inoculated with *R. solani* (Ruppel et. al., 1979). Seed was produced from surviving roots and planted into field isolation pots that were re-inoculated with *R. solani*. This seed was harvested and used for a second round of selection in an inoculated field. This was repeated two more times until the final 121 roots surviving plants had their seed bulked to produce the seed that was released in 1999. FC709-2 had high resistance to RRCR when assessed under strong disease pressure in subsequent field trials between 1994 and 1997, where FC709-2 had statistically equivalent or better RRCR resistance compared to the available resistant check lines.

The pedigree of FC709-2 dates to the very beginning of USDA-ARS Fort Collins, Colorado breeding efforts to improve RRCR resistance. The progenitors of the lineage resulting

in FC709-2 are USDA-ARS Fort Collins pre-breeding lines FC702/5 and FC701/5, which were both released in 1974. Multiple cycles of recurrent phenotypic selection for improved RRCR resistance from this cross resulted in the line FC709, which was released in 1987 (Figure 1). There have been 14 cycles of RRCR selection between the original parental crosses between FC702/5 and FC701/5 and the current seed lot (20121034) for FC709-2. This current seed lot of FC709-2 has served as the resistant check for the disease nurseries that are conducted at the CSU ARDEC research farm since 2012. However, while FC709-2 has regularly had the lowest DI in nearly every RRCR evaluation conducted in the last decade, there is still residual susceptibility in this line. For example, 20121034 seed lot of FC709-2 had 48.44% of roots rated as susceptible with scores 4-7. Presumably, this residual susceptibility is due to a combination of factors, including insufficient selection pressure and the lack of marker assisted selection for RRCR resistance.

Here we report the development of two new sugar beet pre-breeding germplasm derived from FC709-2, named FC709-4 and FC709-5, which both have improved resistance to *Rhizoctonia* Root and Crown Rot. Using artificial inoculation methods in field nurseries over 4 cycles, improved greenhouse seed production methods, and more strict selection criteria compared to past efforts, FC709-4 and FC709-5 represents a useful new resistant check line for field and greenhouse screening, as a potential parent for developing mapping populations, and as a resistant check line to study the efficacy of long term breeding efforts.

## **Materials and Methods**

### **General RRCR nursery screening methodologies**

All RRCR resistance field nurseries results reported here were conducted at the Colorado State University Agricultural Research, Development, and Education Center (ARDEC) in Fort

Collins, Colorado. Specific dates and rates for each yearly RRRCR nurseries are stated below, whereas general methodologies that are consistent across years are as follows. The RRRCR nursery is planted in either a completely randomized or row/column design with 5 replications per entry in one-row plots (76 cm row spacing) 3.7 m long. The soil is a Fort Collins loam, (0 to 1 % slope, pH 7.2). Approximately 2 grams of seed are planted per plot in mid to late May, depending on yearly weather conditions, into beds under a overhead linear irrigation system. When beets reach the appropriate growth stage, stands are thinned to 20 cm between plants resulting in 10-15 plants per plot. Each genotype is replicated 5 times (Dorn and Fenwick, 2022). Plants were be inoculated with *R. solani* infected barley grains (methods described below) Inoculation is typically done when plants are around 8 weeks old (mid to late July). Inoculum was applied using a pull behind implement that has a Gandy Ezee Flow granulate applicator (Gandy Manufacturing Co., Owatona, MN). Inoculum is placed in a 10-cm band over the center of each row and into the beet crowns. Immediately after inoculation, the field was cultivated between the beds to throw soil up onto the crowns creating an advantages environment for infection. After a four-week infection period, beets are uprooted and cleaned off for phenotyping. Each individual beet per plot was rated on a scale of 0-7 (0 = no infection and 7 = plant death) and a disease index value will be designated for each germplasm line being tested (Ruppel and Hecker 1988). Average disease severity per plot was determined to create a disease index (DI) for each entry and DI was treated as a continuous variable. Analysis of variance (PROC GLIMMIX) was performed in SAS (SAS 9.4, Cary, NC. SAS Institute Inc.) on DI. Data are also represented as the percentage of sugar beet roots in classes 0 through 1, considered as healthy and in classes 0 through 3, considered harvestable. Data in classes 0-1 and 0-3 were transformed using arcsine square root to normalize the data for analyses %0-1 and %0-3. Dunnett's one-tailed t-test ( $P = 0.05$ ) was used to compare entries to the resistant

and susceptible checks, or alternatively to the most/least resistant lines tested. To determine statistical difference between the susceptible and resistant groups a Student's t test ( $P = 0.05$ ).

### **2020 RRCR evaluation of USDA-ARS Fort Collins germplasm releases (Metz et al. PDMR)**

Forty-eight sugar beet (*Beta vulgaris* subsp. *vulgaris*) USDA-ARS breeding lines from the Fort Collins Sugar Beet Genetics Lab were screened for resistance to RRCR during the 2020 growing season. The reasoning behind this trial was to determine the variability of all FC700 lines, that have some form of RRCR tolerance, from the Fort Collins sugar beet breeding group. The progenitors of FC709-2 were all involved in this study. The *Rhizoctonia* screening nursery used one highly resistant line (FC709-2) and one susceptible germplasm (20151020) as controls. The field plot was planted to hard red winter wheat in 2018 and corn in 2019. No additional nitrogen was applied for the 2020 field season, however phosphorus (56kg/ha) and Nortron were applied on 18 May. Plots were planted on 21 May with 1.25cm of irrigation applied on 23 May. The field was watered weekly to assist with germination and seedling emergence. Plots were hand weeded and thinned on 26 Jun. An inoculum of dry ground hulless barley grain infested with *Rhizoctonia solani* isolate R-9 (AG-2-2) was applied to the crown of the plants on 15 Jul (8 to 12 leaf growth stage) at a rate of 6.5 g m<sup>-1</sup> of row. A Gandy® electrically driven applicator was used to apply the inoculum and the field was cultivated afterwards to place soil onto the plant crowns. This test was conducted again in 2021 and 2022 with the same parameters (Table 1 and Table 2) (Dorn and Fenwick 2022).

### **Fungal Isolates of *R. solani* and Inoculum Production**

Isolate “R-9” of *Rhizoctonia solani* Kuhn of *R. solani* (anastomosis group 2-IIIB) is

used to create an artificial epiphytotic. This strain has been used in the Fort Collins RRCR nursery for several decades and is also used in multiple other regional sugar beet RRCR nurseries (Pierson and Gaskill, 1961). This isolate is stored on infected barley grains in -80C freezers until needed for inoculum production. To initiate inoculum production, a single infected barley grain is placed on a petri dish with potato dextrose agar. This is left to grow until the mycelia has reached the borders of the petri dish. Mass produced *R. solani* inoculum is curated on moist autoclaved hull-less barley grain. Type 10B polypropylene spawn bag (Unicorn Imp. & Mfg. Corporation) are used with 2 L of hull-less barley grain, plus 550 mL of water that will be placed into the autoclaved to be sterilized. The fungus is allowed to grow in potato dextrose broth for 21 days at 28C before being poured into the sterilized barley that is then sealed and placed into a temperature controlled growth chamber to colonize the barley. After 14 to 21 days (or until the barley is fully colonized by the fungus) the inoculum is air dried at room temperature for 7 days. Dried infected barley grain is ground using a Wiley mill No.1 (Arthur H. Thomas Co., Philadelphia, PA), then stored at 10C until its use.

### **Greenhouse Rhizoctonia Crown and Root Rot Resistance testing**

A single seed ball is sown into a 7-inch pot with featherweight 50-50 soil mix containing 50% coconut coir and 50% sphagnum moss (Paonia Soil Co.). Greenhouse growing conditions are at 18-hour photoperiod, a mean daytime temperature of 26C and a mean nighttime temperature of 18C. The relative humidity was 30-40%. Soil is kept moistened to ensure that germination takes place. When seeds have germinated, they were thinned so that each pot contains only one plant. After 8 weeks the plants will be inoculated with *R. solani* strain R9 inoculum prepared as described in previous sections. Each plant will be inoculated by creating a 3 cm hole next to the

taproot and 6 milligrams of inoculum will be poured into the hole and covered with soil.

Greenhouse conditions were be changed to a mean nighttime temp of 24C for optimal infection.

Plants were then be uprooted after 4 weeks and rated on the same scale as the field trials.

### **Root Selections and Seed Production Procedures**

Root selection is done late summer after beet roots is lifted for RRCR rating. Roots are lifted with the canopy attached that is defoliated soon after for easier handling. Roots that are deemed acceptable are placed into milk crates to be washed before vernalization. Roots are then placed into a cold room that is held at 4°C for 120 days to achieve adequate vernalization. After the vernalization period roots are then shaved of any leaf development and replanted into 7x9 inch fiber pots for greenhouse seed production or are re-planted into field isolation plot.

Greenhouse seed production involves an acclimation period where roots are placed into a cool greenhouse bay (10-15°C) for 10 days before returning to normal growing conditions. Plants are harvested when seed is set, and plants have begun to senesce.

### **FC709-4 Line Development and Testing**

The starting material for the development of both FC709-4 and FC709-5 was the FC709-2 seed lot 20121034 (Figure 2). The seed lot notation of the USDA-ARS Fort Collins sugar beet pre-breeding program is indicated by the first four digits being the year the seed was harvested, followed by an additional four digits assigned to each seed lot in the order in which it was harvested. The 20121034 seed lot was a bulk increase (n=252 mother roots) of the original FC709-2 seed lot that was released, 19921024. All testing and selection for FC709-2 herein is referring to selections from the seed lot 20121034.

The selections to produce FC709-4 began in the 2021 Fort Collins RRRCR nursery. A large selection block of FC709-2 (20121034) was planted, managed, and inoculated as described in the 2021 nursery methods above. The selection block consisted of 13 adjacent rows of FC709-2 planted in 34 meter long plots. A total of 1050 individual roots the two center rows were pulled, and the 84 most resistant individual roots selected, inter-pollinated in the greenhouse to produce seed lot 20221020. In the 2022 RRRCR nursery, the 20221020 seed lot was planted in a large selection block similar to 2021 selection block. The 2022 selection block consisted of 4 rows that stretched the full length of the entire RRRCR nursery (136 m). Approximately 2500 total roots were pulled and rated, and 309 resistant roots (scores of < 2) were selected for subsequent interpollination and seed production, which yielded the 20231008 seed lot, which is referred to FC709-4 herein. FC709-4 was evaluated in the field for RRRCR resistance for the first time in the 2023 nursery.

### **FC709-5 Line Development and Testing**

FC709-5 is directly derived from the FC709-2 seed lot 19921024, with an additional three cycles of mass selection for resistance to RCRR. The first additional cycle of mass selection bottlenecked the original FC709-2 seed lot 19921024 by selecting the most resistance 135 roots out of a population of 616, which were inter-pollinated to yield the 20031009 seed lot. A second cycle of mass selection on the 20031009 seed lot utilized the most resistant 220 of 285 roots to yield the 20041003 seed lot. A final cycle of selection was completed in 2020, when the most resistant 28 of 123 roots were selected in a RCRR field nursery. These 28 roots, all with RCRR scores of 2 or better were interpollinated to yield the 20211020 seed lot, which is referred to as FC709-5 herein. FC709-5 was evaluated for RRRCR resistance in the 2021 RRRCR nursery (Dorn et al. PDMR 16:V162), as well as in greenhouse tests described below.

## **Evaluating FC709-5 and its Progenitor Lines**

Greenhouse RRCR resistance evaluations for FC709-5 and its progenitor seed lots was performed to ensure that genetic resistance has been gained through selection. Seed lots that were selected are 20121034 (FC709-2 original seed lot), 20041003 (three mass selections for resistance from 20121034), 19991018 (FC709 seed lot), and 20211020 (FC709-5 seed lot). One hundred individuals were planted from each line as described in the previous section. These individuals were scored on the 0-7 scale for RRCR resistance. To compare the differences between the four lines a chi squared test was performed. A two-tailed p-value was calculated to determine statistical significance between lines.

## **Results**

### **2020 Evaluation of RRCR resistance in USDA-ARS Fort Collins germplasm releases**

Average disease severity per plot was determined to create a disease index (DI) for each entry and DI was treated as a continuous variable. Analysis of variance (PROC GLIMMIX) was performed in SAS (Ver. 9.4) on DI. Data are also represented as the percentage of sugar beet roots in classes 0 through 1, considered as healthy and in classes 0 through 3, considered harvestable. Data in classes 0-1 and 0-3 were transformed using arcsine square root to normalize the data for analyses %0-1 and %0-3. Dunnett's one-tailed t-test ( $P = 0.05$ ) was used to compare entries to the most resistant (19921022) and susceptible (20151020) lines tested. The entries tested here represented over 50 years of pre-breeding releases from the USDA-ARS Fort Collins program with improved resistance to RCRR. Twenty-two entries were not statistically different than the most resistant entry tested, FC702/7 (Table 1-2). Interestingly, 21 entries were statistically similar to the highly

susceptible check 20151020, which had the highest DI in this test. Of these 21 lines not statistically different than the susceptible check, 16 were originally released with notable enrichment of RCRR resistance (Panella et al, 2015). Determining the homogeneity of each line the number of roots rated 0-1 and 0-3 are determined. Although FC702/7 had the lowest DI it had a high variability of ratings between roots within line.

### **2023 Evaluation of RRCR resistance in USDA-ARS Fort Collins germplasm releases**

Disease severity was uniform and severe with high levels of disease across the entire nursery. The resistant check lines FC709-2 and newly created FC709-4 performed as expected, with the lowest DI ratings in the test of 1.27 and 1.77 (Table 1-3). A total of 10 *Beta vulgaris* subsp. *maritima* entries were statistically similar to FC709-4 (Dorn et. a., 2024). Only one entry (PI 504247) was statistically similar to the susceptible check FC901xC817. A per root rating is reported to understand the heterogeneity of individual accessions. FC704-1 had a 0-1 rating frequency of 70.18% and a 100% 0-3 frequency compared to FC 709-2 at 50.06% 0-1 and 89.9% 0-3.

### **Rhizoctonia Root and Crown Rot Field Selection Trials of FC709-2**

FC709-2 was released in 1999 and has been the resistant check line for annual RRCR disease nurseries around the country. The current seed lot (20121034) was a bulk increase from the released seed lot (19921024). To examine long-term performance of FC709-2 in recent years, we collected per-plant phenotypic distributions and disease index ratings for each year that the 20121034 seed lot was used in the Fort Collins RRCR disease trials (Figure 3). Starting in 2012 and 2013, the original 1992 seed lot of FC709-2 (19921024) was used as the resistant check. In 2012 and 2013, the FC709-2 DI was 1.8 (in both years), with the test average being 4 and 3.2, and

the susceptible checks being 3.5 and 2.9, respectively (Figure 3). The 2014 RRRCR nursery was the first year that the new seed lot 20121034 was used as the resistant check. That year, FC709-2 performed very similar compared to previous years. More recently, FC709-2 regularly demonstrated resistance equivalent to the generally accepted cutoff of D.I. values between 0 and 3. However, starting in 2020, the D.I. levels for FC709-2 increased above this cutoff, and this trend was seen in in 2021, 2022, and 2023.

Heterozygosity within a seed lot is expected to be relatively high as sugar beet is a primarily outcrossing species. Knowing that this line is not a “pure” line, it is expected that there is some degree of variability of resistance in the seed lot. We looked at the phenotypic distribution of individual plant ratings per year to further our hypothesis that there has been a change in the degree of resistance over time (Table 1-1). In Table 1-1 the trend of the number of beets that are rated as “resistant” (0-3) declined over time and those that are rated “susceptible” (3-7) increased over time.

### **Greenhouse RRRCR Resistance Evaluation of FC709-2 Progenitors**

We use FC709-2 for our RRRCR check in most selection assays that are performed in the greenhouse mirroring the field trials. In 2021 we compared the average rating of 709-2 in the greenhouse compared to the field trial. The average ratings were 2.46 in the greenhouse and 4.6 in the field using the student t test.

The FC709-2 pedigree trials comparing the previous bulks, previous releases within its pedigree, and the new FC709-3 line took place in the greenhouse. Here we again looked at the average rating between lines and the phenotypic distribution. The average ratings for the germplasm lines tested were: FC709 (19991018) mean DI= 3.22; FC709-2 with selection

(20041003) mean rating 3.19; FC709-2 (20121034) 2.16; and FC709-5 mean rating 1.4 (Figure 4). The phenotypic distributions from this test also reinforce the fact that with more stringent and frequent selection leads to a more resistant germplasm. To determine if there are differences between lines, a chi-squared test was done to compare each line to one another. The only significant difference found was between 20121034 and the two previous lines in the pedigree, 19991018 and 20041003, with p values equal to 0.0001 and 0.0031, respectively. Seed lots 20041003 and 19991018 were statistically indistinguishable from one another (p value = 0.0913). FC709-4 did not have enough observation to be used in this statistical analysis.

#### **FC709-4 Pedigree, Line development, and Evaluation**

FC709-4 is derived from FC709-2, which has served as the resistant check line for evaluating sugar beet and wild beet germplasm for RCRR since its release in 1999. The FC709 series was developed from a cross between the FC702 and FC701 pre-breeding lines in the 1970s FC709-4 demonstrated more uniform resistance in the 2022 RCRR nursery in Fort Collins, CO compared to FC709-2 (3.6 vs. 4.35, with a nursery average of 6.12).

#### **2023 Field Evaluation of FC709-4**

FC709-4 (20231008) was first evaluated in a field disease nursery in 2023. Following the protocol from previous RCRR nurseries, FC709-4 was used as the resistant check alongside FC709-2 to evaluate its resistance. FC709-4 outperformed FC709-2 with a disease index of 1.26 and 1.93 respectively, however this difference was not statistically significant (Table 1-3). Another observation was that FC709-4 across its five reps did not have a single plant that was rated susceptible (4-7 on the disease scale) whereas FC709-2 still contained susceptible plants. FC709-

4 will be evaluated in both greenhouse and field disease trials for another year before it becomes a new resistant check germplasm line.

## **Discussion**

### **Historical retrospective of RRCCR resistance breeding**

A long-term goal of the USDA-ARS Fort Collins sugar beet pre-breeding program has been to identify resistance from beet and wild beet germplasm, introgress this resistance into sugar beet backgrounds, and release germplasm with enhanced tolerance. This program has spanned nearly a century, and has released many valuable germplasm resources (Panella, Campbell et al. 2016). Perhaps one of the most impactful research outputs of the program to date has been the release of germplasm with improved resistance to *Rhizoctonia solani* infection. The FC700-series of germplasm releases represents pre-breeding lines with notable resistance to RRCCR (Panella, 2019). One of the most impactful germplasm releases is noted to be FC709-2 (Panella 1999). FC709-2 has been the resistant check for many disease field and greenhouse trails since its release in 1999, as well as being highly requested by public and private breeders for evaluation and introgression into their own programs (Wigg et al., 2023).

A comprehensive evaluation of the FC700 series of germplasm in the 2020 RRCCR nursery demonstrated that there has been improvement of RRCCR resistance in the program however this may be due to more efficient and accurate methods of evaluating disease. The introduction of linear overhead irrigation versus the traditional furrow irrigation may have impacted the way disease develops in the field. Year to year variation and how artificial inoculation changed over years of research made comparing data extremely difficult to draw conclusion from hence the 2020 RRCCR nursery. FC709-2 performance has been inconsistent on a year-to-year basis due to multiple factors

including, weather, irrigation method (linear vs furrow), and inoculum dissemination methods. Greenhouses allow for a more controlled and convenient method for RRCR germplasm screening. It also allows for screening and selection to be done during the winter months. This method speeds up the breeding process for selecting and releasing useful germplasm. This and the high heterogeneity of the seed lot could be why FC709-2 inconsistent in its RRCR rating each year. We chose FC709-2 over the more resistant FC702/7 because of its higher number of highly resistant plants. This aids in statistical strength when being used as a resistant check as well as a base for genomic studies. Traits are more than likely “fixed” in this line and there is no need for further inbreeding or using a double haploid method to genetically fix traits. For the first time in the Fort Collins sugar beet breeding program a germplasm line tested in a RRCR disease nursery did not have any individual plants rated in the susceptible category.

### **Phenotypic Heterogeneity of FC709-2**

FC709-2 seed lot is not a very homogenous line, due to the fact that sugar beets are primarily an out-crossing species with evidence inbreeding depression. The manner that this line was selected for in its pedigree using mass selection and very little self-pollination resulted in this highly heterogenous seed lot. Inbreeding depression is present in diploid beets that can result in sterility and other non-advantageous agronomic traits (Hecker 1972). With a heterogenous seed lot not all plants are equally resistant meaning that some years the check can perform poorly just out of chance of seed selection within the lot. With a poor performing resistant check it may be difficult to gauge the success of the disease trial whether that be in the greenhouse or in the field. This began our pursuit of a more resistant homogenous germplasm line derived from FC709-2.

Using the current seed lot, we choose to do a broader mass selection from a field disease trial. Field disease trials tend to experience more disease pressure than greenhouse trails. This is

because of the way inoculum is applied to the soil. The field inoculum is broadcasted over the plants and may not be as evenly distributed as the more precise method in the greenhouse. Some plants may get more inoculum than others throughout the test, but this factor is mitigated by using multiple reps in the same field test. Also, more environmental factors (temp and moisture) come into play in the field that may result in a more highly or lower disease pressure. We chose to do the first selection from the field so that we can start with heavy disease pressure ensuring that beets that were saved for seed production were in fact resistant and no escapes. After the first selection being at 22% (135 out of 616 roots) we wanted to broaden the next selection so that we wouldn't experience any fertility issues when bottlenecking sugar beet germplasm. The final selection was again ~22% (28 of 123 roots) and this time only highly resistant roots (rating 0-2) were saved for seed production. That seed will be used alongside FC709-2 as the resistant check for both field and greenhouse trials over the next few years. More data showing the seed uniformity and increased resistance that FC709-4 possess will make the line a staple in the breeding program.

During this study of the creation of a more homogenous RRCR resistant germplasm line, we were also involved in the creation of the mapping populations used in Wigg et al., 2023 table beet study. The table beet study concluded before this study with the discovery of a QTL on chromosome 2 associated with *Rhizoctonia solani*. This study along with the Lein study (2008) guided our work when we began looking at the genetic inner workings of RRCR resistance because of the common resistant parents used in both studies.

The selection methods in *Beta vulgaris* can be used as a guide for crop wild relatives and other *Beta* species could help uncover important disease resistance traits within those gene pools. *Beta vulgaris* spp. *maritima* is the closest common ancestor to sugar beet and can readily be crossed into existing germplasm lines (Biancardi, Panella et al. 2012). Being able to evaluate those

germplasm lines is crucial as it is more difficult to grow in the field and phenotype accurately. Improving our ability to pre-breed and select from secondary and tertiary gene pools will allow for the discovery of lost genes that have been selected out. These new forms of resistance could be crucial for the future of disease management in sugar beets.

## **Conclusion**

With FC709-4 being created we have a new and improved candidate line to begin to do more advanced genomics to discover the underlying genetic mechanisms of RRCR resistance. The more inbred nature of FC709-4 lends itself well for being a parent in a mapping population and even more selection to continue to improve upon. FC709-4 has performed well in both greenhouse and field trials and will be used as the resistant check line for future disease trials. Beginning to understand the underlying resistant mechanisms of FC709-4 could allow us to discover other novel sources of resistance in the Fort Collins germplasm collection. New sources of resistance can then be mapped and implemented into a marker assisted selection system allowing breeders to stack several forms of resistance into a single germplasm line. Another resource that FC709-4 will provide is being the inbred and highly resistant source of a new sugar beet reference genome. This new genome could provide more insight to the molecular mechanisms that are responsible for *Rhizoctonia solani* resistance in sugar beet

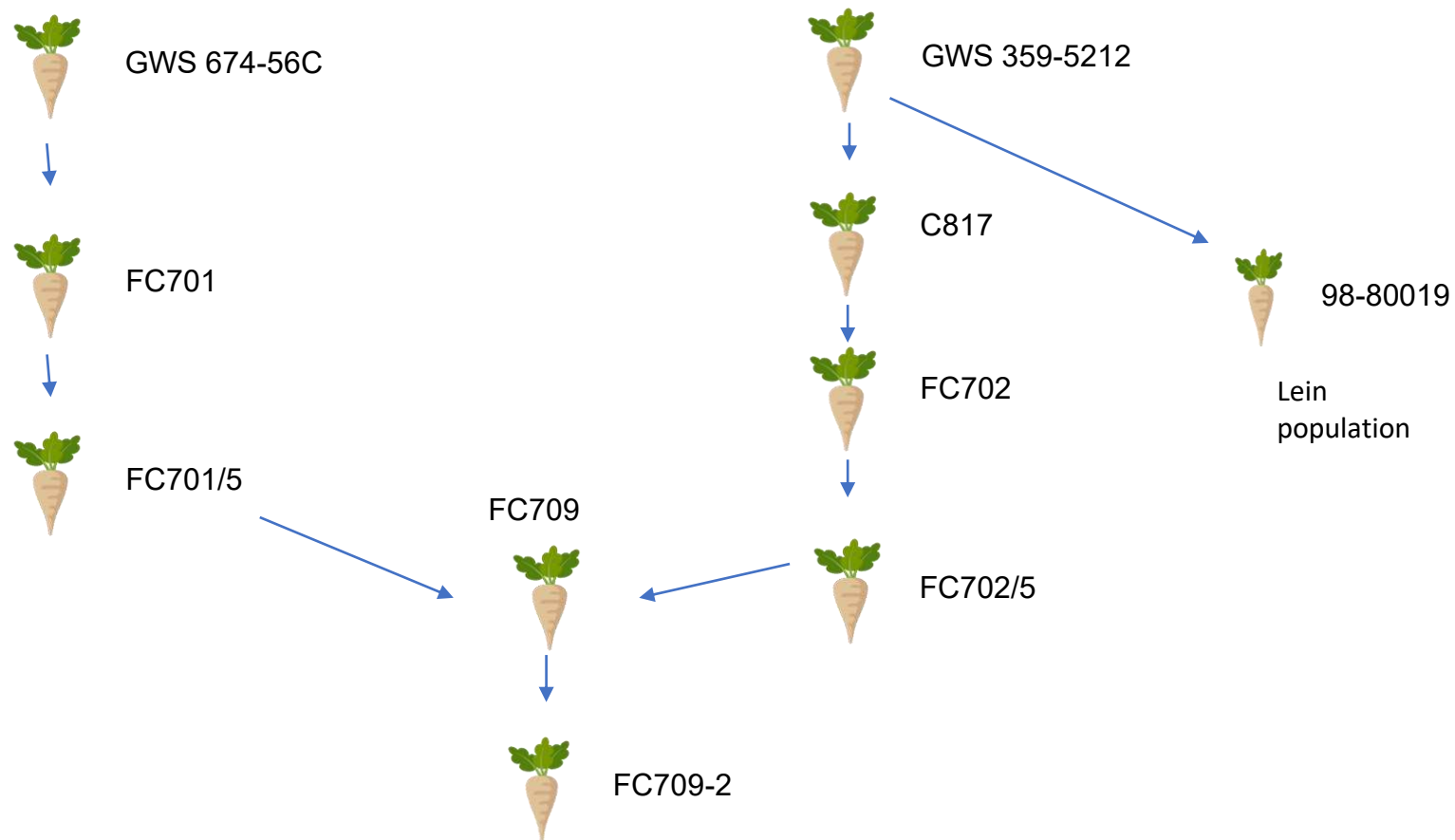


Figure 1: The pedigree for FC709-3. Great Western Sugar varieties 674-56C and GWS 359-5212 are some of the earliest US varieties with high RRCR resistance. GWS 359-5212 is also the progenitor of the population used in the identification of RRCR QTLs in Lien et al's 2008 study. The original parents were the Fort Collins released germplasm lines FC702 and FC701 (Doney 1995) both expressing strong *Rhizoctonia solani* resistance. This gave rise to the next release germplasm line FC709 (Hecker and Ruppel 1988). After multiple round of mass selection for RRCR resistance FC709-2 was released (Panella 1999). Further mass selection with a much higher selection pressure to create a more homogenous resistant line gave rise to FC709-3.

