IMPROVING STEM RUST RESISTANCE AND MALT QUALITY IN THE WASHINGTON STATE UNIVERSITY BARLEY BREEDING PROGRAM

By

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Abstract

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In 2019, a population of the wheat stem rust pathogen (*Puccinia graminis* f. sp. *tritici*, *Pgt*) was collected from barley in eastern Washington. It was observed that 99% of the 100 isolates collected from the Pacific Northwest (PNW) were virulent on the cultivar Morex, which contains the resistance gene *Rpg1*. Furthermore, 10% of the isolates were virulent on Q21861, which contains both resistance genes *Rpg1* and *rpg4/5*. When stacked together, *Rpg1* and *rpg4/5* provided exceptional resistance to all known races and isolates of *Pgt* collected from around the globe, including the African *Pgt* race TTKSK (AKA Ug99) and its lineage. Thus, this remarkable virulence is the first documentation of *Pgt* virulence on the *Rpg1* and *Rpg4/5* gene combination worldwide. This research aims to identify novel sources of seedling resistance against the virulent PNW population of *Pgt*. Screening of 487 accessions from the World Barley Core Collection (WBCC), representing global genetic diversity, was conducted to identify novel seedling resistance sources. A malt barley cultivar, Elliot (PI 592661), was identified to have seedling

resistance to the most virulent PNW isolate, Lsp21. A biparental recombinant inbred line mapping population was developed between Elliot (resistant) and Palmer (susceptible). Utilizing QTL analysis resistance contributed by Elliot was identified as two significant QTL on chromosomes 4H and 5H. A second comprehensive screen was done on the Wild Barley Diversity Collection (WBDC). Furthermore, the WBDC was utilized using GWAS to identify 12 novel loci on chromosomes 1H, 2H, 3H, 5H, 6H, and 7H associated with resistance to *Pgt* isolate Lsp21. Two lines (WBDC-94 and WBDC-238) were identified to have high levels of resistance against *Pgt* isolate Lsp21. Both lines contain the R-gene *Rpg7*. Here, we genetically characterize, fine-map, and identify *Rpg7* candidate genes utilizing a Morex × WBDC-94 biparental population. We successfully used high-resolution mapping to delimit *Rpg7* to a 51 kb region containing two candidate genes on chromosome 3H. We hypothesize that both candidate genes, a RIN4-like protein and a RPM1-like protein, are required for resistance. The resistances are currently being integrated into elite malting barley backgrounds to enhance resistance to the virulent PNW *Pgt* population. Lastly, a diverse panel of 550 experimental malt lines from the WSU malt barley breeding program were used for association mapping to identify loci contributing to malt quality. A total of 44 marker trait associations were identified representing loci across all seven barley chromosomes. These markers will be used for marker-assisted selection to improve malt quality in the WSU breeding program. These quality traits will lead to the expedited development of American Malting Barley Association (AMBA) recommended varieties that can open new markets for WSU malt barley varieties. By developing malt barley varieties that have higher yields than current feed barley varieties, have resistance to stem rust, and AMBA quality standards, growers will have more incentive to grow barley due to increased profit margins.

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Dedication

I want to dedicate this dissertation to my father, James Brooke, my late grandfather, Kenneth, and my first mentor, John Gaffaney, for encouraging me to continue my education in the world of plant sciences.

CHAPTER ONE: LITERATURE REVIEW- MALT BARLEY: STRIVING FOR QUALITY, QUALITY, QUALITY

Barley Overview:

From the high elevations of the Tibetan plains to the short growing seasons of Canada, and the dry plains of the United States, barley (*Hordeum vulgare* L.) genotypes have been identified that are well adapted to diverse growing regions. Barley was domesticated in the Fertile Crescent over 10,000 years ago and is a member of the Poaceae family, the fifth-largest plant family, which contains around 780 genera and 12,000 species (Badr et al., 2000; Zohary and Hopf, 2000). The genus *Hordeum* contains various ploidy levels, including hexaploid, tetraploid, and diploid species (Bothmer et al., 1995). Both cultivated barley (*H. vulgare*) and its wild ancestor (*H. vulgare* ssp. *spontaneum*) are diploids (2n = 2x = 14) representing the primary barley germplasm pool. During the process of domesticating from the wild barley *H. vulgare* ssp. *spontaneum* to *H. vulgare*, the key domestication traits selected were non-brittle rachis, reduced dormancy, and photoperiod insensitivity (Pourkheirandish and Komatsuda, 2007). These traits are straightforward to phenotype and the backbone for domestication and migration. Breeding efforts have brought domesticated barley far from its wild progenitor, *H. vulgare* ssp. *spontaneum*, however, domesticated barley has reduced genetic diversity. Thus, *H. vulgare* ssp. *spontaneum* still contains a diverse reservoir of novel biotic and abiotic stress resistance genes and quality resistance genes that can be introduced into cultivated barley utilizing hybridization (Asfaw and Bothmer, 1990; Fetch et al., 2003; Chen et al., 2021).

Today, barley is utilized predominantly for livestock feed, followed by malt used for brewing and distilling, with a minor fraction for human consumption (Ullrich, 2010). In terms of production, barley ranks fourth (147 million metric tons) in world cereal production behind wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), and rice (*Oryza sativa* L.) (FAOSTAT, 2022). However, total US barley production has steadily decreased from over 13 million acres in 1986 to just over 2 million in 2021, placing the US 10th in world barley production. Currently, barley in the US is primarily grown for the malt industry, with the majority bred and produced to meet the demands of the adjunct brewing, craft brewing, and distilling industries (AMBA, 2021). The US barley industry is estimated at $1.2 billion as a raw grain commodity (AMBA, 2023). The malting, brewing, livestock, and tax revenue activities are all considered added value to the barley industry, making barley worth $384 billion, with the whisky industry accounting for $1.06 billion.

The decrease in barley acreage was mainly due to loss of feed barley acres due to subsidies for corn and soybean. These subsidies incentivized farmers in the Midwest to grow large acreages of corn and soybean in areas previously dominated by feed barley. These crops provide more significant profits as feed for livestock and biofuels; thus, markets for the US feed barley class plummeted. The introduction of large acreages of corn in the Midwest and environments was conducive to the development of the disease Fusarium Head Blight (FHB), primarily caused by *Fusarium graminearum* for which corn is a host; the increased inoculum contributed to devastating FHB epidemics on barley and wheat. The FHB epidemics drastically decreased the quality of malt barley grown in the Midwest. Thus, malt barley production has been pushed west into Montana,

Idaho, Wyoming, and Washington, where dryer, cooler climates are less conducive to FHB disease epidemics.

Washington state ranks 5th in barley production in the US, with over 70 thousand acres harvested in 2021 (USDA - NASS, 2022). However, this is a small fraction of what was produced during the peak of barley production in 1985, when production in Washington state stood at nearly 1.2 million harvested acres (FAOSTAT, 2022). Historically, Washington barley production was predominantly feed barley for foreign and domestic markets. Around 90% of all barley produced in Washington is still grown for the feed market (WGC, 2020). However, an interest in malt barley is growing to supply malt in the region that is being utilized in beer, liquor, malted milk, and flavorings in various foods. Malt is now the second most extensive use of barley, accounting for approximately 10% of the state's production. Barley is considered a favorable rotational crop among farmers in Washington following winter wheat due to its low input and soil ecosystem services. Farmers would choose more malt barley in a crop rotation if adapted high-yielding and quality dryland malt barley varieties allowed for increased profit margins that could compete with spring wheat and garner more contracts through the area's large malting facility, Great Western Malting in the Port of Vancouver, WA.

In an effort to boost barley production in the state, the Washington State University (WSU) barley breeding program began shifting from feed barley variety development to some malt barley just over a decade ago. This research culminated in the recent WSU release of the variety 'Palmer' in 2019, named after Mary Palmer Sullivan, an influential advocate for barley production in the state and the current vice president of the Washington Grain Commission. Palmer was the first, and currently the only, malt barley variety released by WSU. However, Palmer fell short of

meeting the adjunct, craft all malt and distilling grains malt quality standards to make the American Malting Barley Association (AMBA) recommended list, thus it is strictly a niche all-malt craft variety which Great Western Malting does not accept. Craft malt varieties that do not make AMBA recommendations are only planted to small acreage targeted towards toll malting contracts with the smaller craft malt facilities in the region, including LINC malt in Spokane, WA, Cascadia Malts in Nine Mile Falls, WA, Mainstem Malt in Walla Walla, WA and Montana Craft Malt in Butte, MT.

The American Malting Barley Association is a nonprofit organization that collects dues from large brewing, craft brewing, malting, and distilling companies. With these funds, AMBA invests in public malt barley research programs to ensure that the US malting barley industry produces a high-quality product and remains domestically self-sufficient. AMBA has outlined many important traits and benchmarks for breeders to obtain optimal malting and brewing. These traits include low β-glucan, adequate α-amylase, low protein, high extract, friability (indicates adequate modification), soluble protein, color, diastatic power (DP), and free amino nitrogen (FAN) (AMBA, 2021). These traits are critical in making the malting and brewing processes more efficient in large-scale adjunct brewing facilities (Briggs, 1998). Currently, 41 barley varieties (winter and spring) meet the standards and are AMBA-certified. The seed certification is necessary for malting barley to gain large growing acreage within Washington for commercial malting facilities to contract with farmers and open new markets.

What Makes Malt Barley Tick:

A barley variety grown across diverse environments must be consistent and efficient in the malting process to pass the rigorous testing required for AMBA recommendation and to be contracted by large commercial malt facilities. There are three steps in the malting process: steep, germination, and kilning. During the malting process, which is essentially controlled germination, the barley endosperm undergoes complex modifications. Malt modification is the breaking of starches and proteins into simple sugars and free amino acids so that brewer's yeast can utilize these nutrients during the brewing and distilling fermentation processes (Briggs, 1987a; Briggs, 1987b; Briggs, 1992). Thus, the critical malt quality trait is the efficiency and consistency of grain modification to fermentable sugars (extract) during the malting process. The primary end-users of domestically produced quality malting barley are adjunct brewers, craft brewers, and distillers. In the US, adjunct breweries use the majority of the malt produced domestically (60%). Adjunct breweries like Anheuser-Busch InBev and Molson Coors are industrial-scale breweries that include adjunct carbohydrates to their fermentation vats like rice or corn syrup in conjunction with barley malt (Figueroa et al., 1995). The second largest end-users are the 9,118 US craft breweries that traditionally utilize all barley malt brewing, thus barley malt provides all the carbohydrates and enzymes needed for brewing. In craft breweries which utilize ~ 34% of US malt (AMBA, 2021), more barley is used per pint of beer brewed than for adjunct brewers. The third end-user, distilleries, utilizes ~ 6% of US malt. These industries demand different standards from malt barley. Thus, the criteria to meet the different industry standards are set by AMBA and can be categorized into whole kernel, congress mash, and enzyme extract analysis.

The first standard is for whole kernel analysis, which mainly measures grain protein and kernel size. For a malt quality barley variety, grain protein should be between 11 to 13 % (AMBA,

2021). Lower protein in malt barley is desirable for several reasons. The first is that barley grain with higher protein content produces a hazy effect in beer which under most circumstances is considered undesirable (Asano et al., 1982; Briggs, 1998). However, with the popularization of hazy craft beers, higher protein varieties could be better suited for the craft market. Most hazy beers are brewed using raw oats with high protein, brewers use oats instead of high-protein barley to make hazy beer because barley grain with higher protein is correlated with lower available carbohydrates in the endosperm, which are broken down to simple sugars known as extract (Fox et al., 2003). The second reason low protein is desirable is that during the steeping process, high protein content in grain can impede water uptake (Henry and Cowe, 1990). For instance, if a barley cultivar has a higher protein content, the steeping stage will have to be extended until the moisture content reaches roughly 40 %, which is very undesirable by commercial malt houses that have a very regimented malting process (Molina-cano et al., 1995). However, if protein levels are too low, there can be insufficient enzymes needed to break down complex carbohydrates used by yeast during fermentation.

Kernel size is measured using a Pfeuffer Sortimat. This machine uses three sieves, 2.78 mm, 2.38 mm, and 1.98 mm, to sort the kernels into plumps, intermediates, and thins. The target standard for kernel plumps is 90 % of harvested grain at or above 2.38 mm. Plump kernels result in more starch and higher extracts, and uniform seed results in uniform germination and consistent modification during the malting process (Fox et al., 2003).

The second standard is Congress Mash, a process brewers have used for over 100 years. It is the first step in the brewing process, typically called the mash. For the congress mash, malt barley is finely ground and added to water in a 1:4 ratio at 45˚C for 30 minutes (ASBC, 1991).

After 30 minutes, more water is added at 70°C for a ratio of 1:6, and the mash is kept at 70°C for 60 minutes. After cooling, the grist is separated from the water by a process called lautering to obtain the wort. In a standard brewing process, brewers would boil the wort and add hops or other flavorings before cooling and adding yeast to initiate the fermentation process. For malting analysis, the wort is used to analyze malt extract, β-glucan, free amino nitrogen (FAN), soluble protein over total protein (S/T), and color (Briggs, 1998).

Malt extract is the amount of total solid material dissolved during the mash through enzymatic degradation of carbohydrates and proteins to simple fermentable sugars and free amino acids that the yeast can utilize during fermentation (ASBC, 2009; Briggs, 1998). The dissolved material will have a higher density than water, so specific gravity is used to measure extract levels. The equation to determine the specific gravity as % extract is below (Equation 1). P = g extract in 100 g of wort (°Plato) and M = % moisture in malt. Furthermore, 400 g of water is added to 50 g of malt, which converts to 800 g of water per 100 g of malt. Extract is one of the most critical measurements in malt barley because it serves as a reliable estimator of fermentable extract, color, viscosity, and free amino nitrogen (FAN) (Collins et al., 2003; Fox et al., 2003)

$$\%\, Extract \;=\; \frac{P \times (M + 800)}{100 - P}$$

Equation 1: specific gravity for wet malt extract.

β-glucan is a source of fiber in the barley grain that makes up 75% of the endosperm cell walls (Jamar et al., 2011). Maltsters and brewers want low amounts of fiber in malt barley as high fiber results in a failure to degrade cell walls during the malting process, which leads to a decrease

in the final extract (Bamforth, 2003, 2017). Furthermore, higher β-glucan content within the wort will also negatively affect filtration during lautering in the brewing process, which is of the utmost importance during the very standardized large commercial adjunct brewing processes (Vis and Lorenz, 1998; Bamforth, 2003).

Free amino nitrogen or FAN measures proteins that have broken into individual amino acids. Brewers use FAN to predict healthy yeast growth and fermentation efficiency. FAN comprises amino acids and small peptides and is the only source of nitrogen for yeast cells to grow and reproduce during fermentation (Stewart et al., 2013). However, excessive amino acid content in wort has been attributed to off-flavors (Hill et al., 2019). Another measurement related to FAN is soluble protein over total protein (S/T). The S/T ratio, also known as the Kolbach index, is the ratio of soluble protein in wort in relation to the total protein of malt (ASBC, 1990). This ratio can be used as a measurement of modification. For example, the higher the S/T value, the greater the degree of modification of the grain during the malting process.

The third and last standard measured is enzyme extract analysis, which includes α-amylase (AA) and diastatic power (DP), a measurement of enzyme activity. The industry standard emphasizes high diastatic power and α-amylase (AMBA, 2021). To conduct enzyme extract analysis, a ratio of 1:20 of finely ground malt is added to a 0.5 % sodium chloride solution at 20°C for 2 hours. After 2 hours, the grist is separated from the sodium chloride and is used to conduct α-amylase and diastatic power analysis. AA is an endohydrolase that breaks starch down into simple sugars like maltose oligosaccharides and dextrin (Briggs, 1998; Fox et al., 2003). The purpose of AA is to break up long starch chains called amylose at the α-glucoside (1-4) glucose linkages. Within a quiescent barley grain, AA is virtually undetectable. However, the embryo and

aleurone layer synthesize AA during germination in the malting process (Bathgate and Palmer, 1973).

Diastatic power is a measurement that includes all the starch-degrading enzymes. These enzymes include AA, β-amylase (BA), limit dextrinase (LD), and α-glucosidase (AG). As mentioned previously, AA breaks up starch chains at random within a branch, acting as an endo-acting enzyme. At the same time, BA breaks up the disaccharide maltose, a (1-4) glucose linkage, from the non-reducing chain end of amylose (Fox et al., 2003). At each break that AA forms, another non-reducing end is created that the BA can act on. These two enzymes do most of the work to break down amylose and amylopectin into simple sugars like glucose, maltose, and maltotriose. Yeast will not break down larger sugar molecules that remain after the malting process and thus these will be passed on to the final product. The debranching enzymes LD and AG are deployed to hydrolyze the (1-6) linkage bonds within the branched starch to break up complex branched starches like amylopectin. Linking oligosaccharides are formed once these 1-6 linkages are broken, allowing AA and BA to break the starch down further (Stenholm and Home, 1999; Fox et al., 2003). However, AG can also break a 1-4 linkage bond like AA. Unfortunately, AG activity is considerably lower than other starch-degrading enzymes because it is not heat stable during the kilning or mashing process (Agu and Palmer, 1997)

AMBA sets strict standards for malt barley breeders. The amount of grain that can be converted into beer or spirit yield is % extract. For quality malt barley, the extract must be above 80 % (AMBA, 2021). The new WSU Malt Quality Lab will perform the abovementioned analyses. This lab contains state-of-the-art equipment and analysis developed by the American Society of Brewing Chemists (ASBC). Developing malting barley varieties to make AMBA specifications

within a changing climate is challenging. The major bottleneck in the selection process is generating malt quality data, which has been addressed in the program by the development of the WSU malt quality analysis lab within the breeding program.

Stem Rust and Barley:

Throughout history, wheat stem rust, caused by the biotrophic fungal pathogen *Puccinia graminis* f. sp. *tritici* (*Pgt*), has been a significant issue for barley and wheat (*Triticum aestivum* L.) production (Brown and Reeves, 1975). Stem rust has been reported to cause significant epidemics and heavy yield losses in barley and wheat when susceptible varieties are grown under conditions conducive to the development of stem rust epidemics. In North America, stem rust has historically been a problem in the midwestern growing regions of the US and Prairie Provinces of Canada. However, effective stem rust management was successfully implemented mainly through the deployment of effective genetic resistances in wheat and barley and through the program to eradicate barberry (*Berberis vulgaris*), the alternate host of *Pgt* for sexual reproduction (Roelfs, 1982). One of the earliest records of a barberry eradication program was a law in Rouen, France, in 1660 that advocated the destruction of barberry plants to protect the cereal crops (Mckay, 1957). The first barberry eradication program in the US was passed in Connecticut in 1725 (Maloy, 1993). Later, in 1755, Maine and Rhode Island passed similar laws. As the US progressed to 1918, the federal barberry eradication program was started (Roelfs, 1982). The program employed 175 people across nineteen states, including Washington state. By 1955, the program eradicated over a half-billion barberry bushes and 25 different *Pgt* races had been identified. By removing barberry from the environment, the pathogen could not complete its sexual cycle, stabilizing the *Pgt* population as new virulent gene combinations could not arise via meiotic recombination during

the sexual cycle. With the help of stacking resistance genes in wheat and using the single durable resistance gene *Rpg1* in barley, stem rust epidemics became a thing of the past, and the federal barberry eradication program was discontinued in 1977 (Roelfs, 1978; Maloy, 1993). With the recent emergence of virulent populations of *Pgt* arising in sexual populations in Africa, Europe, and the PNW region of North America and their potential to cause major stem rust epidemics, the search for effective resistance genes against these emerging virulent populations has once again become a priority.

Puccinia graminis is a heteroecious and obligate parasite, meaning it completes its life cycle on two unrelated hosts and needs a living host or tissue to survive. In the case of wheat stem rust, which infects both wheat and barley, barberry and mahonia (*Mahonia aquifolium)*, also known as Oregon grape, are the two alternate sexual hosts for this pathogen (Roelfs, 1985). *Puccinia graminis* produces five different spore stages during its life cycle. These spores are called basidiospores, pycniospores, aeciospores, urediniospores, and teliospores and play different roles in *Pgt* evolution, infection, and spread.

In the springtime, teliospores germinate to form basidiospores (Leonard and Szabo, 2005). These basidiospores are then distributed throughout a region by wind and infect barberry or mahonia. Once infection occurs on the upper portion of the leaves, spermogonial structures are formed. Sexual reproduction is initiated, resulting in meiotic recombination, the potential for new virulence gene combinations, and the evolution of new virulent genotypes in the population. From here, aecia are formed. Aecia produce aeciospores and are dispersed by the wind from the underside of the leaves. Aeciospores land on the primary cereal host leaf and stem tissue and germinate, forming a germ tube that extends perpendicular to the leaf ridges until it encounters a

stoma. This germ tube produces an appressorium over the stoma, which produces a penetration peg that enters through the stomatal openings. Once the cereal host is successfully infected, colonization occurs, forming a uredinium that erupts from the epidermis, releasing masses of urediniospores. These urediniospores often act as secondary inoculum for the remainder of the season. Near the end of the growing season, teliospores form. The only part of this fungus that can survive without a living host is teliospores, which can survive a few months and can initiate the cycle the following spring in environmental conditions that allow for their survival over winter and producing basidiospores to infect alternate hosts.

Stem rust can also damage cereal grains without barberry or mahonia present (Maloy 1993). Winds from South America that push up to North America in the summer months can bring urediniospores. The wind carries these urediniospores and starts infection as temperatures and moisture conditions become suitable. By the end of summer, the spores have reproduced asexually and spread into the Upper Midwestern US and Prairie provinces of Canada. The three types of rusts affecting wheat and barley are stripe, leaf, and stem. Each rust has an optimum temperature for urediniospores to infect wheat and barley. For instance, stripe rust's optimum temperature is 7-12˚C; therefore, it can infect the host much earlier in the spring. Meanwhile, stem rust's optimum temperature is around 20◦C, and a later infection starts early to mid-summer (Roelfs, 1988).

Nearly 60 stem rust (*Sr*) resistance genes have been characterized in wheat. However, few of these genes have been utilized to protect wheat worldwide, including the gene *Sr31*, a significant resistance gene deployed in wheat (Pretorius et al., 2000; Hafeez et al., 2021). In 1999, a new *Pgt* race, TTKSK (AKA Ug99), was identified in Uganda with virulence to *Sr31,* which raised the alarm for the return of major stem rust epidemics. TTKSK was virulent on 80% of the world's

cultivated wheat varieties (Singh et al., 2015). Unfortunately, there are only nine characterized stem rust resistance genes in barley; *Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, *rpg6*, *Rpg7*, *RpgBH* (recently renamed *rpg8*), and *RpgU* (Figure 1.1) (Steffenson et al., 2017; Henningsen et al., 2021). Steffenson et al. (2013) also found that TTKSK can infect over 95% of the world's barley cultivars, making it a serious global threat to the barley industry. Therefore, it was crucial to identify new sources of resistance and as many barley resistance genes as possible.

During the early 1900s, the North American Great Plains were plagued by stem rust epidemics of barley and wheat. Early deployment of resistant wheat varieties like 'Marquis' and 'Ceres' were either early maturing, which avoids stem rust damage, or bred with genetic resistances (Dyck and Kerber, 1985; Steffenson, 1992). The resistance in these lines did not hold up to the pathogen populations and gave way to the "boom-bust cycles". Powers and Hines (1933) were the first to characterize stem rust resistance in barley as a single dominant resistant gene found in the variety 'Peatland'. This gene was later designated *Resistance to Puccinia gramins 1* (*Rpg1*). Peatland was developed from a landrace imported in 1914 by the USDA from Canton Lucerne in Switzerland. Another sister selection from the Switzerland landrace that was also found to have stem rust resistance was the cv. Chevron (Shands, 1939). In 1935, a farmer named Sam Lykken, in Kindred, North Dakota, noticed one highly resistant barley plant in a field of cv. Wisconsin 37 that was heavily infected with stem rust (Lejeune, 1951). This one plant was recovered and used to develop the cv. Kindred that was released in 1942. It was hypothesized that a spontaneous mutation in a Wisconsin 37 plant gave rise to this resistance. However, through the cloning of *Rpg1* and allele analysis, it was determined that all three cvs, Chevron, Peatland, and Kindred, had

the same allele that could not have been due to a mutation of Wisconsin 37. Thus, the single plant that gave rise to Kindred was an admixture (Brueggeman et al., 2002).

Rpg1 was and is still the only stem rust resistance gene deployed in North American barley varieties and remained durable in the Midwestern and Canadian Prairie province production regions for over 70 years (Steffenson, 1992; Sun and Steffenson, 2005). This gene has been used so widely because it had broad-spectrum control over all North American races of *Pgt*, including HKHJ, HTMJ, MCCF, and TMMJ (Sun and Steffenson 2005). Several factors have led to the durability of *Rpg1*. The first is due to the eradication of common barbery in the late 1920s. This eradication process stabilized the *Pgt* population (Steffeson 1992). Furthermore, resistant wheat lines that reduced inoculum combined with early maturing barley cultivars were able to escape later season inoculum being wind disseminated from the south.

Rpg1 was first mapped to the short arm of chromosome 7H (Figure 1.1) (Kilian et al. 1994). Later, the *Rpg1* locus was delimited to 110kb, which contained two receptor kinase-like genes (RSB288 and NRG31) (Brueggeman et al., 2002). The RSB228 candidate gene was identified to be *Rpg1* due to a single recombination event within the gene. This was later confirmed when Horvath et al. (2002) transferred *Rpg1* from cv. Morex (resistant) into cv. Golden Promise (susceptible), resulting in resistant transgenic lines. *Rpg1* encodes a protein kinase with dual kinase domains (pk1 and pk2), which at the time was a unique plant disease resistance protein structure (Kleinhof et al., 2009; Case et al., 2018). A closer look at the two domains revealed that only one kinase domain (pk2) was functional. In contrast, the other domain (pk1) was found to be non-functional but still required for resistance (Kleihof et al., 2009; Nirmala et al., 2006).

In 1989, a new race of *Pgt* named QCCJB was identified in North Dakota that was virulent on *Rpg1,* causing minor epidemics on barley in the US (Roelfs et al., 1991; Steffenson 1992). This led to a new search for resistance against this exceptional race of *Pgt* which was the first to show virulence on *Rpg1*. Over 18,000 barley accessions collected from around the globe were screened for resistance. Line Q21861 was identified as the best source of resistance against *Pgt* race QCCJB (Jin et al., 1994a). Borokova et al. (1995) mapped this recessive resistance to chromosome 5H using a double haploid (DH) population derived from the resistant line Q21861 and the susceptible parent Steptoe and designated the gene *rpg4.* Q21861 also showed exceptional resistance to rye stem rust. *P. graminis* f. sp. *secali* (*Pgs*) isolate 92-MN-90. A single dominant gene conferred this resistance, designated *Rpg5,* was genetically mapped in the same population used to map *rpg4* and was shown to cosegregate with *rpg4* (Sun et al. 1996). Brueggeman et al. (2008) utilized high-resolution mapping and post-transcriptional gene silencing to identify *Rpg5,* yet the identity of *rpg4* remained elusive. It was discovered that *Rpg5* encodes a typical nucleotide binding site-leucine rich repeat (NLR) resistance gene yet contains a C-terminal serine-threonine protein kinase (S/TPK) integrated domain. Allele analysis determined that the S/TPK domain is required for pathogen recognition and resistance, and the majority of susceptible alleles contain a protein phosphatase 2C domain in place of the S/TPK. Further characterization of this locus to identify *rpg4* determined that three tightly linked genes, *HvRga1*, *Rpg5*, and *HvAdf3*, were required for *rpg4*-mediated wheat stem rust resistance (Brueggeman et al., 2008; Wang et al., 2013). Thus, *Rpg5* is required for *rpg4*-mediated resistance along with two other genes and was designated the *rpg4*/*Rpg5*-mediated resistance locus (RMRL) (Wang et al., 2013).

The RMRL provides all-stage resistance against important *Pgt* races in the Midwest, including QCCJB and MCCFC (Steffenson et al., 2017). Furthermore, in 1999, South Africa experienced an epidemic of stem rust caused by the new virulent *Pgt* race TTKSK (Ug99), which was also virulent on barley containing *Rpg1* (Sun and Steffenson 2005). Interestingly, line Q21861 containing the RMRL was the only resistance source identified as effective against TTKSK, one of the most prevalent *Pgt* races in African (Nazari et al., 2009). However, RMRL is temperature dependent, showing effective resistance at temperatures between 17-21°C but is ineffective at higher temperatures (Jin et al., 1994b), thus in hot years it is less effective.

An additional stem rust resistance gene *Rpg2* from 'Hietpas-5' (CIho 7124) (Patterson, 1951) was selected by a Wisconsin farmer, similar to *Rpg1*, from the landrace Oderbrucker (CIho 1272) (Patterson et al., 1957). *Rpg2* provides a high level of adult plant resistance (APR) to *Pgt* races QCCJB and MCCFC at the adult stage but little resistance at the seedling stage (Case et al., 2018). Bi-parental mapping was conducted using the resistant line Hietpas-5 and a susceptible line, Hiproly (PI 60693). From this population, *Rpg2* was mapped to chromosome 2H (Figure 1.1).

Barley accession GAW-79 (PI382313) was shown to have moderate to high levels of APR to *Pgt* races QCCJB and MCCFC (Case, 2018; Jedel, 1990; Sun and Steffenson, 2005). This barley accession has also been shown to mediate moderate levels of seedling resistance to *Pgs* isolate 92-MN-90, the rye stem rust pathogen that also infects barley (Steffenson et al., 2017). GAW-79 is an Ethiopian landrace, and this gene was given the *Rpg3* designation. *Rpg3* was first discovered in 1990 and was mapped to chromosome 5H (Figure 1.1) (Jedel, 1990; Case et al., 2018).

The *rpg6* gene was discovered on chromosome 6H of an interspecific barley line 212Y1 developed using an in-situ hybridization method (Fetch et al., 2009). Line 212Y1 is a doubled haploid (DH) derived from a 'Golden Promise' (*H. vulgare*) / Cb29204/4/Colch (*H. bulbosum*) cross, which was backcrossed into Golden Promise. To confirm that *rpg6* conferred resistance to stem rust, an allelism test was conducted by crossing 212Y1 and Q21861 (*Rpg1+* and *rpg4/5+*), and the F_2 progeny were inoculated with *Pgt* isolate QCCJB. The novel gene was found to possess high levels of resistance against *Pgt* isolate QCCJB, that was separate from Q21861. Furthermore, this study determined that *rpg6* is a recessive gene. This study was the first to demonstrate the transfer of a stem rust resistance gene from *H. bulbosum* into *H. vulgare*.

The most recent stem rust resistance gene identified was *Rpg7*, which was mapped to the long arm of chromosome 3H (Sallam et al., 2017; Henningsen et al., 2021). This novel gene was discovered among two wild barley (*H. vulgar* ssp. *spontaneum*) accessions (WBDC-94 and WBDC-238) from Jordan. However, whole genome shotgun sequencing of the wild barley diversity collection (WBDC) and analysis determined that WBDC-94 and WBDC-238 are nearly genetically identical. *Rpg7* has shown seedling resistance against *Pgt* pathotypes MCCFC, HKHJC, QCCJB, TTTTF, and TTKSK (Henningsen et al., 2021). The biparental mapping of *Rpg7* was conducted with a WBDC-238 x Hiproly (PI 60693) population, locating a significant QTL on chromosome 3H. This QTL showed an interval between 683.8 and 693.7 Mb on the Morex v1 genome assembly, and a top candidate gene model HORVU3Hr1G113000.3 was proposed, which is predicted to encode a leucine-rich repeat (LRR) domain.

RpgU and *rpgBH* are the last stem rust resistance genes characterized in barley identified from the lines Peatland and Black Hulless (CIho 666), respectively (Steffenson et al., 1984). *RpgU*

has not been mapped (Sun and Steffenson, 2005; Steffenson et al., 2017). However, *rpgBH*, known to confer some APR and seedling resistance to *Pgt*, was recently mapped and given the *rpg8* gene nomenclature (Brian Steffenson, personal communication).

A new diverse sexual population of *Pgt* was recently identified in Washington state (Upadhaya et al., 2022). It was observed that 99% of 100 single pustule isolates collected from the PNW population were virulent on the cultivar Morex, which contains *Rpg1*. Furthermore, 16% of the isolates were virulent on RMRL. Alarmingly, 10% of the isolates from Washington were virulent on Q21861, which contains *Rpg1* and the RMRL. This event was the first report of any isolate worldwide with virulence on *Rpg1* and RMRL when stacked together. To complicate the situation further, none of the barley grown in Washington is known to contain *Rpg1* or RMRL, so the virulent isolates evolved without *Rpg1* or RMRL exerting selection pressure on the pathogen population. This raises the interesting question of where the selection pressure is coming from which resulted in such a high proportion of diverse individuals with virulence on *Rpg1* and RMRL when the two genes are stacked together. Also, it is unsurprising that the *Rpg1* and RMRL virulence have combined in this population, considering that *Mahonia* spp. are abundant in the PNW and serve as an alternative host to complete its sexual lifecycle. This virulent population is an alarming threat to barley breeders and growers in the PNW or the Upper Midwest. Thus, the identification of new sources of resistance that are effective against the virulent isolates identified in the PNW population is necessary.

A Modern Genetic Approach for Plant Breeders

Breeders select many traits when developing a new cultivar (yield, color, height, disease resistance, etc.). The earliest form of breeding was phenotypic selection (PS); however, PS has limitations. The trait of interest needs to be highly heritable. For qualitative traits like color, one or very few genes will control the trait. Quantitative traits, also called continuous traits, have multiple genes contributing to the trait of interest, making PS more difficult. An example of quantitative traits would be plant yield. Plant breeders use what is called the breeder's equation ($R = \frac{ir\sigma_A}{t}$) to make genetic gains within a breeding program. In this equation, R is the genetic response, i is the selection intensity, σ_A is the square root of the additive genetic variance, *r* is the selection accuracy (h^2), and *t* is cycle time (Falconer and Mackay, 1996; Cobbet et al., 2019). Breeders can manipulate this equation to increase genetic gains by decreasing the denominator (*t*) or increasing a variable in the numerator. With the arrival of modern marker technology during the 1980s, breeders have been able to increase genetic gain by increasing selection accuracy while decreasing cycle time through marker-assisted selection (MAS) and genomic selection (GS) (Lande and Thompson, 1990; Meuwissen et al., 2001).

Breeders always seek new and advantageous alleles to increase genetic gain within a given breeding program. One way to discover beneficial alleles to add to a breeding program would be to conduct a Genome-Wide Association Study (GWAS) or Association Mapping (AM). GWAS tests for a significant association between the observed phenotypes and the genotypes of a large and diverse population. The first GWAS was conducted in humans for macular degeneration (Klein et al., 2005). Later, Aranzana et al. (2005) were the first to perform plant GWAS using *A. thaliana*. Currently, GWAS is common in human, animal, and plant genetic characterization studies. GWAS has also been used within and across barley breeding programs to find new loci

associated with many traits, including disease resistance (Sallam et al., 2017) and the malt quality traits kernel plumpness, friability, barley protein, wort protein, diastatic power, alpha-amylase, beta-glucan, extract, and soluble/total protein (Mohammadi et al., 2015, Looseley et al., 2020).