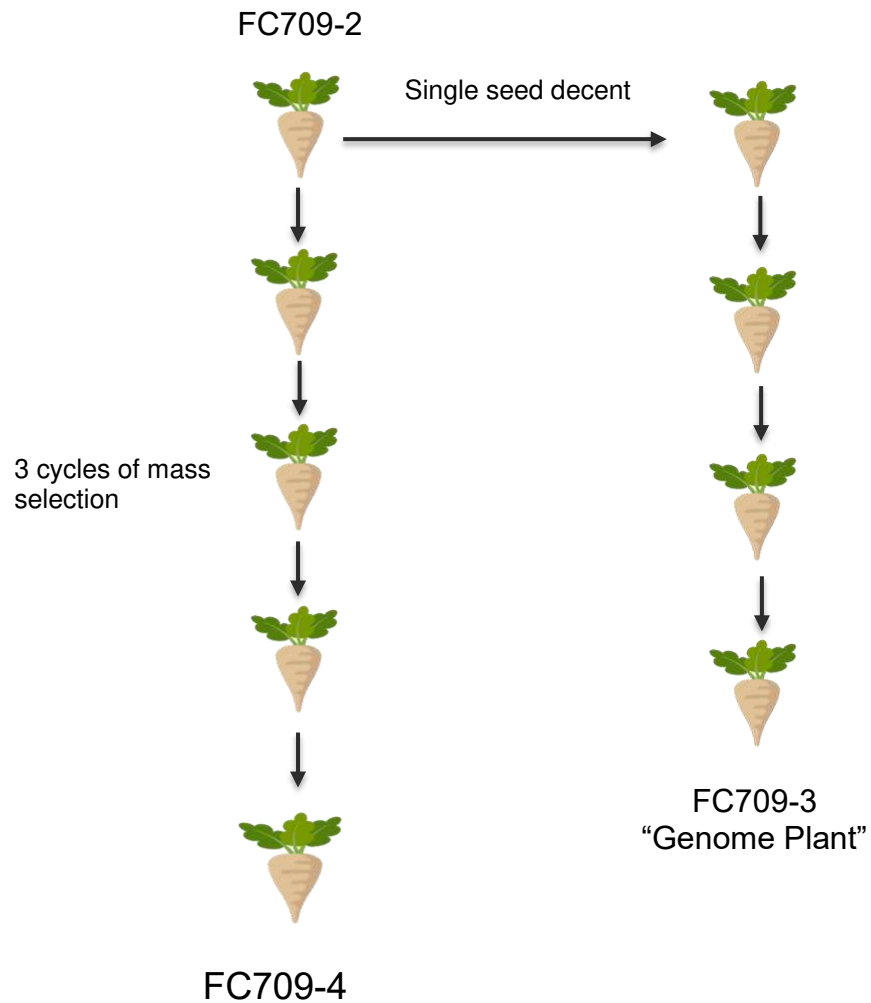


Figure 2: The pedigree of germplasm FC709-4 and genomic stock FC709-3. FC709-4 was derived from 3 cycles of mass selection by only selecting those roots deemed resistant (rated 0-3) and allowed to cross pollinate for each subsequent population. FC709-3 was created using a cycle of single seed decent from a single highly resistant FC709-2 plant followed by two cycles of mass selection. FC709-3 is a single plant rated a 0 for RRCR resistance.

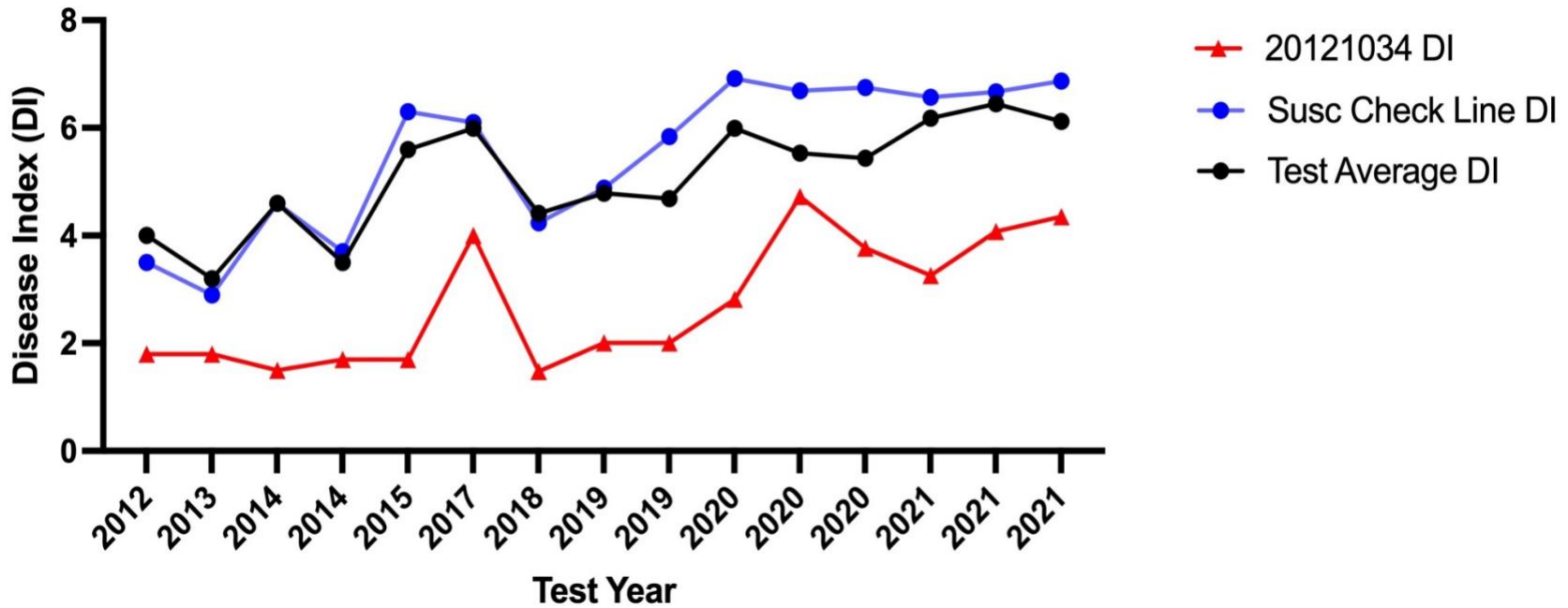


Figure 3. FC709-2 (20121034) has been the long-standing resistant check when evaluating *Rhizoctonia solani* resistance in both field and greenhouse disease studies. The disease index is calculated by giving a weighted per plot entry for each years disease nursery and is used to compare against the test average.

Table 1. Phenotypic distribution of FC709-2 (2014-2023). Rhizoctonia Root and Crown Rot is rated on a scale from 0 to 7 (Ruppel and Hecker 1988). 0 = no disease detected and 7 = plant death. Ratings 0-3 are considered economically resistant and 4-7 are considered susceptible.

Year	Entry	Plants at Rating	Disease Index Rating (# individuals)							
			0	1	2	3	4	5	6	7
2023	20121034	41	3	16	12	7	2	1	0	1
2022	20121034	69	0	10	38	12	4	0	0	5
2021	20121034	58	0	0	7	13	8	10	8	12
2020	20121034	50	0	11	8	9	6	4	4	8
2019	20121034	28	0	9	14	3	1	1	0	0
2018	20121034	65	0	9	20	22	9	2	3	0
2017	20121034	65	0	9	20	22	9	2	3	0
2015	20121034	58	3	34	10	9	2	0	0	0
2014	20121034	54	3	28	10	6	3	2	2	0

Table 2. Evaluation of Rhizoctonia root and crown rot resistance in USDA-ARS Fort Collins 2020:

<sup>z</sup>As per GRIN description, all 30 entries are *Beta vulgaris subsp. maritima*, whereas the 3 check lines are *Beta vulgaris subsp. vulgaris*.

<sup>y</sup>DI = Disease index on a scale of 0 (no damage) to 7 (plant death).

<sup>x</sup>Percent of healthy roots (disease classes 0 and 1 combined) and percent of diseased roots likely to be taken for processing (disease classes 0 through 3 combined); % indicates value after percentages were transformed to arcsine-square roots to normalize the data for analyzes.

<sup>w</sup>Percentages after arcsine-square root transformation for normalization.

<sup>v</sup>These entries were not significantly different (Dunnett's one-tailed t-test / P = 0.05) from the resistant check, FC709-3 (more resistant = smaller DI).

<sup>u</sup>These entries were not significantly different (Dunnett's one-tailed t-test / P = 0.05) from the susceptible check, FC901 x C817).

Entry	Description	DI	0-1	0-3	%0-1	%0-3
19921022	FC702/7; PI 590755	3.25	30	50	27	45
20121034	FC709-2; PI 599668	3.77	20.46	51.56	23.77	46.78
19961014	FC724; PI 632251	3.99	7.07	45.95	13.63	42.58
20021017	FC726; PI 591335	4.23	6.85	36.6	11.71	33.92
19951017	FC727; PI 599669	4.3	6.17	28.87	12.38	32.24
20041005	FC703; PI 590656	4.3	5.45	31.21	8.16	33.61
20011022	FC701/4; PI 590663	4.44	2.71	30.51	6.03	30.36
19991018	FC709; PI 518643	4.56	11.55	29.67	15.42	32.34
20001019	FC705; PI 590660	4.59	6.82	21.42	11.79	27.07
19941024	FC710; PI 542971	4.76	12.39	23.95	16.05	25.95
19911032	FC718; PI 574629	4.86	0	23.6	0	25.98
19921025	FC728; PI 591336	4.87	2.35	25.67	4.01	30.11
19851032H	FC705/1; PI 590754	4.91	7	23	10.75	21.34
20101004	FC708; PI590845	5.02	3.08	23.25	4.62	27.51
20001021	FC707; PI 590702	5.06	4.58	20.38	9.59	24.17
20021016	FC725; PI 591334	5.2	1.05	17.93	2.65	22.2
20151018	F3s of half-sib families from FC907 x FC702-2	5.2	10.57	25.87	14.34	24.62
19771071H	FC701/5; W6 17137	5.21	5	22.7	6.64	24.92
20101009	FC1018; PI 658059	5.21	0	15.73	0	21.69
19971019	FC716; PI 574627	5.38	3.53	10.71	4.97	14.61
19801059H	FC701/6; PI 590756	5.39	1.67	10.67	3.36	14.67
20001020	FC706; PI 590701	5.43	1.33	9.26	2.99	15.55
19911037	FC719; PI 574630	5.49	3.33	11.89	4.82	12.13
19931005HO	FC721; PI 594910	5.72	0	9.52	0	11.49
20071012	FC720; PI 636335	5.76	1.33	7.07	2.99	13.68
20081010	FC717; PI574628	5.77	3.15	11.46	6.5	16.85
20141005	FC715; PI 574625	5.82	2.67	9.35	4.28	15.71
20101011	FC1019; PI 658060	5.85	0	4.82	0	9.87
20071013	FC220; PI 651015	5.87	0.95	5.86	2.52	10.79
20121010	FC1020; PI 658061	5.92	0	5.37	0	10.24
20141004	FC221; PI 651016	6.05	0	2	0	3.69
19931018	FC701; PI 590661	6.08	0	3	0	6.35
20001018	FC704; PI 590659	6.15	0	1.25	0	2.9

20111025	FC1037; PI 665055	6.19	0	3.48	0	6.87
20131007	FC1038; PI 665056	6.21	0	0	0	0
20091006	FC702; PI 590662	6.28	0	2	0	3.69
20121011	FC1036; PI 665054	6.28	0	0	0	0
19951016HO	FC723; PI 639917	6.3	0	0	0	0
20111027	FC1028; PI 665053	6.32	0	0	0	0
20091009	FC1022; PI 658062	6.42	0	0	0	0
19961010HO	FC722; PI 636336	6.43	0	0	0	0
20141002	FC901/C817	6.46	0	2.22	0	3.89
19811055H	FC702/6; PI 590703	6.49	0	0	0	0
20161010	Monohikari	6.53	0	0	0	0
20151020	Susceptible Check - B.I. of 20101013 (½ sib families of LSR population BGRC 45511)	6.75	0	0	0	0
<b>Trial Means</b>		<b>5.44</b>	<b>3.58</b>	<b>14.61</b>	<b>5.11</b>	<b>16.58</b>

Table 3: Evaluation of Rhizoctonia root and crown rot resistance in USDA-ARS Fort Collins 2023

<sup>z</sup>As per GRIN description, all 30 entries are *Beta vulgaris subsp. maritima*, whereas the 3 check lines are *Beta vulgaris subsp. vulgaris*.

<sup>y</sup>DI = Disease index on a scale of 0 (no damage) to 7 (plant death).

<sup>x</sup>Percent of healthy roots (disease classes 0 and 1 combined) and percent of diseased roots likely to be taken for processing (disease classes 0 through 3 combined); % indicates value after percentages were transformed to arcsine-square roots to normalize the data for analyzes.

<sup>w</sup>Percentages after arcsine-square root transformation for normalization.

<sup>v</sup>These entries were not significantly different (Dunnett's one-tailed t-test / P = 0.05) from the resistant check, FC709-4 (more resistant = smaller DI).

<sup>u</sup>These entries were not significantly different (Dunnett's one-tailed t-test / P = 0.05) from the susceptible check, FC901 x C817).

Entry	Description / Origin	Species	DI <sup>y</sup>	0-1 <sup>x</sup>	0-3 <sup>x</sup>	%0-1 <sup>w</sup>	%0-3 <sup>w</sup>
FC 709-4	New resistant check line	<i>Beta vulgaris subsp. vulgaris</i>	1.26 <sup>u,v</sup>	70.18	100	57.83	90
FC 709-2	Resistant check line	<i>Beta vulgaris subsp. vulgaris</i>	1.77 <sup>u</sup>	50.06	89.86	45.43	76.16
PI 504217		<i>Beta vulgaris subsp. maritima</i>	4.81 <sup>u,v</sup>	4.40	30	5.63	30
PI 504227		<i>Beta vulgaris subsp. maritima</i>	4.81 <sup>u,v</sup>	0	24.44	0	23.63
PI 504201		<i>Beta vulgaris subsp. maritima</i>	4.87 <sup>u,v</sup>	0	15.94	0	23.17
PI 562586		<i>Beta vulgaris subsp. maritima</i>	4.88 <sup>u,v</sup>	0	15.03	0	20.37
PI 504251		<i>Beta vulgaris subsp. maritima</i>	4.95 <sup>u,v</sup>	0	28.57	0	26.18
FC901xC817	Susceptible check line	<i>Beta vulgaris subsp. vulgaris</i>	4.96 <sup>u,v</sup>	0	0	0	0
PI 604517		<i>Beta vulgaris subsp. maritima</i>	5.05 <sup>u,v</sup>	6	20.73	6.64	20.71
PI 540610		<i>Beta vulgaris subsp. maritima</i>	5.13 <sup>u</sup>	13.33	28.08	10.95	28.62
PI 604511		<i>Beta vulgaris subsp. maritima</i>	5.21 <sup>u,v</sup>	4.22	17.79	7.58	22.02
PI 518404		<i>Beta vulgaris subsp. maritima</i>	5.45 <sup>u</sup>	3.33	16.67	4.82	16.05
PI 504187		<i>Beta vulgaris subsp. maritima</i>	5.64 <sup>u,v</sup>	1.33	9.33	2.99	11.53
PI 540557		<i>Beta vulgaris subsp. maritima</i>	5.65 <sup>u</sup>	0	7.95	0	10.56
PI 504276		<i>Beta vulgaris subsp. maritima</i>	5.70 <sup>u</sup>	0	6.81	0	11.68
PI 604520		<i>Beta vulgaris subsp. maritima</i>	5.71 <sup>u</sup>	4	8.76	5.31	12.74
PI 504191		<i>Beta vulgaris subsp. maritima</i>	5.72 <sup>u</sup>	0	3.33	0	4.82
PI 540677		<i>Beta vulgaris subsp. maritima</i>	5.73 <sup>u</sup>	2	8.00	3.69	10.33
PI 540584		<i>Beta vulgaris subsp. maritima</i>	5.79 <sup>u</sup>	2.23	7.89	5.46	12.41
PI 504204		<i>Beta vulgaris subsp. maritima</i>	5.83 <sup>u</sup>	0	0	0	0
W6 45840		<i>Beta vulgaris subsp. maritima</i>	5.84 <sup>u</sup>	0	7.58	0	9.84
PI 504268		<i>Beta vulgaris subsp. maritima</i>	5.86 <sup>u</sup>	0	5.93	0	10.93
PI 604515		<i>Beta vulgaris subsp. maritima</i>	5.92 <sup>u</sup>	0	4.25	0	9.19
PI 546413		<i>Beta vulgaris subsp. maritima</i>	5.96 <sup>u</sup>	1.54	4.33	3.22	7.51
PI 518303		<i>Beta vulgaris subsp. maritima</i>	6.03 <sup>u</sup>	2.00	15.02	3.69	17.49
PI 540609		<i>Beta vulgaris subsp. maritima</i>	6.14 <sup>u</sup>	0.83	2.26	2.36	5.46
PI 546385		<i>Beta vulgaris subsp. maritima</i>	6.15 <sup>u</sup>	0	5.54	0	8.53

PI 604528	<i>Beta vulgaris subsp. maritima</i>	6.20 <sup>u</sup>	0	1.25	0	2.90
PI 604536	<i>Beta vulgaris subsp. maritima</i>	6.23 <sup>u</sup>	0	0	0	0
PI 540637	<i>Beta vulgaris subsp. maritima</i>	6.28 <sup>u</sup>	0	2.35	0	4.01
W6 45821	<i>Beta vulgaris subsp. maritima</i>	6.36 <sup>u</sup>	0	0	0	0
PI 504245	<i>Beta vulgaris subsp. maritima</i>	6.44 <sup>u</sup>	0	0	0	0
PI 504247	<i>Beta vulgaris subsp. maritima</i>	6.62	0	0	0	0
	All	5.42	5.02	14.78	5.02	15.96

## FC709- Greenhouse Dist

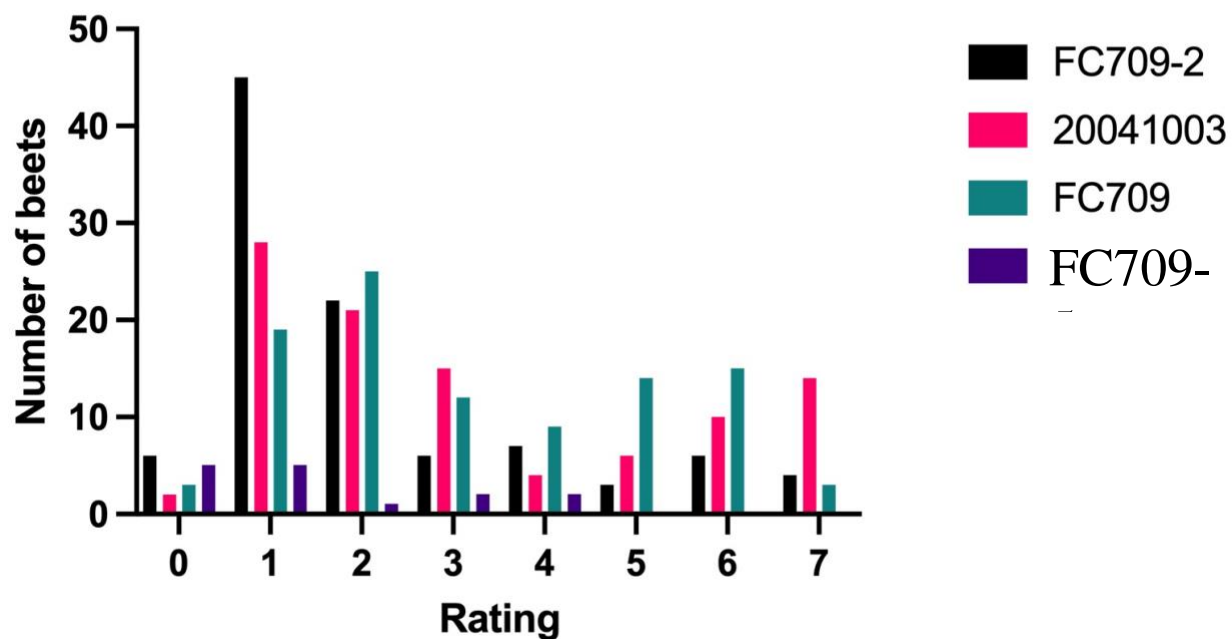


Figure 4. FC709-5 ratings and distributions compared to its predecessors. This was done using the greenhouse disease screening methods with each individual root being rated for RRCR resistance. This experiment was done to determine numerically how many individuals per germplasm line were rated either resistant (0-3) or susceptible (4-7).



Figure 5. Application of artificial *Rhizoctonia solani* inoculum takes place 8 weeks after sowing. Ground hulless barley that has been colonized by *Rhizoctonia solani* is spread on at 2g/M over the top of the crown of the sugar beets. Infection will take place over the course of the next 4 weeks when the beets are lifted and rated for disease resistance.



Figure 6. Above is a representation of the Ruppel scale for *Rhizoctonia solani* in sugar beets. From left to right plants are scored from 0 (no disease present) to 7 (plant death). The bottom row (FC901 susceptible check) represents the complete 0 through 7 scale and the top row (FC09-2) representing the harvestable scale (ratings 0 through 3).



Figure 7. RRCCR field trial at ARDEC research farm 4 weeks post inoculation. Plants that have wilted over multiple days appear brown and dry. Those plants with large green leaves appear to be resistant although above ground phenotypes do not directly correlate with bellow ground phenotypes.



Figure 8. Greenhouse RRCR disease trials take place in the Crops Research Laboratory greenhouses. The controlled environment allows for conducive parameters for disease. Similar to field trials, 8 weeks after sowing plants are inoculated with 0.6 g of ground infected barley grains. 2 weeks post inoculation plants are uprooted, washed, and rated for RRCR resistance.

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## **Chapter Three: A chromosome scale genome assembly for *Beta vulgaris* inbred line FC709-3**

### **Introduction**

High quality genomic resources, including chromosome-scale genome assemblies and gene/repeat annotations, are critical to geneticists and breeders aiming to discover, validate, and deploy genomics-informed strategies like marker assisted and genomic selection. In sugar beet (*Beta vulgaris* ssp. *vulgaris*), the reference genomes RefBeet (a commercial doubled haploid) and EL10 (a USDA-ARS inbred line) have been utilized to develop a limited number of reported molecular markers. New sequencing technologies, combined with assembly and scaffolding approaches, and annotation pipelines continue to rapidly improve, making the development of ‘purpose-built genome assemblies’ almost arbitrary. Here we report the development of a highly contiguous and complete genome assembly of sugar beet inbred line FC709-3 developed specifically for mapping the genomic location of resistance to *Rhizoctonia* Root and Crown Rot, a fungal disease caused by *Rhizoctonia solani*. The FC709-3 genome was constructed using a combination of PacBio HiFi long reads for contig-level assembly, Dovetail Omni-C proximity ligation sequencing for scaffolding, and a combination of Illumina short reads and PacBio IsoSeq reads for annotation. The FC709-3 assembly (USDA\_Bvulg\_FC709-3\_v1.0.1) encompassed 651 Mb, with nine chromosome-sized pseudomolecules. The BRAKER2 annotation pipeline identified 29,463 gene models derived from both short and long read RNA sequencing. Structural comparisons between FC709-3, EL10.2, and RefBeet 1.2.2 revealed significant size and structural variation between these assemblies. The FC709-3 provides foundational genomic and germplasm resources that will enable the identification of precise molecular markers for

RRCR resistance, a platform for understanding molecular plant/pathogen interactions and resistance gene mechanisms.