Prior to utilizing GWAS, genetic characterization and mapping of plant genes relied mainly on bi-parental populations. Bi-parental mapping is relied upon for high-resolution mapping if the gene of interest cannot be delimited to a small physical region in a GWAS study due to confounding issues such as multiple QTL or loci present in the population contributing to the phenotype or the gene is found in the population at very low frequency. Thus, there are advantages and disadvantages between biparental mapping and a GWAS. The downside of a GWAS is that it does not pick up low-frequency QTL within the population, which could be significant. An example of this is a GWAS study that Sallam et al. (2017) conducted with the WBDC and four *Pgt* isolates (TTKSK, QCCJB, MCCFC, and HKHJC). They discovered 45 QTL across all seven barley chromosomes. However, WBDC-94 and WBDC-238 within the collection displayed a strong HR response towards the *Pgt* isolates QCCJB, MCCFC, and HKHJC. This locus was missed because of its low frequency within the population. Later, these two lines were discovered using a biparental QTL population to carry the novel stem rust resistance gene *Rpg7* (Henningsen et al., 2021).

In comparison, a biparental population is time-consuming because parents must be identified, and the population created. Low recombination requires screening large populations for high-resolution mapping. However, bi-parental mapping may be the next step after performing a GWAS if you are interested in identifying or cloning the gene of interest. If the GWAS was unable to delimit the gene to a small physical region and/or identify a manageable number of candidate

genes, it may be required to utilize a high-resolution bi-parental mapping, which is the tried and tested method towards gene identification and positional cloning.

Once loci have been identified, validated, and tightly linked or perfect markers developed, a breeder can utilize MAS. However, high-resolution mapping and/or perfect markers for MAS are not required to be successful as long as markers that are closely linked are available. MAS is possibly one of the most straightforward molecular breeding tools. Breeders will use markers from biparental QTL or perfect markers for pre-breeding to screen large numbers of lines for traits like disease resistance. MAS is best applied to a breeding program when traits are simple/qualitative (Dekkers and Hospital, 2002; Xu and Crouch, 2008). If the trait of interest is quantitative, then MAS becomes inefficient (Kearsey and Farquhar, 1998).

Currently, one of the most advanced tools for breeders is genomic selection, which was first proposed by Muewissen et al. (2001). Unlike MAS, which uses major QTL associated with various traits of interest, GS uses a large subset, or all markers scattered across the genome to predict performance (Muewissen et al., 2001; Bernoado, 2014). The theory behind using all the QTL is that a large percentage of the variance may come from minor QTL when major genes are present and could be overlooked in MAS. The first study to use GS was in dairy cattle (*Bos taurus*) breeding (Schaeffer, 2006). The plant community soon followed with the first GS study in plants conducted in Maize (Massman et al., 2013). Today, GS has been tried on most cereal crops, and most plant breeding programs implement GS.

Genomic selection is and indirect selection method which using all genotypic and phenotypic data to predict traits of future progeny. GS is particularly good at increasing genetic

gain for complex traits with many minor genes affecting the genetic variance, like grain yield, some disease resistances, and malt quality (Massman et al., 2013; Rutkoski et al., 2015; Thorwarth et al., 2017). The first step in GS is to calculate genomic estimated breeding values (GEBV). When calculating GEBV, an extensive training population is needed with accurate phenotypic and genotypic values. This training population is then used to make statistical models that estimate the allele effect at all loci. Another advantage of using GS is its use in early generations to avoid time-consuming and expensive phenotyping (Heffner et al., 2010). Genomic selection has been implemented in barley breeding programs to predict agronomic and end-use malt quality traits in spring and winter barley (Schmidt et al., 2015; Thorwarth et al., 2017; Charmet et al., 2023). These predictive ability accuracies range from 0.14 to 0.80, depending on the trait.

Climate Impact

Across the world, the average temperature has been rising steadily (WMO, 2024). According to records, 2023 was reported as the warmest year in 174 years. As the climate changes, breeders face new challenges in breeding for traits like drought stress, heat tolerance, salinity, and diseases. Furthermore, research shows drought and heat stress can negatively impact malt quality traits like protein content, Grain weight, DP, and AA (Mahalingam et al., 2017; Ye et al., 2020). For malting barley, high growing temperatures can lead to elevated β-glucans (Anker-Nilssen et al., 2008). Elevated temperatures can also increase protein content, reduce starch content, and lower alcohol levels in the final product (Pettersoson and Erckersten, 2007). Research over the last decade predicts global yield loss in barley, ranging from 3 to 17 %, affecting growers, maltsters, brewers, and consumers (Xie et al., 2018).

In Washington, a problem for growers is that recent trends have shown rising average temperatures and unreliable precipitation events. In 2021, average barley yields were reduced by half because of severe drought conditions (USDA-FAS 2022), a complete change of weather pattern from the spring of 2020. The changing and unpredictable environmental conditions also result in more significant variability of disease pressure from year to year. With predicted warmer and wetter springs, fungal infections are expected to start earlier in the growing season, increasing the chances for secondary infections and epidemics (Launay et al., 2014). Prank et al. (2019) determined that *Pgt* inoculum could increase by 40% as temperatures and spring precipitation increase. Over the past two decades, the prevalence of stem rust on barley and club wheat has become much higher in the PNW (Upadhaya et al., 2023a). This region of the US typically did not experience stem rust epidemics because of the dry summers until the last decade. To further strain this problem, our recent characterization of the PNW stem rust population shows the evolution of highly virulent isolates on deployed barley resistance genes that will threaten barley production during years with environmental conditions conducive to disease development.

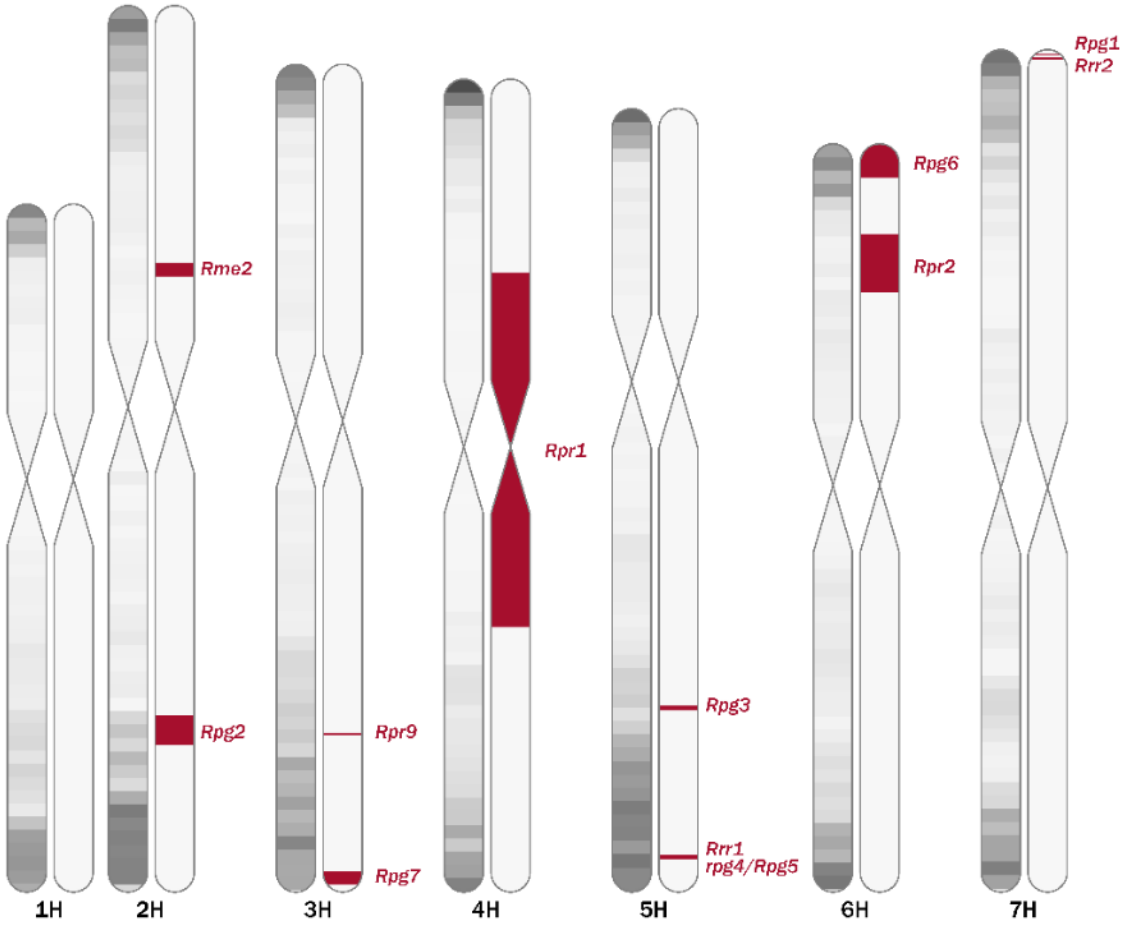

Figure 1.1: The left chromosome in gray each pair depicts the density of Illumnina 50k iSelect SNP markers across the barley (*Hordeum vulgar* L.) genome. The right chromosome in red shows the location of the mapped barley stem rust resistance genes effective against *Puccinia graminis* f. sp. *tritici.*

CHAPTER TWO: QTL ANALYSIS UNVEILS NOVEL SEEDLING RESISTANCE LOCI TO STEM RUST IDENTIFIED FROM THE WORLD BARLEY CORE COLLECTION UTILIZING A BI-PARENTAL RIL POPULATION

Abstract

Wheat stem rust caused by the obligate biotrophic fungal pathogen *Puccinia graminis* f. sp. *tritici* (*Pgt*) is an important disease of barley and wheat worldwide. The Pacific Northwest (PNW) region serves as a center of stem rust diversity in North America due to the completion of its sexual cycle in primary hosts, barley, wheat, and wild grass species (i.e., Elymus), and secondary sexual stage hosts mahonia species and common barberry. There are only a handful of stem rust resistance genes in barley that have been characterized. As stem rust becomes an issue in the PNW, it is crucial to continually characterize and map new R-genes. Alarmingly, the most virulent *Pgt* population on barley was recently discovered in the PNW. From this *Pgt* population, isolate Lsp21 was virulent on *Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, and *rpg8*, but most alarmingly is the virulence on *Rpg1* and the *rpg4*/*Rpg5*-mediated resistance locus (RMRL), when stacked together. This virulence on *Rpg1* and RMRL when combined is unprecedented *Pgt* virulence on barley that had not been previously reported. The line Elliot (PI 592261) was identified from the world barley core collection (WBCC) as containing effective seedling resistance to *Pgt* isolate Lsp21. To genetically characterize this resistance present in Elliot, 129 recombinant inbred lines (RILs) were developed by crossing Elliot (resistant) and Palmer (susceptible) and advancing the population to the $F_{2:6}$ generation by single-seed descent. The RIL population was phenotyped with *Pgt* isolate Lsp21 and genotyped with the Illumina 50K bead express SNP chip, resulting in 7,284 high-quality SNP markers that were used in the QTL analysis. This QTL mapping analysis identified two

significant resistance QTL (EP*Rpg*_4H-1 and EP*Rpg*_5H-1) contributed by Elliot on chromosomes 4H (LOD score of 9.17) and 5H (LOD score of 5.4) at the seedling stage. EP*Rpg*_4H-1 is a novel resistance QTL, while EP*Rpg*_5H-1 mapped to a region of the barley genome known to contain stem rust resistance loci which are near (~6 Mb distal) but distinct from RMRL. These QTL should be useful in developing barley cultivars with resistance to the Pgt virulent population.

Introduction

Stem rust caused by the biotrophic fungal pathogen *Puccinia graminis* f. sp. *tritici* (*Pgt*) induces significant yield loss in barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) when susceptible varieties are grown under conditions conducive to epidemic formation (Roelfs, 1978; Steffenson, 1992; Bhavani et al., 2022). Fungal spores present in grain bins from 10,000 years ago indicate that stem rust epidemics may have been a concern for barley and wheat crops dating back to the time of early crop domestication and the dawn of civilization in the fertile crescent region (Kislev, 1982). Early scientists recognized the importance of environmental conditions for epidemics to occur as Aristotle had described that the "warm vapors" (a warm, humid environment) bring on rust epidemics (Chester, 1946). The Romans believed that Robigo, the god of rust, could be appeased by sacrifices of red animals to save their crops from the scourge of rust epidemics (Chester, 1946). Alas, historical accounts determined that the cereal rusts, including stem rust, caused severe epidemics of barley and wheat that contributed to the demise of the Roman empire (Schumann and Leonard, 2000). During the 19th and early 20th century, prior to the deployment of genetic resistances in barley and wheat, severe epidemics led to complete crop failure in regions of the midwestern United States and Prairie provinces of Canada (Roelfs, 1982). Fortunately, deploying genetic resistances in barley and wheat effectively manages the disease, and severe epidemics had become a problem of the past (Roelfs, 1982; Steffenson, 1992). However, the recent identification of new isolates and races in Africa, Europe, and now North America with virulence on essential deployed resistance genes warrants concern that major epidemics could occur (Singh et al., 2015; Bhattacharya, 2017; Upadhaya et al., 2022). Thus, new resistance genes and combinations of genes must be deployed. Furthermore, *Pgt* population

dynamics must be characterized and monitored for isolates with the potential to overcome deployed *R*-genes.

Puccinia graminis f. sp. *tritici* is a heteroecious obligate fungal parasite that completes its lifecycle on two unrelated hosts (monocot grasses and dicots) and needs living host tissue to survive (Roelfs, 1985). The cereal crops barley, wheat, and wild grasses serve as primary *Pgt* hosts. The two known alternate hosts are common barberry (*Berberis vulgaris*) and mahonia (*Mahonia aquifolium),* also known as Oregon grape (Leonard and Szabo, 2005). The alternate hosts allow for the completion of the *Pgt* sexual cycle, where genetic recombination of virulence loci containing effector genes allows for new virulence specificities to arise. Additionally, the aeciospores coming from the secondary dicot hosts can serve as early-season barley and wheat primary host inoculum, which can start early-season polycyclic infections, resulting in epidemic formation under environments conducive for the disease. Thus, eradication programs aimed at eliminating the alternate hosts, such as the barberry eradication program, eliminate the *Pgt* sexual cycle (Roelfs, 1982), effectively stabilizing *Pgt* populations and making the deployment of genetic resistance more effective and last longer. Fortunately, stem rust disease management strategies, including fungicide application, eradicating the alternate host barberry, and deploying effective resistance genes (R-genes), have made managing this potentially devastating disease possible.

Currently, only eight *resistance to Puccinia graminis* (*Rpg*) *R*-genes have been characterized in barley (*Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, *rpg6*, *Rpg7*, and *Rpg8* (Figure 2.1) (Steffenson et al., 2017; Henningsen et al., 2021). In contrast, nearly 60 stem rust (*Sr*) resistance genes have been characterized in wheat (Hafeez et al., 2021). Alarmingly, *Rpg1* is the only known stem rust *R*-gene currently deployed in commercial barley cultivars in North America. The

exclusive reliance on this remarkably durable *R*-gene in Upper Midwestern and Canadian Prairie Province of Canada barley varieties has made it the best-characterized stem rust R-gene in barley. For over 70 years, varieties have not been released for these growing regions without this source of resistance (Steffenson, 1992; Sun & Steffenson, 2005). The genetic characterization of *Rpg1* localized this gene to the long arm of chromosome 7H, and it was the first barley stem rust resistance gene identified via a map-based cloning strategy (Kilian et al., 1994; Brueggeman et al., 2002). Horvath et al. (2003) also confirmed the identification of *Rpg1* through the stable *Agrobacterium*-mediated stable transformation of the variety Golden Promise. *Rpg1* encodes a protein kinase with dual kinase domains (pk1 and pk2) (Brueggeman et al., 2002). Functional characterization of the *Rpg1* protein determined that only one kinase domain (pk2) was a functional protein kinase, yet the other non-functional pseudo-kinase domain (pk1) was still required for resistance (Kleinhofs et al., 2009; Nirmala et al., 2006). This resistance protein domain structure was a unique plant disease resistance gene architecture at the time it was cloned (Case et al., 2018; Kleinhof et al., 2009). However, with the further identification of resistance genes, it has become a more commonly identified R-gene protein domain structure (Kroj et al., 2016; Sarris et al., 2016).

Although the *Rpg1* gene conferred remarkably durable resistance to all known North American *Pgt* isolates and races for over 50 years, it was inevitable that new isolates that are virulent on this single deployed R-gene would eventually evolve. In 1989, a new race of *Pgt* typed as QCC on the wheat R-gene differential set, later designated QCCJB, was found to be virulent on barley cultivars containing *Rpg1* (Roelfs et al., 1991; Steffenson, 1992; Jin et al., 2008). *Puccinia graminis* f. sp. *tritici* race QCCJB quickly became the dominant *Pgt* isolate in the Midwestern US, threatening commercial barley production in the region (Roelfs et al., 1993). To identify resistance

to *Pgt* race QCCJB, more than 18,000 barley accessions were evaluated, and the unimproved line Q21861 from the International Maize and Wheat Improvement Center (CIMMYT) was identified with effective resistance to *Pgt* race QCCJB designated *rpg4* (Jin et al., 1994a). Interestingly, *rpg4*-mediated resistance was determined to be both recessive and temperature-sensitive (Jin et al., 1994b; Borokova et al., 1995). Borokova et al. (1995) mapped *rpg4* to the long arm of chromosome 5H in a doubled haploid (DH) population derived from a cross of the resistant line Q21861 and the susceptible parent Steptoe using random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) markers.

Barley line Q21861 also contains the R-gene *Rpg5*, which is a single dominant gene that confers resistance to rye stem rust *P. graminis* f. sp. *secalis*, which colocalized with the *rpg4* wheat stem rust resistance gene (Steffenson, 1992). Using a high-resolution mapping approach, Brueggeman et al. (2008) mapped *Rpg5* to the long arm of chromosome 5H within a 70 kb interval. Post-transcriptional gene silencing via barley stripe mosaic virus-virus induced gene silencing (BSMV-VIGS) was used to validate *Rpg5*, which encodes a typical nucleotide binding site-leucine rich repeat (NLR) resistance protein domain structure yet contains a C-terminal serine-threonine protein kinase S/TPK integrated domain. Allele analysis determined that the S/TPK is required for pathogen recognition and resistance, and the majority of susceptible alleles contain a protein phosphatase 2C domain in place of the S/TPK domain (Brueggeman et al., 2008). Further characterization of this locus via BSMV-VIGS mediated post-transcriptional gene silencing identified three tightly linked genes, *HvRga1*, *Rpg5*, and *HvAdf3*, which were all shown to be required for *rpg4*-mediated resistance against *Pgt* race QCCJB and was designated as the *rpg4*/*Rpg5*-mediated resistance locus (RMRL) (Wang et al. 2013). The RMRL gene provides all-

stage resistance against important local *Pgt* races, including QCCJB and MCCFC (Steffenson et al., 2017).

Interestingly, the RMRL locus was also the only known resistance in barley to *Pgt* race TTKSK, one of Africa's most prevalent *Pgt* races (Nazari et al., 2009). TTKSK was found to be virulent on 80% of the world's wheat populations and can infect over 95% of the world's barley cultivars, making it a serious global threat to the barley and wheat industry (Sigh et al., 2007; Steffenson et al., 2013). The RMRL provides broad and effective resistance against diverse *Pgt* races. However, the RMRL is temperature sensitive, providing effective resistances at temperatures between 17-21 °C, but is less effective at higher temperatures (Jin et al., 1994b). Interestingly, the barley line Q21861 containing *Rpg1* and RMRL stacked together provided effective seedling and adult plant resistance against all known stem rust isolates when high temperatures were not a factor (Jin et al., 1994 a). Thus, there was considerable effort in North American breeding programs to stack these two genes together. However, it was determined that when both genes are stacked together in some genetic backgrounds, they both became ineffective, suggesting some functional suppression can occur in the *Rpg1* and RMRL resistance pathways when both genes were present (Sharma Poudel et al., 2018). It was determined by genetic mapping that the *Rrr1* gene must be present for both genes to retain their resistance function when stacked together, such as in the case of Q21861 (Sharma Poudel et al., 2018).

Interestingly, a mutant gene *rpr8* was also identified that inactivates both *Rpg1* and RMRL resistance specificities (Solanki et al., 2019), further suggesting functional redundancy in these two resistance mechanisms. Therefore, it is crucial to identify new sources of resistance as limited stem rust resistance sources have been identified from barley, suggesting that many resistance

genes in barley may be undiscovered. Another hypothesis is that barley is a more recent host of wheat stem rust and thus has not experienced a long evolutionary history of the host-pathogen molecular arms race, and few race-specific resistance genes have evolved in barley, as proposed by Solanki et al. (2019).

The Pacific Northwest (PNW) region serves as a center of *Pgt* diversity in North America due to the completion of the sexual cycle in the primary hosts barley, wheat, and wild grass Elymus and secondary sexual hosts mahonia and common barberry (Upadhaya et al., 2022). An extensive collection of *Pgt* isolates (n = 200) from Eastern Washington and Northern Idaho was generated from the primary cereal hosts barley and wheat and secondary sexual hosts mahonia and common barberry in 2019 and 2020 (Upadhaya et al., 2022). Seedling assays on barley resistance gene differentials showed that 99% of the isolates (n = 100) collected from barley were virulent on the barley stem rust R-gene *Rpg1*, 16% were virulent on the RMRL, and 10% were virulent on both genes when combined in the barley line Q21861. This virulence on the two broad and effective stem rust R-genes, when stacked together, was unprecedented. *Pgt* virulence on barley had never been reported from any isolate collected worldwide. Thus, in regard to barley, the PNW population is the most virulent population ever reported. This finding is puzzling because neither *Rpg1* nor RMRL had ever been deployed in PNW barley varieties, raising the question of why this virulence evolved in the region and became so predominant in the population.

One hypothesis is that virulence evolved, and selection occurred in the natural ecosystem in the life cycle between wild grasses and Mahonia. This sexual population has a high level of diversity due to its ability to recombine and then propagate on the secondary host. Upadhaya (2023) revealed through whole genome sequencing and phylogenetic analysis of 100 isolates from

a PNW population and isolates from the midwestern US that *Pgt* race QCCJB originated in the PNW. Thus, *Pgt* races such as QCCJB that evolved in the PNW with virulence on resistance genes deployed in midwestern barley and wheat varieties can be disseminated over the Rocky Mountains and become established as predominant races in the Midwestern US and Prairie Provinces of Canada. The *Pgt* race QCCJB became the predominant race in the Midwest due to susceptible wheat and barley cultivars, which was the result of the founder effect, where a small population of *Pgt* broke off from the larger population in the PNW (Mayr, 1942). Unlike the situation in the midwestern US, where the introduced common barberry bushes could be eradicated to stabilize the *Pgt* population, mahonia is endemic to the woodland areas of the PNW and impossible to eradicate. Thus, the PNW region will remain a center of stem rust diversity in North America and a hotspot for the evolution of new virulent races. This situation is exacerbated by rising temperatures providing an environment more conducive to stem rust epidemic formation, and mahonia growing near barley and wheat fields can serve as early-season inoculum.

To identify novel sources of stem rust resistance effective against this virulent PNW population, a subset of 488 lines from the world barley core collection representing the genetic diversity present in the complete collection was screened for seedling resistance using the PNW *Pgt* isolate Lsp21, which is virulent on line Q21861 (Upadhaya, 2023). From the stem rust assay, approximately 80 of the 488 lines were identified with some levels of seedling resistance against Lsp21. One of the most effective resistance sources of seedling resistance was identified in the heritage malt barley cultivar Elliot (PI 592261) from Scotland. Elliot was crossed with the susceptible malt barley variety Palmer from the Washington State University breeding program to develop a biparental RIL population to genetically characterize the Elliot resistance.

Material and Methods

Plant materials and pathogen isolates

To genetically characterize the seedling resistance present in Elliot (PI 592261), a biparental recombinant inbred line (RIL) population was developed from an Elliot × Palmer cross via single seed descent to perform quantitative trait locus (QTL) analysis. Elliot is a two-row malting variety developed in the United Kingdom derived from a Trumpf × Hassan cross in 1993 (GRIN). Palmer is a two-rowed malt barley variety released by Washington State University. Palmer has no known R-genes to *Pgt*. From the F_2 generation, single seed descent was utilized to develop an $F_{2:6}$ RIL population of 129 individuals. Parental line Elliot and Oregon State University (OSU) line DH-160748 were used as resistant checks. Morex, Q21861, Palmer, and Steptoe were used as susceptible checks.

The *Pgt* isolate Lsp21 was selected for the biparental analysis because of its virulence on all characterized barley wheat stem rust R-genes (Upadhaya et al., 2022) except *rpg6,* which was not available in the analysis. Lsp21 was collected from the barley variety Lyon in 2019 in Valleyford, WA. *Pgt* isolate Lsp21 is virulent on barley cv Morex (*Rpg1+*), CIho 7124 (*Rpg2+*), PI282313 (*Rpg3+*), HQ1 (*rpg4*/*Rpg5+*), Q21861 (*Rpg1+* and *rpg4*/Rpg5+), and Blackhulless (*rpg8+*) (Supplementary Table S1). The virulence profile on barley *R*-genes represented the most virulent isolates of *Pgt* on barley ever reported.

Plant growth conditions for greenhouse assays

For F_1, F_2, and F_6 seedling resistance analysis, the biparental population was grown in cone containers (6.5 cm diameter by 26.5 cm height). Each cone was filled with a standard soil potting mix (Sun Gro Horticulture, Agawam, MA, USA) supplemented with Osmacote 14-14-14 slow-release fertilizer at a rate of 2 gm per cone. Plants were placed in a growth chamber set to 18°C with a 16 h light (400 $\mu m/m^2$) and 8 h dark cycle as described by Upadhaya et al. (2022). Each F_6 RIL within the population was replicated three times in a randomized complete block design. Three independent replications of the phenotyping experiment were performed.

Seedling and adult stem rust tests

Approximately nine days after planting, when primary leaves were fully expanded, stem rust inoculations were conducted using an atomizer pressured by a pump set at 30kPa (Steffenson et al., 2017). Seedlings were inoculated with fresh urediniospores from *Pgt* isolate Lsp21 and mineral oil at 8 mg of urediniospores per 1 ml of mineral oil. After inoculation, plant leaves were allowed to dry for one hour and placed in a mist chamber for 18 h in complete darkness at 18°C and 100% relative humidity. After 18 h, plants were placed back in the growth chamber at the conditions mentioned previously.

At 14 days after inoculation (DAI), infection types (IT) were assessed on primary leaves. Infections were rated on a modified "0 to 4" scale. This scale was initially developed by Stakman et al. (1962) for wheat, later modified for barley by Miller and Lambert (1955), and further modified by Steffenson et al. (2017). When conducting stem rust analysis on barley, mesothetic reactions of different ITs on the same primary leaf can be observed. These IT values were categorized as 0; = hypersensitive reaction (HR), 1 = Resistance (R), 2 = moderately resistant

(MR), 3- = moderately susceptible (MS), and anything above 3 was considered susceptible (S) (Table 2.1) (Steffenson et al., 2017; Hernandez et al., 2019). To get a more accurate estimation of pustule size, + or – symbols were used after an IT. The categorical IT score of "0 to 4" was converted into a numeric coefficient of infection (CI) value representing quantitative scores of 0 to 5 as described by Zhou et al. (2014). CI values ≤ 2.80 were used to categorize genotypes as resistant, and CI values > 2.80 were used to categorize genotypes as susceptible (Hernandez et al., 2019). If multiple ITs were observed on a single leaf, the CI was calculated by order of their frequency using a weighted average (Zhou et al., 2014).

Tissue collection and genotyping

Tissue from the primary leaf of each $F_{2:6}$ RIL individual and the parents were collected ten days after planting. Approximately 1.5" leaf tissue samples were collected and placed into 96-well plates containing roughly 0.5 g of silica and sent to the USDA cereal genotyping lab in Fargo, ND. There, the population was genotyped as described by Muñoz-Amatriaín et al. (2014) using the 50k Illumina Infinium iSelect SNP chip. SNPs were called using GenomeStudio software (Illumina, San Diego, CA).

Genotypic data curation

The 50k Illumina Infinium iSelect SNP marker genomic positions were updated from the Morex v1 assembly positions to the Morex v3 assembly positions using the Barley T3 database (https://barley.triticeaetoolbox.org/). Markers with >30% missing data were removed prior to imputation using beagle-5.2 (Browning et al., 2018). Following imputation, monomorphic SNPs

were removed. A total of 7,284 high-quality polymorphic markers were used in the QTL analysis (Table 2.2).

QTL mapping and candidate gene identification

The genotype and phenotype data were utilized to construct a genetic mapping using MapDisto v2.1.8. (Lorieux, 2012). Using this software, seven linkage groups were found equal to the seven chromosomes found in barley. Loci were ordered within each linkage group using the Seriation algorithm. The loci order was further refined using the auto ripple function and checked for inversions. Lastly, the bootstrap order was used to test for the stability of the loci order. The constructed map was then added to QGene4.4.0 (Heffelfinger et al., 2017) software to identify seedling resistance loci. This software used composite interval mapping (CIM). CIM performed a QTL test every 15 cM to indicate significant loci, and 1000 permutations were then run to calculate the significant LOD threshold at $\alpha = 0.05$. Significant markers underlying QTL regions were blasted to the current barley genome assembly, Barley_Morex_V3_ psudomolecules_2021, using the GrainGene database (https://wheat.pw.usda.gov/GG3/) (Mascher et al., 2021). Non-significant markers flanking significant QTL were used to delimit the physical region containing candidate genes. High-confidence genes within the delimited QTL regions were considered candidate genes associated with the stem rust QTL and prioritized based on predicted gene functions. The nomenclature for a QTL is as follows: (EP*Rpg*_1H-1) E = Elliot; P = Palmer; *Rpg* = *Resistance to Puccina graminis*; followed by chromosome designation and number of MTA.

Results

Isolate Lsp21 of *Pgt* was utilized to phenotype the Elliot and Palmer parental lines, the Elliot × Palmer F_1 and F_2 individuals, and the $F_{2:6}$ RIL population (Table 2.3). The parental line Elliot was resistant, with CI values ranging from 1.9 to 2.4, and Palmer was susceptible, with CI values ranging from 3.6 to 3.875. All F_1 individuals were moderately susceptible, with an average CI score of 3.46 (Figure 2.2). This suggests the predominant function of dominant susceptibility genes putatively contributed by the susceptible parental line Palmer. From the 98 F_2 individuals phenotyped, 10 were resistant (R), 11 were moderately resistant (MR), 63 were moderately susceptible (MS), and 14 were susceptible (S), further suggesting the partially dominant susceptibility gene function. CI values in the F_2 ranged from 1.63 to 4.00, with a mean of 3.35. Phenotyping of the 129 Elliot × Palmer $F_{2:6}$ RIL individuals resulted in CI values ranging from 2.1 to 3.6 with a mean of 3.0. Of the 129 RILs assayed, 44 exhibited a resistance response to *Pgt* isolate Lsp21, which was skewed toward susceptibility, further suggesting that some of the QTL present may be recessive resistances conferred by Elliot alleles or more accurately dominant susceptibility genes expressed from Palmer alleles. The F_2 and RIL individuals resulted in a normal distribution, suggesting multiple loci segregating in the population and contributing to the resistance/susceptible interactions (Figure 2.3). Thus, the transgressive segregation observed in the F_2 and RIL populations was not unexpected.

Genotyping utilizing the 50k Illumina Infinium iSelect SNP marker panel identified 7,284 high-quality polymorphic markers utilized in the QTL analysis. The analysis identified two significant QTL present on chromosomes 4H (EP*Rpg*_4H-1) and 5H (EP*Rpg*_5H-1) (Figure 2.4). The most significant marker (JHI-Hv50k-2016-262517) ($\alpha = 0.01$) is located in the EP*Rpg*_4H-1

QTL with the physical position of 572,601,355 using the Morex v3 genome assembly (Table 2.5). JHI-Hv50k-2016-262517 accounted for 28% of the phenotypic variation with a LOD score of 9.17. This SNP is present within the annotated high-confidence gene HORVU.MOREX.r3.4HG0406000, which is predicted to encode an alpha/beta-hydrolase superfamily protein. Non-significant flanking markers delimiting the EP*Rpg*_4H-1 are SCRI_RS_233444 and SCRI_RS_157760, located at physical positions 527,470,876 and 576,157,883, respectively. Thus, the resistance locus EP*Rpg*_4H-1 is delimited to a large 49 Mbp region that contains 108 predicted high-confidence gene models representing the candidate genes underlying this locus.

The second significant QTL (EP*Rpg*_5H-1) is located on chromosome 5H. The most significant marker at EP*Rpg*_5H-1 is JHI-Hv50k-2016-358636 ($\alpha = 0.05$), located at the physical position 573,092,342. JHI-Hv50k-2016-358636 accounted for 17.6% of the phenotypic variation with a LOD score of 5.414. Using the Morex v3 genome assembly, the JHI-Hv50k-2016-358636 SNP is present within the annotated high-confidence gene HORVU.MOREX.r3.5HG0530720, which is predicted to encode a ligase-like protein. Flanking non-significant markers 12_21290 and 12_30360, located at the physical positions 569,401,806 and 583,373,814, respectively, delimit the EP*Rpg*_5H-1 locus to a large 140Mbp region containing 722 predicted high confidence gene models.

Discussion

A recently characterized sexual population of *Pgt* collected in Washington state was shown to be the most virulent population of barley worldwide (Upadhaya et al., 2022). This was

determined by 99% of isolates in the population being virulent on the cultivar Morex, which contains *Rpg1*, 16% with virulence on barley line HQ1 containing the *rpg4*/*5*-mediated resistance locus (RMRL), and 10% with virulence on the line Q21861, which contains both *Rpg1* and the RMRL. This virulence leaves barley without known effective resistance genes against this PNW population. Additionally, isolates in the population are also virulent on *Rpg2*, *Rpg3*, and *rpg8*. The evolution of virulence and selection that essentially fixed the *Rpg1* virulence in the PNW population and the high proportion of isolates virulent on RMRL is perplexing, considering that no barley varieties grown in Washington contain *Rpg1* or RMRL. Thus, another source of selection for virulence on these genes must be exerting pressure on the pathogen population. One hypothesis is that selection occurs in the natural ecosystem during the completion of the sexual cycle between wild grasses and *Mahonia* spp. Because *Mahonia* spp. are abundant in the PNW, serving as an alternative host to complete its sexual lifecycle, it is not surprising that the *Rpg1* and RMRL virulence loci have recombined, giving rise to the first isolates ever reported with virulence on *Rpg1* and RMRL when stacked together. These isolates pose an alarming threat to the major barley production regions of North America, which includes the PNW, intermountain regions of the west, the upper midwestern US, and the Prairie provinces of Canada.

However, phenotype analysis of Elliot x Palmer F_1 individuals resulted in susceptibility, and F_2 individuals produced a continuous distribution skewed towards susceptibility, suggesting quantitative recessive resistance genes in Elliot or, more accurately, dominant susceptibility loci contributed by Palmer. The phenotypic distribution of the Elliot x Palmer RIL (F_6) population also showed a continuous distribution. Thus, further supporting the hypothesis that multiple genes contribute to the resistance/susceptibility, and perhaps epistasis among different parental loci. The

focus of the research reported here was to genetically characterize the resistances found in Elliot or susceptibility loci in Palmer at the seedling stage using a biparental mapping population for QTL analysis.

Phenotyping and genotyping of the Elliot x Palmer RIL population followed by QTL analysis identified two significant QTL, EP*Rpg*_4H-1 and EP*Rpg*_5H-1, conferring resistance from Elliot located on chromosomes 4H and 5H. Disease resistance to *Pgt* isolate Lsp21 mapped to a 49 Mbp region on chromosome 4H and a 140 Mbp region on chromosome 5H based on the Morex v3 genome assembly. There have been reports of stripe rust and leaf rust QTL within the EP*Rpg*_4H-1 region identified on chromosome 4H (Hickey et al., 2011; Sandhu et al., 2012; Vatter et al., 2017; Belcher et al., 2018; Dracots et al., 2019; Bettgenhaesuer et al., 2021; Gyawali et al., 2021). Gyawali et al. (2021) reported QTL on chromosome 4H (QPsh-r24-6R-5.2, QPsh-DP-2R-5.3, QPsh-rG-6R-5.2, and QPsh-rG-2R-5.1) that were mapped for stripe rust resistance between markers SCRI_RS_140499 and 11_10736 using GWAS. However, these might be considered one QTL because they are confined to a 4 Mbp region, and no flanking non-significant markers were reported. On chromosome 4H, Mamo et al. (2015) mapped a resistance QTL to Fusarium head blight (FHB) using a GWAS of barley landraces from Ethiopia and Eritrea. The markers identified in their regions were SCRI_RS_148392 and SCRI_RS_157650. However, no stem rust-resistance QTL has been reported in the region, making EP*Rpg*_4H-1 a novel stem rust-resistance locus.

The closest stem rust resistance QTL that had been reported near EP*Rpg*_4H-1 on chromosome 4H are Turuspekov_QTL4H-1, Turuspekov_QTL4-2, and Turuspekov_QTL4-3 (Turspekov et al., 2016) identified in a GWAS study. The 2 QTL (Turuspekov_QTL4H-1, Turuspekov_QTL4-2) are located roughly 13 Mbp proximal and 3 Mbp distal to Elliot's non-

significant flanking markers on 4H (SCRI_RS_233444 and SCRI_RS_157760). However, non-significant markers were not reported, and these three-stem rust QTL could be considered one QTL based on their relative distance from one another. Other seedling and adult QTL for plant resistance to stem rust have also been reported on chromosome 4H (Zhang, 2006; Mamo et al., 2013; Sallam et al., 2017; Case et al., 2018; and Czembor et al., 2022). However, none of these QTL are in the region as the Elliot QTL, EP*Rpg*_4H-1, on 4H. In addition, other QTL have been mapped to EP*Rpg*_4H-1 for waterlogging tolerance and net blotch in wild barley (Vatter et al., 2017; Borrego-Benjumea et al., 2021).

The second most significant QTL, EP*Rpg*_5H-1, mapped to a 140Mbp region on chromosome 5H. The important stem rust resistance genes *rpg4* and *Rpg5*, also known as RMRL, are close to this region of the barley genome (Brueggeman et al., 2008). However, RMRL is located approximately 6 Mbps proximal from EP*Rpg*_5H-1 based on the delimiting non-significant markers 12_21290 and 12_30360. Additionally, multiple other stem rust resistances and specificities have been mapped to the region that has been shown to be distinct from RMRL (Mamo et al., 2014; Sallam et al., 2017; Case et al., 2018; Hernandez et al., 2019). An *Rpg1* homolog, designated ABC1041, is located close to EP*Rpg*_5H-1 (Brueggeman et al., 2006). However, the delimiting non-significant markers (12_21290 and 12_30360) suggest that ABC1041 is approximately 6 Mbps proximal from EP*Rpg*_5H-1. Mamo et al. (2014) and Case et al. (2018) mapped stem rust-resistant QTL to 5H (Mamo_QTL5H-1 and Rpg-qtl-5H-11_10236) that overlap with EP*Rpg*_5H-1, suggesting that these loci could contain the same gene or alleles of the same resistance/susceptibility gene. Furthermore, other stripe rust genes overlap with EP*Rpg*_5H-1 (Dracatos et al., 2015; Clare et al., 2016; Belcher et al., 2018; and Gaywaili et al.,

2021). The data suggests that EP*Rpg*_5H-1 may not be novel but falls into an essential region of the barley genome containing an important stem rust resistance gene, genes, or alleles with broad resistance or several stem rust resistance specificities.

Conclusion

Common barberry has been effectively removed from the Midwest through the barberry eradication program of the early 1900s (Roelfs, 1982). Thus, the stabilization of *Pgt* races present in the Midwestern US and Prairie provinces of Canada contributed to the durability of the *Rpg1* resistance gene that effectively protected Midwestern barley varieties for over 50 years. However, the PNW region is now recognized as a center of *Pgt* diversity in North America due to the completion of the sexual cycle on the secondary dicot host mahonia, as mahonia is native and abundant in the region. Completing the lifecycle allows new races of *Pgt* to arise and overcome disease resistance within wheat and barley. It is not unreasonable to think of a virulent isolate crossing the Rocky Mountains and threatening barley and wheat production in the Midwest. For the US to continue producing high-quality malt barley, resistance to the new *Pgt* isolate population will be necessary. Thus, monitoring the population for new virulences evolving on deployed wheat *Sr* genes and identifying new effective sources of resistance to the population in barley is imperative. Screening the whole WBCC and the Wild Barley Diversity Panel will be necessary to continually discover novel resistance genes against the *Pgt* population in the PNW and deploy effective resistances in barley varieties to prevent future stem rust epidemics.

Table 2.1: Physical description and categorization of infection types (ITs) and coefficients of infection (CI) against stem rust.

IT	CI	Description	Categorized
0	0	No visible sign of infection	
0;	0.5	Hypersensitive flecks, but no uredinia	Hypersensitive Response (HR)
1	2	Miniature uredinia	Resistant (R)
2	3	Small, restricted uredinia	Moderately Resistant (MR)
3-	3.5	Medium-sized uredinia with some restriction	Moderately Susceptible (MS)
3	4	Large uredinia showing no restriction	Susceptible (S)
3+	4.5	Extremely large uredinia	Susceptible (S)
4	5	Extremely large uredinia showing no restriction	Susceptible (S)

Table 2.2: The marker density throughout the barley genome using the Illumina 50k SNP array to map the resistance found in the biparental mapping population Elliot × Palmer.

Chromosome	Number of Markers	Distance (Mbp)	Distance (cM)	Average Marker Density (Mbp)	Average Marker Density (cM)
1H	774	557.9	124	0.72	0.16
2H	1227	665.3	140	0.54	0.11
3H	1301	618.3	138	0.48	0.10
4H	587	604.9	102	1.03	0.17
5H	1344	580.6	170	0.44	0.13
6H	1037	561.7	96	0.54	0.09
7H	1024	632.1	98	0.62	0.10
Total	7,284				

Table 2.3: The number of lines screened and reaction to the PNW *Puccinia graminis* f. sp. *tritici* isolate Lsp21 in the F_1, F_2 and F_6 generations of the Elliot × Palmer population.

Population	Filial Generation	Total Number	R	MR	MS	S
Palmer × Elliot	F_1	9	-	-	9	-
	F_2	98	10	11	63	14
	F_6 (RILs)	129	44	41	38	6

Table 2.4: Average infection types (IT) and coefficients of infection (CI) score on known resistance genes, parental lines, and checks for the biparental population Elliot × Palmer.

Cultivar	Gene	Mode	IT	CI
Morex	*Rpg1*	MS	3-32	3.4
Q21861	*rpg4*/*Rpg5*	MS	3-	3.5
Steptoe	-	S	33-2	3.75
Palmer	-	S	33-2	3.75
Elliot	Unknown	R	21	2.75

Table 2.5 Summary of SNP markers significantly associated with the resistance responses to *Puccinia graminis* f. sp. *tritici* isolate Lsp21 at the seedling stage in the Elliot x Palmer F_6 RIL population.

Chrom[a]	Marker	Position[b] (Mbp)	Distance (cM)	LOD[c]	Variance (%)	Additive effect
4H	JHI-Hv50k-2016-262494	572.55	67.7	9.08***	27.7	-0.113
	JHI-Hv50k-2016-262517	572.60	68.1	9.17***	27.9	-0.113
	JHI-Hv50k-2016-262937	576.15	71.1	7.70***	24.0	-0.104
5H	JHI-Hv50k-2016-358239	571.75	158.2	4.448*	14.7	-0.077
	JHI-Hv50k-2016-358385	573.09	159.1	5.338**	17.4	-0.084
	JHI-Hv50k-2016-358636	573.09	159.1	5.414**	17.6	-1.653

[a]Barley Chromosome (Chrom)

[b]Physical position (bp) of markers according to the Morex v3 genome assembly (Mascher et al., 2021).

[c]Strength of the association expressed as the logarithm of odds (LOD) or -log10(p).

*Significant LOD score at alpha 0.10.

**Significant LOD score at alpha 0.05.

***Significant LOD score at alpha 0.01.

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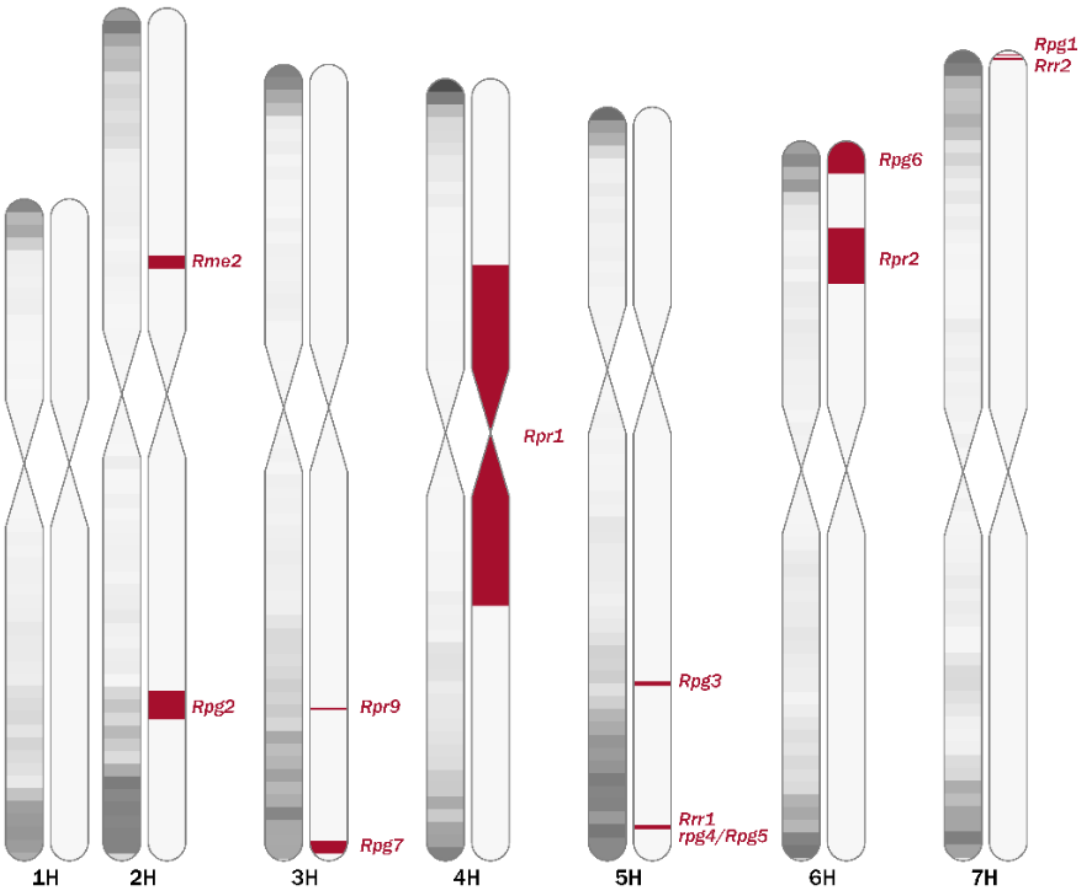


Figure 2.1: The left chromosome in each pair depicts the density of Illumnina 50k iSelect SNP markers across the barley (*Hordeum vulgar* L.) genome. The right chromosome shows the location of the mapped barley stem rust resistance genes effective against *Puccinia graminis* f. sp. *tritici*, which are shown as red regions.

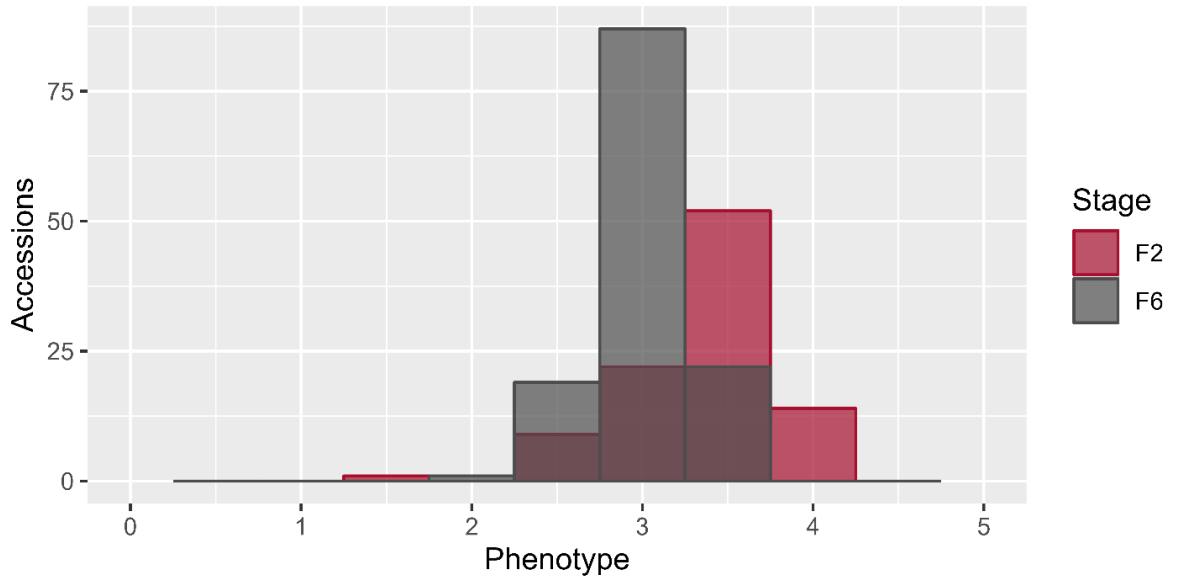


Figure 2.2: Common responses of Elliot x Palmer F_2 and F_6 individuals 14 days after infection (DAI) at the seedling stage assayed with the PNW *Puccinia graminis* f. sp. *tritici* isolate Lsp21. The panel shows typical infections observed that were categorized as resistant (R), moderately resistant (MR), moderately susceptible (MS), and susceptible (S) based on their infection types.

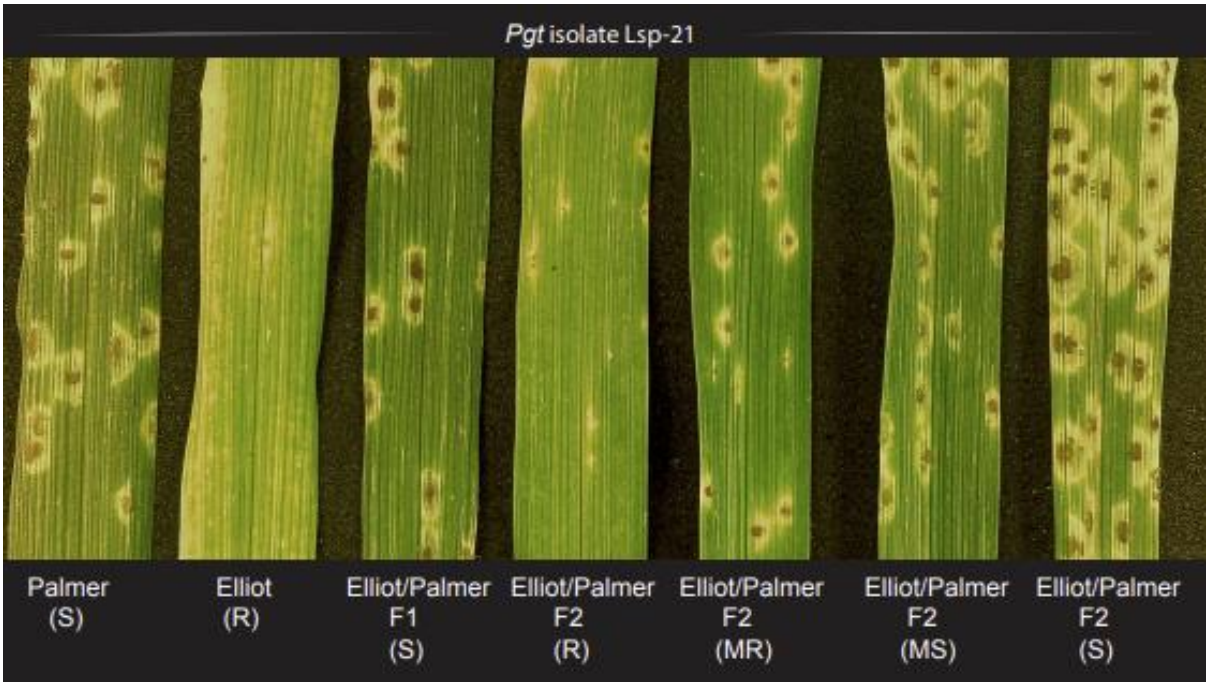


Figure 2.3: Phenotypic distribution 14 days after inoculation with *Puccinia graminis* f. sp. *tritici* isolate Lsp21 at the seedling stage using the coefficient of infection (CI) scores for parental lines Palmer and Elliot and Elliot x Palmer F_1 and F_2 individuals.

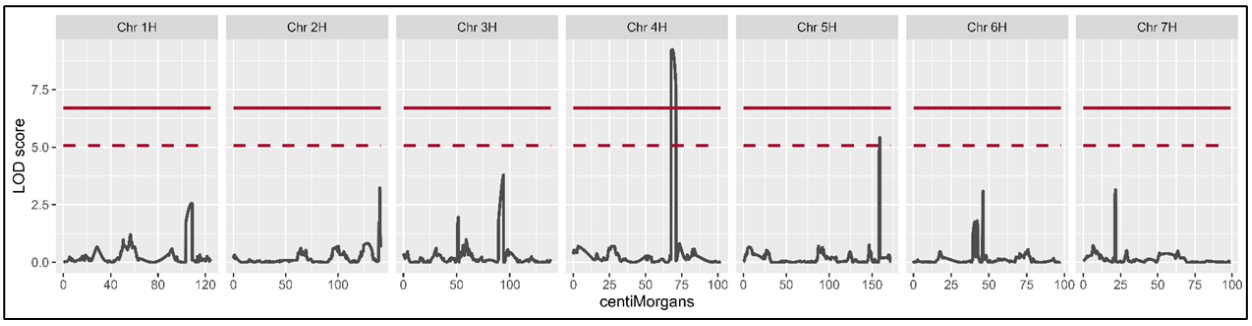


Figure 2.4: QTL analysis of *Puccinia graminis* f. sp. *tritici* isolate Lsp21 responses on the Elliot x Palmer RIL population using composite interval mapping in QGene4.4.0 on all seven barley chromosomes. The solid line represents the LOD threshold obtained at a significance level of 0.01 after 1,000 permutations test. The dotted line represents the LOD threshold obtained at a significance level of 0.05 after 1,000 permutation tests.

CHAPTER THREE: IDENTIFICATION AND ASSOCIATION MAPPING OF SOURCES OF STEM RUST RESISTANCE IN THE WILD BARLEY DIVERSITY PANEL EFFECTIVE AGAINST VIRULENT ISOLATES FROM THE PACIFIC NORTHWEST

Abstract

The identification and genetic characterization of new sources of resistance in barley (*Hordeum vulgare*) effective against the highly virulent wheat stem rust (caused by *Puccinia graminis* f. sp. *tritici*, *Pgt*) population recently identified in the Pacific Northwestern region of North America is important for barley production. Isolates collected from eastern Washington were found to be virulent on barley line Q21861 (*Rpg1+* and *rpg4/5+*). This virulence on the two broadest and most effective stem rust resistance (R)-genes, when combined, is unprecedented *Pgt* virulence that had not been previously reported, representing the most virulent stem rust population on barley worldwide. Utilizing diverse *Hordeum vulgar* subsp. *spontaneum* accessions from the Wild Barley Diversity Collection (WBDC), novel sources of resistance were identified and mapped that are effective against the virulent isolates found in the Pacific Northwest. A total of 277 lines from the WBDC were screened for stem rust reactions at the seedling stage with the most virulent PNW *Pgt* isolate, Lsp21. Only 12% of the accessions showed moderate resistance to resistance reactions at the seedling stage. Two accessions (WBDC-94 and WBDC-238) collected from Jorden displayed an exceptional level of resistance (median infection type of 0;). However, genomic analysis of skim whole genome sequencing of the two accessions determined that they are the same genotype and represented the only two WBDC accessions known to carry the recently characterized *Rgp7* R-gene. Subsequent biparental mapping determined that this resistance is conferred by the single dominant resistance gene *Rpg7*. A genome-wide association study

(GWAS) was conducted to elucidate marker-trait associations using 37,338 single nucleotide polymorphism (SNP) markers generated by restriction-site-associated DNA-genotype-by-sequencing (RAD-GBS) and the phenotyping data generated with *Pgt* isolate Lsp21. Using the GAPIT package in R studio, the BLINK model identified 12 loci associated with resistance to isolate Lsp21 present on barley chromosomes 1H, 2H, 3H, 5H, 6H, and 7H. However, due to the minor allele frequency of *Rpg7*, it was not detected in the GWAS. High linkage disequilibrium decay resulted in small linage blocks averaging 170 bp across the genome, resulting in small intervals delimiting many of the detected loci, which allowed for the detection of a limited number of candidate genes for many loci based on the cv Morex v3 genome assembly. Seven novel loci (WQRpg-2H01, WQRpg-2H02, WQRpg-3H01, WQRpg-5H01, WQRpg-5H03, WQRpg-7H02, and WQRpg-7H03) were identified for stem rust resistance. These new sources of resistance can now be introduced into cultivated barley, and the SNPs utilized to identify the MTAs can be used to track the resistance loci when developing prebreeding lines with stem rust resistance in elite backgrounds. Breeders can utilize these lines to enhance resistance to stem rust in their programs, especially against the virulent races present in the PNW population.

Introduction

Wheat stem rust (*Puccinia graminis* f. sp. *tritici, Pgt*) is a significant foliar disease of barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) worldwide. In 2019, barley stems with susceptible stem rust pustules containing uredinia spores were collected from production fields and experimental plots in eastern Washington. Single spore isolates (n=100 isolates) were generated, representing this wheat stem rust population (Upadhaya et al., 2022). Virulence assays on a barley stem rust differential set determined that 99% of the 100 isolates were virulent on the cultivar (cv) Morex (Clho 15773), which contains the *Resistance to Puccina graminis* 1 (*Rpg1)* gene, 16% of the isolates were virulent on the line HQ1 containing the *rpg4*/*Rpg5*-mediated resistance locus (*RMRL*), and unexpectedly, 10% of the isolates were virulent on barley line Q21861 (PI 584766), which contains both *Rpg1* and *RMRL*. When *Rpg1* and *RMRL* are stacked together, they previously had been shown to provide exceptionally broad resistance effective against all known races and isolates of *Pgt* collected from around the globe, including the virulent African *Pgt* race TTKSK (AKA Ug99) and its lineage (Sun & Steffenson, 2005; Nazari et al., 2009; Steffenson et al., 2017). Thus, this remarkable virulence is the first documentation of *Pgt* virulence on the *Rpg1* and *RMRL* gene combination, and this Pacific Northwest (PNW) stem rust population represents the most virulent population of stem rust on barley ever reported worldwide.

Puccinia graminis f. sp. *tritici*, the causal agent of wheat stem rust, is a heteroecious biotrophic fungal pathogen with a complex life cycle that produces five different spore stages during the completion of its life cycle. These spores are called basidiospores, pycniospores, aeciospores, urediniospores, and teliospores and play unique roles in *Pgt* evolution, infection, and spread (Roelfs, 1985). Furthermore, stem rust completes its life cycle on two unrelated hosts. The

primary monocot hosts are wheat, barley, and wild grasses; the secondary hosts are common barbary (*Berberis vulgaris*) and mahonia (*Mahonia aquifolium*). This pathogen can asexually infect cereal crops such as barley and wheat with urediniospores throughout the summer in its polycyclic disease pattern. As its primary cereal and grass hosts mature in late summer and near senescence, the fungus transitions to forming telia containing teliospores that can overwinter in moderate climates to specifically infect its dicot secondary hosts, common barberry or mahonia, in the spring. In these secondary hosts, the pathogen completes the sexual stages of its life cycle. Here, recombinant gametes can form aeciospores that can cause early infection on its primary grass hosts.

During the early 1900s, the Midwest of the US and the Prairie Provinces of Canada experienced major stem rust epidemics that, in severe years, resulted in nearly 100% crop loss (Dyck and Kerber, 1985). These severe epidemics occurred under conditions conducive to disease formation when susceptible varieties were grown across vast acreage, especially where the secondary host common barberry was growing near wheat and barley fields and served as early-season inoculum. One of the earliest records of a stem rust eradication program was a law in Rouen, France, in 1660 that advocated the destruction of barberry plants to protect cereal crops (Mckay, 1957). However, common barberry was brought to North America by European settlers in the 1800s and became a widespread invasive species (Stakman & Fletcher, 1930; Hill, 2003). Years later, in 1918, the US Federal government implemented the barberry eradication program (Roelfs, 1982) to eliminate the threat posed to wheat and barley production in the US. Over the next half century, the barberry eradication program effectively eliminated susceptible barberry bushes, stopping its contribution of early-season inoculum and the *Pgt* sexual cycle. With the

stabilization of the stem rust population in the midwestern US and the effective deployment of stacked resistance genes in wheat, major stem rust epidemics became a thing of the past, and the federal barberry eradication program was discontinued in 1977 (Roelfs, 1978; Maloy, 1933).

Unfortunately, it was recently discovered that the PNW region of North America now serves as the center of stem rust diversity on the continent due to the endemic presence of mahonia, another secondary sexual host of *Pgt* (Upadhaya, 2023). Mahonia is native to the PNW and found widespread throughout woodland areas; therefore, it is part of the natural ecosystems and would be nearly impossible to control or eradicate. It appears that climate change may be contributing to this problem as warmer winters and increased winter and spring precipitation in the PNW may be contributing to a more conducive environment for stem rust infection and lifecycle completion. This is going to exacerbate the issue of diversity evolution that can and is giving rise to new virulent profiles in this *Pgt* population that not only poses a threat to PNW cereal production but will disseminate to other regions of North America and the world. An example of this virulent isolate dissemination has already occurred with *Pgt* race QCCJB that emerged in the upper great plains of North America in the 1990s with unprecedented virulence on the barley stem rust resistance gene *Rpg1* and has since been shown to have come from the PNW sexual *Pgt* population.

Novel sources of stem rust resistance effective against this virulent PNW population have been identified from the World Barley Core Collection (WBCC) (Upadhaya, 2023). The WBCC is an extensive collection of over 18,000 diverse domesticated barley (*Hordeum vulgare* subsp. *vulgare*) lines collected from around the world, including landraces and cultivated food, feed, and malting barley accessions. Upadhaya (2023) utilized SNP data generated for ~1,500 WBCC lines to organize a 'mini' core collection that represented the diversity present in the collection. Based

on the mini-core collection, a genome-wide association study (GWAS) was conducted using 440 diverse lines from the WBCC to screen for seedling resistance against *Pgt* isolate Lsp21, the most virulent PNW isolate on barley. Of the 440 lines screened, only 6% were resistant, with only three MTAs on chromosomes 3H, 5H, and 6H detected utilizing GWAS.

To identify additional novel resistance genes/loci to the PNW stem rust population, we turned to the wild barley diversity collection (WBDC). The WBDC is a collection of wild barley (*Hordeum vulgare* subsp. *spontaneum*) comprising 318 accessions collected from the Fertile Crescent, Asia, North Africa, and the Caucasus region, which were deposited to the USDA National Small Grains Collection, Aberdeen, Idaho (Steffenson et al., 2007). Cultivated barley was domesticated over 10,000 years ago from the wild barley progenitor *H. vulgare* subsp. *spontaneum* in the fertile crescent region (von Bothmer et al., 2003). Through intensive selection, breeding has narrowed the genetic diversity of barley, losing the diversity that evolved in the natural ecosystems (Wambugu et al., 2018). Thus, *H. vulgare* subsp. *Spontaneum*, which is within the primary barley germplasm pool and is fully compatible with cultivated barley in hybridization, is a rich source of genetic diversity for many traits, including abiotic and biotic stress resistances (Ellis et al., 2000; Fetch et al., 2003; Steffenson et al., 2007; Allam et al., 2017; Liu et al., 2019; Henningsen et al., 2021; Clare et al., 2023). This is especially true for stem rust resistance because wild barley and *Pgt* have been coevolving in a host-pathogen molecular arms race in the Fertile Crescent for thousands of years (Fetch et al., 2003).

The major focus of the research reported in this study was to identify novel sources of seedling resistance against the virulent PNW population of *Pgt*. Only eight stem rust R-genes have been characterized in barley designated *Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, *rpg6*, *Rpg7*, and *rpg8*. The

rpg8 gene was formerly known as *rpgBH* but was recently genetically characterized and given the *rpg8* gene nomenclature (Dr. Brian Steffenson, personal communication). The virulent *Pgt* isolates from the PNW population have been shown to be virulent on *Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, and *rpg8* (Steffenson et al., 2017; Upadhaya et al., 2022). Because of the threat posed by the virulent population of stem rust in the PNW, new barley resistance sources and genes must be identified and characterized. We turned to wild barley because it has coevolved with stem rust in the fertile crescent and could contain novel sources of resistance effective against the PNW *Pgt* population.

Methods and Materials

Plant material and pathogen

The 318 wild barley (*H. vulgare* subsp. *spontaneum*) accessions utilized make up the wild barley diversity collection (WBDC) deposited and obtained from the USDA National Small Grains Collection, Aberdeen, ID (Supplementarily Table S2). These accessions were collected from the Fertile Crescent, Central Asia, North Africa, and the Caucus region, where *H. spontaneum* is native (Steffenson et al., 2007). Domesticated cultivars (*H. vulgare*) were used as susceptible (cv. Morex, Steptoe, and Q21861) and resistant (Elliot and DH160748) checks.

The PNW *Pgt* isolate Lsp21 was selected for this GWAS analysis because it is the most virulent isolate from the PNW population, as determined by assays on a barley stem rust R-gene differential set (Upadhaya et al., 2022). *Pgt* isolate Lsp21 was virulent on barley cv Morex (*Rpg1*+), Clho 7124 (*Rpg2*+), PI282313 (*Rpg3*+), HQ1 (*rpg4*/*Rpg5*+), Q21861 (*Rpg1*+ and *rpg4*/*Rpg5*+), and Blackhulless (*rpg8*+) (Supplementary Table S1). This virulence profile on barley *R*-genes represents the most virulent isolates of *Pgt* on barley ever reported. Cultivars (cv)

Morex, line Q21861, cv Elliot (PI 592261), and cv Steptoe (CIho 15229) were used as susceptible checks, and DH-160748 was used as a resistant check. Morex is a six-row malting line that contains the R-gene *Rpg1*. Q21861 is a two-row barley that contains *Rpg1* and *rpg4*/*Rpg5*-mediated resistance locus (RMRL). Elliot is a two-row malting variety developed in the United Kingdom derived from a Trumpf × Hassan cross in 1993. Elliot contains resistance effective against PNW isolate Lsp21 (Brooke et al., unpublished). Steptoe is a six-row feed line that has no known wheat stem rust R-genes. DH-160748 is a double haploid experimental malt line from the Oregon State University (OSU) barley breeding program that contains uncharacterized resistance effective against the PNW isolate Lsp21.

Plant growth conditions

For the seedling disease reaction analysis, a single seed from each WBDC accession, as well as the susceptible and resistant checks, were planted in three individual 98 well-cone containers (6.5 cm diameter by 26.5 cm height). Each cone was filled with standard potting mix soil (Sun Gro Horticulture, Agawam, MA, USA) supplemented with 2 gm per cone of slow-release Osmicote 14-14-14 fertilizer. Plants were placed in a growth chamber set to 18◦C with a 16 h light (400 μm/m^2) and 8 h dark cycle as described by Upadhaya et al. (2022). Furthermore, the experiment was replicated two times in a complete randomized design. Accessions that gave variable reactions across replicates were phenotyped an additional time.

Stem rust evalution

Approximately nine days after planting, when the primary leaves were fully expanded, stem rust inoculations were conducted using an atomizer pressured by a pump set at 30kPa

(Steffenson et al., 2017; Upendaya et al., 2023). Seedlings were inoculated with fresh urediniospores collected from cv Steptoe seedlings inoculated with *Pgt* isolate Lsp21 and mineral oil at 8 mg of urediniospores per 1 ml of mineral oil. After inoculation, plant leaves were allowed to dry for one hour and then placed in a mist chamber for 18 h in complete darkness at 18°C and 100% relative humidity. After 18 h, plants were placed back in the growth chamber at the conditions mentioned previously.

At 14 days after inoculation (DAI), infection types (ITs) were assessed on primary leaves. Infections were rated on a modified "0 to 4" scale. This scale was initially developed by Stakeman et al. (1962) for wheat, later modified for barley by Miller and Lambert (1955), and further modified by Steffenson et al. (2017). When conducting stem rust analysis on barley, mesothetic reactions of different ITs on the same primary leaf can be observed. These IT values were categorized as 0; = hypersensitive reaction (HR), 1 = Resistance (R), 2 = moderately resistant (MR), 3- = moderately susceptible (MS), and anything above a 3 was considered susceptible (S) (Steffenson et al., 2017; Hernandez et al., 2019). To get a more accurate estimation of pustule size, + or – symbols were used after the corresponding IT. The categorical IT scores of "0 to 4" were converted into numeric coefficient of infection (CI) values, quantitative scores of 0 to 5, as described by Zhou et al. (2014). If multiple ITs were observed on a single leaf, the CI was calculated by order of frequency using a weighted average.

Sequence data

The WBDC had been previously genotyped by Sallam et al. (2017) using a restriction-site-associated DNA-genotyping-by-sequencing (RAD-GBS) approach. This genotyping data was

submitted to the T3 barley database under the project name 2016 GBS_WBDC (https://triticeaetoolbox.org/). The genotyping data was downloaded from the T3 barley database in the vcf file format containing 50,842 SNP markers. Imputation of missing SNP marker data was completed using the Beagle 5.4 software (Pook et al., 2020). Due to the nature of wild barley, the heterozygous SNPs calls were included in the final analysis. SNPs with minor allele frequency (MAF) < 0.05% were removed from the data to address the possibility of detecting false positive MTAs due to minor allele frequency resulting in 37,338 total SNP markers. Linkage disequilibrium (LD) decay was calculated in TASSEL. The final SNP marker density plot and QQ plots are shown in Supplementary Figures S1 & S2.

Model selection and linkage disequilibrium

The GWAS marker-trait association analysis was performed utilizing GAPIT v3 in R, using the Bayesian-information and linkage-disequilibrium Iteratively Nested Keyway (BLINK) model (Huang et al., 2019; Wang & Zhang, 2021). A principal component (PC) analysis of the VanRaden function was used to account for population structure using the findings in GAPIT (Lipka et al., 2012). Next, a Bonferroni adjustment was applied at an α-level of 0.05 to avoid type I errors. Marker-trait associations (MTAs) from the GWAS were considered significant at a *p*-value <0.00000134 corresponding to a LOD [-log10(p-value)] score >5.87. After using the BLINK model, a Manhattan plot was generated using the 'CMplot' package in R to visualize significant MTA (Yin et al., 2021).