Sugar beet (*Beta vulgaris* spp. *vulgaris* L.) is used as a one of the two primary sources of refined sugar in the world (Pennington and Baker 1990). Historically, beet was primarily used for its leafy tops, but in the Middle Ages the enlarged taproot was used as a vegetable. In the 1860s, the first sugar varieties were available for production in Europe (Gayon and Zallen 1998). Since then, many traits have been improved upon through plant breeding programs both in the private and public sectors. One of the more recent tools implemented into these programs is the use of next generation sequencing, genomics, and bioinformatics. This includes the sequencing of the entire *Beta vulgaris* genome. The first reference genome to be published was RefBeet (Dohm, Minoche et al. 2014). Having a reference genome opened numerous opportunities to begin to discover advantageous traits in sugar beet. However, as sequencing technology and bioinformatics improves so can reference genomes. EL10.1 and EL10.2 (McGrath, Funk et al. 2023) implemented PacBio long-read sequencing technology and proximity ligation sequencing for long-range scaffolding (Van Berkum, Lieberman-Aiden et al. 2010) to create a contiguous reference genome assembly. Pseudomolecules with length from 52 to 65 Mb were assembled and annotated, yielding 23,255 high quality gene models on the first public chromosome-scale assembly for beet. These have been extremely valuable tools to uncover the genetic underpinnings of many traits of interest in the crop (McGrath, Koppin and Duckert 2005, Holmquist, Dölfors et al. 2021, Wigg, Brainard et al. 2023).

The USDA-ARS Sugar Beet Genetic Lab in Fort Collins, Colorado focuses on improving disease tolerance within the *Beta vulgaris* germplasm, in particular to *Rhizoctonia* Root and Crown Rot (RRCR). RRCR is caused by the fungus *Rhizoctonia solani* (Herr 1996). This necrotrophic

pathogen is found in all sugar beet growing areas and causes millions of dollars of yield loss (Windels and Nabben 1989). There have been multiple germplasm releases that have some degree of RRCR resistance from the SMSBRU lab (Panella, Campbell et al. 2016). One of the best performing germplasm lines is FC709-2 (Panella 1999). Host resistance in sugar beets has been identified through genetic studies as polygenic trait with at least two loci, with two or three alleles, together with modifying genes (Hecker and Ruppel 1975). The first reported QTL from Lein et al. found three QTL associated with *R. solani* resistance on chromosomes 4, 5 , and 7 using 38 expressed sequence tags (ESTs), resistance reactions of the plants (R-ESTs), and 25 bacterial artificial chromosomes (BACs) containing nucleotide binding site (NBS)-motifs typical for disease resistance genes (Lein, Sagstetter et al. 2008). With the rapid advancement of genomic tools and technology, discovering new QTL regions has become easier and less laborious. Wigg et al used F2:3 families derived from a resistant sugar beet germplasm FC709-2 and a susceptible table beet W357A and a RRCR resistant table beet W365B crossed with a susceptible sugar beet germplasm FC901 to discover a new QTL associated with RRCR resistance. Utilizing genotype-by-sequencing to identify polymorphic markers and interval linkage mapping they identified a new QTL on chromosome 2 for both resistant parents (Wigg, Brainard et al. 2023). The sugar beet germplasm line FC709-2 was selected as a prime candidate to use for discovering RRCR resistance genes.

A primary focus of the work described here aims to identify, fix, map, and characterize RRCR resistance derived from the sugar beet germplasm line FC709-2. However, due to the unique pedigrees of the plants used to create the RefBeet (commercial doubled haploid) and EL10 (highly inbred) genomes, the issue of reference genome bias arises. As DNA sequencing-based genetic mapping often begins with aligning reads to a reference genome, where the reference

genome represented as a linear string of bases (Chen, Solomon et al. 2021). This linearity leads to reference bias, which is a tendency to miss alignments or report incorrect alignments for reads containing non-reference alleles, or completely missing presence-absence variations between the reference genome background and the background of interest. This can lead to confounding or imprecise results. Structural differences between reference genomes and over representation of an individual in a population can result in ascertainment bias. Using a more inclusive and targeted approach might be to look at variation among individuals within the same species. As creating more reference genomes with diverse genetic backgrounds may ultimately result in a pan-genome that will help reduce the effects of reference bias (Della Coletta, Qiu et al. 2021). Another way to reduce reference bias is to select material whose trait of interest is fixed. These purpose-built genomes allow for more accurate trait discovery.

In this report, we describe the selection of an individual derived from the FC709-2 background that was selected for de novo genome assembly and annotation. Generating this new purpose-built genome for RRCR resistance will allow us to discover the genetic and molecular mechanisms that are responsible for resistance in this accession. This new reference genome will also allow for routine inter-cultivar comparisons between accessions for important agronomic traits.

## **Methods and Materials**

### **Doubled Haploid/Homogeneity testing**

Collaborator Dr. Imad Eujayl at the Kimberly, Idaho USDA-ARS research station has developed putative double haploid lines (DHs) from a moderately heterozygous population of the Fort Collins release FC709-2. These putative FC709-2DH lines were screened in the greenhouse

to identify individuals with RRCR resistance (Ruppel RRCR score of 0 or 1). DNA was extracted from four RRCR resistant putative DH individuals using the Qiagen DNeasy Plant Mini Kit (Qiagen Hilden, Germany) and sent to Novogene for Illumina sequencing. We generated high coverage whole genome sequencing reads (Illumina) for each of these RRCR resistant putative doubled haploid plants. Also included in this sequencing experiment was a single FC709-2 individual, which was chosen to represent the background heterozygosity, and a highly inbred individual plant from EL10, which was the result of 7 cycles of inbreeding. Sequencing reads were trimmed and filtered reads (BBduk, part of the BBmap software package <https://sourceforge.net/projects/bbmap/>). K-mer counting derived from the trimmed and filtered read sets was completed using the Jellyfish (v1.1.6) (Marcais and Kingsford, 2011). K-mer analysis using GenomeScope (Vurture et. al., 2017) and standard variant calling was used to determine the heterozygosity of individuals that were selected for sequencing. Single nucleotide polymorphisms (SNPs) were identified and quantified by mapping the trimmed read sets from each individual to the EL10.2 genome using the Whole Map Reads to Reference Tool (v1.7) and variants called using the Basic Variant Detection tool (v2.1) in CLC Genomics Workbench (v20.04).

### **FC709-3 line development and selection of a single plant for sequencing**

The FC709-3 individual plant used for genome assembly was derived from the Fort Collins FC709-2 seed lot 20121034. In 2020, the 20121034 seed lot was grown in the RRCR disease nursery at the Colorado State University Agricultural Research, Development, and Education Center (ARDEC) field research station. A bulk planting of approximately n=1000 plants across 13 rows (42 m in length) were inoculated. 28 plants rated 0 or 1 were collected and

selfed. The plant rated a 0 and with the most vigor after selfing-pollination produced the seed lot 20211028\_1s.

The second cycle of selection was performed in the greenhouse in the fall of 2021. Seeds were sown and thinned to a single plant per 7-inch pot. Plants were grown for 8 weeks before artificial inoculation was performed. Inoculum was infected barley grains with *Rhizoctonia solani* strain R9 (source) that had been ground into a small granule. A small hole about an inch deep was created next to the sugar beet root where 0.6 g of inoculum was placed and covered with soil. Plants were then rated from a scale of 0 to 7 (0 = no detectible disease and 7 = complete plant death) for resistance (disease scale source). Plants that were selected as resistant were then vernalized at 1-2 C for 120 days before being placed back into the greenhouse to inter-pollinate. That seed was then sown into the field for another round of mass selection. Again, following the guidelines from the greenhouse protocol previously described, 8-week-old plants were inoculated with the same *Rhizoctonia solani* strain (R9) and rated after 4 weeks. A single most resistant plant with a rating of 0 was vernalized, then placed into an HEPA-filtered isolation chamber to flower to prevent accidental cross pollination. The progeny (20221014-s) from this selfed individual were then planted in the greenhouse and challenged with *Rhizoctonia solani* infection to identify a single highly resistant inbred plant to be sequenced. One plant from this experiment, which had an RRCR rating of 0, was chosen for sequencing and given the designation 20221014-2s.

The 20221014-2s single plant into the dark for 2 days prior to tissue harvest to reduce plastid and carbohydrates in the tissue to improve high molecular weight DNA isolation and proximity ligation (DoveTail Omni-C) library preparation optimization. Every week two fresh young leaves were harvested, and flash frozen in 50ml conical tubes. This was repeated until 8

grams of fresh tissue was collected and sent to DoveTail Genomics for HMW DNA isolation, PacBio library preparation, and DoveTail Omni-C library preparation.

### **Library preparation and Sequencing**

All sequencing datasets described herein are available at the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under BioProject PRJNA882839 and BioSample SAMN30952620. Individual sequencing read accession numbers for each individual library are listed below.

DoveTail Genomics isolated HMW DNA using the Qiagen blood and cell culture DNA midi kit for extraction. A single PacBio sequencing library was prepared from this HMW DNA, and was sequenced on a single SMRT cell using the HiFi (formerly known as CSS, or Circular Consensus Sequencing) protocol on a PacBio Sequel instrument. These sequencing reads are available via the NCBI SRA under accession SRR21678414.

For Dovetail Omni-C library preparation, the input biological material were the dark-treated leaf samples from the 20221014-2s single plant. Chromatin was fixed in place with formaldehyde in the nucleus and then extracted. Fixed chromatin was digested with DNase I, chromatin ends were repaired and ligated to a biotinylated bridge adapter followed by proximity ligation of adapter containing ends. After proximity ligation, crosslinks were reversed, and the DNA purified. Purified DNA was treated to remove biotin that was not internal to ligated fragments. The sequencing library were generated using NEBNext Ultra enzymes and Illumina-compatible adapters. Biotin-containing fragments were isolated using streptavidin beads before PCR enrichment of each library. The library was sequenced on an Illumina HiSeqX platform (2x150 bp reads) to produce . These sequencing reads are available via the NCBI SRA under

accession SRR21678413.

### **Scaffolding the assembly with Omni-C HiRise**

The input *de novo* assembly and Dovetail OmniC library reads were used as input data for HiRise, a software pipeline designed specifically for using proximity ligation data to scaffold genome assemblies (Putnam et al, 2016). The Dovetail OmniC library sequences were aligned to the draft input assembly using bwa (<https://github.com/lh3/bwa>). The separations of Dovetail OmniC read pairs mapped within draft scaffolds were analyzed by HiRise to produce a likelihood model for genomic distance between read pairs, and the model was used to identify and break putative misjoins, to score prospective joins, and make joins above a default threshold. PacBio CCS (HiFi) reads were used as an input to Hifiasm1 v0.15.4-r347 with default parameters. BLAST results of the Hifiasm output assembly against the nt database were used as input for blobtools2 v1.1.1 and scaffolds identified as possible contamination were removed from the assembly. Finally, purge\_dups3 v1.2.5 was used to remove haplotigs and contig overlaps (purged.fa) (<https://omni-c.readthedocs.io/en/latest>). This assembly was named “USDA\_Bvulg\_FC709-3\_v1.0”.

### **Assembly Characterization, Chromosome Assignment, and Orientation Correction**

Assembly statistics were calculated at the contig and scaffold level using the stats.sh tool within the BBtools software package (citation). To identify the chromosome identities of this assembly versus both EL10.2, resulting 9 largest scaffolds from the USDA\_Bvulg\_FC709-3\_v1.0 genome assembly were aligned pairwise using the Whole Genome Alignment Tool (v20.1) in CLC Genomics Workbench (v20.04). Based on this alignment, these 9 largest

USDA\_Bvulg\_FC709-3\_v1.0 scaffolds were ordered and renamed to match their corresponding EL10.2 chromosome numbering. The resulting set of pseudochromosomes was designated USDA\_Bvulg\_FC709-3\_v1.0.1. Other versions of the FC709-3 assembly were created after the completion of the analyses described herein, namely USDA\_Bvulg\_FC709-3\_v1.3, which is publicly available via NCBI under accession JAVXZB000000000. The v1.3 assembly was derived directly from the v1.0 assembly and includes unanchored contigs from the USDA\_Bvulg\_FC709-3\_v1.0 assembly. The v1.3 assembly differs from the v1.0.1 assembly as the pseudochromosomes were oriented based on alignments to the RefBeet assembly to match historical chromosome orientations, plus the removal or masking of a small number of contaminating adaptor sequences and contaminating contigs identified during NCBI contamination screening.

### **Synteny Analysis between FC709-3 and other assemblies**

The USDA\_Bvulg\_FC709-3\_v1.0.1 and EL10.2 pseudochromosomes were first reoriented to match the linear orientation of the RefBeet v1.2.2 pseudochromosomes to enable synteny visualization. The FC709-3 v1.0.1 pseudochromosomes were aligned to both the RefBeet and re-oriented EL10.2 pseudochromosomes using minimap2 (v2.24) ( Li 2018). The resulting BAM alignment files were indexed using the samtools index command (v1.16.1). Comparisons of the resulting alignments were processed using Syri (<https://github.com/schneebergerlab/syri>, version 1.6.3), implemented using Conda (v4.13.0) on the USDA-ARS SciNet Ceres compute cluster (Goel, Sun et al. 2019). Visualization of alignments was implemented using plotsr (<https://github.com/schneebergerlab/plotsr>, v0.5.4).

## **Genome Annotation**

For gene annotation, both PacBio Iso-Seq long read, and Illumina short read RNA sequencing was used. The PacBio Iso-Seq data was from a full sib plant of 20221014-2s that was infected with *Rhizoctonia solani*. After 14 days post inoculation, approximately 3g of leaf and root tissues were sampled and flash frozen in liquid nitrogen. Tissues were then homogenized together, and RNA was extracted using a QIAGEN Rneasy extraction kit. RNA from the tissue were sent for PacBio IsoSeq library preparation and sequencing at the University of Minnesota Genomics Center (St. Paul, Minnesota). The resulting library was sequenced on a single SMRT cell, which produced 3,071,093 reads (5,903,595,151 bp in total yield). In addition to PacBio IsoSeq long reads, we also generated Illumina short reads to both supplement transcript evidence for annotation, as well as downstream expression analyses. This tissue came from 20121034 seed lot of FC709-2, the progenitor of the FC709-4 individual plant 20221014-2s used to create the USDA\_Bvulg\_FC709-3\_v1.0.1 assembly. An RNA seq experiment consisting of twelve 8-week-old FC709-2 plants being challenged with *Rhizoctonia solani* and twelve plants remaining uninoculated. Tissue was taken at 0 days post inoculation and 10 days post inoculation when symptoms began to show. When sampling tissue was split into two categories: above ground and below ground. The beet was cut perpendicularly at the crown of the plant and tissue above the soil line and below the soil line were separated into their own conical tubes. RNA was extracted as previously described and sent to NovoGene (California, USA) for sequencing on the Illumina NovaSeq platform (2x150 bp reads). Prior to genome assembly, tandem and interspersed repeat sequences were identified and masked using HISAT2 and RepeatMasker (Tarailo-Graovac, M. and Chen, N., 2009). When using RepeatMasker the soft-masking option was used for better results per the BRAKER2 recommendations.

The FC709-3 assembly was annotated using the BRAKER2 pipeline (Gabriel, Bruna et al. 2023). BRAKER2 uses both GeneMark-EX (Bruna et al., 2021) and AUGUSTUS (Camacho et al., 2009) for training and gene prediction using both genomic and RNA-Seq data. The pipeline automatically generates full gene structure annotations in novel genomes.

## **Results**

### **Evaluation of Homozygosity candidate FC709-2 Doubled Haploid Individuals**

K-mer analysis using GenomeScope and standard variant calling indicated large variation in heterozygosity rates between selected lines (Figure 9). For example, EL10 heterozygosity rates were 0.21% (Figure 9E), compared to that of FC709-2, 1.15% (Figure 9F). The candidate DH lines heterozygosity rates ranged from 0.539% to 1.4%. While DH\_08 (Figure 9F) exhibited reduced heterozygosity compared to the FC709-2 background, it would be expected that a true doubled haploid plant would have a heterozygosity rate at least as low as the inbred EL10 individual. The DH\_08 individual plant was allowed to self-pollinate, however, the seed produced was found to be unviable. It is clear we had identified candidate lines that have reduced heterozygosity levels compared to the FC709-2 background (Figure 9, panel F, which are reads from a single FC709-2 plant). We have collected self-pollinated seed from these DH plants; however, these seeds were not viable upon attempting to germinate. These findings were the motivating factor for developing a new partially inbred line from the FC709-2 background for genome sequencing.

### **FC709-3 Genetic Stock Development**

FC709-2 seed was planted in field as the RRCR disease nursery resistant check. A single highly resistant plant rating a 1 was selected. The root was vernalized for 120 days, replanted in

the greenhouse, selfed, and a single seed was selected to plant again to produce single seed decent seed. All seed from the single plant was planted in the greenhouse to perform a RRCCR disease assay and identify individuals that display high resistance (ratings 0 and 1). This cycle was performed twice before selecting a single plant for sequencing. A single plant (FC709-3) that had a rating of 0 and displayed high vigor. After tissue selection was complete for DNA extraction and sequencing the plant was selfed to produce seed for subsequent genetic experiments in the future. Seed was collected and a subset was sent to be stored in the USDA-ARS Germplasm Resources Information Network (GRIN).

### **Genome assembly**

Tissue from the FC709-3 individual had high molecular weight DNA extracted resulting in a majority of the reads in the 10,000 bp to 20,000 bp length (Figure 12). The FC709-3 plant (20221014-2s) was sequenced using PacBio HiFi DNA sequencing technology. A total of 1,922,667 PacBio HiFi reads were obtained from a single sequencing cell, totaling 27,334,524,517 gigabases of data. The HiFiasm assembly pipeline was used to assemble HiFi reads into a contig-level assembly, which totaled 729 Mb (Table 4). Filtered mapped read pairs from the DoveTail Omni-C library (n= 42,955,531 Illumina read pairs) were used to scaffold the contig-level assembly (Figure 11). A total of 288 contig joins were made based on Omni-C reads, along with 3 breaks of inaccurately assembled contigs present in the input assembly, which resulted in the USDA\_Bvulg\_FC709-3\_v1.0 assembly.

USDA\_Bvulg\_FC709-3\_v1.0.1 had 1,529 total scaffolds of which 1,520 were unanchored contigs (11% of total reads). This assembly encompasses 651 Mb across 9 pseudochromosomes. Omni-C (HiC) contact map analysis (Figure 10) demonstrated this assembly is well resolved structurally. Chromosome-by-chromosome dot plot analysis between the USDA\_Bvulg\_FC709-

3\_v1.0.1 and EL10.2 chromosomes (Chr. 1 shown in Figure 10, USDA\_Bvulg\_FC709-3\_v1.0.1 on the x-axis, EL10.2 on the y-axis) shows small structural variations between these two highly contiguous assemblies, with several notable small inversions, plus the addition of multi-megabase segments of additional sequence present in the USDA\_Bvulg\_FC709-3\_v1.0.1 scaffold. It should be noted that EL10.2 was not oriented to the original RefBeet genome so that some chromosomes (Chr1, Chr2, and Chr4) are mis-oriented and have been corrected in later versions on the assembled genomes.

### **Genome Annotation**

Using three different sequencing technologies and bioinformatics tools we annotated the USDA\_Bvulg\_FC709-3\_v1.0.1 assembled genome. Briefly, a single sample consisting of equal total masses of RNA was constructed from each of 6 biological replicates from the FC709-2 background x 2 treatments (*Rhizoctonia* inoculated vs. mock) x 2 tissues (above ground and below ground). A single PacBio IsoSeq library was prepared and sequenced on a single 8M Sequel II cell with a 24-hour movie. Sequencing generated 3,071,093 HiFi reads, with a total HiFi yield of 5,903,595,151 bp. The average length of these HiFi reads was 1,922 bp, with a median quality score of Q44, and a max length of 12.8 kb. For the purposes of genome annotation, these ‘full length’ RNA sequencing reads produced on the PacBio platform represent a novel data type for sugar beet genome annotation. In parallel with the PacBio IsoSeq read generation, Illumina short reads from the 24 individual RNA libraries from the *R. solani* inoculation transcriptomics experiment were also generated. We utilized BRAKER2 pipeline for annotation of the USDA\_Bvulg\_FC709-3\_v1.0.1 genome, utilizing both the PacBio IsoSeq and Illumina RNAseq reads as transcript evidence. Integrating both data types yielded a total of 96,905 gene models.

These gene models were functionally annotated using the Trinotate pipeline (Bryant et al., 2017), and subsequently filtered to eliminate non target species, nonfunctional gene models, and rRNA. After filtering, the final gene set consists of 29,463 gene models. This final annotation of the FC709-3 genome was subsequently used as the reference genome gene set for expression calling of the *R. solani* inoculation RNAseq experiment.

### **Synteny Analysis**

For synteny analysis the program SyRI (Synteny and Rearrangement Identifier) (Goel et. al., 2019) was used to predict genomic differences between related genomes using whole-genome assemblies. Visual analysis of the RefBeet v1.2.2, EL10.2, and USDA\_Bvulg\_FC709-3\_v1.0.1. revealed multi-megabase segments of additional sequence present in the USDA\_Bvulg\_FC709-3\_v1.0.1 particularly in chromosomes 1 and 3. Along with additional sequence, there are multiple small structural variations between these three genomes as well as several small inversions (Figure 13 for all chromosomes and Figures 15-32 for individual chromosomes).

Syntenic relations between USDA\_Bvulg\_FC709-3\_v1.0.1 and EL 10.2 were 666 regions with lengths of 499,480,601 bp in FC709-3 and 494,409,946 bp in EL10.2. Inversions between the genomes resulted in 94 regions totaling in 24,402,745 bp in FC709-3 and 25,314,506 bp in EL10.2. Three-hundred and ninety-five translocations with the lengths of 3,718,092 (FC709-3) and 3,800,210 bp (EL10.2). Duplication regions in each genome were 107 and 298 and the number of reads not aligned were 123,566,552 bp (FC709-3) and 39,319,342 bp (EL10.2).

Syntenic relations between USDA\_Bvulg\_FC709-3\_v1.0.1 and RefBeet v1.2.2 resulted in 290 regions with the length of 279,070,610 bp and 259,332,004 bp. Sixty four inversions with the lengths of 115,043,241 bp and 77,225,534 bp. Translocation regions detected were 363 with the

lengths of 15,519,337 bp and 15,322,953 bp. Lastly the number of duplication regions were 169 in FC709-3 and 153 in RefBeet. The number of sequences not aligned were 240,989,824 bp in FC709-3 and 19,866,609 bp.

## **Discussion**

A new contiguous whole reference genome for sugar beet was created. The new USDA\_Bvulg\_FC709-3\_v1.0.1 assembly contains 9 linkage groups plus the 1520 extra unplaced scaffolds. Each of the 9 chromosomes were captured in this assembly that meets the expectation of the highest of genome standards of using both long and short read technology to get the highest coverage possible. USDA\_Bvulg\_FC709-3\_v1.0.1 captured large regions that were previously unknown in the existing reference genomes EL10.2\_2 and RefBeet.

USDA\_Bvulg\_FC709-3\_v1.0.1 used higher-quality long-read PacBio reads coupled with endonuclease-free chromatin conformation capture provided additional improvements to sugar beet reference genome completions (maximizing actual genome size) and chromosome-level contiguity and accuracy, as shown in recent plant genomes (Sato, Abe et al. 2021).

This is the major difference between this data and the previously published sugar beet genomes. These improvements result in a more complete reference genome that can be used to investigate molecular mechanisms in sugar beet.

Many plant genomes are large because of their highly repetitive nature and many classes of repetitive elements (Bennetzen and Wang 2014). Genome size estimates of the cultivated beets have been variable and have ranged from 633.0 to 875.5 Mb per haploid genome (McGrath, Funk et al. 2023). Genome sizes of sugar beets most recent common ancestor range from 660.1 to 753.1 Mb (Castro, Romeiras et al. 2013). This genome size variation has been a known

occurrence throughout the species and the FC709-4 genome size is again different. Variation in read-depth coverage may be useful in tracking genome size changes as we continue to create whole reference genomes (Castro, Romeiras et al. 2013). Even while adding another sugar beet reference genome, additional sugar beet genomes need to be sequenced and assembled. Creating a pan-genome with a diverse cultivar panel should provide new insights on the repetitive regions and genome size within the *Beta* species.

An increase of high-quality plant genomes in recent years has led to the realization that single reference genomes do not represent the diversity within a species and has led to the concept of pan genomes (Bayer et. al., 2020). Pan genomes represent the genomic diversity of a species and contain a core set of genes that all individuals have. Variable genes may be present or absent in some individuals that can be exploited to discover particular genes that are responsible for a phenotype of interest. Reference genome bias occurs when reads that are mapped in the wrong region or not at all. Using a reference genome whose cultivar contains your trait of interest (disease resistance, yield, ect.) reduces reference genome bias. We created

USDA\_Bvulg\_FC709-3\_v1.0.1 from a germplasm line that showed excellent resistance to *Rhizoctonia solani* infection. This genome will be used to uncover the resistance genes within that germplasm line. Using a variety of different genomic techniques (QTL mapping, bulk segregant analysis, genotype-by-sequencing, ect.) candidate genes that confer for resistance to *Rhizoctonia solani* resistance can be discovered. This resource allows to initiate more careful examination of the genome associations with other pathogen resistance as well.