Candidate gene identification

The physical positions of the GBS markers were initially aligned to the cv Morex v1 genome assembly (Beier et al., 2017; Sallam et al., 2017). All Marker Trait Associations (MTA's) were located on the Morex v1 genome assembly using the GrainGenes browser (https://wheat.pw.usda.gov/GG3/). The flanking sequence (~200 bp) from the Morex v1 assembly of each SNP that was identified as significant MTA's were then aligned to the Morex v3 genome assembly using the BLAST function to identify their physical positions on the updated and most current barley genome assembly (Mascher et al., 2021). The genotype data in hapmap format were converted to plink format files using the Tassesl v5.2.93 software. Then, the LD blocks were estimated using the plink v1.9 tool with the following options: --blocks-max-kb 5000. For block estimation, the method described by Gabriel et al. (2002) was used in the plink tool. Then, the LD blocks were estimated in haploview software using solid spine of LD (Barrett et al., 2005).

Candidate gene models annotated in the Morex v3 genome assembly were then used in BLASTp searches on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to validate predicted gene function given in the Morex v3 assembly as some predicted gene functions or known domain identities have been misannotated on the Morex v3 genome annotations (Blum et al., 2021). Linkage decay was calculated to delimit the region of the MTA. Nomenclature for each MTA is as follows: (*WQRpg1H*-1) W = Wild barley; Q = Quantitative Trait Locus; *Rpg = Resistance to Puccina graminis*; followed by chromosome designation and number of MTA; Loci were considered novel if they did not overlap with other previously reported QTL.

Results

From the original 318 accessions that make up the WBDC, 277 were phenotyped due to poor, inconsistent, or late germination. Of the 277 accessions phenotyped, only 22 were considered resistant or moderately resistant, with CI scores <2.75. The susceptible checks, Morex, Steptoe, and Q21861, were all susceptible, with CI scores averaging 3.33, 3.13, and 2.96, respectively (Table 3.1). The average CI score for all 277 WBDC accessions was 3.51, which was skewed toward susceptibility, with a maximum CI score of 4.41, a minimum of 0.56, and a range of 3.84 (Figure 3.1). The two lines WBDC-94 (PI 681809) and WBDC-238 (PI 681943) displayed a strong resistant response to the virulent *Pgt* isolate Lsp21 and were the two lines with the CI scores of 0.56 (Figure 3.2). Both of these accessions were collected near Mādabā, Jordan, and were previously reported as having an HR response to *Pgt* races QCCJB, MCCFC, HKHJC, and rye stem rust (*P. graminis* f. sp. *secalis*, *Pgs*) isolate 92-MN-90 at the seedling stage (Sallam et al., 2017). All the CI scores of the WBDC accessions can be found in Supplementary Table S2.

GWAS was performed using the BLINK model in GAPIT using R (Huang et al., 2019; Wang & Zhang, 2021) This model identified 12 significant marker trait associations (MTAs) for resistance to *Pgt* isolate Lsp21 spread across the genome on barley chromosomes 1H, 2H, 3H, 5H, 6H, and 7H. (Figure 3.3). The 12 significant markers had a phenotypic variation explained (PVE) by individual markers ranging from 36.5% for 5H_596832560 to 0.55% for 2H_748987056 (Table 3.2). Linkage disequilibrium (LD) decay was used to delimit the regions of each MTA that may contain candidate resistance genes underlying each locus. The average linkage decay calculated in this study was ~170 bp. A depiction of the delimited regions with flanking markers can be found in Supplementary Figure S3.

The two significant MTA 1H_482689583 and 1H_492949440 (Fig. 3.3; Table 3.2) were detected on the long arm of chromosome 1H, with LOD scores of 7.28 and 10.66, respectively. These makers are located at the physical positions 449965275 and 459175825 based on the most recent cv Morex v3 genome assembly. Linkage decay analysis delimited these loci to 208 bp and 251 kb regions, designated WQ*Rpg*-1H01 and WQ*Rpg*-1H02 (Table 2.3). Both MTA 1H_482689583 and 1H_492949440 are located within high-confidence genes HORVU.MOREX.r3.1HG0070200.1 (a predicted transmembrane protein) and HORVU.MOREX.r3.1HG0072680.1 (a predicted respiratory burst oxidase-like protein), respectively. The WQ*Rpg*-1H01 delimited locus is only 208 bp thus only contains the single HORVU.MOREX.r3.1HG0070200.1 candidate gene. The transmembrane proteins have been implicated in many putative functions include energy conversion across organelles, controlling signal transduction, and transport of nutrient molecules (Feng et al., 2021). Transmembrane proteins can also be involved in pathogen defense recognition. (Zhou et al., 2022). In Arabidopsis, transmembrane protein BDA1 is activated when the SNC2 (Suppressor of NPR1, Constitutive2) detects a pathogen-associated molecular pattern (PAMP) (Yang et al., 2012). The activation of BDA1 then functions in a signaling network to induce a WRKY70 transcription factor and salicylic acid (SA) accumulation to increase plant defenses.

The WQ*Rpg*-1H02 delimited locus contains four high-confidence genes, HORVU.MOREX.r3.1HG0072670.1 (a predicted helix-loop-helix transcription factor), HORVU.MOREX.r3.1HG0072680.1 (a predicted respiratory burst oxidase-like protein), HORVU.MOREX.r3.1HG0072700.1 (a predicted transmembrane protein), and HORVU.MOREX.r3.1HG0072710.1 (a predicted TBC1 domain family member 8B). The top

candidate gene in the region is the respiratory burst oxidase-like protein, which is the motivating force behind reactive oxygen species (ROS) (Suzuki et al., 2011; Otulak-Kozieł et al., 2019). ROS is an important factor in plant defense pathways and was first reported in tobacco plants by Doke and Ohashi (1988). ROS increases when pathogen effector molecules are recognized in the cell to induce programmed cell death (PCD), resulting in the hypersensitive response (HR) (Torres et al., 2002; Kwak et al., 2003; Suzuki et al., 2011). However, the predicted helix-loop-helix transcription factor and transmembrane protein are also candidate genes, as both could function in plant defenses.

Chromosome 2H contained the two significant MTA 2H_43141092 and 2H_748987056 (Fig. 3.3; Table 3.2), with LOD scores of 7.55 and 6.72, respectively. Marker 2H_43141092 is located on the short arm of chromosome 2H at physical location 37171396 bp, and 2H_748987056 is located on the long arm of 2H at physical position 649620603 bp based on the cv Morex v3 genome assembly. The LD decay at the 2H_43141092 and 2H_748987056 loci delimited the regions to 54 bp and 308 bp and were designated the WQ*Rpg*-2H01 and WQ*Rpg*-2H02 loci (Table 3.2), respectively. However, no high-confidence genes were discovered in these delimited regions, and these loci have not been previously reported as stem rust resistance loci.

The MTA 3H_623131530 was the only locus identified on chromosome 3H (Fig. 3.3; Table 3.2), and is located at genome position 555008110 bp, with an LOD score of 6.07. The delimited region was only 47 bp and designated the WQRpg-3H01 locus. This MTA is within the high-confidence gene, HORVU.MOREX.r3.3HG 0304090.1 (a predicted ankyrin repeat-containing protein). This predicted ankyrin repeat-containing protein is an excellent candidate gene because ankyrin repeat-containing proteins have been found to play important roles in

responding to biotic stresses. Ankyrin repeat-containing proteins are known to regulate ROS under salt stress and disease infection in Arabidopsis (Lu et al., 2005; Sakamoto et al., 2008; Mou et al., 2013). This class of protein has also been shown to confer resistance to leaf rust (caused by *Puccinia triticina*) in wheat (Kolodziej et al., 2021). WQRpg-3H01 (3H_623131530) is a novel stem rust resistance locus because it is located ~5Mb proximal of qGH_PBIC_3.11 (Dracatos et al., 2019) and 27Mb distal of Sallam_QTL3H-8 (Sallam et al., 2017).

Three significant MTA (5H_435859197, 5H_596832560, and 5H_612544142) were identified on chromosome 5H (Fig. 3.3; Table 3.2). The significant MTA 5H_435859197 was located at physical position 388581717 bp. This locus was delimited to a large 1.2 Mb region but only contained three high-confidence genes: HORVU.MOREX.r3.5HG0473150.1 (a predicted nucleobase-ascorbate transporter-like protein), HORVU. MOREX.r3.5HG0473160.1 (a predicted purple acid phosphatase), and HORVU.MOREX.r3.5HG0473180.1 (a predicted iron-sulfur cluster insertion protein ErpA). The locus was designated WQ*Rpg*-5H01 (Table 3.2). Based on known function, the top candidate gene identified in this region was the predicted purple acid phosphatase (PAP). PAPs play a vital role in plant production, transport, and recycling of inorganic phosphorus (Olczak et al., 2003). This class of genes is also known to function in pathogen defense against *Pseudomonas syringae* in Arabidopsis (Ravichandran et al., 2013; Antonyuk et al., 2014; Ravichandran et al., 2015). WQRpg-5H01 is a potentially novel resistance locus because it is located 17.6 Mb away from Czembor_QTL5H-1 1 (Czembor et al., 2022) and 19 Mb away from Mamo_QTL5H-1 (Mamo et al., 2014), which are the closest previously reported stem rust resistance QTL.

The most significant MTA identified in this GWAS analysis was 5H_596832560 located on the long arm of chromosome 5H at physical position 526215093 bp based on the Morex v3 genome assembly with a LOD score of 11.19 (Fig. 3.3; Table 3.2). Furthermore, 36% of the phenotypic variation was explained by this individual marker. This locus was delimited to a 94 kb region designated the WQ*Rpg*-5H02 locus (Table 3.2) which contains six high-confidence genes: HORVU.MOREX.r3.5HG0510190.1 (a predicted choice-of-anchor C domain protein), HORVU.MOREX.r3.5HG0510200.1 (a predicted protein OBERON 1), HORVU.MOREX.r3.5HG0510 210.1 (a predicted tryptophan RNA-binding attenuator-like protein), HORVU.MOREX.r3.5HG0510220.1 (a predicted seed maturation protein/late embryogenesis abundant protein), HORVU.MOREX.r3.5H G0510230.1 (a predicted dirigent protein), and HORVU.MOREX.r3.5HG0510240.1 (a predicted dirigent protein). The top candidate genes within the WQ*Rpg*-5H02 region are gene models HORVU.MOREX.r3.5HG0510230.1 and HORVU.MOREX.r3.5HG0510240.1, which are predicted to encode dirigent-like proteins. This class of proteins have been shown to be involved in lignin polymerization in secondary cell wall development and pathogen defense (Afzal et al., 2009; Subramanyam et al., 2013). Dirigent proteins have been reported to be involved in resistance against Hessian fly and nematodes in wheat and soybean, respectively (Afzal et al., 2009; Subramanyam et al., 2013).

The last significant MTA detected on 5H is 5H_612544142 (Fig. 3.3; Table 3.2), located at physical position 538786402 bp. This locus was delimited to a 14 kb region and designated as the WQ*Rpg*-5H03 locus (Table 3.2). However, no predicted high-confidence genes are present in the WQ*Rpg*-5H03 delimited region.

Chromosome 6H contained the one significant MTA 6H_576683996 (Fig. 3.3; Table 3.2). This MTA was located at the physical position of 555502895 bp with a LOD score of 9.06 and was designated the WQ*Rpg*-6H01 locus (Table 3.2). This locus was delimited to a region of 5 kb, which contained the single high-confidence gene model HORVU.MOREX.r3.6HG0630630.1, a predicted nucleotide-binding site-leucine-rich repeat (NLR) disease resistance RGA4-like protein. The NLR RGA4 in concerted action with RGA5 (Okuyama et al., 2011; Cesari et al., 2013) were shown to recognize two different avirulence effectors, Avr-Pia and AVR1-CO39 from *Magnaporthe oryza*, the causal agent of the rice blast disease (Pennisi, 2010; Dean et al., 2012). These two NLRs fall within the major class of cytosolic localized disease resistance receptors in plant innate immune systems and elicit effector-triggered immunity (ETI) (Jones et al., 2006) against rice blast in rice. Effector-triggered immunity typically induces localized programmed cell death (PCD) manifested as HR disease resistance responses that are effective at providing resistance against biotrophic pathogens such as rice blast and the rust pathogens (Jones et al., 2006; Van Ooijen et al., 2008; Van Doorn et al., 2011). The NB-LRR domain is typically made up of a central nucleotide-binding domain (NB), and C-terminal leucine-rich repeat domains (LRRs) (Cesari et al., 2014). At the N-terminus there is often a coiled-coil (CC) domain or a TOLL/interlukin-1 receptor (TIR) (Takken & Goverse, 2012). The barley stem rust R-gene *Rpg5* is also an NLR (Brueggeman et al., 2008), which functions by the concerted action of two head-to-head NLRs, which are both required for resistance similar to RGA4 and RGA5 following the integrated sensory domain model first proposed by Kroj et al. (2016).

Lastly, chromosome 7H contained the three significant MTA 7H_107066581, 7H_448221909, and 7H_631891623 (Fig. 3.3; Table 3.2). The 7H_107066581 MTA is located on

the short arm of 7H at the physical position 102573432 bp, with a LOD score of 8.71. This locus designated WQ*Rpg*-7H01 (Table 3.2) was delimited to a large physical region of 1.27 Mb containing seven high-confidence genes: HORVU.MOREX.r3.7HG0667940.1 (a predicted aldo/keto reductase family oxidoreductase), HORVU.MOREX.r3.7HG0668040.1 (a predicted CDP-diacylglycerol--glycerol-3-phosphate3-phosphatidyltransferase), HORVU.MOREX.r3.7HG 0668090.1 (a predicted protein NRT1/ PTR FAMILY 5.5), HORVU.MOREX.r3.7HG0668100.1 (a predicted NAC (No Apical Meristem domain protein)), HORVU.MOREX.r3. 7HG0668110.1 (a predicted myosin protein), HORVU.MOREX.r3.7HG0668120.1 (a predicted major facilitator superfamily domain-containing protein), and HORVU.MOREX.r3.7HG0668180.1 (a predicted O-fucosyltransferase family protein). Based on known functions, the top candidate gene within this locus is the NAC domain transcriptional regulator superfamily protein. NAC transcription factors (TF) link signaling pathways between plant hormones to control disease resistance (Bian et al., 2020). For example, the hormones salicylic acid (SA) and jasmonic acid (JA) play a vital role in signaling in plant defenses (Vlot et al., 2009; Pieterse et al., 2012) and NAC transcription factors, ONAC122 and ONAC131, induce resistance responses in rice when the hormone SA and JA are applied (Sun et al., 2013).

The second significant marker identified on chromosome 7H was 7H_448221909 (Fig. 3.3; Table 3.2) located at the physical position 433462981 bp with an LOD score of 7.48. This locus was designated WQ*Rpg*-7H02 and delimited to a region of 603 kb containing five high-confidence predicted gene models: HORVU.MOREX.r3.7HG0705670.1 (a predicted NAC domain transcriptional regulator superfamily protein), HORVU.MOREX.r3.7HG0705680.1 (a predicted pectinesterase), HORVU.MOREX.r3.7HG0705690.1 (a predicted elongation factor G),

HORVU.MOREX.r3.7HG0705700.1 (a predicted calcium ion-binding protein), and HORVU.MOREX.r3.7HG0705730.1 (a predicted DNA-Apurinic or apyrimidinic site lyase). The top candidate gene for this locus was the NAC domain transcriptional regulator superfamily protein, similar to the top candidate gene proposed for WQ*Rpg*-7H01.

The last and lowest significant marker was S7H_631891623 (Fig. 3.3; Table 3.2), which had a LOD score of 6.01. Marker S7H_631891623 is at the physical position 604620989 bp. This locus designated the WQ*Rpg*-7H03 (Table 3.2) was delimited to a region of 116 kb containing one high-confidence gene, HORVU.MOREX.r3.7HG0740200.1, which is predicted to encode a pectin lyase-like superfamily protein . Pectin lyases are enzymes that can degrade plant cell walls, and are essential for fruit ripening, and in some situations, are necessary for the activation of plant defense mechanisms (De Lorenzo et al., 1991; Marín-Rodriguez et a., 2003; Hugouvieux et al., 2014)

Discussion

Seven of the 12 significant MTAs found in this study (WQRpg-2H01, WQRpg-2H02, WQRpg-3H01, WQRpg-5H01, WQRpg-5H03, WQRpg-7H02, and WQRpg-7H03) potentially represent novel stem rust resistance loci. Interestingly, four MTA's (WQ*Rpg*-1H01, WQ*Rpg*-1H02, WQ*Rpg*-6H01, and WQ*Rpg*-7H03) were delimited to regions with only one candidate gene. WQ*Rpg*-1H02 and WQ*Rpg*-6H01 were intriguing loci because they contained a respiratory burst oxidase-like protein (RBO) and an NLR, respectively. These genes play direct roles in disease resistance within plants. RBO proteins regulate ROS production upon pathogen attack, serving as signaling molecules to activate defense genes and programmed cell death (Torries et al., 2005;

Mittler et al., 2011). The production of ROS can trigger both PTI and ETI responses; thus, RBO proteins play an essential role in initiating plant defense responses against pathogens (Zhang et al., 2009; Kadota et al., 2015). Furthermore, the NLRs comprises the largest family of disease-resistant genes within plants (Dangl & Jones, 2001). Thus, this GWAS analysis effectively delimited regions to a single candidate gene which demonstrates the power of GWAS when robust phenotyping and high-density saturation with molecular markers is available.

Three of the loci identified (WQRpg-2H01, WQRpg-2H02, and WQ*Rpg*-5H03) were delimited to genomic regions based on the cv Morex v3 genome assembly that did not contain any high-confidence gene models/candidate genes. However, the lack of candidate genes identified in these regions could be due to the use of the cv Morex v3 genome assembly as the reference genome. Due to the diversity present between domesticated and diverse wild barley lines, there is the possibility that the region could be expanded in the WBDC lines and contain indels and or translocations that contain additional genes absent in cv Morex. For example, our genome comparative analysis utilizing the barley pangenome sequences shows that the genomic region at the *Rpt5*-mediated net form net blotch resistance locus on barley chromosome 6H has additional genes in the *H. sponteneum* lines (Effertz, 2023). However, further comparative analysis of these regions will be possible when the new barley pangenome v2 is released shortly. The pangenome v2 will allow for diversity analysis, genome architecture collinearity, and differences that occur within these regions of the genome as it will contain more wild barley accessions. However, both WQ*Rpg*-2H01 and WQ*Rpg*-2H02 overlapped with previously identified MTAs for spot form net blotch (*Pyrenophora teres* f. *maculata*) and Fusarium head blight (*Fusarium graminearum*)

resistance, suggesting that these regions contain other disease resistance genes (Mamo & Steffenson, 2015; Burlakoti et al., 2017).

The WBDC has been extensively studied using the diverse *Pgt* races QCCJB, MCCFC, HKHJC, TTKSK, and *Pgs* isolate 92-MN-90 to identify novel sources of stem rust resistance (Sallam et al., 2017; Steffenson et al., 2017; Case et al., 2018). However, none of these races, even the virulent African *Pgt* race TTKSK, have virulence on *Rpg1* and RMRL when stacked together, as shown with several of the PNW *Pgt* isolates (Upadhaya et al., 2022), including the most virulent PNW *Pgt* isolate Lsp21. Thus, this isolate was chosen for the study to identify novel resistance sources and to genetically characterize these loci that are effective against the virulent PNW isolates. Sallam et al. (2017) screened 314 lines from the WBDC against *Pgt* races QCCJB, MCCFC, HKHJC, and TTKSK and reported five novel MTAs on chromosomes 1H, 3H, 5H, and 7H using GWAS. On chromosome 1H, Sallam_QTL1H-5 was located between the physical genomic positions 449965067 bp (1H_482689791) and 467954151 bp (S1H_503256550) using the Morex v3 genome assembly. Both the WQ*Rpg*-1H01 and WQ*Rpg*-1H02 loci identified here fall within the Sallam_QTL1H-5 region. The Sallam_QTL1H-5 locus is associated with resistance against *Pgt* races MCCFC, TTKSK, and *Pgs* isolate 92-MN-90. On Chromosome 5H, Sallam et al. (2017) also reported the Sallam_QTL5H-4 (S5H_596737839) locus at position 526348308 bp, which is only ~133 kb away from WQ*Rpg*-5H02 (5H_596832560) which was the most significant MTA identified in this study. The Sallam_QTL5H-4 locus was associated with resistance against *Pgt* races TTKSK, QCCJB, MCCFC, and *Pgs* isolate 92-MN-90. Two candidate genes were reported for Sallam_QTL5H-4: HORVU5Hr1G094700 and HORVU5Hr1G094710. These genes are predicted to encode disease resistance-responsive dirigent-like proteins, which are the same as

the top candidate genes HORVU.MOREX.r3.5HG0510230.1 and HORVU.MOREX.r3.5HG0510240.1 proposed for the WQ*Rpg*-5H02 locus identified in this GWAS. Lastly, Sallam et al. (2017) reported the Sallam_QTL7H-3 locus on chromosome 7H identified by the significant MTA S7H_112969908 at position 107674001 bp. Sallam_QTL7H-3 is ~5Mb from the WQRpg-7H01 (7H_107066581) locus identified here, and based on LD decay of 1.27 Mb at this region, it is probable that WQRpg-7H01 is distinct from the Sallam_QTL7H-3 locus and represents a novel resistance locus/gene. Also, the candidate genes associated with the Sallam_QTL7H-3 locus providing resistance to *Pgt* race MCCFC near the WQRpg-7H01 locus contained distinct candidate resistance genes. The candidate gene for Sallam_QTL7H-3 is HORVU7Hr1G041160, which is a membrane attack complex and perforin (MACPF) gene that was not present within the WQRpg-7H01 delimited region.

Linkage disequilibrium

Based on the linkage disequilibrium (LD) decay values for each significant SNP we can delimit the region of interest and identify candidate genes (Supplementary Figure 3). LD decay is typically lower in wild barley populations than in domesticated and landrace populations due to outcrossing events over a long evolutionary period, resulting in high levels of recombination events (Morrell et al., 2005). Rapid LD decay has been reported in wild barley populations within only a few hundred base pairs (Caldwell et al., 2006; Sallam et al., 2017). Sallam et al. (2017) reported low levels of association between adjacent markers within the 318 accessions of the WBDC. These levels of LD decay are similar to those reported for outbreeding species such as *Zea mays* (Morrell et al., 2005).

Resistance to Lsp21

This study aimed to identify novel stem rust resistance loci in the WBDC effective against the most virulent *Pgt* isolate ever reported on barley, *Pgt* isolate Lsp21. The PNW *Pgt* population collected in 2019 is the most virulent *Pgt* population reported worldwide (Upadhaya et al., 2022; Upadhaya, 2023). The PNW isolate Lsp21 is virulent on barley cv Morex (*Rpg1*+), CIho 7124 (*Rpg2*+), PI282313 (*Rpg3*+), HQ1 (RMRL+), Q21861 (*Rpg1*+ and RMRL+), and Blackhulless (*rpg8*+) (Supplementary Table S1). It was observed that 99% of the 100 isolates collected from the PNW were virulent on the cultivar Morex, which contains the resistance gene *Rpg1*. Furthermore, 10% of the isolates were virulent on Q21861, which contains both resistance genes *Rpg1* and RMRL. Thus, this remarkable virulence is the first documentation of *Pgt* virulence on the *Rpg1/rpg4/5* gene combination worldwide. The six MTA present on chromosomes 5H and 7H did not colocalized with RMRL or *Rpg1*, and both genes were ineffective against the isolate Lsp21. Thus, we have identified novel resistance genes that do not appear to represent different alleles of RMRL and *Rpg1*. However, 22 lines from the WBDC do contain a function RMRL domain (Sallam et al., 2017; Steffenson et al., 2017)

Twenty-two lines showed resistance to the virulent *Pgt* isolate Lsp21. Interestingly, the WBDC accessions containing the newly discovered R-gene, *Rpg7*, showed a hypersensitive response towards *Pgt* isolate Lsp21. *Rpg7* provides remarkable stem rust resistance in barley as all previously identified and characterized resistance genes in barley typically display a lower level of resistance more in line with the non-race specific resistance sources mediating a slow rusting phenotype. *Rpg7* is resistant to *Pgt* races QCCJB, MCCFC, HKHJC, TTKSK, and *Pgs* race 92-MN-90 but not TTKSA at the adult plant stage (Sallam et al., 2017; Henningsen et al., 2021), thus

still displays a broad range of resistance as demonstrated by many known barley *Rpg* genes. However, only two accessions, WBDC-94 and WBDC-238, contain the resistance gene *Rpg7* within the whole WBDC. Because the frequency of this gene is low in the population, it did not show up in the GWAS as a significant MTA. The inability to pick up MTAs in low frequencies is a common characteristic of a GWAS (Bernardo, 2008). Thus, this gene is an excellent candidate for high-resolution bi-parental mapping and positional cloning based on previous studies showing that the single dominant *Rpg7* R-gene confers this remarkable resistance (Henningsen et al., 2021).

Conclusion

Through the domestication of cultivated barley (*H. vulgare*), there has been a significant bottleneck of genetic diversity that is still present in wild barley *H. vulgar* subsp. *spontaneum*. One way to reverse this is the characterization of *H. vulgar* subsp. *spontaneum* diversity collections to identify novel genes contributing to traits of interest. Here, we utilized the world barley diversity collection and a GWAS approach to identify stem rust resistance genes that are effective against a virulent isolate from the PNW population for which barley has no known resistance. The WBDC GWAS analysis identifies seven novel loci on chromosomes 2H, 3H, 5H, and 7H associated with resistance to *Pgt* isolate Lsp21. However, the previously characterized loci are also of interest as we begin introgressing and stacking these resistances into domesticated elite barley germplasm. Mapping these new sources of stem rust resistance genes in barley is crucial for integrating diverse R-genes into elite barley backgrounds to enhance resistance to the virulent PNW *Pgt* population. Also, the phenotyping of the WBDC identified the remarkable resistance presumably conferred by the *Rpg7* gene, which we utilized for a positional cloning strategy and putatively identified *Rpg7* candidate genes via high-resolution bi-parental mapping.

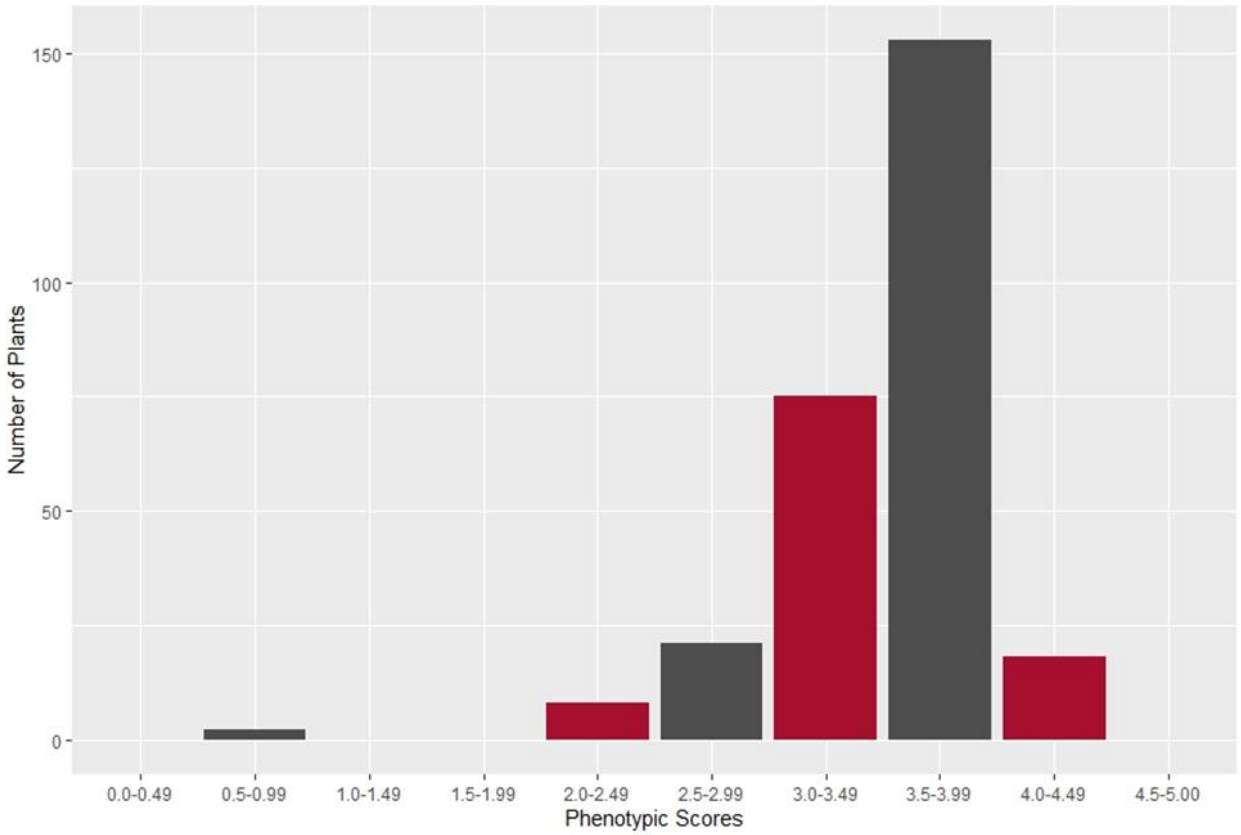


Figure 3.1: Phenotypic distribution of coefficient of infection (CI) scores of 277 wild barley diversity collection (WBDC) accessions using to *Puccinia graminis* f. sp. *tritici* isolate Lsp21 from the Pacific Northwest.

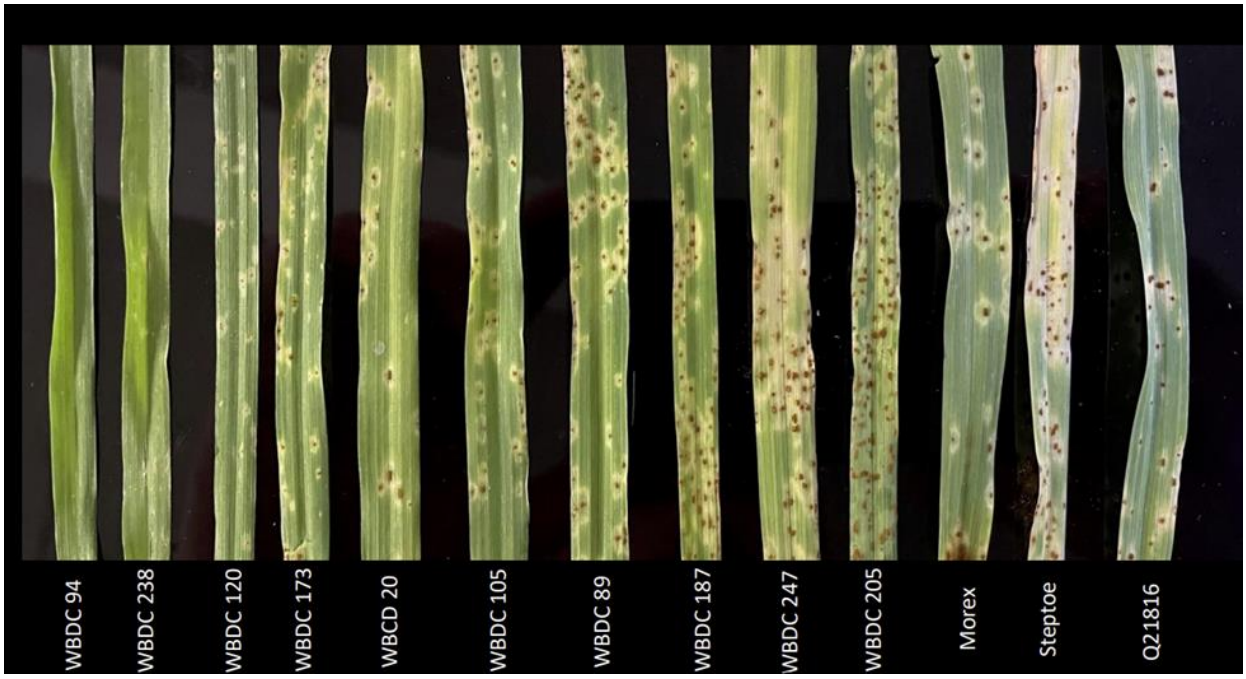


Figure 3.2: Seedling stage stem rust assay on barley from the Wild Barley Diversity Collection (WBDC) against *Puccinia graminis* f. sp. *tritici* isolate Lsp21 at 14 days after inoculation. The panel shows a typical disease assay with the virulent *Pgt* isolate, Lsp21. This isolate is virulent to important resistance genes *Rpg1* and RMRL when they are stacked together, Morex (*Rpg1*+) and Q21861 (*Rpg1*+ and *RMRL*+).

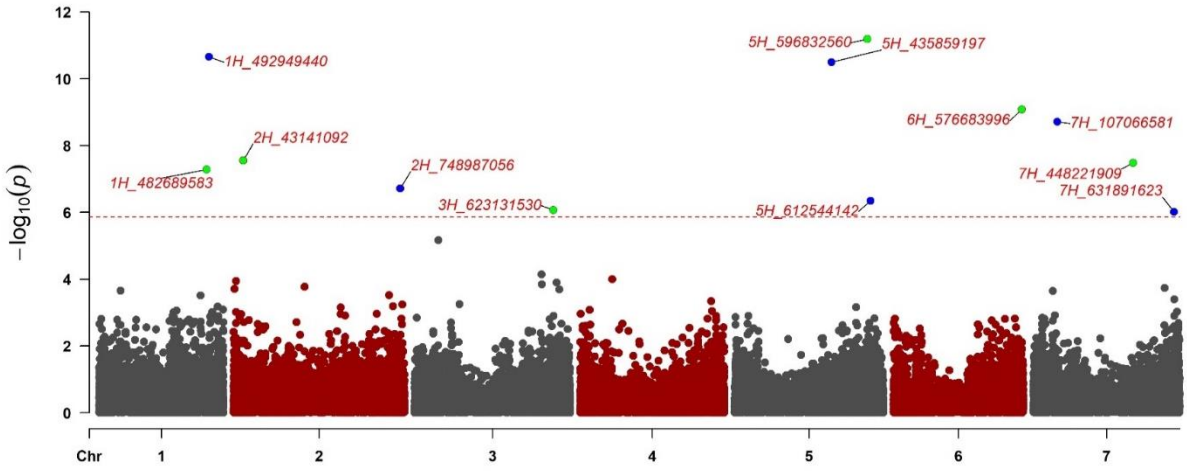


Figure 3.3: Manhattan plot displaying the seven chromosomes of barley labeled below. SNPs are shown distributed across the chromosome based on positions on the cv Morex v1 genome assembly as grey or crimson dots. The 12 MTA associated with resistance to *Puccinia graminis* f. sp. *tritici* isolate Lsp21 are above the significance threshold (horizontal dashed red line) and labeled according to their genome positions on each chromosome based on the cv Morex v1 genome assembly.

Table 3.1: Summary of the median Infection types (IT-M), range (IT-R), and average coefficient of infection (CI) on barley accessions from the WBDC against the Pacific Northwest (PNW) stem rust isolate Lsp21. Resistant (Elliot and DH160748) and susceptible (Morex, Steptoe, and Q21861) checks are also included.

Name[a]	Accession	IT-M[b]	IT-R[c]	CI[d]	Country of Origin
WBDC-094	PI 681809	0;	0; to ;1	0.56	Jordan, Mādabā
WBDC-238	PI 681943	0;	0; to 0;1	0.56	Jordan, Mādabā
WBDC-123	PI 681833	12;	1; to 12;	2.09	Khorāsān-e Raẕavī, Iran
WBDC-157	PI 681866	21;	;12 to 21;	2.19	Nīnawá, Iraq
WBDC-120	PI 681830	21;	0;1 to 21;	2.23	Sughd, Tajikistan
WBDC-173	PI 681880	21;	1;2 to 2	2.25	Hamadān, Iran
WBDC-330	PI 682022	21;	1;2 to 23-1	2.40	Balkan, Turkmenistan
WBDC-213	PI 681919	21	1;2 to 2	2.46	Uzbekistan, Samarqand
WBDC-214	PI 681920	21	1;2 to 2	2.47	Uzbekistan, Samarqand
WBDC-020	PI 681744	21	21; to 2	2.60	Turkey, Şanlıurfa
WBDC-243	PI 681947	21	21; to 213-	2.63	Jordan, Al Balqā’
WBDC-119	PI 681829	12;	12; to 213-	2.64	Uzbekistan, Jizzax
WBDC-305	PI 682003	21	21; to 3-2	2.65	Syria, Rīf Dimashq
WBDC-013	PI 681737	21;	21; to 23-	2.69	Iraq, As Sulaymānīyah
WBDC-002	PI 681727	21	21 to 2	2.75	Syria, Ḩalab
WBDC-017	PI 681741	21	21 to 213-	2.75	Syria, Dimashq
WBDC-246	PI 681950	21;	21; to 23-	2.80	Jordan, Irbid
WBDC-105	PI 681817	2	21 to 2	2.83	Jordan, Irbid
WBDC-260	PI 681962	21	21 to 2	2.85	Jordan, Ma‘ān
WBDC-170	PI 681877	21	21 to 2	2.88	Lebanon, Al Biqā ‘
WBDC-209	PI 681915	213-	213- to 3-	2.88	Uzbekistan, Jizzax
WBDC-137	PI 681847	3	21; to 3	2.89	Lebanon, Al Biqā ‘
Morex	Ciho 15773	3-2	23- to 3-	3.33	United States
Steptoe	Ciho 15229	3-2	21 to 3-2	3.19	United States
Q21861	PI 584766	23-	21- 23-	2.93	Australia
Elliot	PI 592261	21	21; to 23-	2.87	United Kingdom
DH-160748	-	21	21 to 21	2.75	United States

[a] Top resistance accessions from the wild barley diversity collection (WBDC) against PNW isolate Lsp21.

[b] IT-M represents the most common or frequent infection type observed in multiple experiments (2 to 3). Infection types (ITs) were based on the modified 0-4 rating scale initially developed for wheat by Stakman et al. (1962) and modified based on uredinia sizes on barley as described by Miller and Lambert (1955).

[c] IT-R represents the range of infection types.

[d] The coefficient of infection (CI) represents a numeric, quantitative disease score of 0 to 5, calculated from a categorical phenotype score of 0 to 4, as described by Zhou et al. (2014). CI values are means across experiments.

Table 3.2: Markers significantly associated with seedling resistance in the WBDC to the PNW *Puccinia graminis* f. sp. *tritici* isolate Lsp21 using the BLINK model.

Loci	Marker	Allele	Chr[a]	Position[b]	LOD[c]	MAF[d]	PVE[e]	LD Decay (kb)	Candidate Gene	Gene Description
WQ*Rpg*-1H01	1H_482689583	C/G	1H	449,965,275	7.28	0.20	0.96	0.208	HORVU.MOREX.r3.1HG0070200.1	Transmembrane protein
WQ*Rpg*-1H02	1H_492949440	C/T	1H	459,175,825	10.66	0.05	6.61	251	HORVU.MOREX.r3.1HG0072680.1	Respiratory burst oxidase-like protein
WQ*Rpg*-2H01	2H_43141092	G/A	2H	37,171,396	7.55	0.08	1.63	0.054		
WQ*Rpg*-2H02	2H_748987056	T/C	2H	649,620,603	6.72	0.47	0.55	308		
WQ*Rpg*-3H01	3H_623131530	C/G	3H	555,008,110	6.07	0.05	2.00	0.047	HORVU.MOREX.r3.3HG0304090.1	Ankyrin repeat-containing protein
WQ*Rpg*-5H01	5H_435859197	G/C	5H	388,581,717	10.49	0.23	2.31	1,539	HORVU.MOREX.r3.5HG0473160.1	Purple acid phosphatase
WQ*Rpg*-5H02	5H_596832560	G/A	5H	526,215,093	11.19	0.06	36.50	94	HORVU.MOREX.r3.5HG0510240.1	Dirigent-like protein
WQ*Rpg*-5H03	5H_612544142	A/G	5H	538,786,402	6.35	0.09	1.07	14		
WQ*Rpg*-6H01	6H_576683996	G/C	6H	555,502,895	9.08	0.07	2.83	5	HORVU.MOREX.r3.6HG0630630.1	RGA4 (NB-LRR)
WQ*Rpg*-7H01	7H_107066581	C/A	7H	102,573,432	8.71	0.11	1.92	1,268	HORVU.MOREX.r3.7HG0668120.1	NAC domain transcriptional regulator superfamily protein
WQ*Rpg*-7H02	7H_448221909	G/A	7H	433,462,981	7.48	0.14	0.93	603	HORVU.MOREX.r3.7HG0705680.1	NAC domain transcriptional regulator superfamily protein
WQ*Rpg*-7H03	7H_631891623	A/G	7H	604,620,989	6.01	0.05	5.45	116	HORVU.MOREX.r3.7HG0740200.1	Pectin lyase-like superfamily protein

[a] (Chr) Chromosome number in which the marker is located.

[b] Physical position of markers according to Morex v3 genome assembly (Mascher et al., 2021)

[c] (LOD) logarithm of odds or $\log_{10}(p)$ significantly expressed for association.

[d] (MAF) Minor allele frequency for each SNP marker. [e](PVE) Phenotypic variation explained by individual markers.

CHAPTER FOUR: TITLE: GUARDIANS OF THE GRAIN: UNLOCKING THE POTENTIAL OF WILD BARLEY BY FINE MAPPING THE *RPG7* BARLEY STEM RUST RESISTANCE GENE

Abstract

The recent virulence characterization of a diverse sexual population of the stem rust pathogen (*Puccinia graminis* f. sp. *tritici*, *Pgt*) collected from the Pacific Northwestern (PNW) region of North America on a barley resistance gene differential set determined that this is the most virulent *Pgt* population ever reported on barley worldwide. This was determined by individual isolates, including the isolate Lsp21 collected from Valleyford, WA, with virulence to the barley stem rust resistance genes (*R*-genes) *Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, and *rpg8*. The lack of resistance sources identified against this virulent population in the domesticated barley (*Hordeum vulgare* L.) germplasm pool directed our efforts to screen the wild barley (*Hordeum* ssp. *spontaneum*) diversity collection (WBDC) for new sources of resistance effective against the virulent *Pgt* isolate Lsp21. A previous study identified two WBDC accessions (WBDC-94 and WBDC-238) that had exceptional resistance to diverse stem rust isolates, and subsequent genetic characterization of this resistance mapped the dominant resistance to an ~10 Mbp region on the long arm of chromosome 3H and was designated *Rpg7*. Our phenotypic screening of the WBDC also identified WBDC-94 and WBDC-238 as having exceptional resistance to *Pgt* isolate Lsp21. To fine-map *Rpg7*, a biparental population was developed between cv. Morex (susceptible) and WBDC-94 (resistant). 649 F_2 individuals were screened with *Pgt i*solate Lsp21, resulting in a 3:1 resistance to susceptible ratio, indicating a single dominant R-gene presumed to be *Rpg7*. Using 45 single nucleotide polymorphisms (SNPs) identified by comparative analysis between the cv

Morex v3 genome assembly and whole genome skim sequencing of WBDC-94, a polymerase chain reaction-genotype-by-sequencing (PCR-GBS) marker panel was designed to saturate the 10 Mbp *Rpg7* region. The PCR-GBS panel was used to genotype 474 of the F_2 individuals representing 948 recombinant gametes. From this marker saturation of the region and phenotyping data, the gene conferring resistance to Lsp21 was confirmed as *Rpg7*, and this high-resolution mapping delimited *Rpg7* to ~130 kb based on the cv Morex v3 genome assembly. A total of 26 individuals were identified with recombination within the region and were advanced to the $F_{2:3}$ generation. $F_{2:3}$ homozygous critical recombinant lines were identified by genotyping with seven PCR Allele Competitive Extension (PACE) markers, further delimiting the *Rpg7* region to ~53 kb containing two predicted candidate gene models, an RPM1-like nucleotide binding site-leucine rich repeat (NLR) disease resistance-like protein and a RPM1 interacting protein 4 (RIN4)-like protein. The high-resolution mapping suggests that both genes are required for resistance and that the HvRPM1-like NLR protein may guard the HvRIN4-like protein to detect pathogen manipulation following the 'guard' model similar to the RPM1/RIN4-mediated resistance against *Pseudomonas syringae* in Arabidopsis.

Introduction

Wheat stem rust caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*) is an important foliar disease of both wheat and barley worldwide. The pathogen is an obligate fungus, requiring living host cells to colonize, acquire nutrients, and complete its life cycle by sporulating. Historically, stem rust epidemics caused significant yield losses of both wheat and barley and also caused significant decrease of grain quality (Dill-Macky et al., 1990; Steffenson, 1992; Chen, 2012; Mwando et al., 2012; Shamanin et al., 2016). The primary *Pgt* inoculum infecting wheat and barley are urediniospores disseminated from other cereal or grass hosts during polycyclic infection. However, aeciospores that form from the completion of the pathogen's sexual cycle in the secondary dicot hosts barberry and mahonia also infect wheat and barley. If the environmental conditions early in the growing season are conducive to disease formation, this early season inoculum coming from barberry or mahonia can start the polycyclic infection, resulting in severe epidemic formation (Roelfs, 1982). Once spores land on leaves with adequate moisture and temperature, they germinate during the night, and the germ tubes grow perpendicular to the leaf ridges until they encounter a stoma (Leonard & Szabo, 2005; Kolmer et al., 2009). At the stomate, an appressorial structure forms, which produces an infection peg that enters the host through the stomatal opening. Host entry induces intracellular hyphal growth where invagination of mesophyll cells results in the formation of haustoria that serves as the biotrophic feeding structure from which virulence effectors are secreted to manipulate host physiology and ultimately absorb nutrients (Staples &

Macko, 1984; Wiethölter et al., 2003). Once haustoria are formed, they serve as the powerhouse for the pathogen, which facilitates further colonization and, ultimately, completion of its life cycle by producing uredinia that erupt through the plant epidermis to release urediniospores that reinfect in the polycyclic disease pattern (Leonard & Szabo, 2005).

Unlike humans or animals, plants do not have adaptive immune systems to defend against pathogens and instead rely on a two-layer innate immune system (Jones & Dangle, 2006; Sekhwal et al., 2015). The first line of defense relies on transmembrane cell surface receptors known as pattern recognition receptors (PRRs) that evolved to recognize conserved pathogen-associated molecular patterns (PAMPs) at the leaf surface (Zipfel & Felix, 2005). PRRs contain an apoplast-localized extracellular receptor domain anchored to the plasma membrane by a transmembrane domain with an intracellular serine/threonine protein kinase signaling domain (Jones and Dangl, 2006). These proteins with the extracellular receptor, transmembrane, and intracellular signaling domain are known as receptor kinases (RKs). This early recognition of the pathogen leads to pattern-triggered immunity (PTI) (Zipfel, 2009; Dodds and Rthjen, 2010) which results in papilla formation for cell reinforcement (Hauck et al., 2003), pathogenicity-related (PR) gene induction (Dangle & Jones, 2001), rapid stomata closure which inhibits the pathogen from host entry and colonization (Melotto et al., 2008), and accumulation of reactive oxygen species (ROS) (Zipfel et al., 2008). PAMP-triggered immunity is considered the early layer of basal resistance (non-race specific resistance) that recognizes a broad spectrum of pathogens because it is elicited by conserved pathogen molecules. For example, common and well-characterized PAMPs include fungal chitin, which is a major component of fungal cell walls (Couto & Zipfel, 2016), and bacterial flagellin present in most motile bacteria (Dodds and Rathjen, 2010). The recognition of

these conserved PAMP molecules by different classes of PRRs initiates pattern-triggered immunity (PTI) (Dodds and Rathjen, 2010).

During the host-pathogen evolutionary arms race, pathogens are pressured to evolve virulence effectors to overcome these early basal PTI-mediated defenses (Jones and Dangl, 2006; Sharma Poudel et al., 2019). Many virulence effectors have been functionally characterized to suppress PTI responses, leading to effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). Once a pathogen has overcome these broad-spectrum PTI-mediated defenses, it effectively becomes a host-specific pathogen that is typically successful at colonizing specific plant species. The pressure exerted on the host by these virulence effectors leads to the evolution of race-specific R-genes. These R-genes directly recognize these effectors, or their manipulation of the host susceptibility targets, leading to the higher amplitude effector-triggered immunity responses proposed in the zig-zag model explaining host-pathogen genetic evolution (Jones and Dangl, 2006). The largest class of cytoplasmically localized plant R-genes encode proteins with a nucleotide-binding site and leucine-rich repeat (NLR) protein domain structure (Takken and Groverse, 2012). This second layer of ETI-mediated plant defenses increases ROS in cells at and adjacent to the infection site, inducing a highly regulated programmed cell death (PCD) response known as the hypersensitive response (HR). The HR effectively arrests the colonization of biotrophic pathogens such as *Pgt* that require living cells to feed and colonize the host.

A well-characterized example of ETI that is relevant to the *Rpg7* candidate genes discovered in this research is the NLR R-protein RPM1-mediated recognition of manipulation of the RPM1 interacting protein 4 (RIN4) susceptibility target by the *Pseudomonas syringae* pv. *maculicola* effector AvrRpm1 in Arabidopsis (Kim et al., 2009). Within this pathosystem, the

bacterial effector AvrRpm1 targets RIN4, inducing RIN4 phosphorylation, which was hypothesized to suppress the function of RIN4 in PTI-mediated defense responses. Once phosphorylation of RIN4 occurs, the RPM1 NLR recognizes this effector-mediated modification activating ETI defense responses, resulting in HR and race-specific resistance (Mackey et al., 2002) following H.H. Flors gene-for-gene hypothesis (Flor, 1956). Interestingly, *P. syringae* also evolved a second effector (AvrRpt2) that also targets the RIN4 protein by cleaving RIN4 at two cysteine protease cleavage sites near the N- and C-termini designated the RCS1 and RCS2 cleavage sites. Following the host-pathogen molecular arms race zig-zag model (Jones and Dangl, 2006), Arabidopsis counter evolved *Rpt2,* another NLR R-gene that recognizes RIN4 cleavage by the AvrRpt2 protease, and upon recognition of RIN4 cleavage activates ETI-mediated HR defense responses (Mackey et al., 2003).

The fact that *P. syringae* evolved two different effectors to manipulate a single host susceptibility target infers that RIN4 plays an essential role in PTI-mediated early basal defenses. This hypothesis was supported by a study showing that RIN4 functions in stomata regulation during pathogen attacks by regulating the plasma membrane H+ -ATPases AHA1 and AHA2 in Arabidopsis (Liu et al., 2009). It was hypothesized that upon pathogen challenge, RIN4 activation via PRRs induces AHA1 and AHA2 for stomatal aperture closure, which is a vital PAMP-triggered defense response that stops the bacterial pathogen from entering through the stomates. Thus, *P. syringae* is hypothesized to have evolved AvrRpm1 and AvrRpt2 effectors to suppress this important PTI defense response. However, RIN4 also negatively regulates PTI (Kim et al., 2005), thus playing a complex role in plant innate immunity at an intersection between PTI and ETI responses, yet its role as a negative regulator of PTI remains unknown. These two interactions

follow the guard model that was first proposed by Dangl and Jones (2001). This model states that R-proteins (the ‘guards’) monitor a second protein (the ‘gaurdees') that are targeted and modified by pathogen virulence effectors. Specifically, in the *Arabidopsis thaliana – P. syringae* pathosystem, the guard proteins, the NLRs RPM1 and RPT2, monitor RIN4, the guardee protein, to detect pathogen manipulations mediated by the avirulence effectors AvrRpm1 and AvrRpt2. Once the virulence effectors modify the 'gaurdee' protein or susceptibility target to suppress PTI responses, the R-genes recognize this manipulation to trigger the higher amplitude ETI responses, which rely on HR to stop or inhibit further pathogen colonization and disease formation.

Over the last two decades, the characterization of stem rust populations in North America has shown that the PNW region is the center of stem rust diversity in North America (Roelfs and Growth, 1980; Jin et al., 2014; Upandaya et al., 2022). It was hypothesized that this is due to the completion of the *Pgt* sexual cycle in primary grass hosts, barley, wheat, and wild grass *Elymus* (Leonard & Szabo, 2005; Park, 2007), and the alternate dicot hosts barberry and mahonia (Oregon grape) species. In 2023, we collected wheat stem rust from the wild grass *Elymus trachycaulus* spp. *Trachycaulus* near heavily infected mahonia bushes on Whidbey Island in western Washington. These isolates collected off *Elymus* were particularly aggressive on barley, producing ITs of 4, which is rare on barley. The virulence spectra of these isolates have not been completed, but these observations confirm that the *Pgt* disease cycle is occurring between mahonia and *Elymus* in the PNW. Virulence analysis has not been completed on the isolates collected from *Elymus* but alarmingly, single PNW isolates (i.e., *Pgt* isolate Lsp 21) have been recently identified with virulence on the barley stem rust *R*-genes *Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, and *rpg8*, which represent the most virulent isolates on barley ever reported (Upadhaya et al., 2022).

To identify novel sources of stem rust resistance in wild barley (*H. vulgare* ssp. *spontaneum*) effective against this virulent PNW population, the wild barley diversity collection (WBDC) was phenotypically screened with the virulent PNW *Pgt* isolate Lsp21, identifying two accessions (WBDC-94 and WBDC-238) with remarkable resistance at the seedling stage (Brooke et al., unpublished) (Figure 4.1). *Rpg7* provides broad and exceptionally effective seedling resistance in barley, a strong HR reaction producing median IT scores of 0;. This resistance was initially identified in the WBDC accessions WBDC-94 and WBDC-238 by screening the population with the diverse *Pgt* races MCCFC, QCCJB, TTTTF, and TTKSK as well as rye stem rust (*P. graminis* f. sp. *secalis*) isolate 92-MN-90 (Sallam et al., 2017). However, comparative genomics of WBDC-94 and WBDC-238 utilizing recent whole genome skim sequencing of the WBDC population as part of the WBDC pangenome sequencing project determined that WBDC-94 and WBDC-238 are nearly genetically identical. Thus, due to the minor allele frequency of *Rpg7* in the WBDC, essentially a single accession, it was not identified via GWAS analysis. Thus, a biparental mapping population was developed between Hiproly x WBDC-238, and *Rpg7* was mapped to a ~10 Mbp region on the long arm of chromosome 3H (Henningsen et al., 2021).

Here, we report on the high-resolution mapping of *Rpg7* using a cv Morex x WBDC-94 biparental population utilizing 474 F_2 individuals representing 948 recombinant gametes. This high-resolution mapping localized the *Rpg7* gene to an ~53 kb region on chromosome 3H based on the latest cv Morex v3 genome assembly (Mascher et al., 2021). This genomic region in cv. Morex contains two candidate gene models predicted to encode a barley (*H. vulgare*; *Hv*) *HvRpm1*-like NLR homolog and a *HvRin4*-like gene. Both genes contain a high level of polymorphism in their predicted primary nucleic acid and amino acid sequences between Morex and WBDC-94

alleles, further suggesting that they could be the gene/s underlying *Rpg7*-mediated resistance. Interestingly, two of our critical recombinant lines also suggest that both genes may be required for resistance and thus may function in barley similarly to how RPM1 and RIN4 function in Arabidopsis following the 'guard' model. This study was the first step in the positional cloning of *Rpg7*, and further gene validation and subsequent functional analysis are underway, which will fill critical knowledge gaps in the understanding of how and why the PNW *Pgt* population is so virulent on barley and why *Rpg7* provides such broad and effective resistances to these virulent isolates. It is also important to understand these functional mechanisms for the effective deployment of *Rpg7* in barley varieties in order to be good stewards of our limited *Rpg* genes in barley.

Methods and Materials

Plant materials and pathogen isolates

To conduct high-resolution mapping to genetically characterize the *Rpg7* stem rust resistance gene present in the WBDC-94 and WBDC-238 accessions, a biparental population was derived from a cross between Morex and WBDC-94. Morex is a six-row malting variety developed by the University of Minnesota in 1978 (Rasmusson and Wilcoxson, 1979). Morex does contain the *Rpg1* gene (Brueggeman et al., 2002); however, *Rpg1* is ineffective against the PNW isolate Lsp21 and should not contribute to resistance (Upadhaya et al., 2022). Morex was chosen as the susceptible parent because it is the variety for which the cv Morex v3 reference genome assembly was constructed. This allows for the efficient development of polymorphic SNP markers for saturating genomic regions in this high-resolution mapping study. The *H.* ssp. *spontaneum* wild

barley accession WBDC-94 was chosen because it is one of the two WBDC accessions collected from Jordan that contains the *Rpg7* gene (Henningsen et al., 2021). Also, we were permitted early access to the WBDC-94 whole genome sequencing data as members of the WBDC pangenome sequencing consortium, which allowed for the identification of polymorphic SNP markers and allele analysis of candidate genes utilizing genome comparative analysis against the cv Morex reference genome assembly. The WBDC-94 plant morphology is a two-row barley line with typical *H.* subsp. *spontaneum* growth habit, including prostrate plant growth with thin leaves, long stems, rough awns, and a shattering head. Six seed from the F_1 progeny derived from a single head of the Morex x WBDC-94 cross were planted and allowed to self-pollinate to generate the F_2 seed used for the high-resolution mapping. All F_1 plants showed the two-rowed phenotype with an intermediate phenotype between domesticated barley and *Hordeum* ssp. *spontaneum* determining that these were all hybrids as cv Morex was used as the female parent and is a six six-rowed malt barley line, and the two-row trait from WBDC-94 is dominant and primarily contributed by the wildtype *Vrs1* gene (Franckowiak et al., 1997).

The *Pgt* isolate Lsp21 was collected from a barley production field in Valleyford, WA, planted with the variety Lyon in 2019. Lsp21 was chosen for the high-resolution mapping of *Rpg7* because it is representative of the most virulent and aggressive PNW *Pgt* isolates in our characterized collection and shows virulence on nearly all known barley stem rust R-genes (Upadhaya et al., 2022). Lsp21 is virulent on barley cv. Morex (*Rpg1+*), CIho 7124 (*Rpg2+*), PI282313 (*Rpg3+*), HQ1 (*rpg4+* and *Rpg5+*, A.K.A. RMRL+), Q21861 (*Rpg1+*, *rpg4+* and *Rpg5+*) and Blackhulless (*rpg8+*). Our previous phenotyping analysis, as part of a GWAS utilizing the WBDC, determined that WBDC-94 has a high level of resistance to isolate Lsp21.

Plant growth conditions

F_1 seeds were grown in 6" pots to maximize F_2 seed yield. In contrast, the F_2 and F_3 seedlings used for phenotyping and progeny selfing and advancement were grown in 98 well-cone containers (6.5 cm diameter by 26.5 cm height). Each pot and cone was filled with a standard soil potting mix (Sun Gro Horticulture, Agawam, MA, USA) supplemented with Osmicote 14-14-14 slow-release fertilizer at a rate of 2 gm per well-cone. Plants were placed in a greenhouse set to 20˚C with a 16 h light (400 μm/m^2) and 8 h dark cycle as described by Upadhaya et al. (2023).

F_2 and F_3 stem rust tests

Seedlings were inoculated with rehydrated *Pgt* isolate Lsp21 urediniospores using the protocol from Upadhaya et al. (2023). All urediniospores were stored at -80˚C for long-term storage. Approximately 100 mg of spores were removed from storage and placed in a 50˚C water bath for 10 minutes of heat shock. Spores were rehydrated over a saturated KOH (Potassium hydroxide) solution for 3 h at 80% relative humidity (RH) immediately prior to inoculation.

Approximately nine days after planting, when primary leaves were fully expanded, stem rust inoculations were conducted using an atomizer pressured by a pump set at 30kPa (Steffenson et al., 2017; Upadhaya et al., 2023). Seedlings were inoculated with rehydrated urediniospores of *Pgt* isolate Lsp21 and Soltrol oil (Soltrol 170) at 8 mg of urediniospores per 1 ml of Soltrol oil. After inoculation, plant leaves were allowed to dry for one hour and placed in a mist chamber for 18 hr in complete darkness at 18˚C and 100% relative humidity. After 18 hrs, plants were placed back in the greenhouse at 20˚C with a 16 h light (400 μm/m^2) and 8 h dark cycle. For the

phenotyping inoculation experiments, parental lines were randomized throughout the cones as resistant and susceptible checks.

At 14 Days after inoculation (DAI), infection types (IT) were assessed on primary leaves. Infections were rated on a modified "0 to 4" scale. This scale was developed initially by Stakman et al. (1962) for wheat, later modified for barley by Miller and Lambert (1955), and further modified by Steffenson et al. (2017). When conducting stem rust analysis on barley, mesothetic reactions of different ITs on the same primary leaf can be observed. These IT values were categorized as 0; = hypersensitive reaction (HR), 1 = Resistance (R), 2 = moderately resistant (MR), 3- = moderately susceptible (MS), and anything above a 3 is considered susceptible (S) (Table 1) (Steffenson et al., 2017; Hernandez et al., 2019). To get a more accurate estimation of pustule size, + or – symbols were used after an IT. F_2 and F_3 progeny with ITs of 0 to 23- were considered resistant, while progeny with ITs from 3-2 to 4 were considered susceptible (Henningsen et al., 2021).

Tissue collection

Tissue from the primary leaves of each F_2 and $F_{2:3}$ seedlings from the biparental population were collected ten days after planting. All leaf tissue from F_2 seedlings were cut into approximately 3.81 cm strips, collected in a 96-well DNA silica collection plate, and sent to the USDA cereal genotyping lab in Fargo, ND, for DNA extraction. The gDNA for all 649 F_2 lines was normalized to 10 ng/ul. Plant tissue from 12 $F_{2:3}$ individuals derived from each of the 26 F_2 critical recombinants identified by the PCR-GBS genotyping was collected from the newest emerged

tissue available. The DNA was isolated from the $F_{2:3}$ individuals using a BioSprint 96 DNA Plant Kit (1536) following the protocol in the BioSprint DNA Plant Handbook (www.Qiagen.com).

Identification of Polymorphic SNPs

WBDC-94 whole genome shotgun sequencing files in fastq format generated by Illumina DNA sequencing were obtained as a part of the WBDC pangenome sequencing consortium. IIIumina adaptor sequences were trimmed from the raw sequencing reads and quality filtered using fastp 0.22.0 (Chen et al., 2018), based on quality reports produced using fastqc 0.11.9 (Andrews, 2010) and MultiQC 1.12 (Ewels et al., 2016). The fastp parameters included trimming 10 bp from the ends, low complexity filter, default cut right settings, paired-end correction, and a minimum length of 10 bp. Trimmed reads were mapped to the delimited *Rpg7* region on the long arm of chromosome 3H using the cv Morex v3 genome assembly as the reference sequence (Henningsen et al., 2021). This mapping of WBDC-94 reads to the reference sequence was performed using a custom piping loop with bwa-mem2 2.2.1 (Vasimuddin et al., 2019) and samtools (Li et al., 2009) to generate sorted bam and index files. Variant calling was performed on individual files using HaplotypeCaller within GATK 4.2.1.6 (Poplin et al., 2017) using sample g.vcf files. The g.vcf files were imported into a GenomicsDB database using GenomicsDBImport within GATK 4.2.1.6 and joint variants called using GenotypeGVCF. The population vcf file was filtered using vcftools 0.1.16 (Danecek et al., 2011) for biallelic polymorphisms with a minimum quality of 30, maximum missing data of 30%, a minimum depth of 1, and a maximum depth of 25 to prevent read pileup of homologous reads. Polymorphisms were further filtered for exonic polymorphisms using snpEff 5.1 and snpSift 5.1 (Cingolani et al., 2012). SNP calls were visualized against the 10 Mb *Rpg7*

region on the long arm of chromosome 3H, which was reported by Henningsen et al. (2021), using Geneious Prime 2024.0 (https://www.geneious.com).

F_2 Primer design

In order to genotype the Morex x WBDC-94 F_2 individuals, primer pairs were designed across identified SNPs within exons of predicted high-confidence genes located across the 10 Mb *Rpg7* region on chromosome 3H using the Primer-3 Plus primer design plugin (Untergasser et al., 2012) in Geneious Prime 2024.0 (https://www.geneious.com). A total of 60 primer pairs designed to produce ~90 to 110 bp PCR amplicons containing the SNPs of interest were initially designed. The predicted gene-specific PCR-GBS markers were designed in ~ 225 kb intervals spanning the ~10Mb *Rpg7* region. Each of the 120 primers was tested for specificity using BLAST against the barley Morex v3 genome assembly using EnsemblPlants (https://plants.ensembl.org/index.html) to predict if they would produce off-target amplicons. All 60 primers were run through MFEPrimer-3.0 to predict hairpins or dimers that may form when the primers were used in a multiplexed PCR pool (Wang et al., 2019). After this step, 49 robust primer pairs remained. NEBNext® Adapter sequences were added to each forward and reverse primer in order to form hairpin-loop structures on each end of the PCR products to minimize adaptor dimmer formation before barcoding.

NEBNext Adapter for forward primer – 5’-ACACTGACGACATGGTTCTACA-3'

NEBNext Adapter for reverse primer - 5’-TACGGTAGCAGAGACTTGGTCT-3’

Each primer pair was diluted to 100µM with PCR-grade water and verified to produce specific PCR amplicons using Morex and WBDC-94 parental genomic DNA as templates (DATA NOT SHOWN). After testing all primer pairs on the parents, 45 primer pairs remained (Supplementary table S3).

PCR-GBS library preparation

For the first round of amplification (PCR 1) in the production of the PCR-GBS sequencing libraries, 5 µl at 100 µM of each of the 90 primers targeted to produce the 45 amplicons were pooled and then brought to a final concentration of 450 µl by bringing the volume to 1 ml with PCR-grade water. 1.5 µl of gDNA from each of the 474 F_2 individuals and parental controls at a concentration of 10ng/µl was pipetted into individual wells in a 96-well PCR plate. To each reaction, 2.5 µl of Platinum® Multiplex PCR Master Mix and 1 µl of the primer pool was added for a total volume of 5 µl per reaction. Thermocycler conditions for PCR1 can be found in Table 4.1, and amplification was performed in a BIO-RAD CFX384 Touch Real-Time PCR Detection System thermocycler.

Table 4.1: Thermocycler protocol for PCR1

Step	**Temp. (°C)**	**Time**
Initial	94	10 min
10 Cycles	94	20 sec
	64 – 0.8/cycle - 56	1 min
20 Cycles	94	20 sec
	57	1 min
	68	30 sec
Final	72	3 min
Hold	4	∞

For the second PCR (PCR2), unique forward and reverse barcodes were added to each individual reaction. To attach the barcodes, 2 µl of the PCR product from PCR1 was added to a new 96-well plate, followed by 1 µl of unique forward and reverse multiplex oligo index primer pairs (barcode adaptor primers) for Illumina® from NEBNext®. Lastly, 17 µl of Q5® 2x buffer master mix was added to each well for a total of 20 µl per reaction sample. Thermocycler conditions for PCR2 can be found below and amplification was performed in a BIO RAD CFX384 Touch Real-Time PCR Detection System thermocycler.

Table 4.2: Thermocycler protocol for PCR2

Step	**Temp. (°C)**	**Time**
Initial	98	30 sec
10 Cycles	98	10 sec
	72 – 0.8/cycle - 62	30 sec
	72	30 sec
20 Cycles	98	10 sec
	62	30 sec
	72	30 sec
Final	72	2 min
Hold	4	∞

After the barcoding PCR2 reaction was completed, 15 µl of water was added to each well. Next, 5 µl of each diluted PCR2 reaction was transferred to a clean 1.5 ml tube to develop the multiplexed PCR-GBS sequencing library. Once the multiplexed library was pooled, the PCR DNA was purified using a Monarch® PCR Cleanup kit (New England Biolabs, https://www.neb.com cleanup). According to manufacturer instructions, the PCR-GBS library was

quantified with the Qubit™ DNA BR assay kit (ThermoFisher). An agarose gel was used to separate the DNA fragments from pre-barcode amplicons and the PCR-GBS library to determine if barcodes were successfully added during PC2 based on the amplicons shift in size.

The PCR-GBS library was sequenced on an Illumina MiSeq DNA sequencer using a Micro Reagent Kit at the Washington State University Laboratory of Biotechnology and Bioanalysis at Pullman, Washington. The DNA sequencing data was obtained in the Fastq file format and aligned to the 10 Mb region of *Rpg7* on chromosome 3H to delimit the region and identify critical recombinant using the protocol previously described in the *"Identification of Polymorphic SNPs"* section.

PACE primer design

Seven PCR Allele Competitive Extension (PACE^{TM}) primer combinations (Forward 1, Forward 2, and common reverse) were designed for each SNP within the ~130 kb region delimiting *Rpg7* after the analysis of the 474 F_2 recombinants using Geneious Prime software 2023.0.1 (https://www.geneious.com) (Supplementary Table S4). Each primer was aligned to the cv Morex v3 assembly to identify any off-target binding sites to ensure the specificity and efficiency of amplicons for each PACE marker. Tail sequences were attached to each Forward 1 and Forward 2 PACE markers for allelic identification.

Tail Sequence (F1): GAAGGTGACCAAGTTCATGC

Tail Sequence (F2): GAAGGTCGGAGTCAACGGAT

Each PACE marker was first tested with a 10 µl reaction on the parents, and a synthetic heterozygote containing 50% WBDC-94 and 50% Morex parental DNA, and water controls to validate amplification and determine the optimal cycle time for each PACE marker. After $F_{2:3}$ plants from each F_2 critical recombinant family were phenotyped with *Pgt*, isolate Lsp21, the PACE markers were used to genotype ~12 individuals from each of the 26 critical recombinant $F_{2:3}$ families.

Table 4.3: Thermocycler protocol for seven PACE markers.

Step	Temp (°C)	Time
Initial	94	15 min
10 cycles	94	20 sec
	65 (-0.8/cycle)	1 min
26 cycles	94	20 sec
	57	1 min
Final	4	∞

Candidate gene identification

Linear nanopore sequencing (Oxford Nanopore Technology, Oxford, United Kingdom) provided by Plasmidsaurus (SNPsaurus, Eugene, Oregon, United States) was used to validate critical recombination within $F_{2:3}$ lines identified via PCR-GBS and PACE markers. Primers were designed using Geneius Prime software to amplify the entire predicted coding region of the HvRPM1-like and HvRIN4-like candidate genes (Supplementary Table S5). Each primer was aligned to the cv Morex v3 genome assembly to verify amplification specificity and predict that no off-target amplification would occur. For quality control, each primer pair was used to amplify the candidate genes from parental gDNA template Morex and WBDC-94 to test for PCR amplification. These full-length parental amplicons were sequenced to ensure that the amplicons

were gene-specific. PCR protocol Q5® High-Fidelity DNA Polymerase (M0491) (New England Biolabs) was used. A water control was used for quality control. Critical recombinants were amplified using forward and reverse primers in 50 µl reactions. A Monarch® PCR Cleanup kit (New England Biolabs, https://www.neb.com cleanup) was used to purify amplicons. According to manufacturer instructions, PCR DNA was quantified with the Qubit™ DNA BR assay kit (ThermoFisher) before the linear amplicon sequencing using MinION Nanopore (Oxford Nanopore Technologies) sequencing service provided by Plasmidsaurus (SNPsaurus, Eugene, Oregon, United States). Fastq files were then obtained and aligned in Geneious Prime software 2023.0.1 (https://www.geneious.com) using the MAFFT multiple sequence alignment algorithm function with default setting.