With these new contiguous, well-annotated genome sequences, we can look at the synteny between species and within the *Beta* family. Following the syntenic organization of each of the reference genomes we showed just how many missing regions were in the previous genomes.

Sugar beet is one of the few plant species with multiple highly contiguous genomes and in the future will have more to do further investigation of synteny between species and subspecies.

## **Conclusion**

The new reference of USDA\_Bvulg\_FC709-3\_v1.0.1 is a new highly contiguous genome of a highly *Rhizoctonia solani* resistant sugar beet germplasm line. FC709-3 consists of 651.4 Mb of which (89%) was contained in nine chromosomes. Compared to the previous genome assemblies (RefBeet 1.2 and EL10.2\_2) our approach improved chromosome resolution and coverage of highly repetitive regions. A total of 29,463 gene models were predicted using the BRAKER2 pipeline. Comparing to other sugar beet genomes we found that genome size variability continued in the USDA\_Bvulg\_FC709-3\_v1.0.1 genome (McGrath et. al., 2023). This size variability came from high quality long reads that contained more base pairs than the previously reported genomes. Variation can also be attributed to multiple other factors including types of sequencing reads (long vs short), individual bias, and bioinformatics program being used to process the sequencing data. Genome comparisons revealed many structural differences but most importantly a substantial amount of new sequence in the new USDA\_Bvulg\_FC709-3\_v1.0.1 genome. The genomic data presented here will enable further molecular research of sugar beet and other *Beta* species.

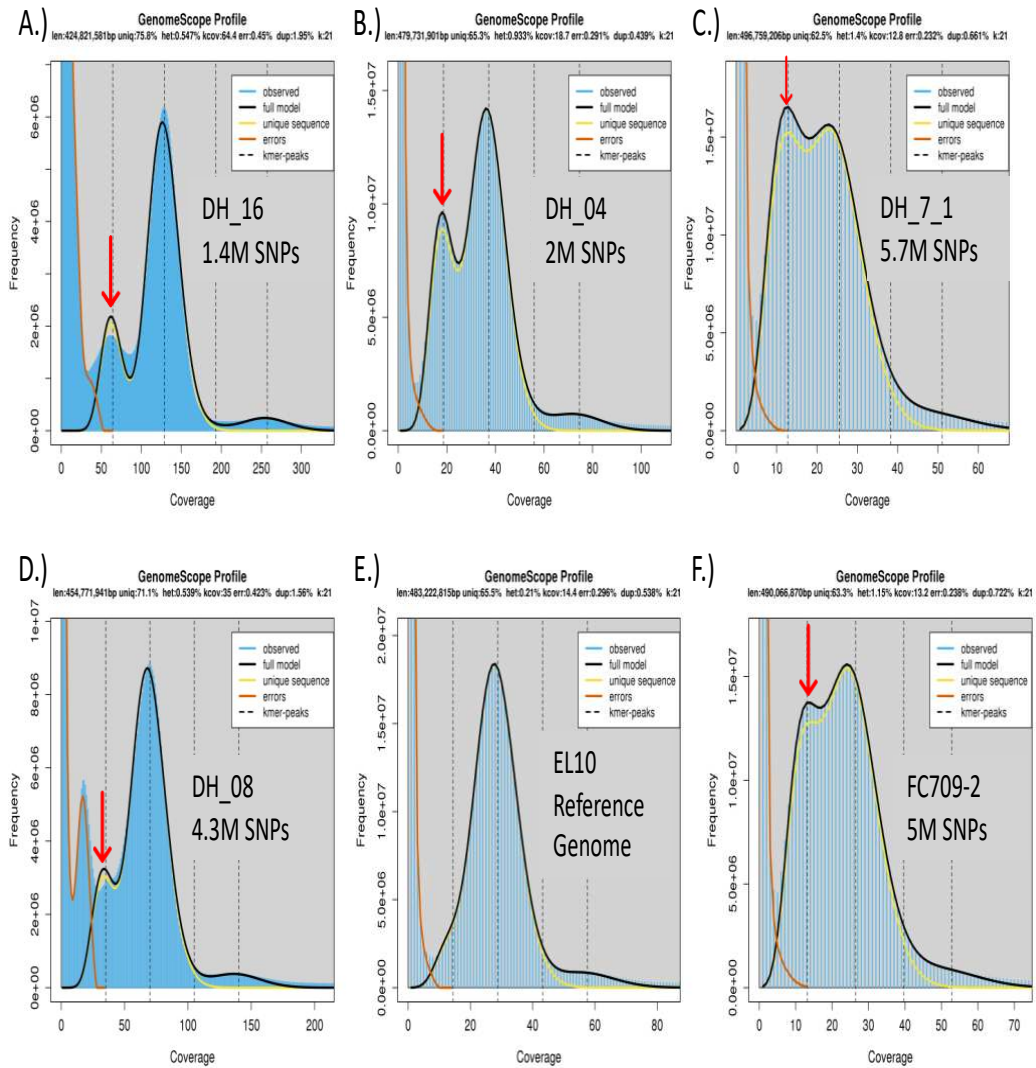


Figure 9: GenomeScope profiles of candidate doubled haploid lines derived from FC709-2 and control lines. The amplitude of the second peaks, noted with a red arrow, indicate varying levels of heterozygosity detected via k-mer abundance analysis. A higher second peak indicates a higher level of heterozygosity. The ‘control’ plants, EL10 (highly inbred) and FC709-2 (expected high heterozygosity) are shown in panels E and F. The number of SNPs listed indicates (in millions) the number of heterozygous SNPs present in that sample when mapped against the EL10 reference genome.

Table 4: Assembly statistics of FC709-3 v1.0.1 assembly of all of the scaffolds before arranging into pseudochromosomes in the left column. The right column contains the assembly statistics when arranged into 9 pseudochromosomes representing the total chromosomal content of the *Beta vulgaris* genome.

<b>Table 4: FC709-3 genome assembly statistics</b>		
	<b>USDA_Bvulg_FC709-3_v1.0.1 assembly (all scaffolds)</b>	<b>USDA_Bvulg_FC709- 3_v1.0.1 (pseudochromosomes only)</b>
<b>Scaffold Total</b>	1529	9
<b>Contig Total</b>	1823	302
<b>Scaffold Total Assembly Size</b>	729,400,531	651,430,459
<b>Contig Total Assembly Size</b>	729,371,131	651,401,159
<b>Scaffold N/L 50</b>	5 / 70.993 MB	5 / 70.993 MB
<b>Contig N/L 50</b>	50 / 3.826 MB	42 / 3.946 MB
<b>Scaffold N/L 90</b>	10 / 6.579 MB	8 / 67.06 MB
<b>Contig N/L 90</b>	219/538.484 KB	156 / 1.073 MB
<b>Max Scaffold Length</b>	81.771 MB	81.771 MB
<b>Max Contig Length</b>	16.51 MB	16.51 MB

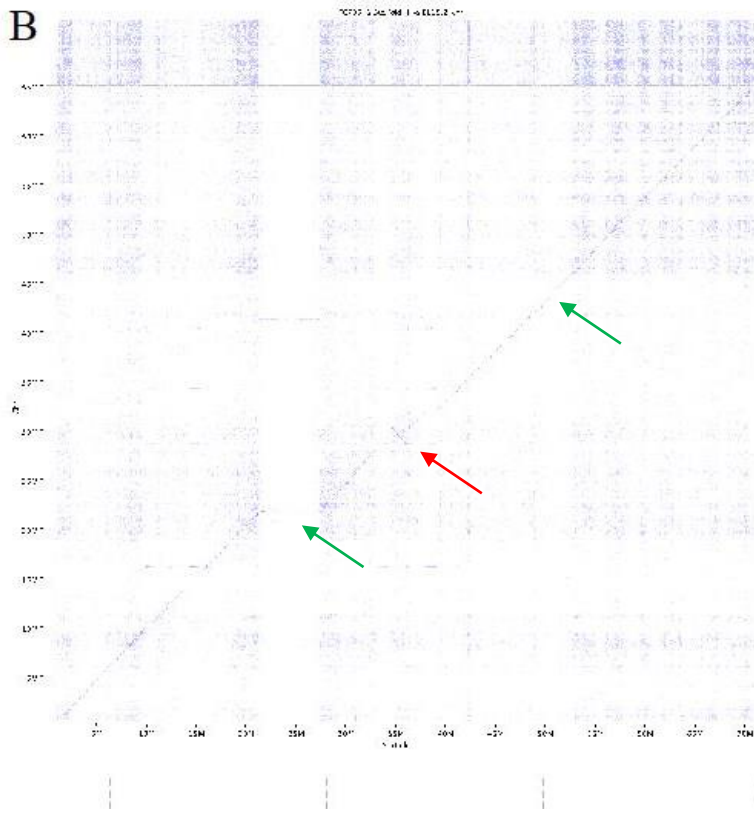


Figure 10. Chromosome-by-chromosome dot plot analysis between the USDA\_Bvulg\_FC709-3\_v1.0.1 and EL10.2 chromosomes (Chr. 1 shown in Figure 2, USDA\_Bvulg\_FC709-3\_v1.0.1 on the x-axis, EL10.2 on the y-axis) shows small structural variations between these two highly contiguous assemblies, with a notable small inversion (red arrow), plus the addition of multi-megabase segments of additional sequence present in the USDA\_Bvulg\_FC709-3\_v1.0.1 scaffold (green arrows).

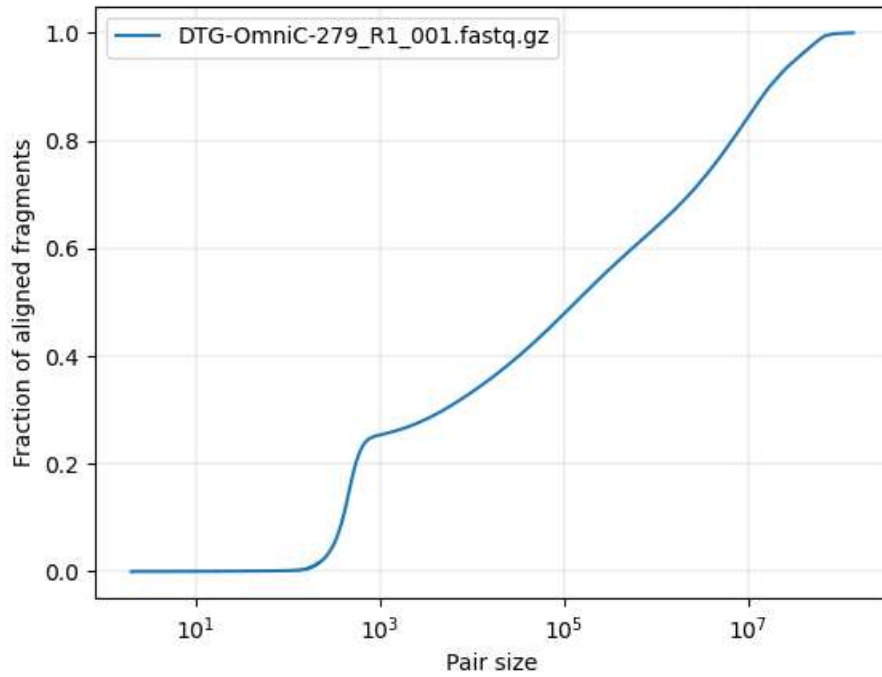


Figure 11. The Omni-C reads received from DoveTail Genomics. Omni-C is a nuclease-free version of Hi-C sequencing. This results in large (>10 Mb) links that allow for more coverage. The size of paired reads that were aligned to the reference genome.

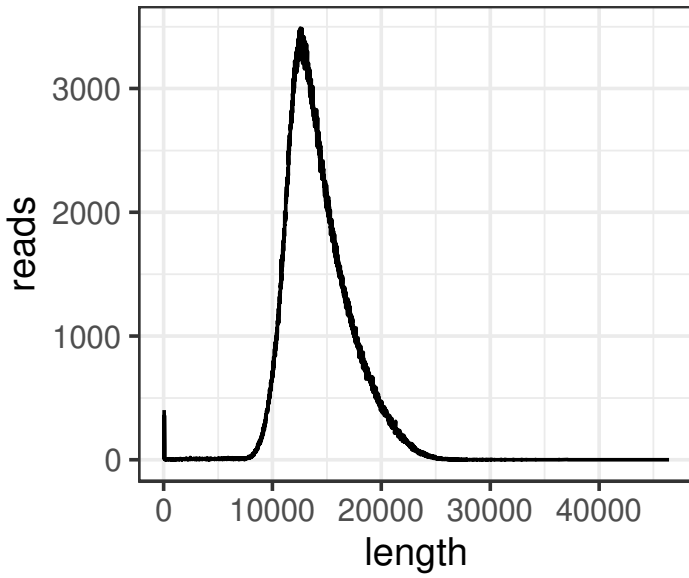


Figure 12. High molecular weight extraction done by DoveTail Genomics. Quality control is done on the reads to ensure that the length is of high quality. This allows for more accurate and complete genomic analysis and assembly.

Table 5. Comparing genome assemblies to previously published sugar beet reference genomes. The assembly statistics comparing the previously reported reference genomes RefBeet 1.2.2 and EL10.2.2 to FC709-3 genome assembly. Differences in the number of contigs, assembly size, and contig N/L50 between the three genomes represent the differences in genomic techniques and bioinformatic tools over time. Advanced sequencing technologies allow for more base pairs to be sequenced and aligned resulting in a more complete genome (FC709-3).

Assembly Name	# of Contigs	Assembly Size	Contig N/L50 (# fragments/length)
RefBeet 1.2.2 chromosomes	14,364 (in 9 scaffolds)	Scaffold: 376 Mb Contig: 342 Mb	Contig: 2289/45 Kb Scaffold: 4/44 Mb
EL10.2.2 chromosomes	2,946 (in 9 scaffolds)	Scaffold: 564 Mb Contig: 532 Mb	Contig: 118/1.3 Mb Scaffold: 8 / 56 Mb
FC709-3	302 (in 9 scaffolds)	Scaffold: 651.43 Mb Contig: 651.40 Mb	Contig: 42/3.9 Mb Scaffold: 67 Mb

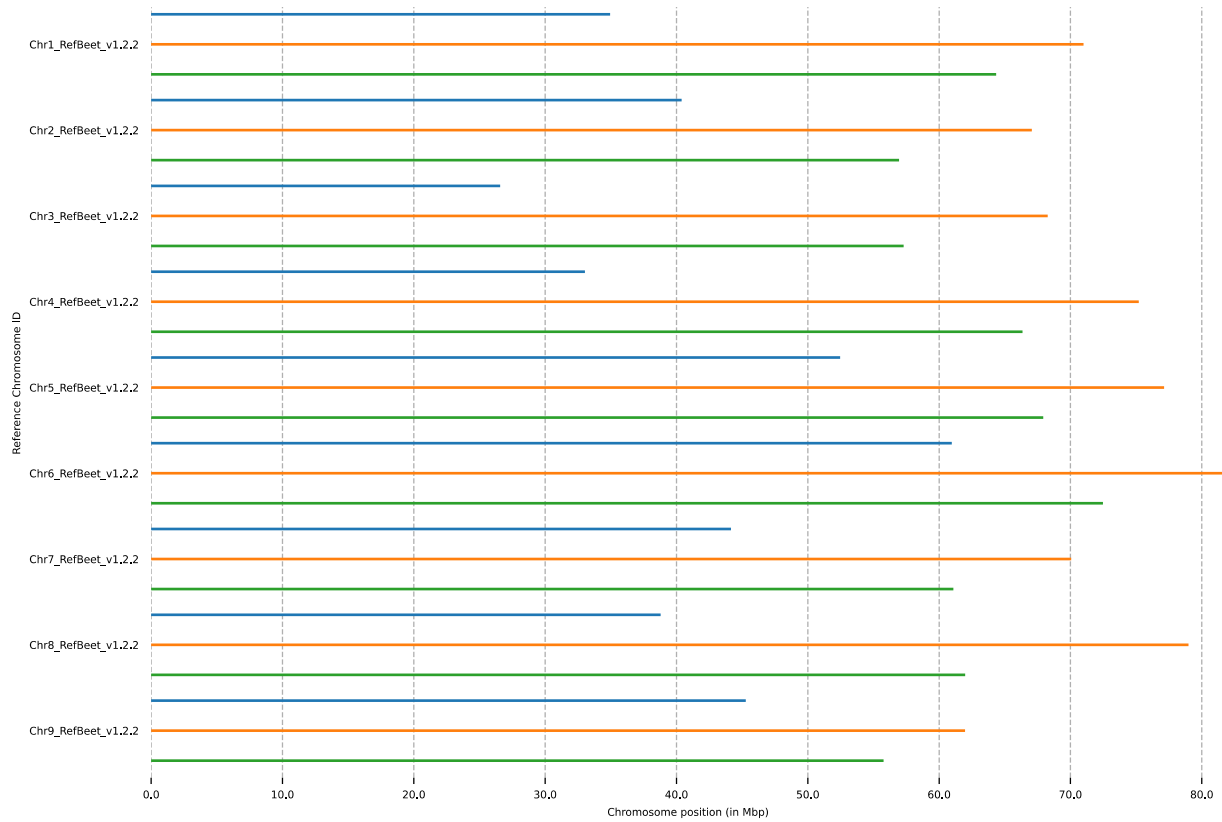


Figure 13: Three-way alignment of RefBeet v1.2.2 (blue) versus FC709-3 (orange) versus EL10.2 showing syntenic regions (grey), inversions, translocations, and duplications across each genome assembly.

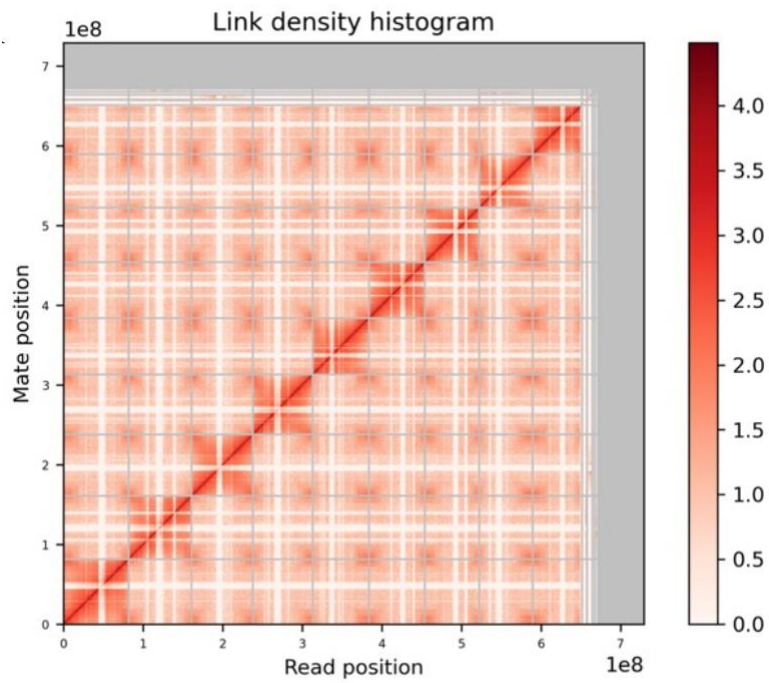


Figure 14: Hi-C contact map of the physical contacts between chromatic regions in cell nuclei. Hi-C allows for the correct orientation of chromosomes.

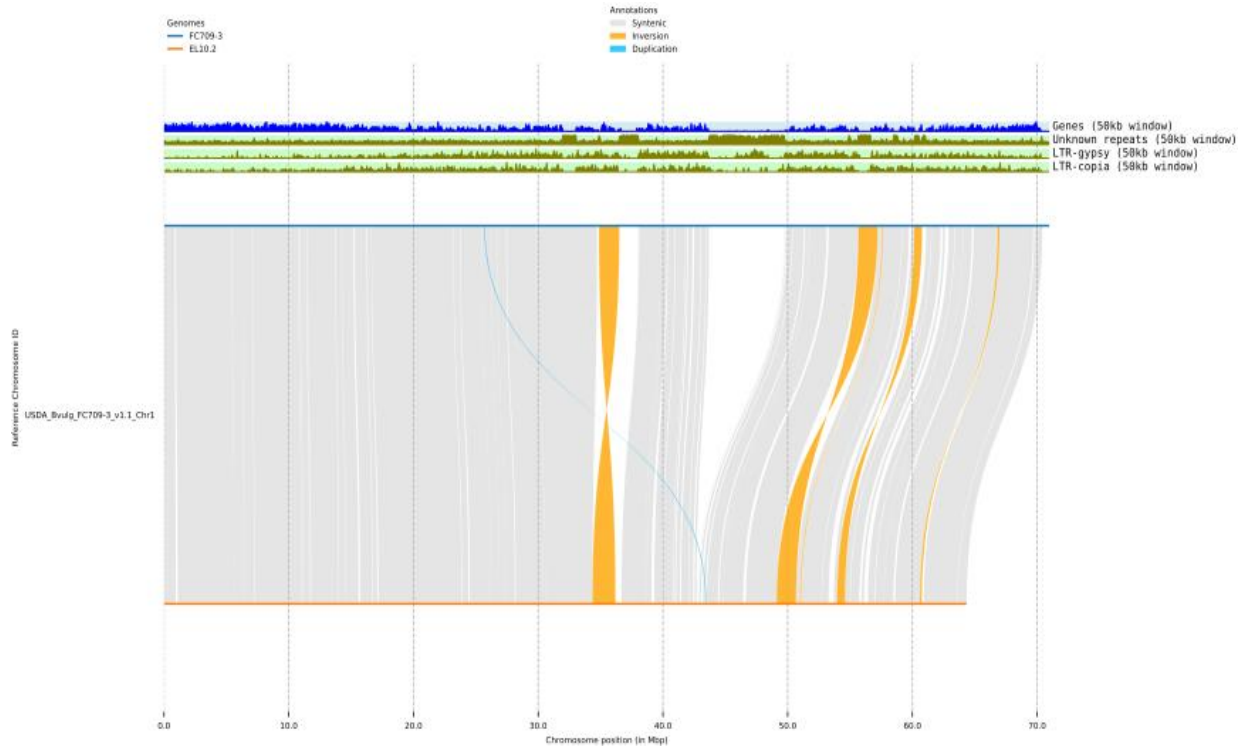


Figure 15: FC709-3 genome compared to EL10 genome on chromosome 1. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the EL in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and EL10.

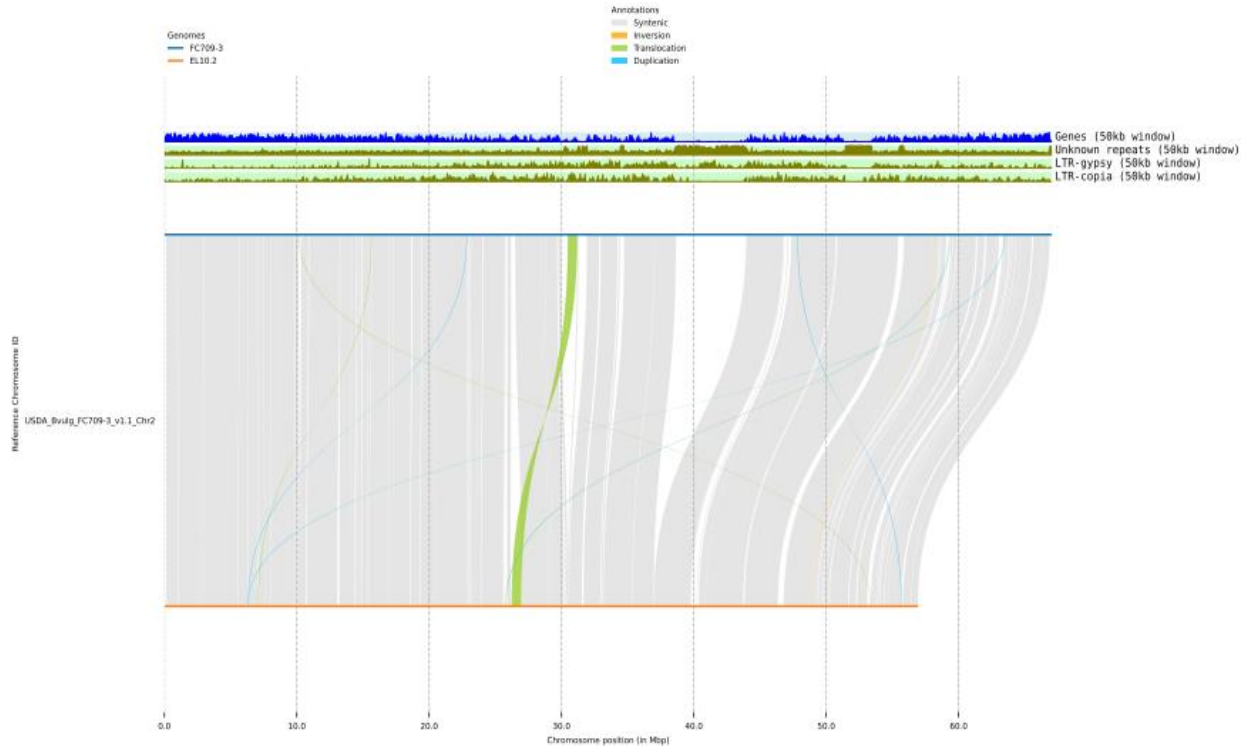


Figure 16: FC709-3 genome compared to EL10 genome on chromosome 2. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the EL in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and EL10.

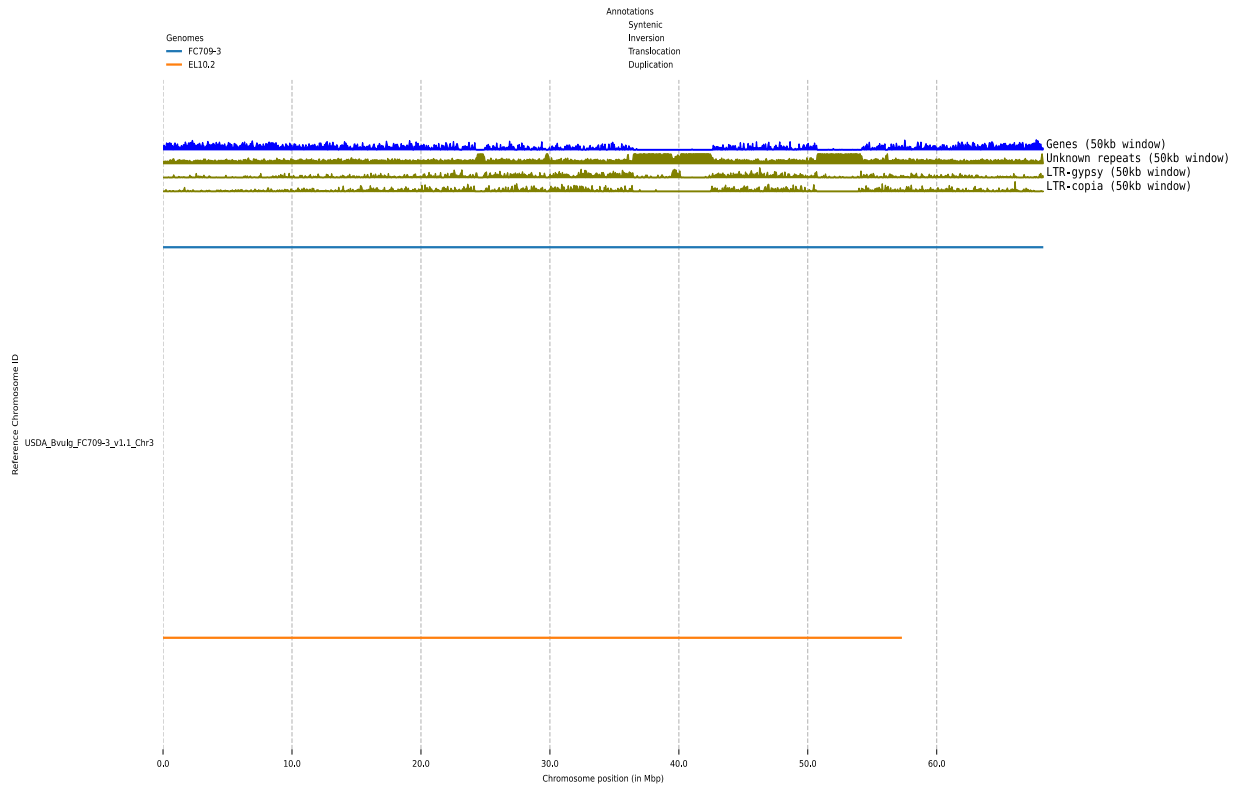


Figure 17: FC709-3 genome compared to EL10 genome on chromosome 3. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the EL in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and EL10.

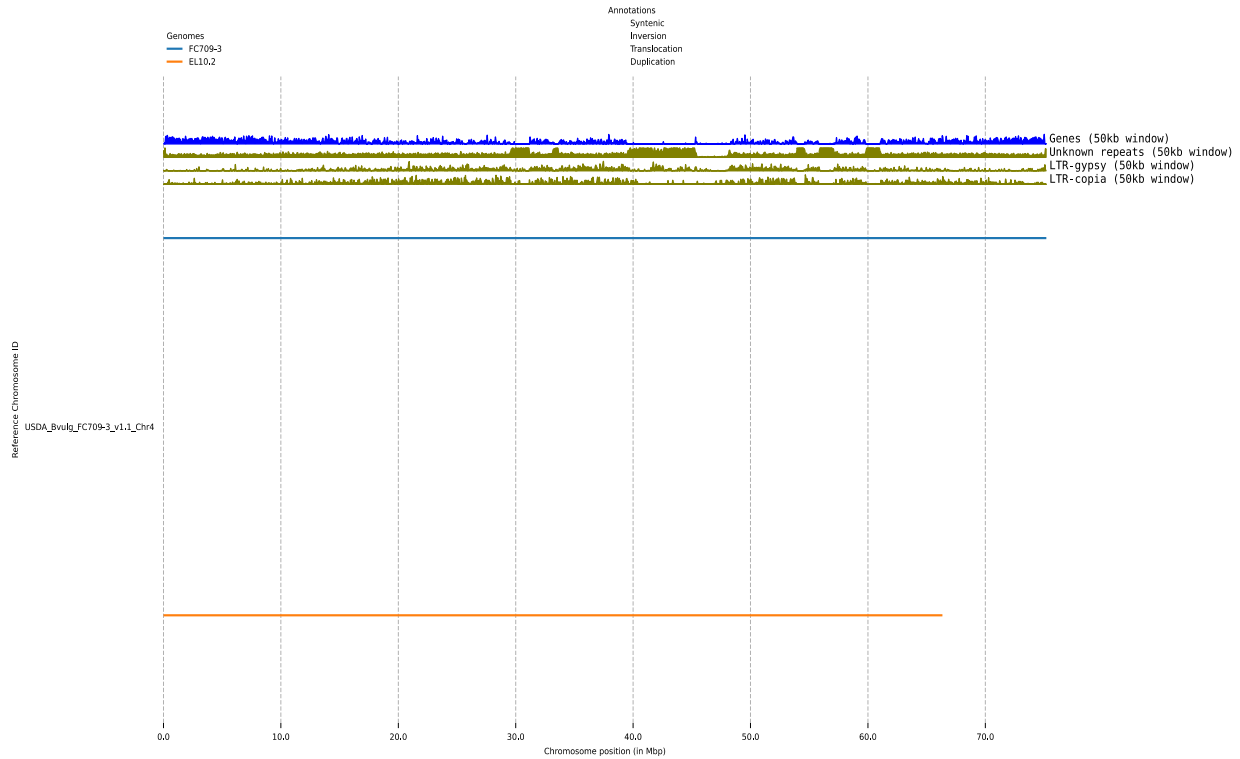


Figure 18: FC709-3 genome compared to EL10 genome on chromosome 4. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the EL in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and EL10.

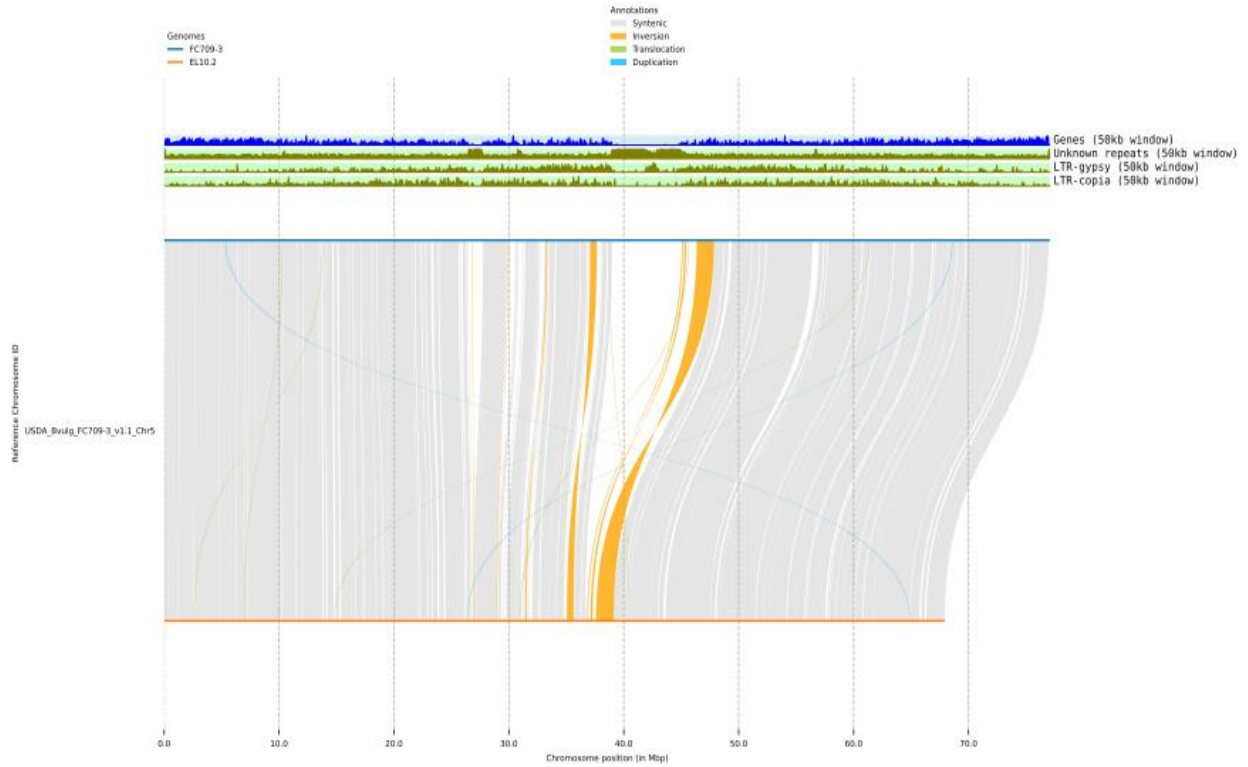


Figure 19: FC709-3 genome compared to EL10 genome on chromosome 5. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the EL in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and EL10.

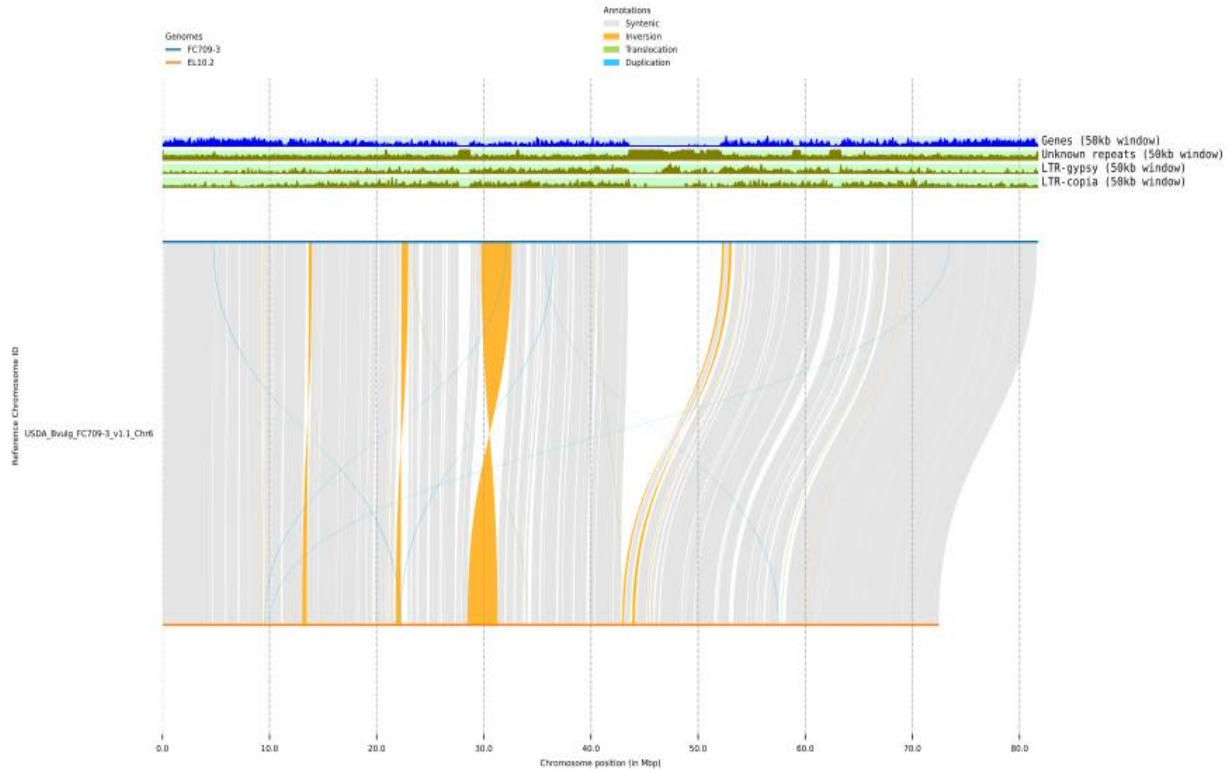


Figure 20: FC709-3 genome compared to EL10 genome on chromosome 6. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the EL in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and EL10.

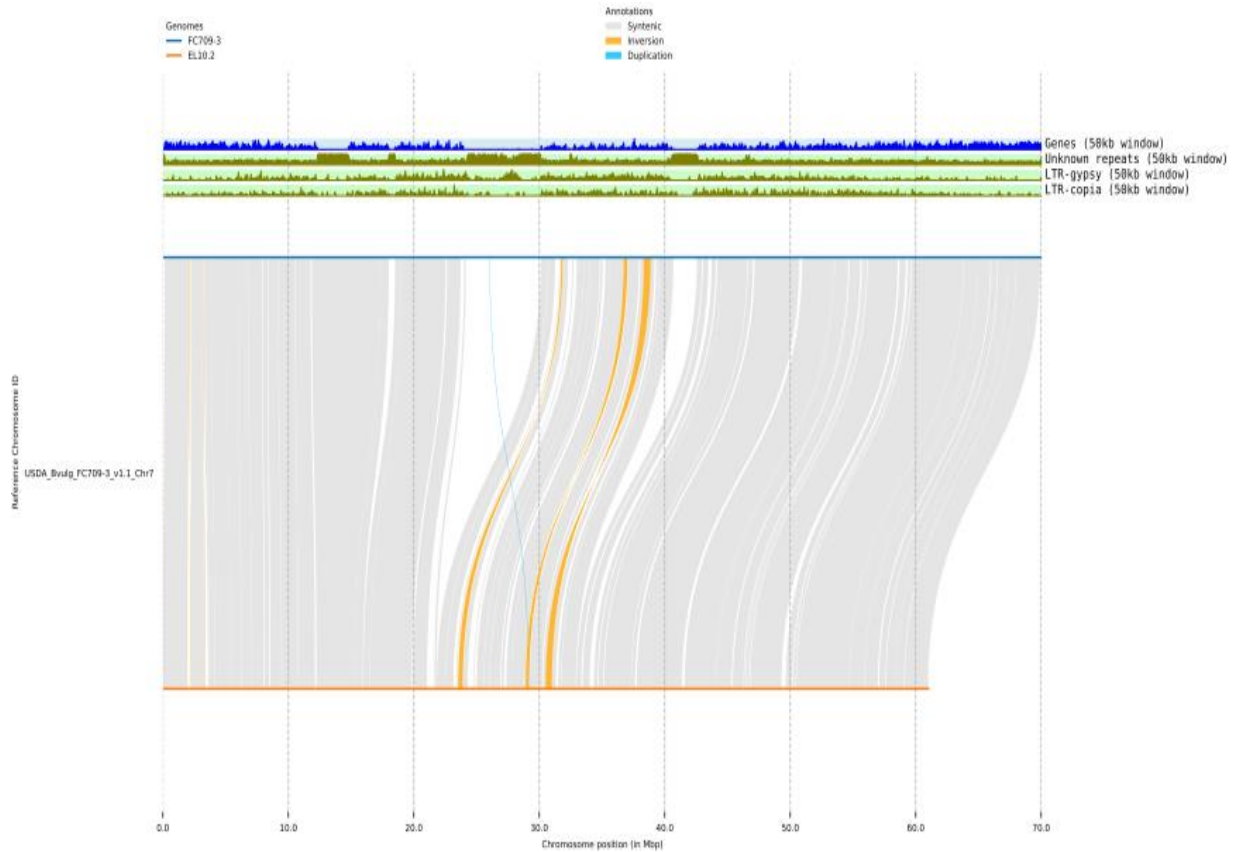


Figure 21: FC709-3 genome compared to EL10 genome on chromosome 7. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the EL in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and EL10.

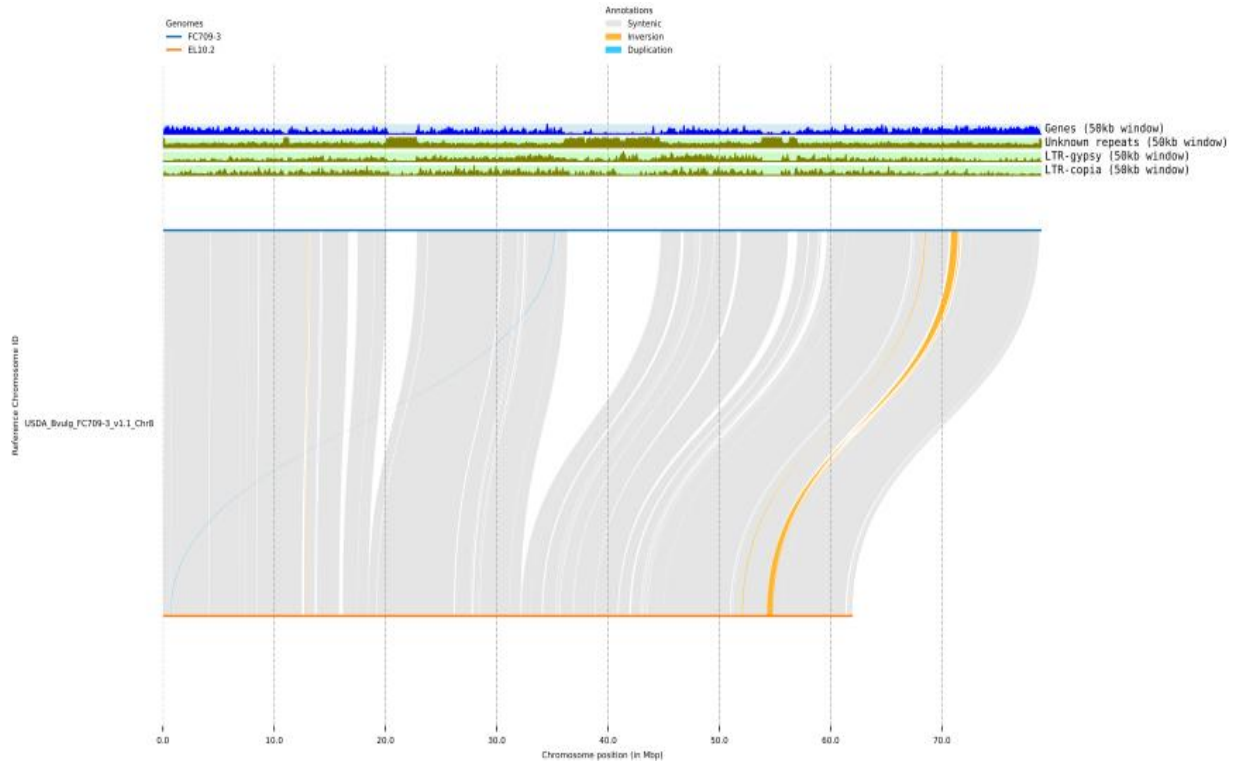


Figure 22: FC709-3 genome compared to EL10 genome on chromosome 8. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the EL in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and EL10.

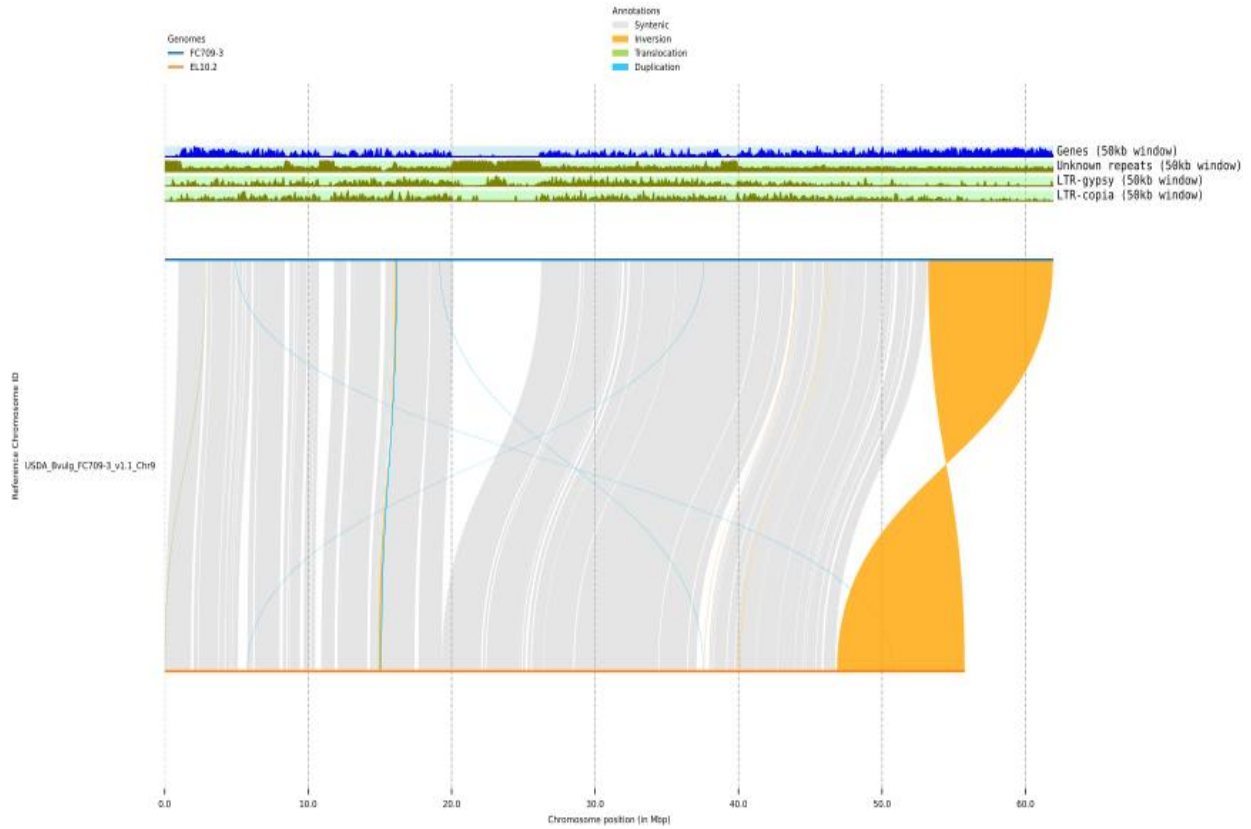


Figure 23: FC709-3 genome compared to EL10 genome on chromosome 9. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the EL10 in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and EL10.

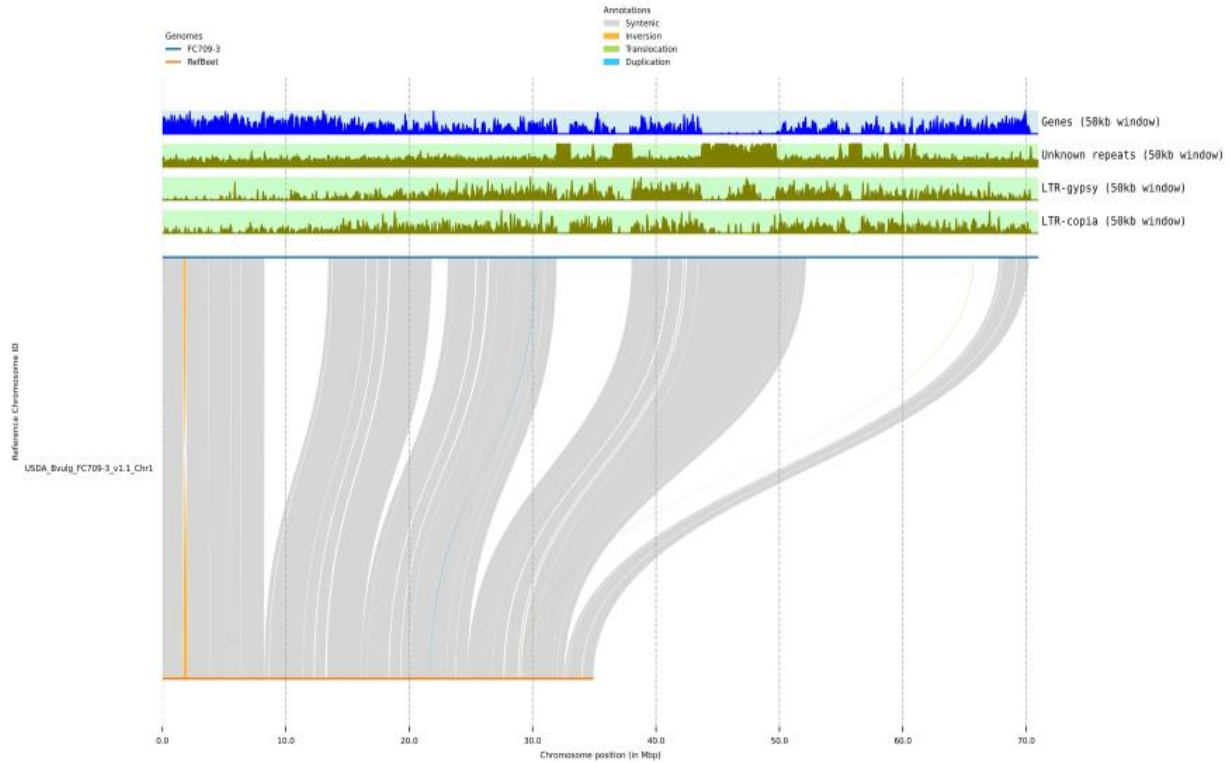


Figure 24: FC709-3 genome compared to RefBeet genome on chromosome 1. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the RefBeet in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and RefBeet.