Results

Phenotyping of 649 F_2 progeny from a Morex × WBDC-94 cross inoculated with the virulent PNW *Pgt* isolate Lsp21 resulted in a ratio of 483 resistant: 166 susceptible individuals. A χ^2 analysis of the segregation data (0.11) at an $\alpha < 0.05$ resulted in a *P*-value of 0.70144; thus, the null hypothesis of a 3:1 resistant to susceptible segregation ratio was accepted, indicating a single dominant resistance gene underlying the resistance conferred by the parent WBDC-94. Parental checks reacted as expected, with cv Morex displaying a susceptible reaction with a median reaction type of 3- and WBDC-94 displayed a resistance reaction with a median reaction type of 0; in response to *Pgt* isolate Lsp21. Previous low-resolution genetic mapping of the *Rpg7* gene, which is only present in the WBDC accessions WBDC-94 and WBDC-238, delimited the *Rpg7*-mediated resistance to an ~10 Mb region between the flanking markers S3H_682867653 and S3H_693707636 on barley chromosome 3H (Henningsen et al., 2021). Within this 10 Mb region,

45 PCR-GBS SNP markers were developed with polymorphic SNPs between WBDC-94 and Morex within the delimited *Rpg7* region (Supplementary Table S3). This custom SNP marker panel was used to construct a PCR-GBS library to genotype 474 F_2 progeny from the Morex × WBDC-94 population representing 948 recombinant gametes (Supplementary Table S4). For sequencing quality control results the average reads per sample before filtering was 5,345, and after filtering was 4,456 per sample.

The initial high-resolution map utilizing all 474 F_2 individuals delimited *Rpg7* to an ~ 129 kb region between PCR-GBS markers_002 and marker_008 (Figure 4.1). Five high-confidence candidate genes and six low-confidence genes were identified within this delimited region. Furthermore, 26 F_2 critical recombinant individuals containing recombinant gametes within the region were identified. Each of the 26 F_2 critical recombinants were advanced and allowed to self-pollinate, and twelve $F_{2:3}$ individuals from each of the 26 F_2 critical recombinants were planted and phenotyped with *Pgt* isolate Lsp21 and genotyped. The $F_{2:3}$ families were genotyped with seven PACE markers designed over the PCR-GBS markers 002, 003, 004, 005, 006, and 007 located within the ~ 129 kb region delimited by the GBS-PCR genotyping and phenotyping of the F_2 individuals (Supplementary Table S4). The $F_{2:3}$ genotyping allowed for the identification of homozygous critical recombinants, and phenotyping of these individuals allowed for the delimiting of the *Rpg7* region to an ~53 kb region. This region contained two high-confidence genes, HORVU.MOREX.r3.3HG0326090 and HORVU.MOREX.r3.3HG0326080. These genes are predicted to encode a RPM1-like NLR disease resistance protein and an RPM1-interacting protein 4-like (RIN4) protein. Three low-confidence genes were also identified in the region as well: HORVU.MOREX.r3. 3HG0326040 (a predicted volume-regulated anion channel subunit

LRRC8E), HORVU.MOREX.r3.3HG0326060 (a predicted E3 ubiquitin-protein ligase), and HORVU.MOREX.r3.3HG0326070 (a predicted polyribonucleotide nucleotidyl-transferase).

Candidate genes

Based on the high-resolution genetic mapping, the two candidate *Hv*RIN4-like and *Hv*RPM1-like genes were the top two candidate genes (Table 4.4). PCR primers were developed, and Min-Ion long-read sequencing technology was utilized to sequence the full-length predicted coding regions of both candidate genes from the $F_{2:3}$ derived homozygous critical recombinants to validate candidate genes (Supplementary Table S5). After sequencing across the *Hv*RPM1-like and *Hv*RIN4-like candidate genes from six $F_{2:3}$ susceptible homozygous critical recombinants (274.1, 274.4, 274.11, 274.12, and 344.3) and aligning the sequence reads to both Morex and WBDC-94 parents, it was hypothesized that both WBDC-94 gene alleles may be necessary for resistance (Figure 4.2). The four homozygous critical recombinants derived from F_2 line 274 all contained identical WBDC-94 *Hv*RPM1-like genes. However, they all had a Morex-like coding region of the *Hv*RIN4-like gene. The opposite was true for the crucial recombinant 344.3. The *Hv*RIN4 allele was identical to the WBDC-94 *Hv*RIN4 but had a Morex allele of the HvRPM1-like gene. Analysis of the predicted amino acid sequence of the WBDC-94 *Hv*RIN4-like gene predicted a premature stop codon as compared to the Morex allele. This premature stop codon is also the same for critical recombinant 344.3. Thus, we hypothesize that both the HvRIN4-like and HvRPM1-like genes from WBDC-94 may be required for resistance.

Discussion

High-resolution mapping was utilized to delimit the barley *Rpg7* stem rust resistance gene/s to an ~ 53kb region containing a high confidence *Hv*RPM1-like NLR R-gene and an *Hv*RPM1 Interacting 4 (RIN4)-like protein. Two critical recombinants determined that both genes may be required for *Rpg7*-mediated resistance. Based on the well-characterized roles of RPM1 and RIN4 in *P. syrinage* resistance in Arabidopsis, we hypothesize that these two barley homologs of RPM1 and RIN4 may function together similarly in stem rust resistance following the guard model. The guard model states that R-proteins (the guards) monitor a second protein (the guardee), which are targeted by pathogen virulence effector proteins to suppress PTI-mediated basal defense responses (Dangl and Jones, 2001). Once a virulence effector modifies its host susceptibility target (the guardee), the NLR R-protein (the guard) activates effective ETI-mediated disease resistance that renders the pathogen as avirulent. Thus, this virulence effector gene in the presence of the race specific R-gene becomes a pathogen avirulence (Avr) gene (Van der Biezen and Jones, 1998; Dangl and Jones, 2001).

The guard model was first proposed based on functional characterization of the *P. syrinage* pathogen effector (AvrPto), which targets and modified the tomato protein kinase (Pto), which activates the NBS-LRR protein (Prf), resulting in an incompatible (disease resistance) interaction (Dangle and Jones, 2001). RIN4-like proteins have been shown across multiple species, including apple, barley, soybean, tobacco, and Arabidopsis to be targeted by avirulence effectors and probably guarded by cognate NLR proteins (Luo et al., 2009; Selote et al., 2013; Prokchorchik et al., 2020, Gill et al., 2016). However, additional studies in the tomato-*Psuedomonas* pathosystem have determined that the actual host susceptibility target of AvrPto is the serine/threonine protein

kinase signaling domain of the FLS2 PRR receptor-like kinase that recognized the PAMP bacterial flagellin to elicit PTI-mediated resistance which includes stomate aperture-closing to inhibit pathogen entry into the plant (Xiang et al., 2008; Zong et al., 2008). It was determined that Pto functions as a mimic of the FLS2 protein kinase signaling domain and when AvrPto targets the FLS signaling domain to suppress the PTI defense responses, it also targets the Pto mimic protein kinase which is guarded by the PRF NLR immunity receptor activating ETI defenses, which gave rise to the 'decoy' model (Zhou & Chai, 2008; Zipfel & Rathjen, 2008). Another example of a pathogen decoy is the RCR3 (required for *Cladosporium* resistance 3) protein in tomato. The pathogen *Cladosporium fulvum* pv. *tomato*, secretes the Avr2 effector into tomato cells, targeting the decoy protein RCR3 as it intends to target PIP1 triggering Cf-2-mediated resistance (Shabab et al., 2008). The hypothesis is that RCR3 evolved from the PIP1 via a duplication event and subsequently evolved the new decoy function because they are paralogous genes with similar sequences located adjacent to one another in the tomato genome (Tian et al., 2007).

In contrast to the guardee, which is the original susceptibility target, the decoy evolved to resemble the actual virulence target in the host to possibly remove the NLR resistance complex away from the essential function of the original virulence target in PTI defense responses (van der Hoorn and Kamoun, 2008). The hypothesized benefit of guardee or decoy proteins is that they allow the recognition of pathogen-effector molecules while allowing the plant to maximize the limited numbers of immune receptors (Contreras et al., 2023). Furthermore, because decoy and guardee proteins do not retain the essential function of their progenitor proteins, which are required for basal PTI defenses, a higher level of mutation is permitted, allowing for co-evolution with the rapidly evolving pathogen effector repertoire (Contreras et al., 2023).

In our current barley *Rpg7* functional model, we hypothesize that the *Pgt* AvrRpg7 effector targets the truncated WBDC-94 *Hv*RIN4-like protein, which may function as a decoy of a functional RIN4 barley homolog as there are several present in the barley genome. Thus, it is the actual RIN4 susceptibility gene in barley that is targeted by AvrRpg7 to induce stomate opening for pathogen entry. We have come to this hypothesis based on the predicted HvRIN4 gene structure in WBDC-94, which does not resemble a functional RIN4 protein but could function as a decoy similar to Pto and RCR3. Interestingly, this is not the first barley R-gene that appears to function by guarding a HvRIN4 homolog. A yeast-two-hybrid experiment with the Rpg1 protein identified another HvRIN4 homolog in barley that interacts with *Rpg1*; thus, *Rpg1* may also function in a role to monitoring the action of an AvrRpg1 protein to suppress RIN4 mediated PTI defenses (Gill et al., 2015).

Conclusion

To date, only eight stem rust resistance genes have been identified and named in barley, including *Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, *rpg6*, *Rpg7*, and *rpg8*; six from *H. vulgare*, one from the wild barley *H. bulbosum*, and one from the wild barley *H. spontaneum*. Of the three effective stem rust resistance genes in barley (*Rpg1*, *rpg4*, and *Rpg7*), two have been cloned and characterized, *Rpg1* and *rpg4* (Brueggeman et al., 2002; Brueggeman et al., 2008; Wang et al., 2013). However, the only stem rust resistance gene deployed in commercial barley cultivars is *Rpg1*. This limited number of stem rust R-genes leaves barley pathologists and breeders with few options for genetic resistance when it comes to emerging new virulent isolates. However, wild barley may represent a rich source of new stem rust resistance genes. The virulent PNW *Pgt* population, which contained isolates that were virulent to all known stem rust resistance genes, was used to screen the wild

barley diversity panel, and it was determined that *Rpg7* provided effective resistance to these virulent isolates. Here, we reported on the high-resolution mapping of *Rpg7* utilizing a Morex × WBDC-94 population for positional cloning. We identified two candidate *Rpg7* genes: an *Hv*RPM1-like nucleotide binding site-leucine rich repeat (NLR) disease resistance-like protein and a *Hv*RPM1 interacting protein 4 (RIN4)-like protein. Based on two critical recombinants in the region, we hypothesize that both genes may be required for resistance. Our current functional model that will be tested moving forward is that the HvRPM1-like NLR protein may guard the HvRIN4-like protein to detect pathogen manipulation following the 'guard' model similar to the RPM1/RIN4-mediated resistance against *P. syringae* in Arabidopsis. As our first step in functional validation of these candidate genes, we developed an EMS mutant population to identify *Rpg7* mutants, which will be characterized to determine if our HvRpm1/HvRin4 hypothesis is correct. It is crucial to identify and characterize *Rpg7* to determine how it can be deployed for more effective and durable resistance against the virulent isolates emerging in the PNW and may allow us to begin determining why the PNW population is so virulent and evolved to overcome all previously known barley stem rust resistance genes.

Table 4.4: List of candidate genes for *Rpg7* after high-resolution mapping using the $F_{2:3}$ critical recombinant families from the biparental population of Morex × WBDC-94.

Gene Name	Description	Length (bp)	Position (Morex v3; 3H)
HORVU.MOREX.r3.3HG0326080	RPM1-Interacting Protein 4	481	610008276 - 610008756
HORVU.MOREX.r3.3HG0326090	Disease resistance protein RPM1	4,552	610055444 - 610059995

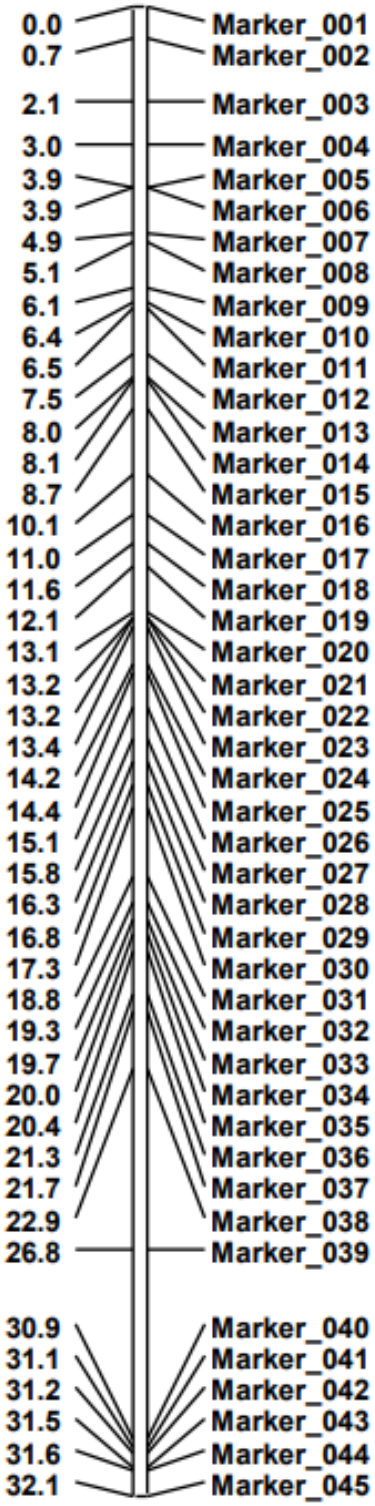


Figure 4.1: Physical map of all 45 GBS-PCR primer pairs across the 10 Mpb region of *Rpg7* on chromosome 3H.

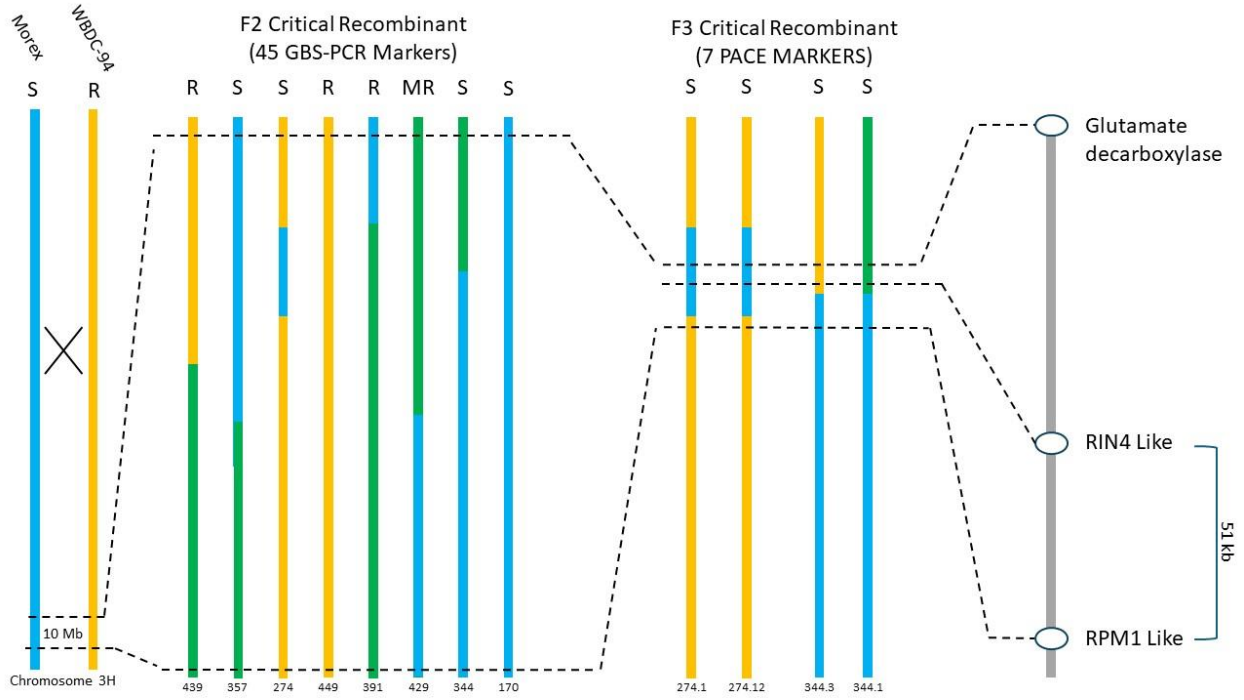


Figure 4.2: Genetic and physical map showing the results of the Morex × WBDC-94 high-resolution mapping using F_2 and $F_{2:3}$ critical recombinants. The vertical blue bars represent homozygous barley cultivar Morex regions, vertical orange bars represent homozygous WBDC-94 regions, and vertical green bars represent heterozygous regions. The dotted black bars represent the position of markers within the high-resolution mapping on the critical recombinants within the region. The vertical grey bar is the delimited region of the high-resolution mapping delimiting the candidate genes to two genes shown in white ovals.

Figure 4.3: Annotation of *Rpg7* genomic region and predicted protein structures from a high-resolution biparental population of Morex × WBDC-94. Barley cultivar Morex (susceptible), wild accession WBDC-94 (resistant), and two critical recombinants, CR_274.1 and CR_344.3 (susceptible), are labeled on the left. Scale shown above in kilobases. The annotations in blue represent alleles from Morex. The annotation in orange represents alleles in WBDC-94. Annotation in black represents alleles from both Morex and WBDC-94.

CHAPTER FIVE: UTILIZING ASSOCIATION MAPPING TO DEVELOP WSU SPRING MALT BARLEY VARIETIES

Abstract:

Barley (*Hordeum vulgare* L.) is an economically important cereal crop in the United States (US) that is mainly used for malt production to support the brewing and distilling industries. The American Malting Barley Association (AMBA) provides the malt barley recommended variety list for the US, which primarily determines which varieties the large malting companies will contract producers to grow. To determine if a barley variety has the malt quality standards required of the brewing and distilling industries, AMBA coordinates a malt quality analysis program that requires a barley variety to meet strict standards for 18 malt quality traits across diverse environments. This can be a difficult standard for the breeder as these end-use quality traits are under complex, highly quantitative genetic control. Furthermore, phenotyping experimental lines for malt quality traits is time-consuming and expensive. To address these limitations and to assist the development of WSU malt barley lines, the top yielding 250 experimental lines from the Washington State University (WSU) barley breeding single replicated yield trials in 2021 and 2022 were selected and malted at the USDA-ARS Cereal Crops research unit in Madison, WI. Malt quality phenotypes tested were kernel weight, kernel plumpness, kernel protein, malt β-glucan, free amino nitrogen, soluble protein over total protein, extract, barley color, wort color, wort clarity, wort protein, α-amylase, and diastatic power. These lines were also genotyped using the Illumina iSelect 50k platform. After filtering the single-nucleotide polymorphism (SNP) data by removing SNPs with >30% missing data, minor allele frequencies <5%, and heterozygotes >3.25%, the final number of high-quality SNPs was 19,166. The phenotyping and genotyping data were utilized in genome-wide association

studies (GWAS) to identify marker-trait associations (MTAs) with 13 malt quality traits. Using the GAPIT package in R studio, the BLINK model was used to identify 44 MTAs with malt quality across all seven chromosomes anchored to the cv Morex v3 genome assembly. The most significant marker was SCRI_RS_193456, which was associated with β-glucan, free amino nitrogen, soluble protein over total protein, wort protein, and α-amylase in the 2021 field season. These MTAs will assist breeding programs in identifying lines enhanced for malt quality by utilizing marker-assisted and genomic selection.

Introduction

The United States (US) harvested roughly 185 million bushels of barley (*Hordeum vulgare* L.) in 2023 (UDSA-NASS, 2024). North Dakota, Montana, Idaho, Wyoming, Colorado, and Washington State were the top barley-producing states. Barley that is produced in the US is primarily used for malt (68%) and livestock feed (28%), with little going towards human consumption (4%) (USDA-NASS, 2022). Barley was historically grown on large acreage throughout the US, and at the peak of barley production in 1942, the US harvested over 16 million acres of barley (USDA-NASS, 2019). However, there has been a steady decline in barley acreages over the last 80 years due to competition from other feed crops like corn and soybean, which are more profitable for producers. Despite this national trend of declining production, malting barley still plays an important economic role in the US and is essential to an industry embedded in our social fabric. Although the estimated worth for barley grain in 2024 was over $1 billion (AMBA, 2024), this is relatively small compared to other cereal grains such as wheat and corn. However, because barley is a critical raw material in beer brewing, it currently supports over 9,000 breweries in the US and over 120 malt plants. To this end, barley has an estimated value-added economic

impact in the US brewing industry alone of $409 billion, supporting 2.37 million jobs and generating 63.8 billion in tax revenue (AMBA, 2023). These numbers do not include the distilling industry. In 2023, there were more than 1000 distillers in the US, with 378 producing American single malt whiskey, and according to the American Single Malt Whiskey Commission, American single malt whiskey must be produced from 100 percent malted barley (ASMWC, 2024). New data suggests that Kentucky alone sold enough bourbon to support a $9 billion industry where malt barley is essential but not the main distilling ingredient (Kornstien & Luckett, 2014; Coomes & Krnstien, 2019).

For barley to make the journey from the farm field to the malt house, then to a brewery or distillery, and finally into a glass, it must pass rigorous quality standards. The American Malt Barley Association (AMBA) is a non-profit organization that sets malt quality standards to ensure that the US produces a stable, efficient, and reliable domestic supply of quality malt barley to support the needs of the brewing and distilling industries. The criteria to meet the different industry standards are set by AMBA and can be categorized into whole kernel, congress mash, and enzyme extract analysis. These categories can be broken down further into 13 phenotypic parameters:

1) **Whole Kernel**: Barley Protein (BP), Barley Color (BC), Kernel Weight (KW), Grain Plumps (PL)
2) **Congress Mash**: Extract, (EX), β-Glucans (BG), Free Amino Nitrogen (FAN), Wort Protein (WP), Soluble Proteins (S/T), Wort Color (WC), Wort Clarity (WC)
3) **Enzyme Extract**: α-Amylase (AA), Diastatic Power (DP)

These quality traits make barley more efficient at the malting plant while providing the proper enzyme package for brewers and distillers (Fox et al., 2003). The malting process is a tightly controlled germination process containing three steps: 1) steeping, 2) germination, and 3) kilning. The process of malting is a time-consuming and expensive value-added procedure that can take up to 7 days to complete (Schwarz and Li, 2011). During this controlled germination process, the barley endosperm undergoes complex modifications. Malt modification consists of breaking starches and proteins into simple sugars and free amino acids so that brewer's yeast can utilize these nutrients during the brewing and distilling fermentation processes (Briggs, 1987a,b, 1992). Thus, the critical malt quality trait is the efficiency and consistency of grain modification to fermentable sugars (extract) during the malting process.

Breeding for malt quality can be difficult because of environmental factors, agronomic inputs, and the quantitative genetics controlling the traits that affect the end-use malt quality (Fox et al., 2003). Furthermore, phenotyping malting samples within a breeding program is time-consuming and expensive. Breeders are beginning to overcome some of these limitations by genetically characterizing loci positively contributing to malt quality and identifying marker-trait associations (MTAs) with malt quality. Genotype and phenotype data are used to associate regions of the genome to a specific trait or multiple traits (Bernardo, 2008).

Much of the past research characterizing the genetic loci contributing to malt quality traits has focused on bi-parental and double haploid populations to map quantitative trait loci (QTL) (Hayes et al., 1993; Kleinhofs et al., 1993; Han et al., 1997; Mather et al., 1997; Hayes and Jones, 2000; Marquez-Cedillo et al., 2000). Many of these populations consisted of parents with contrasting phenotypes for the malt quality trait(s) of interest. Hays et al. (1993) were the first to

utilize a bi-parental population to genetically map malt barley quality traits using a Morex (a six-row malt barley variety) crossed with Steptoe (a six-row feed barley variety). They generated a genetic map utilizing restriction fragment length polymorphism (RFLP) markers and identified 62 QTL corresponding to eight malting traits across multiple environments. Interestingly, all malt quality traits were relatively stable across environments except grain protein, in which a significant G x E interaction was detected. A bi-parental mapping population was also used from a cross between Morex, a spring six-row malt barley variety, and Dicktoo, a winter barley (Oziel et al., 1996). Interestingly, on chromosome 5H (7), there was a complex of malt-quality QTL representing a multi-locus cluster of genes. This malt-quality gene cluster on chromosome 5H was first reported by Ullrich et al. (1993).

Mather et al. (1997) used the parents Harrington (a two-row malt variety) and TR306 (a two-rowed experimental line) to develop a double haploid (DH) population to map alleles associated with total grain protein using a genetic map generated with RFLP markers. Harrington alleles were associated with lower grain protein levels, and QTL were mapped to chromosomes 4H, 5H, and 7H. Lower protein is vital in malt barley varieties because excessive grain protein has been found to inhibit H_2O uptake during the malting process, lower malt extract, and increase wort viscosity, which all affect either the malting or brewing processes (Bishop, 1930; Burger & Laberge, 1985; Bamforth & Barclay, 1993). Lastly, Marquez-Cedillo et al. (2000) found 17 QTL associated with malt quality traits across multiple environments using a biparental population between Harrington (a two-row malt barley variety) and Morex (a six-row malt barley variety).

As single nucleotide polymorphism (SNP) marker technologies became more robust and affordable and plant geneticists began to adopt genome-wide association study (GWAS)

technologies that were initially developed in human and animal systems, barley geneticists also began utilizing GWAS to discover marker-trait associations (MTA) for malt quality within diverse population (Matthies et al., 2014; Mohammadi et al., 2015; Gyawali et al., 2018; Looseley et al., 2020; Jensen et al., 2023). Genome-wide association studies differ from quantitative trait loci analysis utilizing a bi-parental recombinant inbred line (RIL) or double haploid (DH) mapping populations because RIL or DH populations are developed using two parental genotypes with contrasting phenotypes. Thus, these QTL studies contained limited loci and recombination events, identifying limited MTA at low resolution. Diverse populations utilized for GWAS allow for the mapping of diverse loci associated with the trait of interest, and the high levels of recombination and linkage disequilibrium (LD) decay can allow for higher resolution mapping dependent upon the population and level of marker saturation (Bernardo, 2008). Thus, GWAS can take advantage of genetic diversity and ancient recombination events to identify more loci contributing to quantitative traits.

This study aims to discover loci positively contributing to malt quality within the WSU malt barley breeding program. The MTAs identified in this study utilizing a GWAS approach will be used for marker-assisted selection (MAS) and genomic selection to improve malt quality in the Washington State University barley breeding program with a significant focus on lowering β-glucan and protein levels and raising enzyme α-amylase activity, and total malt extract. These quality traits will expedite the development of American Malting Barley Association (AMBA) recommended varieties. By developing high-quality malt barley varieties that consistently meet AMBA standards and the yield requirements of PNW producers, we strive to provide growers with dual-purpose malt/feed varieties that may allow growers to produce a malting variety off contract

that may still make malt grade and increase profit margins on years when there are deficits in malt barley such as 2022 when the US malting industry had to import substantial amounts of malt barley from Argentina.

Methods and Materials

Germplasm and experimental design

The germplasm utilized in this study was from the Washington State University Barley Breeding and Molecular Genetics Lab. Germplasm consisted of $F_{4:5}$ derived single replicated (SR) yield trials of two-row spring malt barley through single seed decent single. The field trials were set up in a randomized complete block design for 2021 and 2022 at Spillman Farms near Pullman, Washington. Each line was planted in a 4' x 16' plot row at 80 g per plot. After harvest, a 250 g sub-sample was collected from each plot to be malted. Two malting checks were replicated each year to account for environmental effects between years. These checks consisted of malting barley varieties CDC Copeland and AC Metcalfe.

Malting traits and parameters

The approximately 300 lines selected each year to be malted were chosen based on their yield. All barley grain samples were malted to a standard base malt by the USDA-ARS Cereal Crops Research Unit (CCRU) in Madison, WI. The malting procedure followed the protocols of the American Society of Brewing Chemists (ASBC, 1992). Approximately 170 g samples of each line were screened through a 5/64" slotted screen before malting. The malting protocol included a steeping, germination, and kilning cycle. Each sample was steeped in water for 4 h at 16°C,

followed by a 4 h air rest at 18°C and a 4 h water immersion at 16°C. The target grain moisture or steep-out was 45%. After the samples had finished steeping, they were germinated for 120 h at 17°C with >98% humidity. During the germination cycle, the samples were turned for 3 min every half hour to prevent the rootlets from clumping. After the germination cycle was completed, the samples were kilned to arrest germination and preserve the enzyme activity of the malt. Hot air at 49°C was forced through the samples for 10 h, 54°C for 4 h, 60°C for 3 h, 68°C for 2 h, and 85°C for 3 h until the final target malt moisture was ~4.0%.

After the kilning process, each sample was derooted and cleaned. To undergo full analysis, each sample was subjected to quality measurements using congress mash and salt extract analysis as described by the American Society of Brewing Chemists (ASBC) (Budde et al., 2010). Sample was analyzed for 13 traits, including barley protein (BP), β-glucan (BG), free amino nitrogen (FAN), soluble protein over total protein (S/T), extract (EX), kernel weight (KW), kernel plump (KP), barley color (BC), wort color (WC), wort clarity (WC), wort protein (WP), α-amylase (AA), and diastatic power (DP).

Tissue collection and genotyping

After field harvest, three seeds from each experimental line were planted in 98 well containers (6.5 cm diameter by 26.5 cm height). Each cone was filled with a standard soil potting mix (Sun Gro Horticulture, Agawam, MA, USA), and supplemental slow-release fertilizer Osmicote 14-14-14 was used at a rate of 2 mg per well-cone. Plants were placed in a greenhouse at 21˚C with a 16 h light (400 μm/m^2) and 8 h dark cycle. Seven days after planting, leaf tissue was cut into approximately 1.5" strips and collected in 96-well silica plates with approximately 0.5 g

of silica per well. The tissue was then sent for DNA isolation and genotyping at the North Central Small Grains Genotyping Lab in Fargo, North Dakota. The genotyping platform used was the Illumina iSelect 50k SNP chip (Muñoz-Amatriaín et al., 2014), which provides saturated SNP coverage of the barley genome.

Association mapping

SNP data were initially filtered by removing SNPs with >30% missing data. Then, data imputation was performed using Tassel (Bradbury et al., 2007). Following imputation, markers with minor allele frequency (MAF) <5% were eliminated to minimize the likelihood of type I error in the GWAS analyses. A principal component analysis, explaining at least 25% of the variation, was performed using the VanRaden function of genomic association prediction integrated tool (GAPIT) at default settings in R (Lipka et al., 2012). Next, a Bonferroni adjustment was applied at an α-level of 0.05 to avoid type I errors. Marker-trait associations (MTAs) from the GWAS were considered significant at a *p*-value <0.00000303 corresponding to a LOD [-log10(p-value)] score >5.60. Furthermore, using GAPIT version 3 in RStudio, marker-trait associations were tested using BLINK (Huang et al., 2019; RStudio Team, 2020; Wang and Zhang, 2021). Manhattan plots were generated using the cmplot package in R (Yin, L. et al., 2021) (Supplementary Figure S5).

Linkage disequilibrium

The genotype data in hapmap format were converted to PLINK format files using the Tassesl v5.2.93 software. Then, the LD blocks were estimated using the PLINK v1.9 tool with the following options: --blocks-max-kb 100000 and --blocks-min-maf 0.01. For block estimation, the method described by Gabriel et al. (2002) was used in the PLINK tool. LD blocks were used to

estimate and delimit the significant loci detected and to identify high-confidence candidate gene models within the physical regions defined by significant MTA and nonsignificant MTA both distally and proximally. Then, the LD blocks were estimated in haploview software using solid spine of LD (Barrett et al., 2005).

MTA and candidate gene identification

The regions delimiting predicted high-confident candidate gene models underlying malt quality loci identified by significant MTA were anchored to a barley physical map utilizing the cv Morex v3 genome assembly genome browser housed on Grain Genes (https://wheat.pw.usda.gov/GG3/). The physical positions of the SNP markers were initially aligned to the Morex v1 genome assembly and updated to the Morex v3 assembly positions using the CSV file option from the T3 Barley triticeae toolbox (https://barley.triticeaetoolbox.org/). All SNP markers identified as significant marker-trait associations (MTAs) were located using sequence tag files and the BLASTn function on the cv Morex v3 genome assembly genome browser housed on GrainGenes (https://wheat.pw.usda.gov/GG3/). Delimited genomic regions for each locus were determined by the nearest nonsignificant markers proximal and distal to the significant marker. The predicted candidate gene models within the delimited regions were submitted to BLASTp in the National Center for Biotechnology Information (NCBI) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify putative gene function based on significant homology to genes with known function and domains (Blum et al., 2021). Linkage disequilibrium decay was also calculated to predict the delimited region of each MTA. The nomenclature for each MTA was as follows; (*WA-AA_1H*-1) where W=Washington; A=Association, followed by the abbreviation for the malt quality trait, Kernel weight (KW), wort clarity (CL), kernel plumpness

(Plump), extract (EX), barley protein (BP), wort protein (WP), soluble/total ratio (S_T), free amino nitrogen (FAN, diastatic power (DP), α-amylase (AA), and β-glucan (BG), followed by the chromosome designation and number of MTA on the chromosome associated with the MTA.

Results

Malt analysis phenotype

The GWAS analyses were conducted to identify MTA for 12 different malt quality traits utilizing diverse Washington State University experimental lines derived from 61 crosses and 25 different parental lines. From the 2021 and 2022 field seasons, 279 and 261 lines, respectively, were phenotyped for malt quality traits and genotyped using the Illumina iSelect 50k SNP chip (Muñoz-Amatriaín et al., 2014) for a total of 540 lines. Initially, we wanted to combine both years of data for the GWAS analysis. However, due to the inconsistent barley checks that were malted in 2021, we could not perform an ANOVA to account for environmental differences between years (data not shown). Thus, years were separated in these two GWAS analyses. The USDA-ARS Cereal Crops Research Unit (CCRU) in Madison, WI, malted the single replicated lines and checks. The phenotypic distributions separated by year for each trait were utilized to generate histograms (Figure 5.1 A & B). The Kernel Weight (mg) averages for 2021 and 2022 were 35.2 and 36.58 mg, respectively. Grain plumpness (%) averages for 2021 and 2022 were 91.4 and 81.16%. Grain plumpness was probably lower in 2022 due to reoccurring heat domes during grain fill, as adequate soil moisture and precipitation were not an issue. Barley Color (Agtron) averages were consistent across years at 77.6 in 2021 and 78.0 in 2022. Extract (%) averages in 2021 and 2022 were 77.0 and 75.98%. Each year's average grain protein (%) was 15.3 and 15.33%, and

Wort Protein (%) was 5.8 and 5.63%. The soluble over total protein S/T (%) was 38.9 and 37.88%, remaining consistent across years. Diastatic Power (°ASBC units) between the two years were 136.6 and 144.07. A-Amylase (20° dextrinizing units or DU) was also consistent with an average of 77.7 and 77.04 in 2021 and 2022, respectively. There was a drop in the average of b glucan (ppm) in 2022 from 289.9 to 262.77 ppm. Lastly, Free Amino Nitrogen (ppm) averaged 209.0 in 2021 and was lower in 2022 at 194.61 ppm.