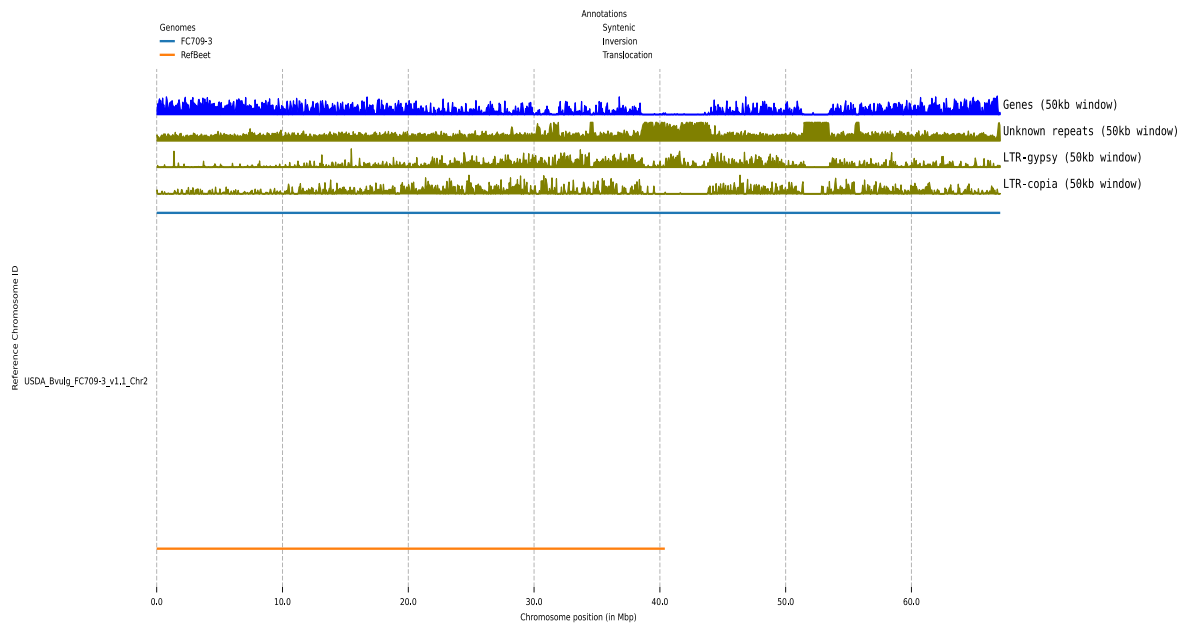


Figure 25: FC709-3 genome compared to RefBeet genome on chromosome 2. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the RefBeet in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and RefBeet.

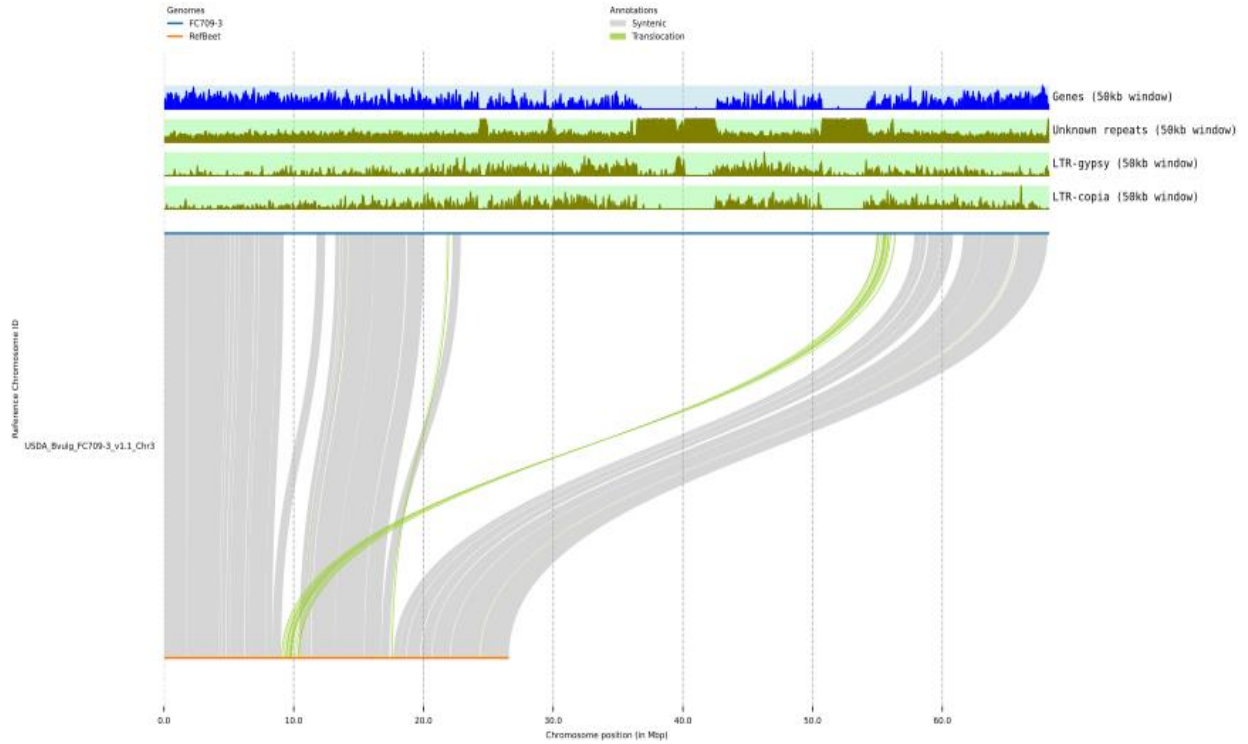


Figure 26: FC709-3 genome compared to RefBeet genome on chromosome 3. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the RefBeet in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and RefBeet.

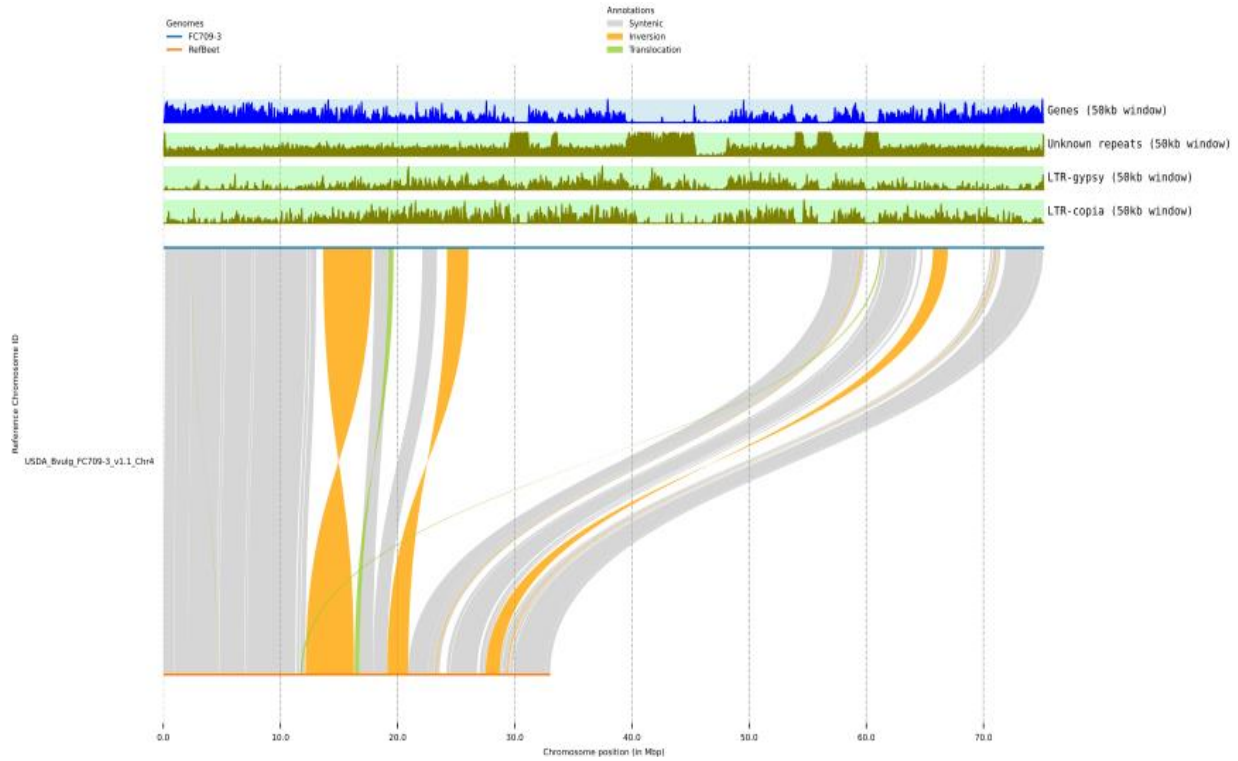


Figure 27: FC709-3 genome compared to RefBeet genome on chromosome 4. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the RefBeet in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and RefBeet.

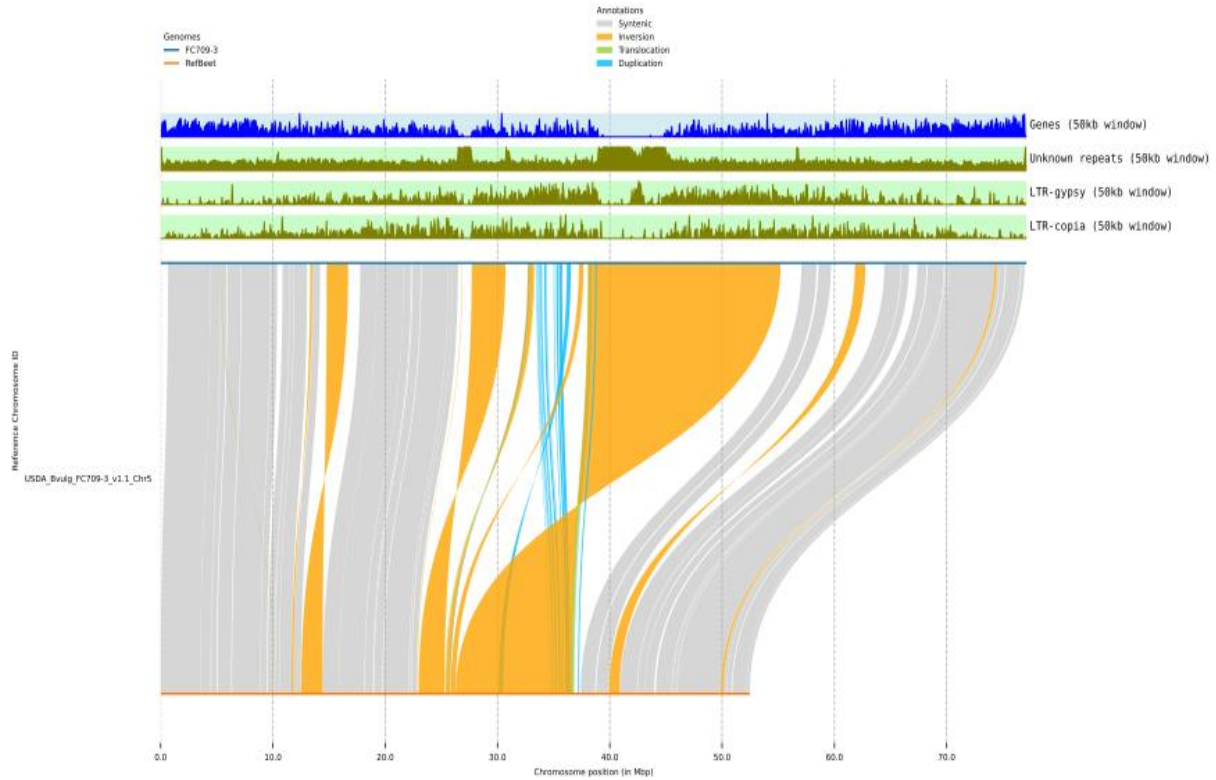


Figure 28: FC709-3 genome compared to RefBeet genome on chromosome 5. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the RefBeet in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and RefBeet.

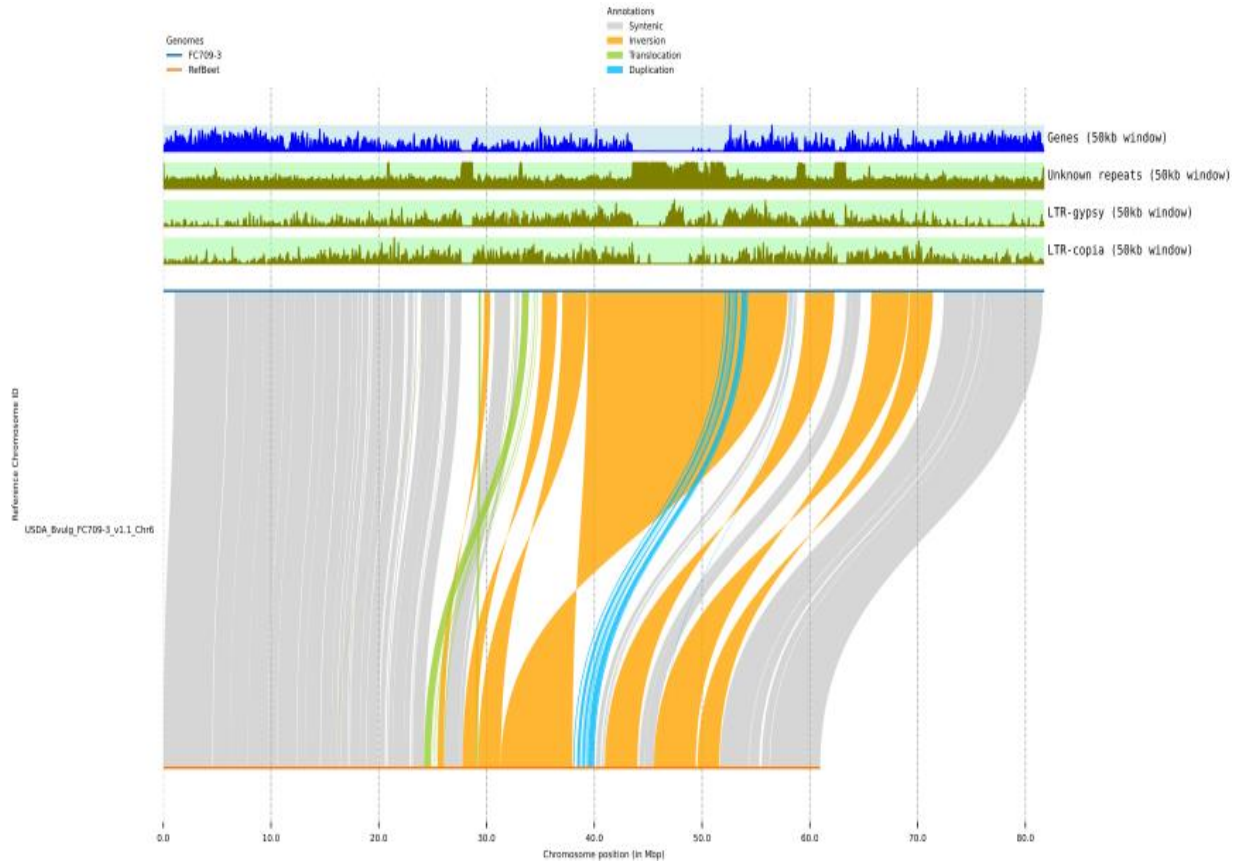


Figure 29: FC709-3 genome compared to RefBeet genome on chromosome 6. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the RefBeet in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and RefBeet.

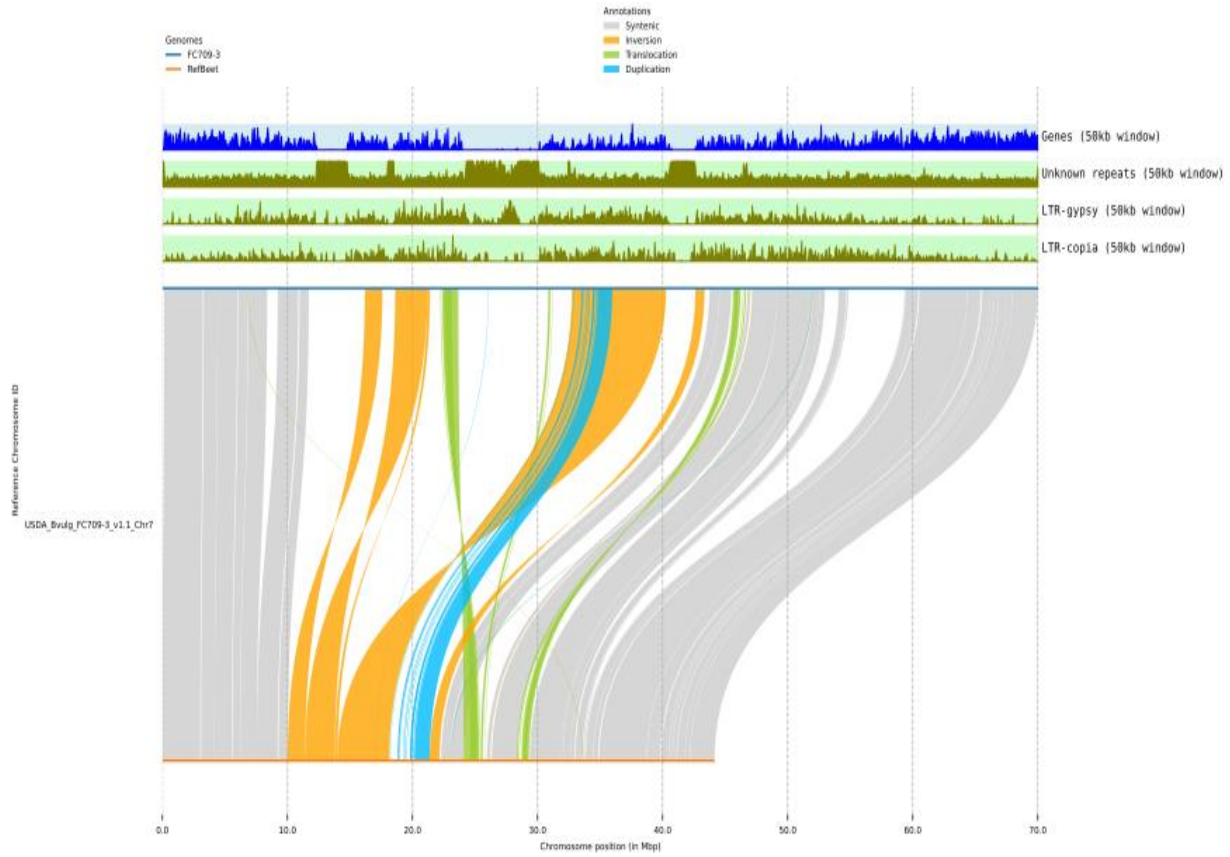


Figure 30: FC709-3 genome compared to RefBeet genome on chromosome 7. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the RefBeet in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and RefBeet.

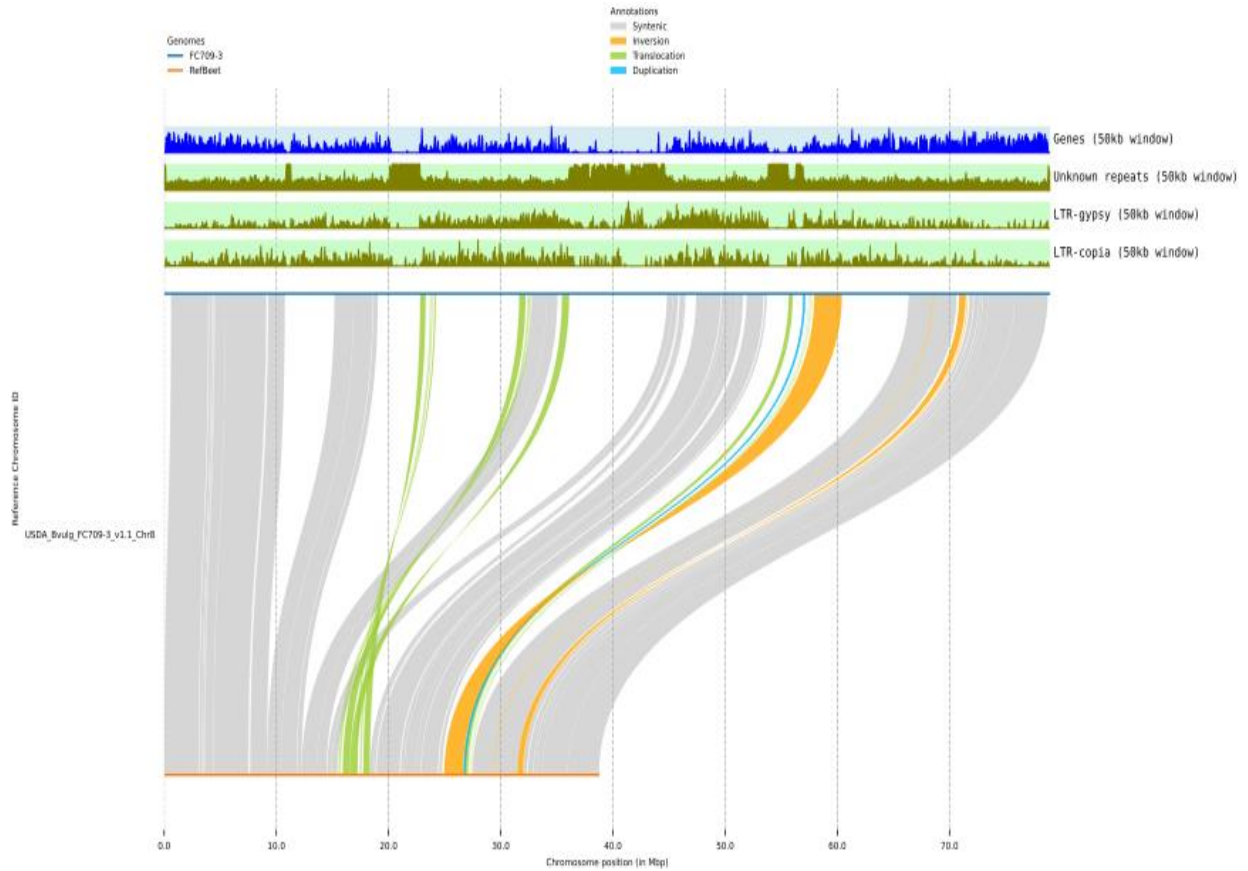


Figure 31: FC709-3 genome compared to RefBeet genome on chromosome 8. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the RefBeet in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and RefBeet.

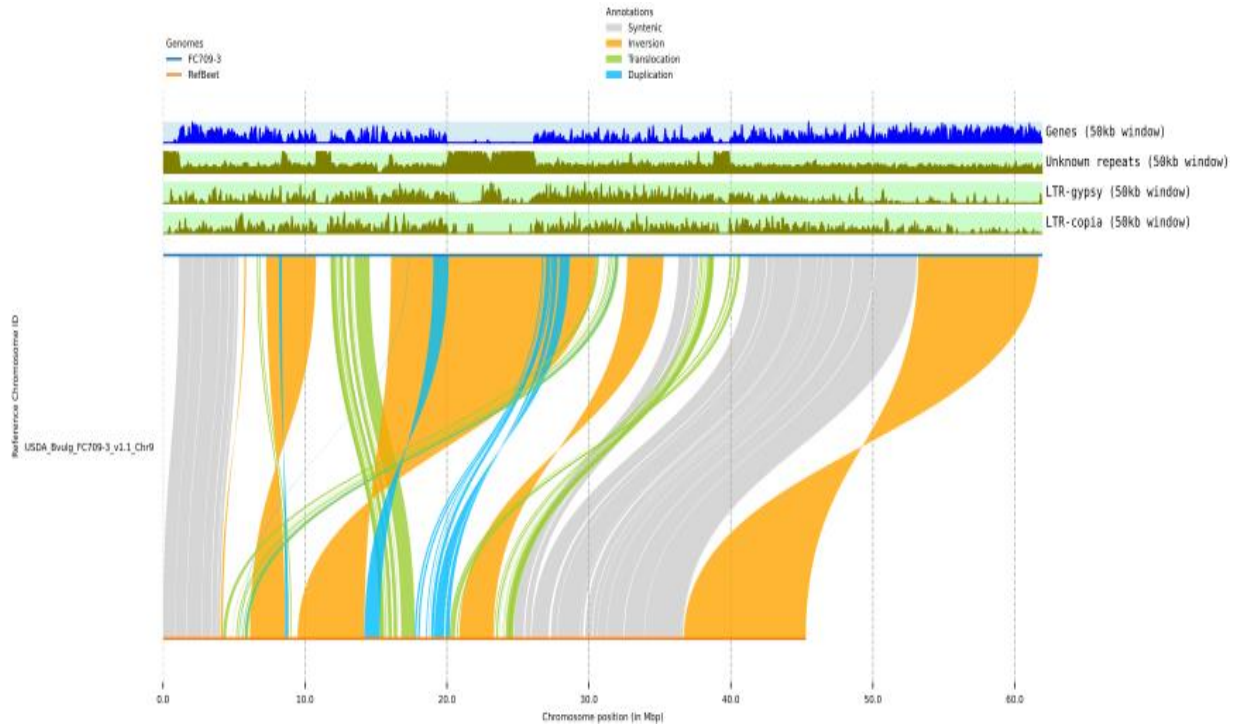


Figure 32: FC709-3 genome compared to RefBeet genome on chromosome 9. The blue line above representing the annotated genes in the FC709-3 genome compared to those of the RefBeet in the bottom two green lines. The unknown repeats between genomes are represented in the second green line. The figure below is showing syntenic regions (grey), inversions (orange), and duplications (blue) between FC709-3 and RefBeet.

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# **Chapter Four: Identification of a sugar beet *Rhizoctonia* Root and Crown Rot Resistance QTL on Chromosome 2 derived from FC709-**

## **2**

### **Introduction**

Sugar beets are attacked by several fungal pathogens that cause root damage, including *Rhizoctonia solani*, which causes the disease Rhizoctonia Root and Crown Rot (RRCR). Several Quantitative Trait Loci have been reported to harbor resistance genes to RRCR, but no RRCR resistance gene has been cloned from sugar beet or a crop wild relative. Discovering the identity of the genetic elements responsible for resistance will enable more precise molecular assisted breeding alongside a better understanding of the plant-pathogen interaction to drive future RRCR research efforts. In this study, we report a multifaceted genomics approach to map and characterize RRCR resistance from a highly resistant sugar beet pre-breeding line utilizing biparental mapping. F2 families were produced by crossing a RRCR susceptible parent line (03-124) with the highly resistant FC709-2. Multiple F2 families were screened in both field and greenhouse disease trials to study phenotypic segregation. A single greenhouse-based RRCR trial on one F2 family was the focus of a sequencing-based approach to identify the genomic locations underlying RRCR resistance. Two contrasting phenotypic pools of resistant and susceptible F2 individuals were sequenced. A new reference genome derived from the FC709-3 background was used to avoid reference genome bias. Bioinformatic analyses identified a strong QTL signal on chromosome 2. This QTL is in a similar genomic position as a recently identified RRCR QTL in table beet. Candidate genes within the QTL interval have been identified with

differential expression in an RNA sequencing experiment. Molecular markers are being developed to validate causal variants. Collectively, these results will allow breeders to more accurately select for RRCR resistance and incorporate them faster into elite hybrid lines.

Sugar beet (*Beta vulgaris* ssp. *vulgaris*) is a dicot crop grown in the temperate zone of the US and Europe as the major production regions (Draycott 2008). The crop is cultivated for its sucrose enriched taproot. Sugar beet is a biennial crop where carbon is translocated from the leaves to the root during the first year vegetative state and vice versa during the generative phase (Fondy, Geiger and Servaites 1989). With its relatively long growing season, sugar beets are particularly vulnerable to soil borne pathogens that are attracted to the carbohydrate enriched taproots. The soil-borne basidiomycete *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*) has become an increasing threat to commercial sugar beet production. The fungus resides in the soil as mycelium or sclerotia and can remain viable for multiple years (Menziez 1970). *Rhizoctonia solani* is classified by anastomosis groups and only groups AG-1, AG-2-1, AG-2-2, AG-3, AG-4 and AG-5 have been recorded on sugar beet (Windels and Nabben 1989). In this study we focus on a Colorado isolate R9 (AG-2-2 IIIB) which has been used by breeders for several national screening nurseries.

US growers typically use partially resistant cultivars and apply fungicides in a timely manner to manage *R. solani* in production sugar beet fields (Panella, Ruppel and Hecker 1995, Kiewnick, Jacobsen et al. 2001). In European sugar beet growing regions the strict regulation on the use of agrochemicals prohibits treatment of the soil or the canopy to decrease *R. solani* damage. The only way known to manage the disease is by implementing various cultivation practices and deploy available resistant varieties (Buhre, Kluth et al. 2009). There is a continuing need for stable and robust genetic resistance or tolerance to many of sugar beet's pathogens. Almost all of

the public breeding programs direct their efforts towards identifying and introgressing disease resistance into germplasm releases that can be used by commercial seed companies in their private breeding programs (McGrath and Panella 2018). To discover resistant germplasm, protocols for disease evaluation both in the field and in the greenhouse, have been developed and refined for accurate selection (Scholten, Panella et al. 2001, Büttner, Pfähler and Märländer 2004). This has resulted in many useful germplasm lines released by the USDA-ARS public breeders, however, there is still little information of the sources and mechanisms of *Rhizoctonia* Root and Crown Rot (RRCR) resistance in sugar beet.

The first regions that were genetically mapped in association with *R. solani* resistance were on chromosomes 4, 5, and 7 (Lein, Sagstetter et al. 2008). A total of 67 molecular markers were used to identify these QTL and respective regions on a molecular map and genes within this region were identified as potential pathogen recognition and resistance genes. As sequencing technology has continued to drop in price, its application has identified additional genomic regions and potential candidate genes for *R. solani* resistance. In 2020, the use of RNA sequencing and transcriptome analysis revealed that major latex coding proteins (*BvMLP1*, *BvMLP2* and *BvMLP3*) all located on chromosome 3 (Holmquist, Dörfors et al. 2021). In table beet, (*Beta vulgaris* ssp. *vulgaris*) the discovery of a resistance QTL on chromosome 2 resulted from crosses between resistant and susceptible table beet and sugar beet lines (Wigg, Brainard et al. 2023). F<sub>2:3</sub> families were evaluated for RRCR resistance in greenhouse experiments. Every individual was subjected to genotyping-by-sequencing and those reads were mapped back to a new table beet reference genome (W357B). The sugar beet line used for the RRCR resistant parent happened to be FC709-2. The QTL found on chromosome 2 of FC709-2 accounting for 30% of the phenotypic variance was at position 60, 853,362 bp and had an interval of 56,099,742

bp to 62,113,791 bp. The reference genome that reads were mapped to in the study was the RefBeet 1.2.2. This experiment helped guide this study with improved sequencing and genomic methods.

In this study, we utilize bi-parental mapping and DNA sequencing approaches to screen sugar beet lines, identify regions of the sugar beet genome associated with resistance to *Rhizoctonia solani*, and identify candidate genes that are responsible for the resistance. In order to find “new” genes within the *Beta vulgaris* germplasm we have to know what we already have within the current germplasm lines. The National Plant Germplasm System has curated and collected several sugar beet and sugar beet crop wild relative lines that could be exploited by using genic markers to understand genetic diversity in the *Beta* family. Markers associated with resistance will also be identified to be used in marker assisted selection in sugar beet breeding programs around the world.

## **Materials and Methods**

### **Plant Material/ Mapping population**

The parental populations used to create this mapping population were resistant and susceptible checks used in field trials. 03-124 is a RRCR susceptible line developed in Salinas, California that was used as the susceptible parent in the cross. USDA-ARS Fort Collins, Colorado line FC709-2 was used as the resistant parent. This line has been used as a resistant check line for decades and has undergone 7 cycles of RRCR mass selection and 1 cycle of self-pollination to homogenize the seed lot. To ensure that the resistant parental plant was resistant FC709-2 plants were subjected to *Rhizoctonia solani* infection and a single resistant plant was selected to cross with an 03-124 individual plant whose seed lot was homogenous for RRCR susceptibility. Seed

was collected and planted out as F1 plants to be self-pollinated into F2 families. Six individual F2 families were harvested and stored for future plantings while some of the seed was planted and self-pollinated into F3 families. In 2018 we sowed the six F2 families along with ten F3 families into the RRCR disease field nursery to be evaluated for *Rhizoctonia solani* resistance. In 2019 we conducted the same disease nursery trial but in the greenhouse with two of the F2 families that displayed segregation for resistance within the seed lots of 20161044-2S and 20161044-4S. 204 individuals from 20161044-2S and 215 individuals from 20161044-4S were infected with *Rhizoctonia solani* to be further evaluated for RRCR resistance in a future study.

### **Tissue collection/DNA extraction**

Leaf tissue was collected, and flash frozen in liquid nitrogen from every F2 individual from both families. In addition to the F2s, we also included 50 individuals from each of the parental lines. These were collected in ThermoFisher DNAflex 96 deep well tubes to be extracted at a later date. DNA extraction was done using ThermoFisher DNA flex 96 well extraction kit and completed with the ThermoFisher KingFisher robot. Quality and quantity of each sample were evaluated for each sample using Nanodrop. Equimolar pools of 50 susceptible plants and 38 resistant plants were created and sent to NovoGene to be deep sequenced for analysis.

### **Field RRCR F2 disease trials**

*Rhizoctonia solani* field disease nurseries have been the gold standard for evaluation of germplasm for decades. In 2019, six F2 families from the cross of RRCR resistant parent FC709-2 and susceptible parent 03-124 were sown into the field in late spring. Field design was five 16-foot plots per F2 family containing approximately 10-15 plants spaced 6-12 inches apart.

Inoculum consisting of *Rhizoctonia solani* strain R9 infected and ground hulless barely was spread over sugar beet plants using a gandy implement pulled by a tractor. Approximately 6 grams per meter of inoculum is applied for ample amount of disease pressure. 28 days post inoculation beets are lifted for disease evaluation. The sugar beets are rated using the establish 0-7 scale (Ruppel and Hecker 1988). Each individual is given a rating as well as an overall germplasm line rating which is the average of all individuals in the study.

### **Greenhouse RRRCR F2 disease trials**

Greenhouse disease trials were conducted following the field evaluation of F2 families to determine segregating families for resistance. The two families 20161044-2S and 20161044-4S were selected for greenhouse trials. 200 seed balls from each F2 family were planted into soil in 7-inch plastic pots and grown for 8 weeks before inoculation. 50 seed balls from the resistant parent (FC709-2) and susceptible parent (03-124) were included as checks for the experiment. After 8 weeks (roughly 6-8 leaf stage) a small hole was created next to the tap root and 0.6g of R9 *Rhizoctonia solani* infected ground barley is placed into the whole and buried. 14 days post inoculation plants were uprooted and washed to be rated for resistance.

### **Bulk Segregate Population sampling and sequencing**

A single F2 family was selected from the previously described greenhouse survey. Pools of DNA were created that consisted of 38 individuals in the resistant pool and 50 individuals in the susceptible pool. The unbalanced population size was the result of non-normal phenotypic distributions of the F2 individuals. These pools were sequenced using Illumina DNA sequencing (2x150 bp on the NovaSeq 6000 platform by NovoGene).

## **QTL analysis**

The R package QTLseqR is a program used for bulk segregant analysis of next generation sequencing data (NGS) (Mansfeld and Grumet 2018) . Raw reads were trimmed and filtered using the bbdut in the BBTools suite. Trimmed and filtered reads were then mapped to the USDA\_Bvulg\_FC709-3\_v1.0.1 genome using default parameters in the HISAT2 program. Variant calling between the resistant and susceptible pool is done using Samtools/BCFtools to generate variant calls in VCF format. This program uses two different statistics: deltaSNP and G prime (G') (de la Fuente et al., 2022). These approaches use a simulation method and a tricube smoothed G statistic, to identify and assess statistical significance of a QTL. We performed both methods starting with the QTL-seq approach. This pipeline is used to calculate a tricube-smoothed delta SNP-index in a “sliding window fashion”. The tricube smooths out noise while accounting for LD between SNPs in the window. In a similar approach, G' counts the number of SNPs within the genomic interval and estimates the tricube-smoothed G' statistic of each SNP within that window. A primary benefit of running the G' method is that p-value and genome-wide Benjamini-Hochberg false discovery rate (FDR) adjusted p-values are calculated for each SNP. Running the analysis on our bulks consisting of 38 resistant F2 individuals (highBulk) and 50 susceptible individuals (lowBulk). Each analysis was ran using the parameters to filter SNPs at reference allele frequency of 0, minimum total read depth of 5, maximum read depth of 500, a depth difference of 100, a minimum sample depth of 25, and a minimum genome quality score of 99. The QTLseq and G' analysis was both run with a window size of 3 million base pairs, an F2 population structure, replication set at 1000, and plotted with 90 and 99 percent confidence intervals.

### **Expression profiling of RRCR infection via RNA sequencing**

The resistant and susceptible parents, FC709-2 and 03-124 respectively, were used in this experiment. Following the previously described greenhouse disease trial parameters 24 plants from each line were planted. After 8 weeks half of the plants were inoculated, and half were not being treated as controls. Time point 0 tissues were taken from both inoculated and uninoculated plants. Samples were further divided between “above ground” and “below ground”. Above ground tissues consisted of the top 1cm of the crown and all leaf tissues. Below ground tissues consisted of taproot and root hair tissue. Ten days post inoculation both tissues were taken again. All tissue sampled were flash frozen in liquid nitrogen and placed into a -80 C° freezer to be extracted at a later date. These 48 samples were sequenced using Illumina RNAseq short read platform. Approximately 40 million 2x150bp sequencing reads were generated in each sample. Reads were subjected to standard quality control checking and trimming (FASTQC and BBduk). Using HISAT2 (Kim et al., 2019), reads were mapped to the USDA\_Bvulg\_FC709-3\_v1.0.1 reference genome. Mapped reads were quantified using stringtie (Pertea et. al., 2015) before differential gene expression analysis was done in R (v 4.3.1) using the DESeq2 package (Love et. al., 2014). Differential expression was conducted with resultant reads for each gene feature using the DESeq2 package (version 1.28.1) in the statistical software R (version 4.2 (R Core Team, [2020](#))). Reads were transformed to logarithmic fold change log<sub>2</sub> and compared across biological replicates for each population. For each population, the untreated condition was compared to either the 0 or 10 day timepoint to determine expression. Mean normalized counts per gene, an adjusted pvalue of < .05, and log<sub>2</sub> fold change > .5 were the pre-filtering parameters used by DESeq2 for optimal significant genes below the false discovery rate (FDR) of <.05. For

a particular gene, a log<sub>2</sub> fold change of -1 for condition treated vs untreated means that the treatment induces a multiplicative change in observed gene expression level of  $2^{-1}=0.5$  compared to the untreated condition. If the variable of interest is continuous-valued, then the reported log<sub>2</sub> fold change is per unit of change of that variable.

### **QTLseqr analysis**

A total of 261,061,266 paired-end reads were generated for the resistant pool (79.3 gigabases total), whereas 263,286,965 paired-end reads were generated for the susceptible pool (79 gigabases total). Standard bioinformatics approaches were used to QC, trim, and filter the sequencing reads (FastQC and Bbduk), followed by mapping of each of the phenotypic pool to the FC709-3 genome using HiSAT2 (Kim et al., 2019). Genomic variants, along with allelic depth and total depth statistics per site were generated using bcftools mpileup, and subsequently filtered for minimum depth (DP>20) and genotype call quality (GQ>50). The GATK VariantsToTable script was used to create the final variant call table for the input into the QTLseqR R package with a LOD score cutoff of 4.5 (Takagi et al., 2013, Magwene PM., 2011).

### **Transcriptomic Analysis**

Differential gene expression was done using the R package DESeq2 (v1.20.0). Counts from the stringtie pipeline and a meta data file comparing resistant and susceptible tissues, that had been treated or untreated, were used to create a matrix for DESeq2 determine statistically significant differences in gene expression. Each comparison resulted in the number of total nonzero read counts, up regulated gene expression, and down regulated gene expression. DESeq2 uses a default log<sub>2</sub> fold change to estimate adjusted p-values. Statistical significance was determined

with an adjusted p-value of  $>0.05$ . Gene names of those genes that were most highly differentially expressed and genes that may be involved with plant immunity were entered into a BLAST search to discover regions of local similarity between the gene of interest and the data base.

## **Results**

### **Field RRCR F2 disease trials**

The 6 F2 families in 2019 were rated for RRCR resistance 4 weeks post inoculation. Each individual beet was scored from 0-7 following (Ruppel and Hecker 1988) guidelines. Some F2 families suffered from poor germination rate and number of individuals evaluated per family was not equal (Table 6). F2-1 had 38 individuals evaluated with an average disease index (DI) of 3.3 and phenotypic ratio resulting in a normal distribution suggesting little to no segregation within the line. F2-2 had 69 individuals evaluated with an average DI of 4.3 and showed no signs of segregation. F2-3 had 19 individuals rated with an average DI of 2.4 with many of the individuals rated at 1 or 2 but still containing susceptible individuals as well. F2-4 and F2-5, 20 and 26 individuals evaluated, were the only families where all individuals were rated in the resistant category. The test average DI was 4.69 with the susceptible check (FC901) being 5.84. These two families were selected to be further tested in a greenhouse disease trial to increase the number of individuals screened as well as tissue sampled to be used for genomic studies in the future.

### **Greenhouse RRCR F2 disease trials and bulks**

The two F2 families that were selected for further evaluation were F2-4 seed lot 20161044-2S

and F2-5 seed lot 20191044-4S. These seed lots were planted out resulting in 204 individuals for 20161044-2S and 216 individuals for 20161044-4S as well as 39 FC709-2 resistant check and 37 03-124 susceptible check individuals. After 14 days post inoculation plants were uprooted and washed to be phenotyped for RRCR resistance (Table 7). The overall test DI was 5.39 with the susceptible checking being 6.89 and resistant check being 2.46 respectively. F2-4 had an average DI of 5.88 with a phenotypic ratio of 13% resistant (rated 0-3) F2-5 had an average DI of 6.35 and a phenotypic ratio of 7% resistant.

### **Bulk segregant QTLseqr analysis**

QTLseqr analysis and G' analysis pipelines revealed a strong signal of disease resistance associated at a location on chromosome 2 (Figure 33). Plotting the delta SNP-index with 90% confidence interval thresholds identified significant QTLs. G's values were plotted allowing for the identification of significant regions that passed a FDR (q) threshold of 0.01. The significant statistically significant region on chromosome 2 is approximately 2 MB wide with the peak at position 50,479,341 (Figure 34). 24 Genes associated with resistance, cell wall synthesis, or cell signaling were within the QTL boundaries.

### **RNAseq analysis**

Transcript data from USDA\_Bvulg\_FC709-3\_v1.0.1 annotated reference genome generated 29,463 gene models. Data from 3 resistant and 3 susceptible plants with comparisons of all tissue types and only root tissue was used to differentiate each line. During the time-course of day 0 to day 10 post inoculation, there was an overall increase of transcriptionally affected genes in the resistant genotype compared to the susceptible (Table 8). All tissue types for both resistant and

susceptible plants were also analyzed for up and down regulation (Figure 36). Candidate genes that were highly expressed in the resistant genotype at day 10 versus day 0 were considered for further analysis (Table 8). BLAST (Basic Local Alignment Search Tool) was used for the candidate genes to determine their possible function. Many of these genes including FC709\_g18127.t1, FC709\_g18372.t1, FC709\_g18767.t1, FC709\_g16402.t, FC709\_g19006.t, FC709\_g18446.t2, FC709\_g18648.t1 and FC709\_g16519.t are potentially involved with plant defense responses. Raw root tissue only counts were used to determine an average, standard deviation, and standard error of the mean to determine significance between timepoints and genotypes. The top 6 candidate genes that may be involved in disease resistance (Figures 36,37, and 38) were analyzed for gene expression difference. The genes FC709\_g18373.t1 and FC709\_g18374.t1 were significantly different for both genotype and days post inoculation. The gene FC709\_g18127.t1 encodes for the RPP13 resistance like protein that is located within the peak of the QTL on chromosome 2. Other candidate genes were significantly different in either genotype or days post inoculation would need further to be further studied to be conclusive in their resistance roles.

## **Discussion**

Novel QTL associated with resistance *Rhizoctonia solani* in a bi-parental mapping population was discovered in the resistant germplasm line FC709-2. The resistant parent FC709-2 is a resistant check within disease nursery trials throughout the US. This line may have already been introgressed into seed company elite varieties used by growers around the world. Uncovering the QTL will lead to molecular markers to be used in breeding programs. SNPs that are either located within the QTL or near it will be tested for marker development. To validate a candidate

SNP a diverse germplasm panel segregating with the trait of interest will be genotyped. Markers with high association of the trait can be used in breeding programs for selection. Kompetitive Allele-Specific PCR (KASP) is a high-throughput SNP genotyping platform that is the modern trait-specific marker used by plant breeders (He et al., 2014). KASP markers utilize homogeneous fluorescent genotyping system that easily identifies allele specific SNPs. These markers will be useful to begin to look at past germplasm releases and determine if they contain the QTL on chromosome 2. Interestingly is that this QTL is not any of the previously reported QTLs from the Lein et al. 2008 study. FC709-2 and the resistant parent in their study share a common parent but each study had different QTLs that were associated with RRCR resistance. This may be due to mass selection and that the QTLs segregated away from one another. The chromosome 2 QTL was the same QTL reported in the Wigg et al., 2023 paper that the Fort Collins group was a part of. This result was not surprising as the resistant parent in the study was FC709-2 the same as this study. All three of these studies are derived from some original Great Western Sugar lines that were bred from RRCR resistance in the early 1900s. There is potential that with these three studies concluded that all forms of RRCR resistance may be captured within this long-standing population. Understanding how resistance has been integrated into past germplasm releases will enable breeders to look for new forms of resistance in sugar beet and its crop wild relatives. We would want to investigate previous FC700 *R. solani* resistant lines to see if the QTL on chromosome 2 has just been reselected for over time or if there are other forms of resistance within those germplasm lines. Novel forms of resistance can then be stacked into a single germplasm line that then can be released to be integrated into seed company elite varieties. In addition to the genetic background of the QTL, size of the QTL must also be considered. With the ability to map the sequencing reads to the newly form FC709-4 genomes that is a descendent

of the resistant parent FC709-2 we refined the QTL that was previously described (Wigg, Brainard et al. 2023). Refining QTLs may result in more accurate markers that directly select for the trait of interest with little to no negative effects. Marker assisted selection (MAS) can also be helpful for the selection of traits that are impacted by environmental conditions. The disease triangle concept in plant pathology demonstrates the importance of environmental impact for diseases development (Davis 1960). If there is a suitable environment with a enough inoculum there will be disease pressure, however if one factor is missing the possibility of a “disease escape” can happen and alter the disease screening process (Agrios 2005). Using marker assisted selection in plant systems that have this available to them eliminates disease escapes all together (Francis and Asher 2000). Without MAS steps are needed to be taken in order to provide the most accurate disease screening results possible. This can be done in the field by ensuring the inoculum is applied at the proper growth stage and at an even rate. Also providing the best possible environment that is advantageous for disease, but this can only be controlled to an extent in the field. Greenhouse disease trials can help eliminate the environmental factor by controlling for variables such as temperature, humidity, and day length. This is the best way to do small scale disease trials while reducing the risk of disease escapes, however the field trials are needed to screen a larger number of varieties and number of individuals per variety. MAS could also reduce the need for large scale trials as seedlings could be tested early and culled if they do not contain the trait of interest while also reducing the time and money needed to perform these trials.

*Rhizoctonia solani* resistance is polygenic in nature and this can be difficult to breed without the use of molecular markers. Compared to those traits that are simply inherited with one major resistance gene such as sugar beet cyst nematode (*Heteroder schachtii* Schmidt) and Rhizomania

(BNYVV) *Rhizoctonia* will have a difficult time overcoming genetic resistance (Lewellen, Skoyen and Erichsen 1987, Stevanato, Trebbi et al. 2015). MAS can be used to test for multiple genes simultaneously (Francis and Asher 2000) and the would lead to being able to stack or pyramid the resistance traits into a single germplasm line. Developing sugar beet lines with multiple sources of resistance increases the robustness of the resistance and prevents *Rhizoctonia* from overcoming the resistance. MAS is useful for stacking diseases resistance traits for other diseases and can lead to sugar beet varieties with multiple advantageous traits thus reducing disease overall in both field and storage.

Future studies may focus on refining the RNAseq experimental design using more time points to find the exact time when these traits are being expressed at their highest levels. The candidate genes reported have some interesting roles in plant defense. FC709\_g18127.t1 candidate gene when searched in BLAST resulted in a disease resistance protein RPP13. RPP13 encodes for a nucleotide binding site leucine rich region (NBS-LRR) type R protein that confers resistance to the biotrophic oomycete, *Peronospora parasitica* (Bittner-Eddy et. al., 2000). Another interesting gene reported in the expression data was the FC709\_g18648.t1. This gene when searched in the BLAST database came up to encode the PERK1 gene. The proline-rich, extension-like receptor kinase-1 (PERK1) gene has been shown to be rapidly induced by wounding (Silva and Goring, 2002). This gene may show a possible role in plant defense responses triggered by tissue wounding by an outside force either mechanical or pathological. Applying this gene to the system being looked at in this study it would be plausible to see this gene upregulated when *Rhizoctonia solani* creates external wound to enter the root cells of the sugar beet. Taking the candidate genes that were discovered in this study and using them for functional validation via hairy root transformation. This has been done when conferring a

rhomboid-like protease gene from an interspecies translocation for resistance to sugar beet cyst nematodes (Kumar, Harloff et al. 2021). Further transcriptomic studies may unveil the exact gene or genes responsible for resistance.

The experiments here identified and refined a QTL associated with resistance to *R. solani* AG2-2 IIIB that can be used to screen sugar beet germplasm. Accessions with the markers have been identified, greenhouse and field screens can be used to obtain a more precise evaluation of the level of resistance within other germplasm lines.