Pearson's correlation

Trait correlations for each year were calculated using Pearson's correlation coefficient for all 12 malting quality traits (Figure 5.2). High whole-grain protein content was negatively correlated with high malt extract but positively associated with higher diastatic power in 2021 and 2022. This correlation is consistent with previous findings (Rasmusson and Glass, 1965; Ullrich et al., 1981; Eagles et al.,1995; Fox et al., 2003; Mohammadi et al., 2014). High grain protein is considered low quality in malting barley due to the correlation with lower carbohydrate levels, thus reducing extract levels (Bishop, 1930). High protein is one of the number one criterion that will result in malt barley being rejected at the grain elevator and being reduced to feed-grade barley which results in the producer losing malt barley premium prices. Thus, lower protein is a major trait of selection in malt barley breeding programs. In both years, high α amylase activity positively correlated with diastatic power, wort protein, FAN, extract, and S/T.

Furthermore, the plumpness of barley grain had a positive correlation with extract levels within the grain. This is because there is an increase in starch, thus increasing the potential for extract (Fox et al., 2003). However, if the grain is too large, there can be a negative impact on

water uptake during malting (Fox et al., 2003). Interestingly, high b-glucan negatively correlated with all traits except barley color (2021) and wort clarity (2022). High levels of b-glucan can impact extract potential, wort viscosity, and filtration time (Stewart et al., 1998; Stewart et al., 2000). Thus, low b-glucan is essential for malt barley quality as a brewing base malt because high b-glucan can cause major issues during laundering in the brewing process and is an unacceptable characteristic to the brewing industry yet is not a major concern to the distilling industry.

Thousand-kernel weight (KW), wort clarity (Clarity), kernel plumpness (Plump), extract (EX), barley protein (BP), wort protein (WP), soluble/total ratio (S_T), free amino nitrogen (FAN, diastatic power (DP), α-amylase (AA), and β-glucan (BG) were assayed. Magnitudes of correlation were color-coded to depict positive and negative correlations from low to high. Dark blue indicates a positive correlation, while red indicates a negative correlation. A significant test of Pearson's correlation was performed. Non-significant was denoted by "ns".

Associations

For the GWAS analyses, malt quality data was generated for all 540 lines and the number of SNP markers included in the final GWAS analyses was 16,517. Using the BLINK model in GAPIT and barley lines harvested in the 2021 and 2022 field seasons, 44 significant MTAs were identified present on all seven barley chromosomes (Table 5.1). The average linkage disequilibrium (LD) decay was 3.6 Mb across all chromosomes. Markers associated with malt quality traits ranged from zero (barley protein) to eight (b-glucan) (Table 5.1). The marker effect for each significant MTA identified ranged from 0.3% (wort clarity) to 50% (α amylase). The most significant MTA detected using the 2021 data was SCRI_RS_193456 on chromosome 5H,

associated with AA, BG, FAN, S_T, and WP, with the highest LOD score of 39.23. In 2022, the two most significant MTA were SCRI_RS_207382 (LOD = 27.93) and JHI-Hv50k-2016-366325 (LOD =28.02), which were associated with FAN and AA, respectively. These markers are located at ~2.2 Mb and ~2.3 Mb distal from SCRI_RS_193456, which was the most significant MTA identified in 2021 AA, BG, FAN, S_T, and WP. Between 2021 and 2022 no MTA was consistent between years. Manhattan Plots were generated to visualize the MTA between years (Supplementary Figure S4).

Of the 44 MTA identified in this GWAS study representing 44 distinct loci 24 had been previously reported QTLs using the bi-parental mapping populations Diktoo × Morex 'DiMo' (Oziel et al., 1996), Harrington × Morex 'HaMo' (MarquezCedillo et al., 2000), and Steptoe × Morex 'StMo' (Hayes et al., 1993) and a GWAS study utilizing breeding lines from six North American barley breeding programs (Mohammadi et al., 2015). Thus, 20 of the malt-quality loci were considered novel. Of these novel loci, three were detected for α amylase (WA-AA_2H-1, WA-AA_5H-4, WA-AA_7H-7), two were detected for barley color and β-glucan (WA-BC_6H-2, WA-BC_7H-3, WA-BG_5H-8, WA-BG_7H-11), five were detected for wort clarity (WA-CL_1H-1, WA-CL_6H-2, WA-CL_6H-3, WA-CL_6H-4, WA-CL_6H-5), two were detected for extract (WA-EX_1H-1 WA-EX_2H-2), one for kernel weight, plumpness, diastatic power, and FAN (WA-KW_2H-1, WA-PL_5H-3, WA-DP_5H-2, WA-FAN_5H-2) and lastly three for wort color (WA-WC_5H-1, WA-WC_6H-2, WA-WC_6H-3) (Table 5.1). Of the 44 loci identified for malt quality traits 16 were located on chromosome 5H. Only 1 of these 16 loci was not on the long arm of chromosome 5H, suggesting that this region of the barley genome is a malt-quality gene-

rich region, which is consistent with previous studies reporting on the genetic mapping of malt quality traits (Ullrich et al., 1993; Hayes et al., 1993; Oziel et al. 1996; Mohammadi et al., 2015)

Discussion

The ultimate goal of a breeding program is to increase genetic gain within the program (Hazel & Lush, 1942). Breeders can accomplish this goal by increasing selection accuracy or increasing the cycle time of the breeding program (Lande & Thompson, 1990; Meuwissen et al., 2001; Cobb et al., 2019). By identifying MTAs for malt quality, malt barley breeders can make more accurate selections within their programs, thus increasing genetic gain. However, utilizing the malting process and malt quality analyses for 13 different traits can be time-consuming and resource-draining, presenting a significant phenotyping bottleneck that slows down the selection process. Utilizing malt quality phenotyping data can even further hinder a winter malt barley breeding program when generation times are longer due to the vernalization requirement. The turnaround for the selection of winter barley has to be very quick compared to spring barley, which has all winter to malt and test new barley lines. Winter barley breeding programs harvest in June-July, then must turn around and plant in October, making the window for time-consuming malt quality phenotyping even more of a bottleneck compared to spring malt barley selections. Thus, GWAS can be utilized to identify malt quality MTA within both spring and winter malt barley breeding populations, which allows for an additional tool that can be utilized for early-generation selections. GWAS and the identification of significant marker-trait associations specific to a breeding program are imperative when developing a program-specific genotyping marker panel for marker-assisted selection (MAS) and genomic selection (GS). MAS and GS would not negate the need to generate malt-quality phenotyping data for advanced and elite later-generation

experimental lines; however, they would allow the program to cull lines that have apparent malt quality inadequacies at the early generations to alleviate the bottleneck presented by running complete malt analysis on all high-yielding experimental lines coming down the pipeline.

Marker-assisted selection (MAS) is helpful when selecting and fixing major effect malt quality loci using significant MTA specific to a breeding program when limited genes control the trait (Heffner et al., 2009). In this study, the loci identified by the MTA WA-FAN_5H-1, WA-FAN_5H-2, WA-AA_5H-3, and WA-AA_5H-4 would be candidates for MAS of early-generation material. Interestingly, these MTAs have been consistently associated with malt barley quality in other barley breeding programs; thus, should be reliable markers for malt quality. However, this is not surprising considering that the WSU malt barley breeding program is relatively new and has spent the last decade bringing in new malt quality genetics from malt barley lines that originated from many of the programs from which these QTL and MTA were identified. However, grain protein content is a critical trait that would be difficult to select for utilizing MAS. This is mainly due to the highly quantitative nature and genetic complexity, including many minor loci/genes controlling this trait (Ullrich, 2002; Emebiria et al., 2005).

Furthermore, grain protein content is heavily influenced by environmental factors (Tester et al., 1991; Högy et al., 2013; Yu et al., 2017). The acceptable range for malt barley protein is 11.0% - 13.0% (Fox and Bettenhausen, 2023). If the protein is too low, enzymatic activity can be limited, and modification during malting will be slowed down due to the low enzyme activity (Bishop, 1930; Fox et al., 2003). However, when protein content within the grain is higher than the acceptable range of 13.0 % it will decrease extract, affect mouthfeel and foam stability, and limit starch degradation.

The analyses using GWAS and subsequent MAS can miss important alleles that are rare or are small effect loci, contributing to a highly quantitative trait (Bernardo, 2014). To overcome some of the difficulties breeders encounter when selecting highly quantitative genetically complex traits, programs are beginning to utilize genomic selection (GS) (Montesinos-López et al., 2015). Genomic selection allows for the utilization of historic phenotyping and genotyping data sets within a program to predict and make selections with accuracy levels that allow for genetic gains over time versus traditional 'breeders eye' and selections based on expensive and time-consuming phenotyping analysis (Heffner et al., 2009; Ceron-Rojas et al., 2015). An accurate and reliable training population must be developed to implement GS within a breeding program. A GS training population is used to generate a prediction model to estimate allele effects at all loci concurrently. Typically, a training population has multiple years of genotypic and phenotypic data across environments (Merrick & Carter, 2021). Breeders use the training population to predict and select new lines in the breeding pipeline. The challenge with GS is introducing new diverse alleles within the breeding program while increasing genetic gain (Rutkoski et al., 2015).). Implementing GS in a malt barley program could expedite the selection process for malt quality by skipping the expenses that come with malting each line at the early generations, thus increasing selection efficiency.

Results from this study utilized GWAS to characterize genetic loci contributing to malt quality traits in the WSU barley breeding program. The MTA reported in this study were all identified from two-row spring barley from crosses focused on introducing malt quality into the program from diverse germplasm sources. Interestingly, 11 MTAs were found on chromosome 5H for both the 2021 and 2022 data. Marker SCRI_RS_193456 found on the long arm of chromosome

5H was associated with AA, BG, FAN, S_T, and WP. A host of other MTA from previous studies are also associated with this region on 5H (Hayes et al., 1993; Ullrich et al., 1993; Oziel et al., 1996; Mohammadi et al., 2015). Thus, this region of barley chromosome 5H is a malt-quality gene-rich region, possibly due to multi-gene clusters and pleiotropic effects (Hayes et al., 1996; Fox et al., 2003). Mohammadi et al. (2015) reported on a GWAS among six malt barley breeding programs within the US in which the Washington State University barley breeding program participated. However, new MTAs were found in this study because of the increase in the number of lines screened from this program and the new alleles introduced into the program.

Conclusion

Barley is an excellent alternate grain crop that fits well into the PNW dryland winter wheat rotation. Producers can receive premium prices for malt barley over feed barley, making malt barley more profitable within rotation. This research focuses on improving malting quality in barley by utilizing GWAS analysis. By identifying important MTAs within a breeding program, early-generation screening can be conducted to save valuable time and money. Future directions of this research will focus on marker-assisted selection and genomic selection strategies to select for low protein, low β-glucan, high α-amylase, free amino nitrogen, and enzyme extract in the early generation selection of breeding materials. Thus, advancements in malt barley agronomics and quality will provide a viable and profitable rotation crop, making this research an added value in PNW dryland cropping systems.

Table 5.1: The distribution of significant malt quality marker-trait associations utilizing GWAS with the Washington State University Barley Program germplasm grown during the 2021 and 2022 field seasons.

Year	Traits[a]	MTA[b]	Marker	Allele[c]	Chr[d]	Pos[e]	LD Decay (kb)[f]	LOD[g]	Marker Effect	Previous Reported MTA[h]
2021	AA	*WA-AA_2H-1*	JHI-Hv50k-2016-135537	G/**A**	2H	644050076	877.078	6.8	0.5	
2021	AA	*WA-AA_4H-2*	JHI-Hv50k-2016-235068	G/**A**	4H	37263926	na	6.37	0.48	*QGpc.DiMo-4H*
2021	AA	*WA-AA_5H-3*	SCRI_RS_193456	A/**C**	5H	585327502	1762.17	39.23	0.49	*QAa.StMo-5H.2; QS/T.WA2-5H; QWp.WA2-5H; QWp.AB2-5H; QAa.HaMo-5H; QS/T.HaMo-5H; QS/T.MN6-5H; QAa.MN6-5H; QAa.WA2-5H; QMe.WA2-5H; QBgnm.WA2-5H; QAa.AB2-5H*
2022	AA	*WA-AA_5H-4*	JHI-Hv50k-2016-366325	**A**/G	5H	587765271	1943.41	27.93	0.45	
2022	AA	*WA-AA_6H-5*	JHI-Hv50k-2016-415097	**G**/A	6H	513243883	1375.41	6.26	0.22	*QAa.MN6-6H*
2022	AA	*WA-AA_6H-6*	JHI-Hv50k-2016-418690	A/**G**	6H	528110016	723.801	7.59	0.33	*QAa.StMo-6H*
2022	AA	*WA-AA_7H-7*	JHI-Hv50k-2016-518057	**G**/A	7H	626764314	5969.76	5.99	0.1	
2022	BC	*WA-BC_2H-1*	JHI-Hv50k-2016-74681	G/**A**	2H	28819579	2628.21	8.65	0.45	*QMe.DiMo-2H*
2021	BC	*WA-BC_6H-2*	SCRI_RS_173641	**A**/G	6H	391628322	12872.6	6.81	0.21	
2021	BC	*WA-BC_7H-3*	JHI-Hv50k-2016-436857	**A**/C	7H	272681	1334.16	7.49	0.21	
2021	BG	*WA-BG_ 3H-4*	SCRI_RS_108543	G/**A**	3H	498882116	3089.71	7.05	0.46	*QKp.StMo-3H*
2021	BG	*WA-BG_ 3H-5*	JHI-Hv50k-2016-202076	**G**/A	3H	552646274	3272.26	12.91	0.09	*QAa.StMo-3H; QGpc.StMo-3H.1*
2021	BG	*WA-BG_ 5H-6*	JHI-Hv50k-2016-305632	**G**/C	5H	412035997	22295.2	6.37	0.16	*QGpc.HaMo-5H*
2022	BG	*WA-BG_ 5H-7*	JHI-Hv50k-2016-348170	**A**/G	5H	554007152	51.766	6.64	0.04	*QMe.DiMo-5H.3*
2022	BG	*WA-BG_5H-8*	JHI-Hv50k-2016-355569	**G**/A	5H	568253558	759.523	6.51	0.27	
2022	BG	*WA-BG_ 5H-9*	JHI-Hv50k-2016-365417	**G**/A	5H	582993918	183.919	11.52	0.16	*QAa.StMo-5H.2; QS/T.WA2-5H; QWp.WA2-5H; QWp.AB2-5H; QAa.HaMo-5H; QS/T.HaMo-5H; QS/T.MN6-5H; QAa.MN6-5H; QAa.WA2-5H; QAa.AB6-5H*

2021	BG	*WA-BG_ 5H-10*	SCRI_RS_193456	**A**/C	5H	585327502	1762.17	8.49	0.49	*QAa.StMo-5H.2; QS/T.WA2-5H; QWp.WA2-5H; QWp.AB2-5H; QAa.HaMo-5H; QS/T.HaMo-5H; QS/T.MN6-5H; QAa.MN6-5H; QAa.WA2-5H; QMe.WA2-5H; QBgnm.WA2-5H; QAa.AB2-5H*
2021	BG	*WA-BG_7H-11*	SCRI_RS_222330	G/**A**	7H	18719352	130.51	8.03	0.28	
2022	CL	*WA-CL_1H-1*	JHI-Hv50k-2016-37607	**C**/A	1H	449924982	3.043	7.45	0	
2022	CL	*WA-CL_6H-2*	JHI-Hv50k-2016-377985	A/**G**	6H	18209747	1712.76	6.71	0.08	
2021	CL	*WA-CL_6H-3*	JHI-Hv50k-2016-402539	A/**G**	6H	388441153	12872.6	5.78	0.11	
2022	CL	*WA-CL_6H-4*	JHI-Hv50k-2016-414749	**G**/A	6H	511402405	0.205	17.07	0.11	
2022	CL	*WA-CL_6H-5*	JHI-Hv50k-2016-415394	**G**/A	6H	514939792	3289.49	6.69	0.1	
2021	DP	*WA-DP_2H-1*	JHI-Hv50k-2016-80864	**A**/G	2H	59607064	3989.17	5.9	0.4	*QMe.DiMo-2H; QGpc.StMo-2H.2*
2021	DP	*WA-DP_2H-2*	JHI-Hv50k-2016-113582	G/**A**	2H	601553437	1194.09	6.6	0.29	
2022	DP	*WA-DP_3H-3*	JHI-Hv50k-2016-166617	**A**/G	3H	120559678	86764.9	7.28	0.04	*QBgnm.StMo-3H*
2021	DP	*WA-DP_3H-4*	JHI-Hv50k-2016-202869	**A**/G	3H	554655625	912.384	5.67	0.28	*QKp.StMo-3H, QGpc.StMo-3H.1, QGP_Ctrl.World-3H*
2022	EX	*WA-EX_1H-1*	JHI-Hv50k-2016-37855	G/**A**	1H	459175233	600.789	6.3	0.41	
2021	EX	*WA-EX_2H-2*	JHI-Hv50k-2016-71819	C/**G**	2H	21557091	263.489	8.06	0.12	*QGP_WLog.World-2H; QKwp_WLog.World-2H.1; QBio_Ctrl.World-2H.1; QHt_Ctrl.World-2H.1*
2022	EX	*WA-EX_2H-3*	JHI-Hv50k-2016-132462	**A**/C	2H	638154181	178.66	7.61	0.2	
2021	FAN	*WA-FAN_5H-1*	SCRI_RS_193456	A/**C**	5H	585327502	1762.17	20.6	0.49	*QAa.StMo-5H.2; QS/T.WA2-5H; QWp.WA2-5H; QWp.AB2-5H; QAa.HaMo-5H; QS/T.HaMo-5H; QS/T.MN6-5H; QAa.MN6-5H; QAa.WA2-5H; QMe.WA2-5H;*

										QBgnm.WA2-5H; QAa.AB2-5H
2022	FAN	*WA-FAN_5H-2*	SCRI_RS_207382	G/**A**	5H	587603103	1943.41	28.02	0.44	
2021	KW	*WA-KW_2H-1*	JHI-Hv50k-2016-118574	A/**G**	2H	613449982	5245.45	5.68	0.33	
2022	PL	*WA-PL_1H-1*	SCRI_RS_149683	**G**/A	1H	4883803	472.169	5.99	0.34	*QGpc.DiMo-1H, QKp.HaMo-1H.1*
2022	PL	*WA-PL_3H-2*	JHI-Hv50k-2016-204992	T/**A**	3H	562587513	4800.45	8.53	0.28	*QKp.StMo-3H, QGpc.StMo-3H.1, QGP_Ctrl.World-3H*
2021	PL	*WA-PL_5H-3*	SCRI_RS_155555	G/**A**	5H	4880406	312.539	11.31	0.06	
2021	S/T	*WA-S/T_5H-1*	SCRI_RS_193456	A/**C**	5H	585327502	1762.17	11.69	0.49	*QAa.StMo-5H.2; QS/T.WA2-5H; QWp.WA2-5H; QWp.AB2-5H; QAa.HaMo-5H; QS/T.HaMo-5H; QS/T.MN6-5H; QAa.MN6-5H; QAa.WA2-5H; QMe.WA2-5H; QBgnm.WA2-5H; QAa.AB2-5H*
2022	S/T	*WA-S/T_5H-2*[$]	JHI-Hv50k-2016-367564	**A**/G	5H	586108262	1943.41	8.94	0.43	*QAa.StMo-5H.2; QWp.WA2-5H; QWp.AB2-5H; QAa.HaMo-5H; QS/T.HaMo-5H; QAa.WA2-5H; QMe.MT2-5H; QAa.AB2-5H; QS/T.AB2-5H;QWp.MT2-5H; QS/T.MT2-5H; QAa.MT2-5H; QMe.MT2-5H*
2022	WC	*WA-WC_5H-1*[$]	SCRI_RS_207382	G/**A**	5H	587603103	1943.41	11.28	0.46	*QAa.StMo-5H.2; QWp.WA2-5H; QWp.AB2-5H; QAa.HaMo-5H; QS/T.HaMo-5H; QAa.WA2-5H; QMe.MT2-5H;*

										QAa.AB2-5H; QS/T.AB2-5H;QWp.MT2-5H; QS/T.MT2-5H; QAa.MT2-5H; QMe.MT2-5H
2021	WC	*WA-WC_6H-2*	SCRI_RS_222315	**G**/A	6H	388090060	12872.6	6.35	0.09	
2022	WC	*WA-WC_6H-3*	JHI-Hv50k-2016-415229	**G**/C	6H	513852758	3289.49	14.37	0.07	
2021	WP	*WA-WP_5H-1*	SCRI_RS_179582	**A**/C	5H	575033160	5105.1	5.52	0.19	*QAa.StMo-5H.2; QS/T.WA2-5H*
2022	WP	*WA-WP_5H-2*	SCRI_RS_105868	**A**/G	5H	583472928	433.607	12.12	0.44	*QAa.StMo-5H.2; QS/T.WA2-5H; QWp.WA2-5H; QWp.AB2-5H; QAa.HaMo-5H; QS/T.HaMo-5H; QS/T.MN6-5H; QAa.MN6-5H; QAa.WA2-5H; QAa.AB6-5H; QMe.WA2-5H; QBgnm.WA2-5H; QWp.MN6-5H; QAa.AB2-5H*
2021	WP	*WA-WP_5H-3*	SCRI_RS_193456	A/**C**	5H	585327502	1762.17	22.68	0.49	*QAa.StMo-5H.2; QS/T.WA2-5H; QWp.WA2-5H; QWp.AB2-5H; QAa.HaMo-5H; QS/T.HaMo-5H; QS/T.MN6-5H; QAa.MN6-5H; QAa.WA2-5H; QMe.WA2-5H; QBgnm.WA2-5H; QAa.AB2-5H*

[a] Abbreviated trait names: Kernel weight (KW), wort clarity (CL), kernel plumpness (PL), extract (EX), barley protein (BP), wort protein (WP), soluble/total ratio(S_T), free amino nitrogen (FAN, diastatic power (DP), α-amylase (AA), and β-glucan (BG).

[b] (MTA) Marker trait association nomenclature (SNP) Single nucleotide polymorphism marker

[c] Allele that is responsible for higher trait levels, shown in bold.

[d] (Chr) Chromosome number in which the marker is located.

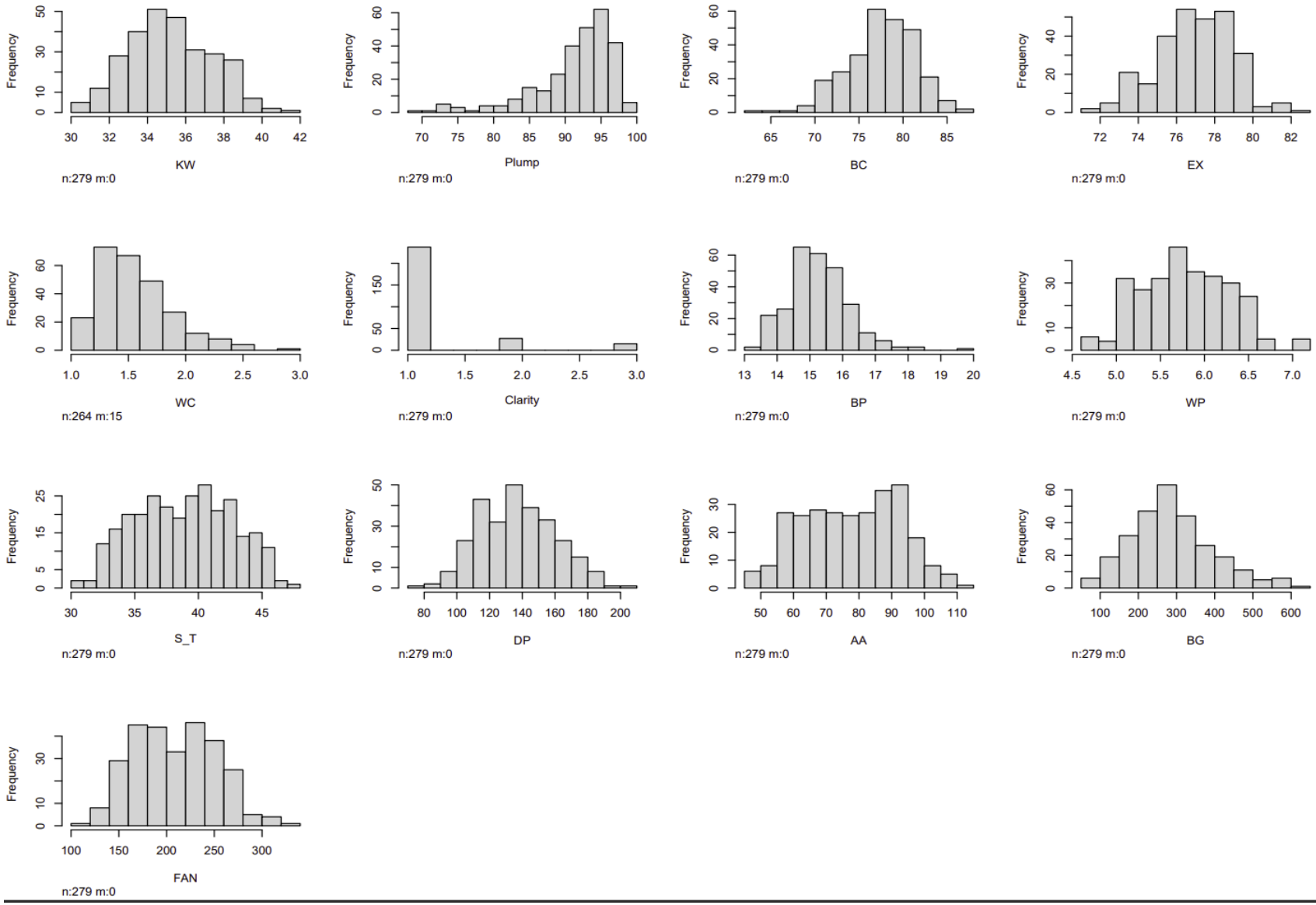
[e] Physical position of markers according to Morex v3 genome assembly (Mascher et al., 2021)

[f] Linkage Decay calculated using PLINK software

[g] (LOD) logarithm of odds or log10(p) significantly expressed for association.

[h] Previously identified MTAs in bi-parental mapping populations Diktoo × Morex' DiMo' (Oziel et al., 1996), Harrington × Morex' HaMo' (MarquezCedillo et al., 2000), Steptoe × Morex' StMo' (Hayes et al., 1993), and GWAS populations (Mohammadi et al., 2015). The locations of MTAs were found on the grain gene database.

A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BC
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EX
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WC
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Clarity
n:279 m:0
BP
n:279 m:0
WP
n:279 m:0
S_T
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DP
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AA
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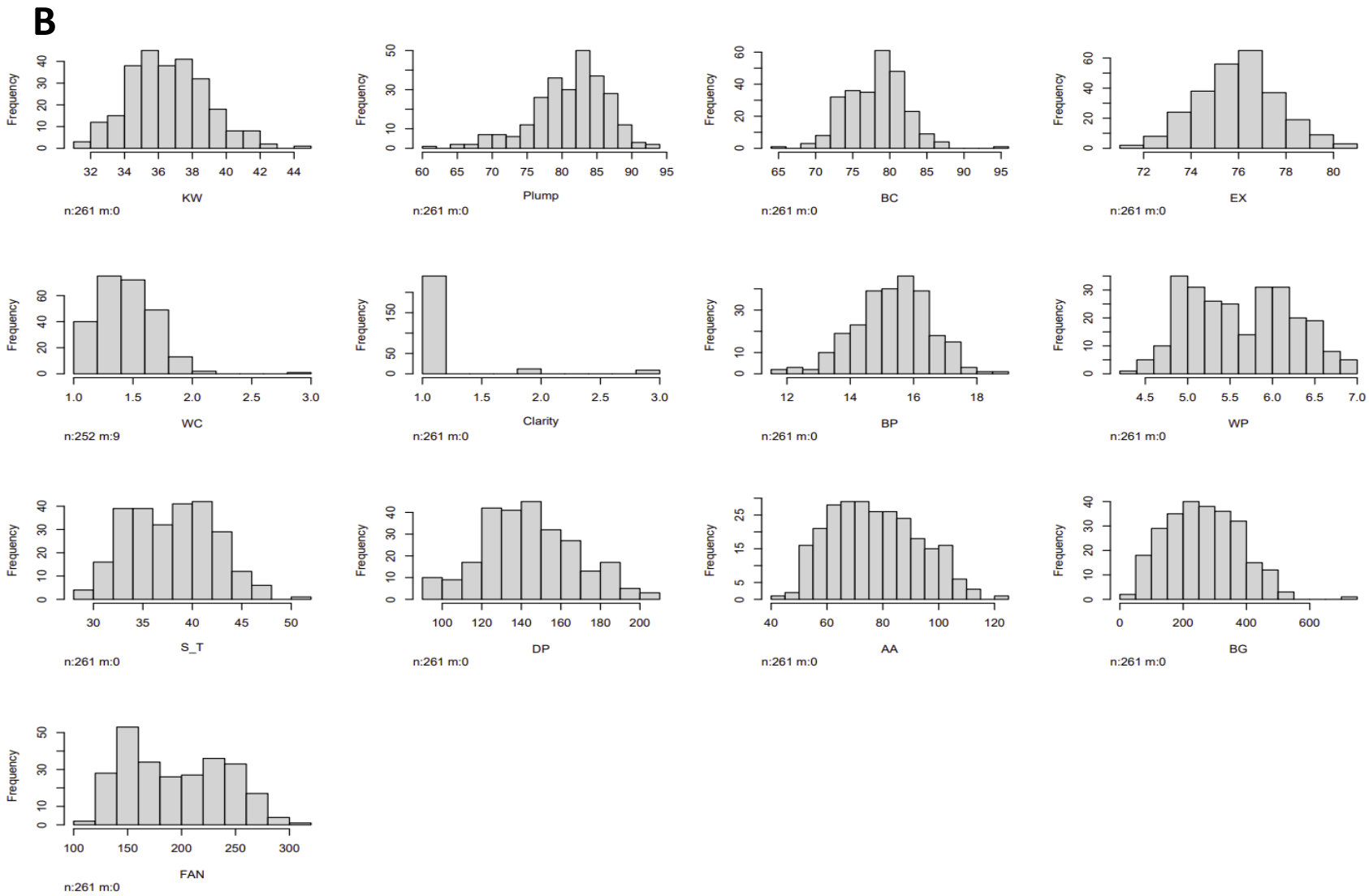

Figure 5.1: Histogram of 2021 (**A**) and 2022 (**B**) malting data. Kernel weight (KW), wort clarity (Clarity), kernel plumpness (Plump), extract (EX), barley protein (BP), wort protein (WP), soluble/total ratio (S_T), free amino nitrogen (FAN, diastatic power (DP), α-amylase (AA), and β-glucan (BG)

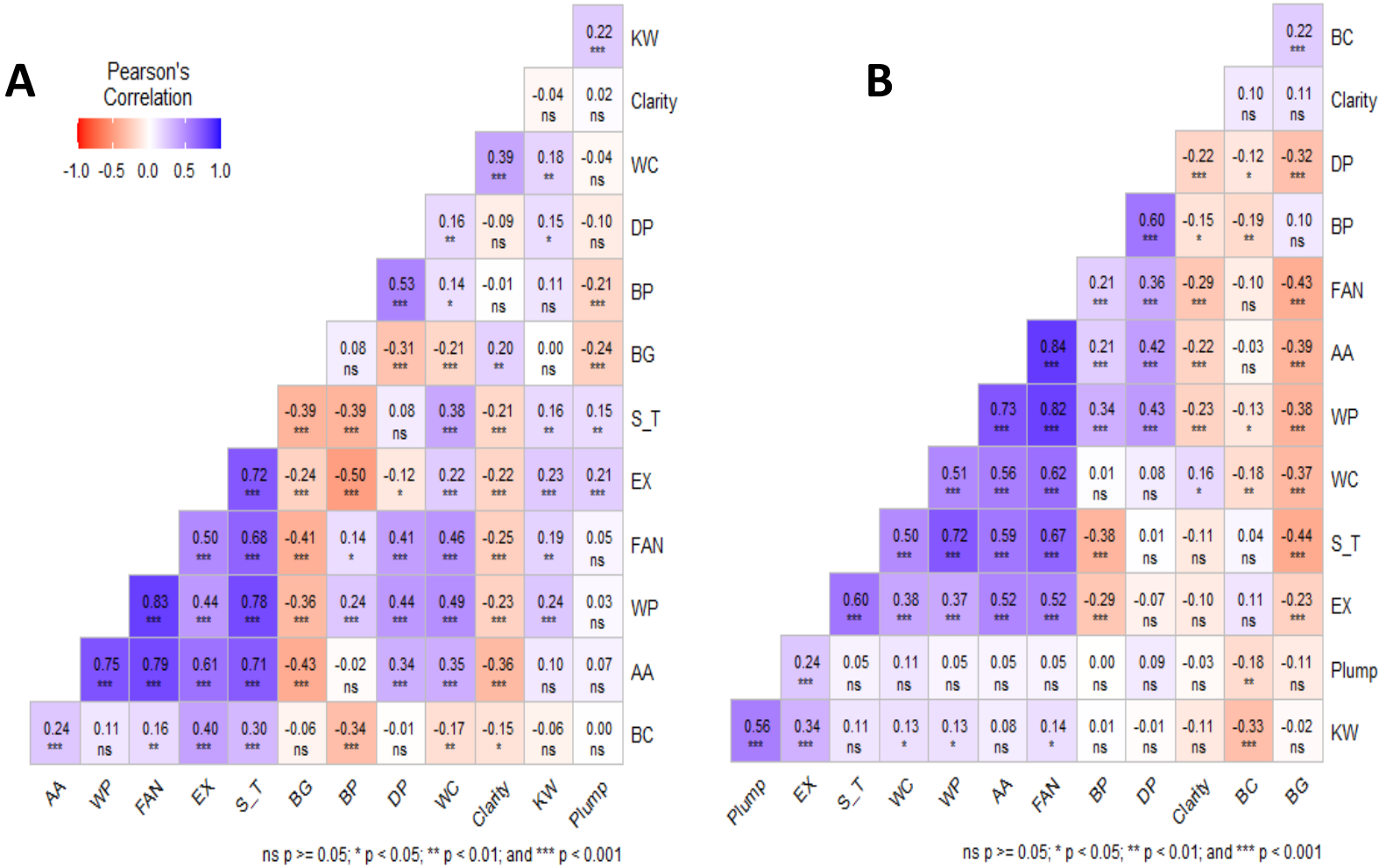

Figure 5.2: Pearson's correlation coefficient of 13 malting quality traits for single replicated individuals between 2021 (A) and 2022 (B). Trait abbreviations were used as follows: Kernel weight (KW), wort clarity (Clarity), kernel plumpness (Plump), extract (EX), barley protein (BP), wort protein (WP), soluble/total ratio (S_T), free amino nitrogen (FAN, diastatic power (DP), α-amylase (AA), and β-glucan (BG). Magnitudes of correlation were color-coded to show correlation from low to high positive. Dark blue indicates a positive correlation, while red indicates a low correlation. A significant test of Pearson's correlation was performed. Non-significant was denoted by "ns"

CHAPTER SIX: GENERAL CONCLUSION

In conclusion, the investigation into malt quality and stem rust resistance through a biparental marker-trait association (MTA) mapping, two genome-wide association studies (GWAS), and high-resolution mapping has provided valuable insight into the genetic basis of these traits in barley. By identifying critical genomic regions associated with resistance to the most virulent isolate of stem rust (Lsp21) and traits contributing to malt quality, this research lays the foundation for targeted breeding efforts to develop barely cultivars with durable resistance to this disease while improving malt quality attributes.

There are many exciting and interesting key findings throughout this dissertation. In the bi-parental mapping population between Elliot and Palmer, the analysis identified two significant MTAs (EP*Rpg*_4H-1 and EP*Rpg*_5H-1) contributed by Elliot on chromosomes 4H and 5H at the seedling stage. Data suggest that these MTAs have a dominant susceptibility gene function due to segregation ratios in the F_6. This biparental population serves a dual purpose for the breeding program at WSU because not only does it map disease resistance, but Elliot and Palmer are both malting barleys. Accessions from this population are currently in malt quality evaluations and showing promise malting traits.