## **Conclusion**

Identifying QTLs associated with resistance to *R. solani* AG 2-2 IIIB will allow breeders to select elite germplasm more easily and accurately. In this study we identified and refined a QTL on chromosome 2 using both traditional and new genomic breeding tools. A bi-parental mapping population was used to establish F2 population that were then bulked into resistant and susceptible bulks. These bulks were then sequenced and mapped against the resistant parent FC709-2 to ensure that the resistant QTL regions were present. To begin to understand the molecular resistance RNAseq technology was used to look for differentially expressed genes that may be responsible for resistance to *R. solani*. Having molecular markers and understanding how resistance is achieved by the host will not only enrich our knowledge of plant/pathogen interaction but allow for breeders to look back at the decades of germplasm to determine if there are other novel forms of resistance. Continuing to identify new forms of resistance will allow for stacking of these disease traits and achieve greater disease management for growers around the world.

Table 6: F2 families created from the crossing of RRCCR resistant parent FC709-2 and RRCCR susceptible parent 03-124. 5 families were selected and tested in the 2019 RRCCR disease nursery to determine levels of resistance. Disease indexes were calculated per family and 20161044-2S and 20161044-4S were selected for further evaluation.

Germplasm line	Entry	Plants at Rating	Disease Index Rating (# individuals)								DI
			0	1	2	3	4	5	6	7	
20161044-2S	F2-1	38	0	6	7	3	14	6	2	0	3.34
20161044-4S	F2-2	69	0	4	11	11	7	10	15	10	4.37
20161045-1S	F2-3	19	0	3	13	0	1	1	0	1	2.32
20161045-2S	F2-4	20	0	7	9	2	1	0	0	0	1.84
20161045-3S	F2-5	26	0	12	14	0	0	0	0	0	1.53
Trial mean											2.68

Table 7: Greenhouse *R. solani* screening of selected F2 families. Those F2 individuals ranked 0 through 3 from each of the families were used to create the resistant bulks used in the QTL analysis.

Germplasm line	Entry	Plants at Rating	Disease Index Rating (# individuals)								DI
			0	1	2	3	4	5	6	7	
20161044_2S	F2-4	204	1	16	8	3	9	15	15	137	5.88
20161044_4S	F2-5	215	3	9	3	1	2	11	17	169	6.35
20121034	Res Parent	39	15	9	1	0	2	0	7	5	2.46
03-124	Susc Parent	37	0	0	0	0	0	0	4	33	6.89
Trial mean											5.39

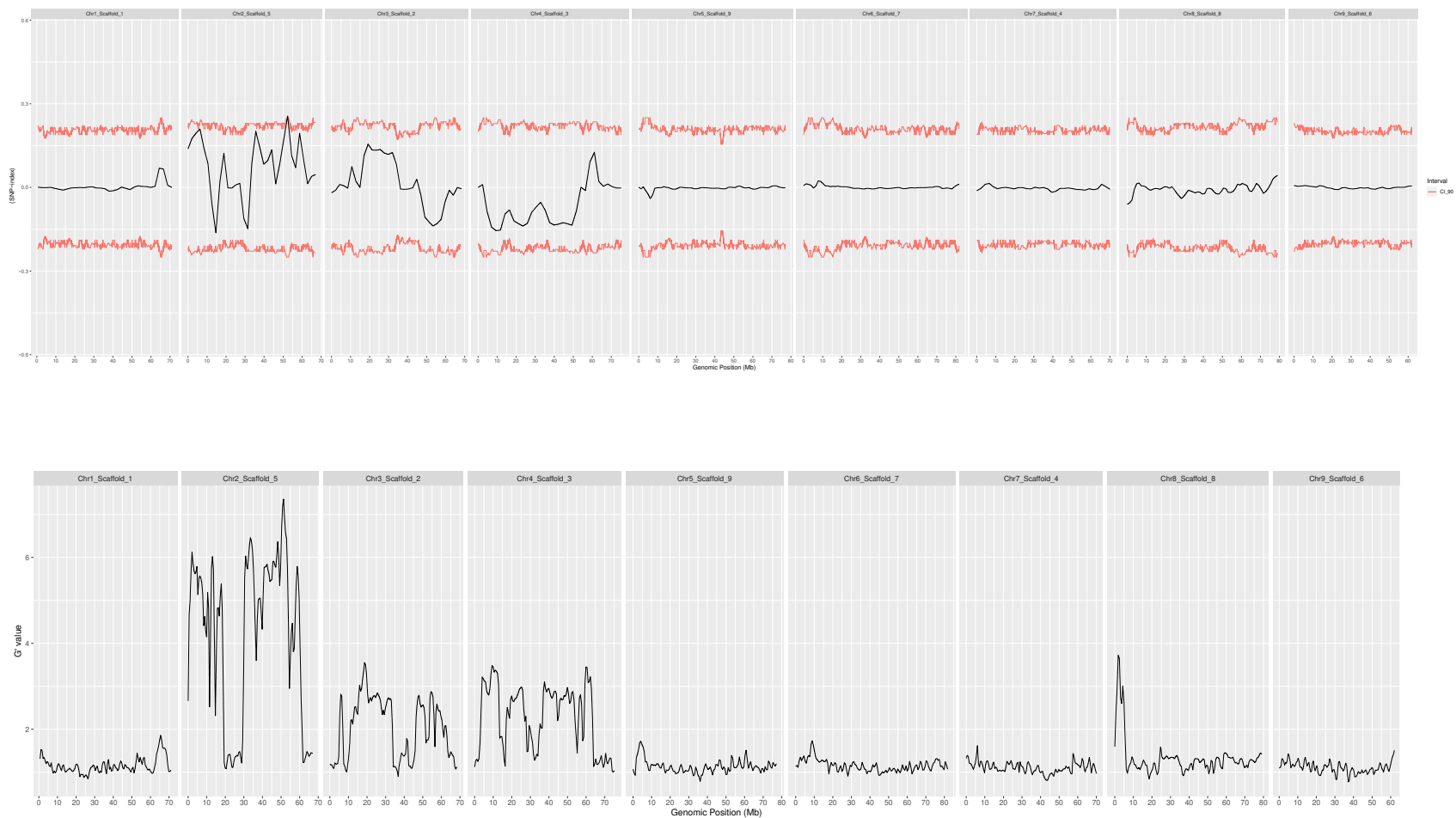


Figure 33: QTLseq (top) and  $G'$  (bottom) methods were used in the R package QTLseqr. The QTLseq plot has 90% confidence intervals represent by red lines and  $G'$  has the  $G$  value cutoff set by the program (4.5). Both methods resulted in a QTL on chromosome 2 associated with RRCR resistance.

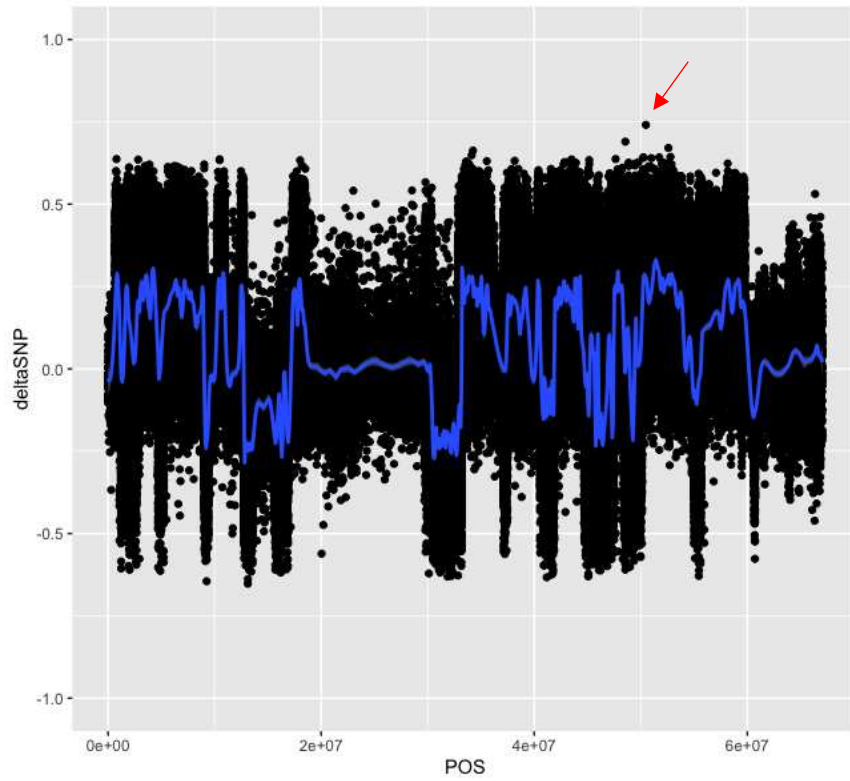


Figure 34: A subset of filtered SNP markers only on chromosome 2. Delta SNP plotted with regards to its chromosome position. This resulted in the exact peak position at 50,479,34 (red arrow).

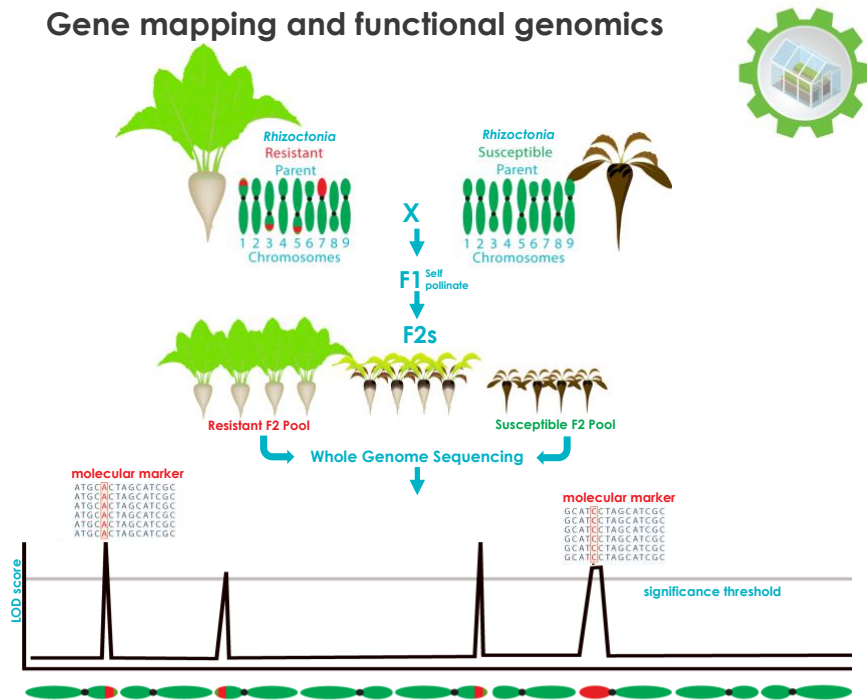


Figure 35: Bi-parental mapping population diagram between FC709-2 and 03-124 used to create F2 phenotypic bulks. A resistant bulk (n = 38 with ratings 0-3) and a susceptible bulk (n = 50 with ratings of 7) were created from several individuals and sequenced. Sequencing reads were mapped back to the resistant parent and the R package QTLsegr regions associated with RRCR resistance were identified.

Table 8. Comparisons between the treated (Trt) untreated (Unt) resistant (R) susceptible (S) timepoint 0 days after inoculation (T0) and timepoint 10 days after inoculation (T10). These comparisons were chosen to capture genes that may be up or down 4 regulated during the infection process on both resistant and susceptible plants. Upregulated genes had a LFC >0.50 and downregulated genes had a LFC value <-0.50.

Comparison	Tissue Type	Read Count	LFC >0.50 (up regulated)	LFC < -0.50 (down regulated)
T0_Unt_R vs T10_Trtr_R	All	19548	995	1468
T10_Trtr_S vs T10_Trtr_R	All	19548	28	4
T0_Unt_R vs T0_Unt_S	All	19548	403	1183
T0_Unt_R vs T0_Trtr_R	All	19548	456	1274
T0_Trtr_R vs T0_Trtr_S	Root	18188	754	859
T0_Unt_S vs T0_Trtr_S	Root	18188	90	98
T10_Trtr_R vs T0_Unt_R	Root	18188	732	329
T0_Trtr_R vs T10_Trtr_R	Root	18188	604	502

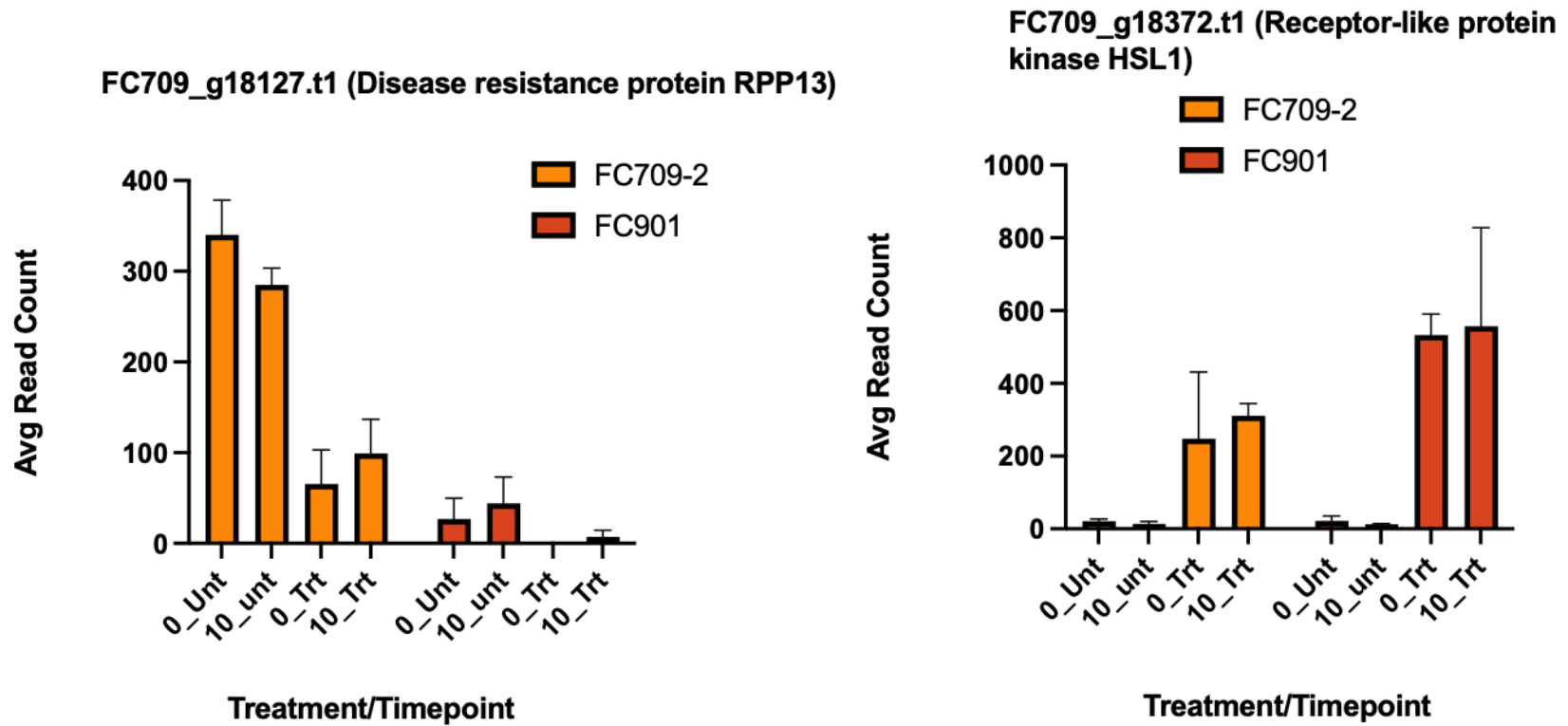


Figure 36. Candidate genes were examined in both the susceptible (FC901) and resistant (FC709-2) lines. Each line had an untreated and treated trial that were sampled at 0 DPI and 10 DPI. These time periods were used to determine significance in expression between each variable.

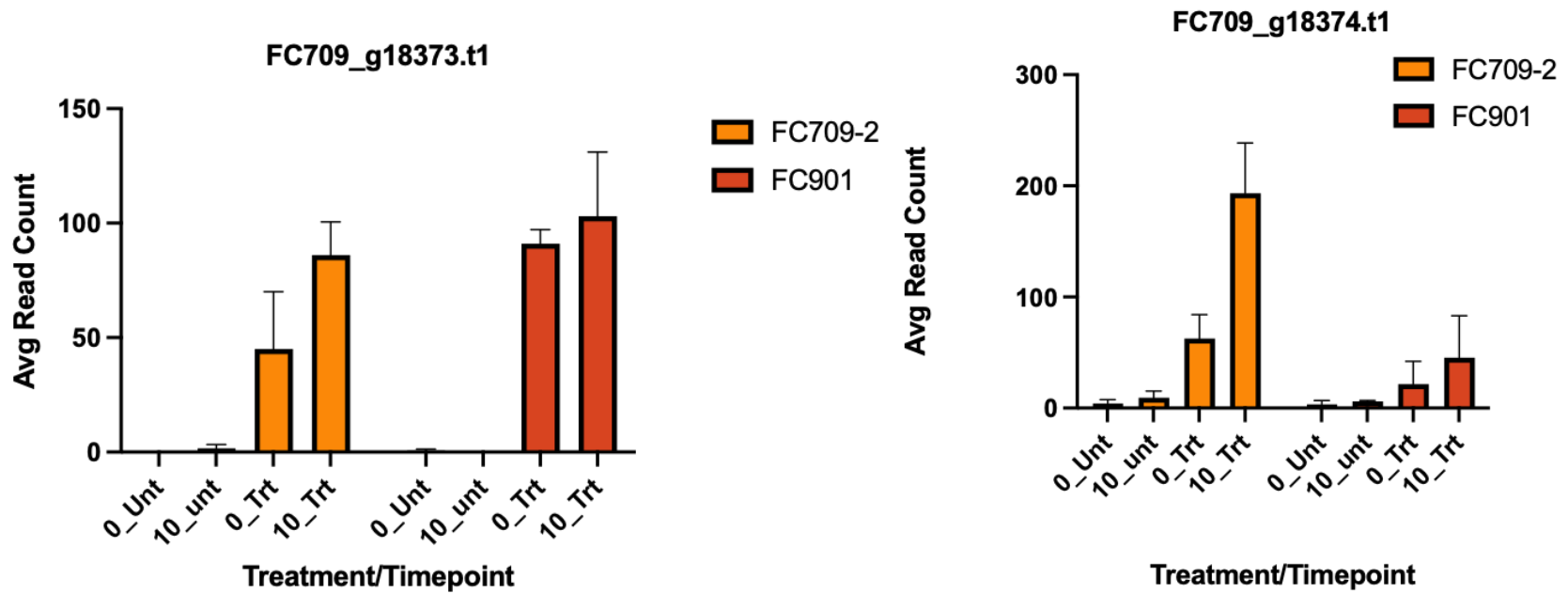


Figure 37. Candidate genes were examined in both the susceptible (FC901) and resistant (FC709-2) lines. Each line had an untreated and treated trial that were sampled at 0 DPI and 10 DPI. These time periods were used to determine significance in expression between each variable.

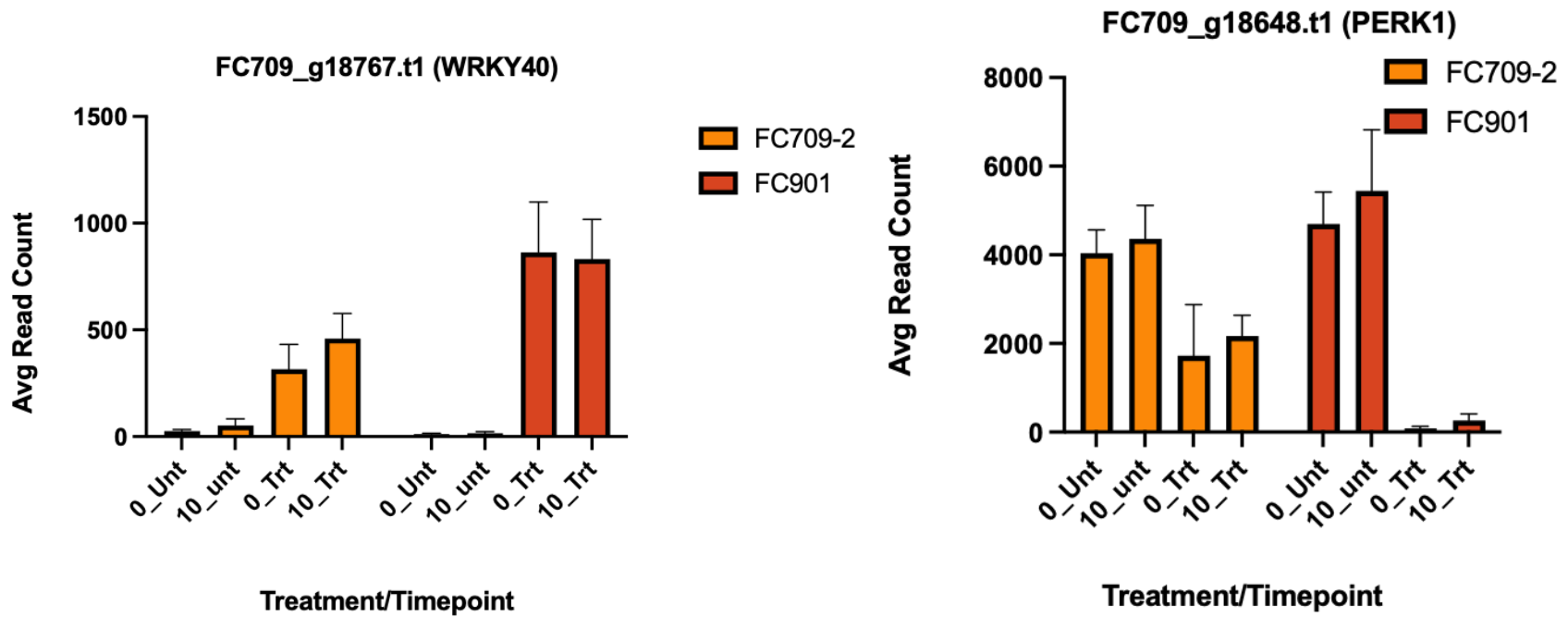


Figure 38. Candidate genes were examined in both the susceptible (FC901) and resistant (FC709-2) lines. Each line had an untreated and treated trial that were sampled at 0 DPI and 10 DPI. These time periods were used to determine significance in expression between each variable.

Table 9. Candidate genes in the chromosome 2 QTL associated with RRCR resistance. Genes names are derived from the FC709-3 genome assembly. Each gene was searched using the BLAST tool to find the common name and function. Genes that may be involved in resistance, cell wall formation, or cell signaling were reported.

Location	Gene name	Start Pos bp	Stop Pos bp	Name	Function
Chr 2	FC709_g18113.t1	52456953	52460985	Alpha-galactosidase 1	Cell Wall
Chr 2	FC709_g18113.t2	52456953	52460985	Alpha-galactosidase 1	Cell Wall
Chr 2	FC709_g18115.t1	52479569	52479898	Uncharacterized mitochondrial protein	Cellular Component
Chr 2	FC709_g18117.t1	52485873	52486580	Retrovirus-related Pol polyprotein from transposon	Nucleic Acid Binding
Chr 2	FC709_g18122.t1	52506140	52509226	Copia protein	Nucleic Acid Binding
Chr 2	FC709_g18123.t1	52513756	52516356	Putative disease resistance RPP13-like protein 3	Plant Defense
Chr 2	FC709_g18124.t1	52521510	52521896	Retrovirus-related Pol polyprotein from transposon TNT	Nucleic Acid Binding
Chr 2	FC709_g18127.t1	52542803	52545424	Disease resistance protein RPP13	Plant Defense
Chr 2	FC709_g18129.t2	52578134	52578886	Protein transport protein Sec61 subunit alpha	Membrane transporter
Chr 2	FC709_g18132.t1	52611968	52615396	Retrovirus-related Pol polyprotein from type-1 retrotransposable element	Nucleic Acid Binding
Chr 2	FC709_g18140.t1	52691066	52695598	Fe(2+) transport protein 3	Transporter
Chr 2	FC709_g18141.t1	52700226	52706389	Probable beta-1,3-galactosyltransferase 12	Transporter
Chr 2	FC709_g18143.t1	52723419	52725077	Retrovirus-related Pol polyprotein from transposon TNT	Nucleic Acid Binding
Chr 2	FC709_g18149.t1	52764279	52767761	Putative GATA transcription factor 22	Signaling Pathway
Chr 2	FC709_g18160.t1	52801981	52803156	Retrovirus-related Pol polyprotein from type-2 retrotransposable element R2DM	Nucleic Acid Binding
Chr 2	FC709_g18162.t1	52824611	52829974	Transposon Ty3-I Gag-Pol polyprotein	Nucleic Acid Binding
Chr 2	FC709_g18165.t1	52865109	52872261	Glycine dehydrogenase (decarboxylating)	Mitochondria
Chr 2	FC709_g18167.t1	52883838	52897271	Probable serine/threonine-protein kinase SIS8	Signaling Pathway
Chr 2	FC709_g18169.t1	52924567	52924797	Probable protein phosphatase 2C	Signaling Pathway
Chr 2	FC709_g18170.t1	52956558	52957862	Protein REVERSION-TO-ETHYLENE SENSITIVITY1	Signaling Pathway
Chr 2	FC709_g18176.t1	53023478	53024938	Transposon Tf2-11 polyprotein	Nucleic Acid Binding
Chr 2	FC709_g18181.t1	53039322	53039600	Uncharacterized mitochondrial protein AtMg00820	Mitochondria

Chr 2	FC709_g18182.t1	53042043	53046803	Calcineurin B-like protein 10	Cellular Component
Chr 2	FC709_g18183.t1	53047594	53049162	Retrovirus-related Pol polyprotein from transposon TNT	Nucleic Acid Binding

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