The Wild Barley Diversity Collection (WBDC) was one of the most interesting populations to work with. This population will test whether you love barley or hate barley. A total of 277 of the 318 lines from the WBDC were screened for stem rust reactions at the seedling stage with the most virulent PNW *Pgt* isolate, Lsp21. Only 12% of the accessions showed moderate resistance-to-resistance reactions at the seedling stage. Of the 12 percent that were resistant, most of the lines

were from the Jordan region. GWAS results showed seven novel loci (WQ*Rpg*-2H01, WQ*Rpg*-2H02, WQ*Rpg*-3H01, WQ*Rpg*-5H01, WQ*Rpg*-5H03, WQ*Rpg*-7H02, and WQRpg-7H03) were associated with stem rust resistance. Breeders can utilize these novel loci to enhance resistance to stem rust in their programs, especially against the virulent races present in the PNW population. However, due to the nature of wild barley, alternative breeding strategies will have to be developed so as not to bring deleterious alleles from wild barley into a domestic breeding program.

From this, two WBDC accessions (WBDC-94 and WBDC-238) provided exceptional resistance towards the most virulent isolate of stem rust in the world, Lsp21. Both of these accessions contained the newly discovered resistant gene *Rpg7*. This gene is becoming increasingly important to the Pacific Northwest (PNW) and the Midwest because of the exceptional resistance to not only the stem rust population in the PNW but also resistance to significant races of stem rust in the Midwest, like QCCJB, MCCFC, HKHJC, and rye stem rust isolate 92-MN-90. Because of this strong resistance response, high-resolution mapping was utilized to illuminate the mechanism behind *Rpg7*. The high-resolution mapping was successful in delimiting the region to ~53 kb containing two predicted candidate gene models, an RPM1-like nucleotide binding site-leucine rich repeat (NLR) disease resistance-like protein and an RPM1 interacting protein 4 (RIN4)-like protein.

The current hypothesis is that both genes are required for resistance and that the HvRPM1-like NLR protein may guard the HvRIN4-like protein to detect pathogen manipulation following the 'guard' model similar to the RPM1/RIN4-mediated resistance against *Pseudomonas syringae* in Arabidopsis. Another aspect of *Rpg7* that was not shown in this study was that it was not temperature-sensitive. Resistance was still holding at temperatures above 28ºC. This is the only

temperature-stable R-gene against *Pgt*. Further evaluation of this temperature-stable gene is ongoing.

An interesting question about *Rpg7* is when and where to release domesticated barley that contains this resistant gene. If a popular malting line was released in the PNW with only *Rpg7* for a resistant gene, I believe this would pressure the *Pgt* population to overcome the resistance and render this gene susceptible over time. Thus, the zig-zag theory would apply where avirulence effectors would be pressured to mutate to a non-functional or unrecognizable effector to avoid detection by resistance gene. However, if stem rust becomes a significant problem in the PNW, growers will want cultivars with resistance. We already see stem rust completing its lifecycle on mahonia and wild grasses in western Washington on Whidbey Island. Furthermore, there have been reports of the natural stem rust population overcoming Q21816 (*Rpg1* and RMRL) in northern California.

One strategy would be to stack resistance genes together to achieve broader resistance. The genes I would suggest stacking for this strategy would be *Rpg1*, RMRL, and *Rgp7*. The resistance from a newly mapped gene (*rpg8*) would be excellent to stack. However, due to the recessive nature of *rpg8*, this might be a more difficult gene to integrate once this gene is validated. Staking resistance genes could be done by making a MAGIC population or a double haploid population between Q21861 and WBDC-94 and then crossing resistance lines into ACC connect, which has *Rpg1*.

Another strategy would be to release barley lines with only *Rpg7* as a resistant gene. To avoid the pressure of the diverse *Pgt* population in the PNW, this line might only be grown in the

Midwest, much to the dismay of Washington grain growers and the Washington Grains Commission. Because the population of *Pgt* is more stable in the Midwest, this resistance might be more durable. However, there is always a chance of an isolated crossing the Rocky Mountains and devastating the Midwest. Strategies similar to this have been implemented in the United States before with other crops. For instance, the state of Montana only grows seed potatoes. No commercial potato production is allowed in Montana to avoid devastating diseases like late blight or potato mosaic virus in seed potatoes. Likewise, sugar beet seed production takes place in Oregon and Washington, so seed production is kept away from commercial sugar-beet production in North Dakota.

Advancements in fungicide chemistry and mechanical application strategies have been implemented over the last 40 years, and *Rpg7* could be very successful in the PNW. Either way, stacking these resistant genes and releasing a quality malting line could prove to be difficult. This is because there might be minor alleles that are strategic to resistance, as in the case of *Rrr1* for *Rpg1*. Furthermore, the deleterious alleles from wild barley would take more time in the breeding pipeline to overcome for domestic use.

Key findings for the malt quality traits have been identified in WSU malting barley. A total of 44 markers were identified within association mapping of the diverse experimental lines on all seven chromosomes. These markers will be used for marker-assisted selection to improve β-glucan, α-amylase, enzyme extract, and protein. These quality traits will lead to the expedited development of American Malting and Barley Association (AMBA) recommended varieties that can open new markets for WSU malt barley varieties.

These studies could take a couple of directions for future research. The first would be to clone and validate *Rpg7*. EMS mutant populations of WBDC -94 and WBDC-238 have already been created, and the first round of screening M_1 seeds has already been conducted (Supplementary Figure S5). From these M_1 screenings, susceptible lines have been identified. Whether or not the RIN4 or the RPM1 has been made non-functional is to be determined for a future study. The outcome of this would be to provide breeders with perfect markers to integrate *Rpg7* into a breeding program. As for malting quality, the GWAS in this study will lead to marker-assisted selection strategies and genomic selection as more data comes in.

The work in this dissertation covers barley stem rust resistance and malting quality, but ultimately, the goal is to develop a barley line with exceptional quality and disease resistance. The arms race for disease resistance continues. Plants and diseases have coevolved for millions of years before plant pathologists and breeders intervened. It is a delicate dance between the two parties, and the music never stops. As an aspiring plant breeder and stand-in pathologist, I find myself controlling the tempo and volume of the music but never finding a way to stop the music completely. So, on must the dance continue. If you have made it this far into my dissertation, thank you, and I hope you enjoyed reading it. The work I have outlined in this dissertation has been built on the backs of many hard-working scientists, students, and mentors.

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APPENDIX

APPENDIX A: SUPPLEMENTARY TABLES

Supplementary Table S1: Five stem rust isolates from the inland northwest and barley and wheat's resistance and susceptibility reactions (Data derived from Upenday et al., 2022).

Accession	R-gene	----------Local Isolates----------				
--------*WHEAT*--------		MoPu6	Mopu4	Psp8	Lsp21	Brcx6
GSTR 501	*Sr5*	S	S	R	R	S
GSTR 502	*Sr21*	S	S	S	S	S
GSTR 503	*Sr9e*	R	R	R	R	R
GSTR 504	*Sr7b*	S	S	S	S	S
GSTR 505	*Sr11*	R	R	R	R	R
GSTR 506	*Sr6*	R	R	R	R	R
GSTR 507	*Sr8a*	S	S	S	R	S
GSTR 508	*Sr9g*	S	S	S	R	R
GSTR 509	*Sr36*	S	R	R	S	R
GSTR 510	*Sr9b*	S	S	S	S	S
GSTR 511	*Sr30*	S	R	S	S	R
GSTR 512	*Sr17*	R	R	S	S	S
GSTR 513	*Sr9a*	S	S	S	R	S
GSTR 514	*Sr9d*	S	S	R	R	S
GSTR 515	*Sr10*	S	S	S	S	S
GSTR 516	*SrTmp*	S	S	R	R	S
GSTR 517	*Sr24*	S	R	S	S	S
GSTR 518	*Sr31*	R	R	R	R	R
GSTR 519	*Sr38*	S	S	S	S	R
GSTR 520	*SrMcN*	S	S	S	S	S
--------*BARLEY* --------		----------		----		
CIho15773	*Rpg1*	S	S	S	S	S
CIho 7124	*Rpg2*	S	S	S	S	S
PI 382313	*Rpg3*	S	S	S	S	S
HQ-1	*Rpg4/5*	R	R	S	S	S
PI 584766	*Rpg1 & Rpg5*	R	R	S	S	R
GSHO103	*rpg8*	S	S	S	S	S

Supplementary Table S2: List of all *Hordeum vulgare* subsp. *spontaneum,* the accessions from the WBDC used for the GWAS analysis.

Accession	Name	Origin	Average CI Score
PI 681726	WBDC 001	Syria, Ḥalab	3.34
PI 681727	WBDC 002	Syria, Ḥalab	2.75
PI 681728	WBDC 004	Syria, Idlib	3.21
PI 681730	WBDC 006	Jordan, Al Mafraq	4.00
PI 681731	WBDC 007	Jordan, Irbid	3.38
PI 681732	WBDC 008	Jordan, Irbid	3.78
PI 681733	WBDC 009	Jordan, Mādabā	3.78
PI 681734	WBDC 010	Afghanistan, Jowzjān	3.52
PI 681735	WBDC 011	Iraq, Arbīl	3.68
PI 681736	WBDC 012	Afghanistan, Badakhshān	3.19
PI 681737	WBDC 013	Iraq, As Sulaymānīyah	2.69
PI 681740	WBDC 016	Iran, Khūzestān	3.83
PI 681741	WBDC 017	Syria, Dimashq	2.75
PI 681742	WBDC 018	Afghanistan, Bādghīs	4.00
PI 681743	WBDC 019	Iran, Āẕārbāyjān-e Ghārbī	3.81
PI 681744	WBDC 020	Turkey, Şanlıurfa	2.60
PI 681745	WBDC 021	Iraq, Diyālá	2.91
PI 681746	WBDC 022	Turkey, Eskişehir	3.13
PI 681747	WBDC 023	Iran, Īlām	3.91
PI 681748	WBDC 024	Iran, Fārs	3.63
PI 681749	WBDC 025	Pakistan, Balochistan	4.05
PI 681750	WBDC 026	Tajikistan, Khatlon	3.94
PI 681753	WBDC 029	Israel, HaDarom	3.50
PI 681754	WBDC 030	Israel, HaTsafon	3.66
PI 681755	WBDC 031	Israel, Ḥefa	4.13
PI 681756	WBDC 032	Israel, Yerushalayim	3.38
PI 681757	WBDC 033	Israel, HaDarom	3.33
PI 681758	WBDC 034	Israel, HaMerkaz	3.24
PI 681759	WBDC 035	Israel	2.94
PI 681762	WBDC 038	Israel, Yerushalayim	3.73
PI 681764	WBDC 040	Israel, Ḥefa	3.55
PI 681765	WBDC 041	Israel, HaDarom	3.88
PI 681766	WBDC 042	Israel, HaTsafon	3.61
PI 681767	WBDC 043	Israel, HaTsafon	3.56
PI 681768	WBDC 044	Israel, Tel Aviv	3.48
PI 681769	WBDC 045	Jordan, Al ʻĀşimah	3.17

PI 681770	WBDC 046	Jordan, Irbid	3.69
PI 681772	WBDC 048	Turkey, Hakkâri	3.04
PI 681773	WBDC 049	Turkey, Hakkâri	3.88
PI 681775	WBDC 051	Syria, Ḥimş	4.31
PI 681776	WBDC 052	Jordan, Al Mafraq	3.84
PI 681777	WBDC 053	Pakistan, Balochistan	4.08
PI 681778	WBDC 054	Syria, Ḥimş	3.48
PI 681780	WBDC 056	Turkey, Kilis	3.13
PI 681781	WBDC 057	Syria, Rīf Dimashq	3.38
PI 681782	WBDC 058	Cyprus, Ammochostos	3.51
PI 681784	WBDC 060	Egypt, Maţrūḥ	4.02
PI 681785	WBDC 061	Syria, Idlib	3.15
PI 681786	WBDC 062	Syria, Ḥalab	4.21
PI 681787	WBDC 063	Syria, Ḥalab	3.96
PI 681788	WBDC 064	Syria, Idlib	3.60
PI 681790	WBDC 066	Syria, Rīf Dimashq	3.90
PI 681791	WBDC 067	Syria, Rīf Dimashq	3.50
PI 681792	WBDC 068	Syria, As Suwaydā'	4.13
PI 681794	WBDC 070	Syria, Al Lādhiqīyah	3.57
PI 681795	WBDC 072	Libya	3.63
PI 681796	WBDC 073	Libya	3.75
PI 681797	WBDC 074	Libya	3.46
PI 681798	WBDC 075	Libya	3.69
PI 681799	WBDC 078	Syria, Al Ḥasakah	3.38
PI 681800	WBDC 079	Jordan, Jarash	3.56
PI 681803	WBDC 082	Jordan, ‘Ajlūn	3.51
PI 681804	WBDC 083	Jordan, Jarash	3.77
PI 681805	WBDC 085	Jordan, Al Balqā’	3.50
PI 681806	WBDC 089	Jordan, Al ‘Āşimah	3.41
PI 681807	WBDC 092	Jordan, Al Mafraq	3.69
PI 681808	WBDC 093	Jordan, Az Zarqā’	4.06
PI 681809	WBDC 094	Jordan, Mādabā	0.56
PI 681810	WBDC 095	Jordan, Al Karak	3.60
PI 681811	WBDC 097	Jordan, Al Karak	3.63
PI 681812	WBDC 100	Jordan, Aţ Ţafīlah	3.69
PI 681813	WBDC 101	Jordan, Aţ Ţafīlah	3.67
PI 681816	WBDC 104	Jordan, Al Karak	3.58
PI 681817	WBDC 105	Jordan, Irbid	2.83
PI 681818	WBDC 106	Syria, Tarţūs	3.69
PI 681819	WBDC 107	Syria, Ḥimş	3.45
PI 681820	WBDC 108	Syria, Rīf Dimashq	3.06

PI 681821	WBDC 109	Syria, As Suwaydā'	3.72
PI 681822	WBDC 110	Syria, As Suwaydā'	3.57
PI 681823	WBDC 111	Syria, Rīf Dimashq	3.10
PI 681824	WBDC 112	Syria, Rīf Dimashq	3.11
PI 681825	WBDC 113	Turkmenistan, Ahal	3.00
PI 681826	WBDC 115	Turkmenistan, Balkan	3.95
PI 681827	WBDC 116	Turkmenistan, Balkan	3.51
PI 681828	WBDC 117	Turkmenistan, Balkan	3.31
PI 681829	WBDC 119	Uzbekistan, Jizzax	2.64
PI 681830	WBDC 120	Tajikistan, Sughd	2.23
PI 681831	WBDC 121	Iran, Fārs	3.81
PI 681832	WBDC 122	Iran, Lorestān	3.76
PI 681833	WBDC 123	Iran, Khorāsān-e Raẕavī	2.09
PI 681835	WBDC 125	Uzbekistan, Qashqadaryo	3.90
PI 681836	WBDC 126	Lebanon, Al Janūb	3.52
PI 681837	WBDC 127	Syria, As Suwaydā'	3.69
PI 681838	WBDC 128	Syria, Rīf Dimashq	2.94
PI 681839	WBDC 129	Syria, As Suwaydā'	3.60
PI 681840	WBDC 130	Syria, Dar'ā	4.03
PI 681842	WBDC 132	Lebanon, Al Biqā‘	3.26
PI 681843	WBDC 133	Lebanon, Al Biqā‘	4.06
PI 681844	WBDC 134	Lebanon, Al Biqā‘	3.88
PI 681846	WBDC 136	Lebanon, Al Biqā‘	3.45
PI 681847	WBDC 137	Lebanon, Al Biqā‘	2.89
PI 681848	WBDC 138	Lebanon, An Nabaţīyah	3.10
PI 681849	WBDC 139	Lebanon, Al Biqā‘	3.54
PI 681853	WBDC 143	Lebanon, Al Biqā‘	3.43
PI 681854	WBDC 145	Lebanon, Al Biqā‘	3.32
PI 681855	WBDC 146	Iran, Āẕārbāyjān-e Ghārbī	3.69
PI 681856	WBDC 147	Iran, Āẕārbāyjān-e Ghārbī	3.79
PI 681857	WBDC 148	Iran, Āẕārbāyjān-e Ghārbī	4.06
PI 681858	WBDC 149	Iran, Āẕārbāyjān-e Ghārbī	3.79
PI 681859	WBDC 150	Iran, Āẕārbāyjān-e Shārqī	4.00
PI 681860	WBDC 151	Syria, Ḥalab	3.50
PI 681861	WBDC 152	Iran, Tehrān	3.47
PI 681862	WBDC 153	Iran, Markazī	3.85
PI 681864	WBDC 155	Iraq, Nīnawá	3.71
PI 681865	WBDC 156	Iraq, Nīnawá	3.73
PI 681866	WBDC 157	Iraq, Nīnawá	2.19
PI 681868	WBDC 159	Syria, As Suwaydā'	3.70
PI 681869	WBDC 160	Syria, As Suwaydā'	4.04

PI 681870	WBDC 161	Syria, Ḥalab	3.43
PI 681871	WBDC 164	Syria, Al Ḥasakah	3.71
PI 681872	WBDC 165	Syria, Al Ḥasakah	3.30
PI 681874	WBDC 167	Syria, Al Ḥasakah	3.61
PI 681875	WBDC 168	Lebanon, Al Biqā‘	3.60
PI 681876	WBDC 169	Lebanon, Al Biqā‘	3.77
PI 681877	WBDC 170	Lebanon, Al Biqā‘	2.88
PI 681878	WBDC 171	Lebanon, Al Biqā‘	3.34
PI 681879	WBDC 172	Iran, Hamadān	4.06
PI 681880	WBDC 173	Iran, Hamadān	2.25
PI 681881	WBDC 174	Iran, Kordestān	4.13
PI 681882	WBDC 175	Iran, Kordestān	3.83
PI 681883	WBDC 177	Iraq, Nīnawá	3.63
PI 681884	WBDC 178	Iraq, Nīnawá	3.00
PI 681885	WBDC 179	Libya	3.75
PI 681886	WBDC 180	Libya	3.19
PI 681887	WBDC 181	Jordan, Az Zarqā’	2.94
PI 681888	WBDC 182	Jordan, Al Mafraq	4.08
PI 681889	WBDC 183	Jordan, Irbid	3.84
PI 681890	WBDC 184	Libya	3.50
PI 681891	WBDC 185	Libya	3.56
PI 681892	WBDC 186	Turkey, Kilis	4.04
PI 681893	WBDC 187	Turkey, Kilis	3.55
PI 681894	WBDC 188	Turkey, Gaziantep	3.71
PI 681895	WBDC 189	Turkey, Kilis	3.63
PI 681896	WBDC 190	Turkey, Kilis	3.69
PI 681897	WBDC 191	Turkey, Gaziantep	3.21
PI 681898	WBDC 192	Turkey, Gaziantep	3.30
PI 681899	WBDC 193	Turkey, Gaziantep	3.77
PI 681900	WBDC 194	Turkey, Gaziantep	3.92
PI 681901	WBDC 195	Turkey, Kilis	3.80
PI 681902	WBDC 196	Turkey, Gaziantep	3.37
PI 681903	WBDC 197	Syria, Ḥalab	3.58
PI 681904	WBDC 198	Syria, Ḥimş	3.77
PI 681905	WBDC 199	Syria, Ḥamāh	4.02
PI 681906	WBDC 200	Syria, Ḥamāh	3.11
PI 681907	WBDC 201	Syria, Idlib	3.08
PI 681908	WBDC 202	Syria, Idlib	3.50
PI 681909	WBDC 203	Syria, Idlib	3.72
PI 681910	WBDC 204	Turkmenistan, Ahal	3.32

PI 681911	WBDC 205	Russian Federation, Dagestan, Respublika	3.93
PI 681912	WBDC 206	Syria, As Suwaydā'	3.63
PI 681913	WBDC 207	Uzbekistan, Farg‘ona	3.75
PI 681914	WBDC 208	Uzbekistan, Toshkent	3.29
PI 681915	WBDC 209	Uzbekistan, Jizzax	2.88
PI 681916	WBDC 210	Uzbekistan, Jizzax	4.00
PI 681917	WBDC 211	Uzbekistan, Jizzax	3.42
PI 681918	WBDC 212	Uzbekistan, Jizzax	3.44
PI 681919	WBDC 213	Uzbekistan, Samarqand	2.46
PI 681920	WBDC 214	Uzbekistan, Samarqand	2.47
PI 681921	WBDC 215	Turkmenistan, Balkan	3.48
PI 681922	WBDC 216	Turkmenistan, Ahal	2.97
PI 681923	WBDC 217	Armenia, Erevan	3.64
PI 681925	WBDC 219	Kazakhstan, Ongtüstik Qazaqstan oblysy	3.90
PI 681926	WBDC 220	Kazakhstan, Ongtüstik Qazaqstan oblysy	3.43
PI 681927	WBDC 221	Tajikistan, Sughd	3.25
PI 681929	WBDC 223	Tajikistan, Sughd	3.54
PI 681930	WBDC 224	Tajikistan, Sughd	3.63
PI 681933	WBDC 228	Azerbaijan	3.56
PI 681936	WBDC 231	Azerbaijan	3.60
PI 681937	WBDC 232	Azerbaijan	3.58
PI 681938	WBDC 233	Afghanistan, Baghlān	3.89
PI 681939	WBDC 234	Cyprus, Ammochostos	3.40
PI 681941	WBDC 236	Jordan, Mādabā	3.63
PI 681943	WBDC 238	Jordan, Mādabā	0.56
PI 681944	WBDC 240	Jordan, Al ‘Āşimah	3.84
PI 681945	WBDC 241	Jordan, Al ‘Āşimah	3.71
PI 681946	WBDC 242	Jordan, Jarash	3.93
PI 681947	WBDC 243	Jordan, Al Balqā’	2.63
PI 681948	WBDC 244	Jordan, Al Balqā’	3.45
PI 681949	WBDC 245	Jordan, Jarash	3.88
PI 681950	WBDC 246	Jordan, Irbid	2.80
PI 681951	WBDC 247	Jordan, Irbid	3.64
PI 681952	WBDC 248	Jordan, Irbid	3.63
PI 681953	WBDC 250	Jordan, Irbid	3.85
PI 681955	WBDC 253	Jordan, ‘Ajlūn	2.92
PI 681956	WBDC 254	Jordan, Irbid	4.02
PI 681957	WBDC 255	Jordan, Irbid	3.05
PI 681958	WBDC 256	Jordan, Irbid	3.90

PI 681959	WBDC 257	Jordan, Al Karak	3.44
PI 681960	WBDC 258	Jordan, Al Karak	4.00
PI 681962	WBDC 260	Jordan, Ma‘ān	2.85
PI 681966	WBDC 265	Jordan, Aţ Ţafīlah	3.94
PI 681967	WBDC 266	Jordan, Aţ Ţafīlah	3.52
PI 681968	WBDC 267	Jordan, Aţ Ţafīlah	3.05
PI 681969	WBDC 268	Jordan, Az Zarqā’	4.00
PI 681970	WBDC 269	Lebanon, An Nabaţīyah	3.51
PI 681971	WBDC 270	Israel, Yerushalayim	3.73
PI 681972	WBDC 271	Israel, HaTsafon	3.78
PI 681973	WBDC 274	Israel, HaDarom	3.59
PI 681974	WBDC 275	Israel, HaDarom	3.24
PI 681975	WBDC 276	Israel, HaTsafon	3.41
PI 681976	WBDC 277	Israel, HaMerkaz	3.48
PI 681977	WBDC 278	Israel, HaMerkaz	4.00
PI 681978	WBDC 279	Israel, HaMerkaz	3.68
PI 681980	WBDC 281	Israel, HaTsafon	3.60
PI 681981	WBDC 282	Israel, HaMerkaz	4.17
PI 681982	WBDC 283	Israel, HaTsafon	3.31
PI 681983	WBDC 284	Israel, HaTsafon	3.63
PI 681985	WBDC 286	Israel, HaTsafon	3.66
PI 681986	WBDC 287	Israel, West Bank	3.66
PI 681987	WBDC 288	Israel, HaTsafon	3.07
PI 681988	WBDC 289	Israel, HaTsafon	3.20
PI 681989	WBDC 290	Israel, Yerushalayim	3.11
PI 681990	WBDC 291	Israel, Yerushalayim	3.20
PI 681991	WBDC 292	Israel, Yerushalayim	3.48
PI 681992	WBDC 293	Israel, Yerushalayim	3.10
PI 681993	WBDC 294	Israel, Yerushalayim	3.27
PI 681994	WBDC 295	Syria, Al Ḩasakah	3.78
PI 681995	WBDC 296	Syria, Al Ḩasakah	3.78
PI 681996	WBDC 297	Syria, Al Ḩasakah	3.91
PI 681997	WBDC 298	Syria, Al Ḩasakah	3.63
PI 681998	WBDC 299	Syria, Ḩalab	3.94
PI 681999	WBDC 300	Syria, Ḩalab	4.00
PI 682000	WBDC 302	Syria, Rīf Dimashq	3.02
PI 682001	WBDC 303	Syria, Rīf Dimashq	3.68
PI 682002	WBDC 304	Syria, Rīf Dimashq	3.65
PI 682003	WBDC 305	Syria, Rīf Dimashq	2.65
PI 682004	WBDC 306	Syria, Rīf Dimashq	3.50
PI 682005	WBDC 307	Syria, Rīf Dimashq	4.00

PI 682006	WBDC 308	Syria, Dar'ā	3.73
PI 682007	WBDC 309	Syria, Ḥamāh	3.85
PI 682009	WBDC 311	Syria, Ḥimş	3.05
PI 682010	WBDC 312	Syria, Ḥimş	3.63
PI 682011	WBDC 314	Syria, Ḥimş	3.92
PI 682012	WBDC 315	Syria, Ḥimş	3.55
PI 682014	WBDC 317	Syria, Ar Raqqah	3.72
PI 682015	WBDC 318	Syria, As Suwaydā'	3.85
PI 682016	WBDC 319	Syria, As Suwaydā'	3.41
PI 682017	WBDC 320	Syria, As Suwaydā'	3.56
PI 682018	WBDC 323	Turkmenistan, Ahal	3.58
PI 682019	WBDC 324	Turkmenistan, Ahal	4.00
PI 682021	WBDC 329	Turkmenistan, Balkan	3.97
PI 682022	WBDC 330	Turkmenistan, Balkan	2.40
PI 682023	WBDC 331	Turkmenistan, Balkan	3.18
PI 682025	WBDC 333	Turkmenistan, Balkan	3.05
PI 682027	WBDC 335	Turkmenistan, Balkan	3.63
PI 682028	WBDC 336	Turkmenistan, Ahal	3.37
PI 682029	WBDC 337	Turkey, Gaziantep	3.52
PI 682030	WBDC 338	Turkey, Gaziantep	3.90
PI 682031	WBDC 340	Turkey, Gaziantep	3.82
PI 682032	WBDC 341	Turkey, Kilis	4.00
PI 682033	WBDC 342	Turkey, Kilis	3.93
PI 682034	WBDC 343	Turkey, Kilis	3.66
PI 682035	WBDC 344	Turkey, Şanlıurfa	4.12
PI 682036	WBDC 345	Uzbekistan, Farg‘ona	3.56
PI 682037	WBDC 346	Uzbekistan, Samarqand	3.83
PI 682038	WBDC 347	Uzbekistan, Surxondaryo	3.87
PI 682039	WBDC 348	Israel	3.56
PI 682040	WBDC 349	Israel	3.34
PI 682041	WBDC 350	Israel	3.73
PI 682042	WBDC 354	Israel	3.55
PI 682043	WBDC 355	Azerbaijan	3.62

Supplementary Table S3: List of all GBS-PCR Primers that were used in this study to conduct highresolutiion mapping of *Rpg7* on chromosome 3H.

Primer	Name	Sequence (5' -->3')	Length (bp)	GC (%)	Tm (Â°C)	Dg (Kcal/mol)	Tail
32984_F	Rpg7_F1	CTTTTGCTGCATCTGCTCTCC	21	52.38	60.38	-22.77	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
33084_R	Rpg7_R1	TGCAGTGAGTGGATGTCAGG	20	55	60.19	-21.98	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
411207_F	Rpg7_F2	CCAGGGAGCTGCTGTTTCTC	20	60	61.19	-22.51	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
411304_R	Rpg7_R2	GCCACCTACTGCTAACACCTT	21	52.38	60.52	-22.57	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
621019_F	Rpg7_F3	TAAAGTGCATGGCATCACCG	20	50	59.41	-21.72	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
621106_R	Rpg7_R3	TAAGGATGCGCAACCACTGA	20	50	60.19	-21.93	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
621124_F	Rpg7_F4	TTGTTGCACTGCATTGCGAG	20	50	60.82	-22.57	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
621237_R	Rpg7_R4	GCCTGGCAGTCATAGATGCT	20	55	60.4	-22.06	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
1242067_F	Rpg7_F5	AAGGCACCTGCTACCGTTAC	20	55	60.55	-22.18	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
1242173_R	Rpg7_R5	ATCGTCGCATGGAGGTGTTT	20	50	60.55	-22.16	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
2964639_F	Rpg7_F6	GAGAGGGACGTCTTACCCCA	20	60	60.84	-22.13	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
2964740_R	Rpg7_R6	TGAGGCTACAACTACCCTCC	20	55	58.65	-21.08	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
2965787_F	Rpg7_F7	GCGGAAGACGTCATACTGCT	20	55	60.68	-22.47	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
2965887_R	Rpg7_R7	CGTTCTGCCACCTCCTTCAT	20	55	60.55	-22.13	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
3169624_F	Rpg7_F8	TGGCCGAGCATTGCTTAAGT	20	50	60.84	-22.21	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
3169735_R	Rpg7_R8	ATATCCCCCGGCTACACCAA	20	55	60.93	-21.96	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
3732149_F	Rpg7_F9	AGTCCTCAGTCCTCGCCC	18	66.67	60.87	-21.17	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
3732256_R	Rpg7_R9	CACAACCATCAATGCAGGCG	20	55	60.96	-22.65	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
3822174_F	Rpg7_F10	ACTTTGCAGGTTCCATGGCA	20	50	60.99	-22.14	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
3822253_R	Rpg7_R10	TCATACCCTGCCTCCGGTAC	20	60	61.28	-22.38	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
3881025_F	Rpg7_F11	AGCCTCGGCCATTTGTTCAT	20	50	60.85	-22.12	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
3881114_R	Rpg7_R11	GGGAGAGAGTTTACTAGGGGC	21	57.14	59.48	-22	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
3891267_F	Rpg7_F12	TGACACAGAAGAGCGTCCTG	20	55	60.19	-22.12	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
3891369_R	Rpg7_R12	GCGTCTGACCATGTCCTTCA	20	55	60.54	-22.25	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT

3951110_F	Rpg7_F13	GGTTGTGGTTCATGTGCCTT	20	50	59.47	-21.58	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
3951186_R	Rpg7_R13	GGGCTGACGTTCAAAGCTAG	20	55	59.41	-21.82	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
3954580_F	Rpg7_F14	GCTGACCAGATATCCCGTGG	20	60	60.47	-22.21	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
3954661_R	Rpg7_R14	GTAGGTGGCATCATGGGCTT	20	55	60.63	-22.05	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
4002341_F	Rpg7_F15	GCTCAACAGCTTTGCCAAGAA	21	47.62	60.45	-22.67	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
4002457_R	Rpg7_R15	CAGGTCAGCATTCAAGGGCT	20	55	60.84	-22.18	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
4053235_F	Rpg7_F16	GCAGCAACTTAAACCGGCAA	20	50	60.47	-22.24	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
4053346_R	Rpg7_R16	GGTTGTTGCCAAGCTGCTAC	20	55	60.54	-22.35	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
4090867_F	Rpg7_F17	GGCATATGTACTTGGTGCTGG	21	52.38	59.56	-22.28	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
4090968_R	Rpg7_R17	ACCTGGCGATATTCCCCTTC	20	55	59.75	-21.6	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
4094174_F	Rpg7_F18	AGCTTCACTGAACGGTTTGC	20	50	59.83	-21.96	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
4094235_R	Rpg7_R18	CAGCCAGCTCCAGTTCTTGT	20	55	60.76	-22.22	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
4103834_F	Rpg7_F19	CGACCGAACACCATGATCTCT	21	52.38	60.38	-22.72	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
4103934_R	Rpg7_R19	ACAAAAGATTGTCCGAGCACC	21	47.62	59.63	-22.26	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
4105098_F	Rpg7_F20	CATGGCTCTCCACTTGTTGC	20	55	59.98	-22.04	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
4105187_R	Rpg7_R20	ATGAAGGCTTGCCTGATCGT	20	50	60.26	-21.9	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
4147882_F	Rpg7_F21	TTTGGGGCCATTCGGTTTCT	20	50	60.71	-21.91	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
4147967_R	Rpg7_R21	GTTTATGTCGCTCGGGGTGT	20	55	60.9	-22.45	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
4425721_F	Rpg7_F22	AGCATCACTAGAACGTCCAC	20	50	57.84	-20.98	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
4425802_R	Rpg7_R22	AGGCAGTGATGTAGACCGAG	20	55	59.4	-21.65	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
5191213_F	Rpg7_F23	ATGGCGAAGTTGGACGGAAG	20	55	61.19	-22.54	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
5191301_R	Rpg7_R23	CGGCAGCTCGTAGGTCTTTT	20	55	60.9	-22.47	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
5503226_F	Rpg7_F24	ATGGATGGCTATGTCGCGAA	20	50	60.12	-21.96	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
5503307_R	Rpg7_R24	TGCACCACAACTGAATAGCC	20	50	58.97	-21.42	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
5503798_F	Rpg7_F25	ACTGTCAAGGCACGGAAACA	20	50	60.62	-22.13	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
5503906_R	Rpg7_R25	TCTGCCAGAGGAAAATCGGG	20	55	60.26	-21.9	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
5712374_F	Rpg7_F26	TAACTCCCCGGAATCAGTGC	20	55	59.97	-21.81	ACACTCTTTCCCTACACGACGCTCTTCCGATCT

5712441_R	Rpg7_R26	ATTGCGGGGATGATGCACTC	20	55	61.33	-22.55	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
5718809_F	Rpg7_F27	GTCTTGGCTTCTCGATCTGCT	21	52.38	60.65	-22.81	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
5718898_R	Rpg7_R27	CAACAAGCACCGGCATCCAA	20	55	62.32	-23.07	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
5781329_F	Rpg7_F28	GTGTGCAGAGCTGTTAACACC	21	52.38	60.24	-22.78	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
5781398_R	Rpg7_R28	GGGAAGGAATTCGGCGGAAA	20	55	61.2	-22.39	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
5783642_F	Rpg7_F29	CACAGCCGATTGAGCGTACT	20	55	60.96	-22.61	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
5783705_R	Rpg7_R29	GACGGCCATCAGCATTGGTA	20	55	60.97	-22.41	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
6142007_F	Rpg7_F30	GCATTGCAAGGTGGATAGACC	21	52.38	59.83	-22.39	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
6142104_R	Rpg7_R30	TCTCCACATTTCATTTATCCGCAC	24	41.67	60.19	-24.06	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
6239650_F	Rpg7_F31	CCGTGTCCTCGACGAATCTT	20	55	60.33	-22.28	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
6239746_R	Rpg7_R31	ATCCTTGCTGCCATCAGTGA	20	50	59.9	-21.66	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
7220083_F	Rpg7_F32	GCGGTGTTTCTATGTCGTCC	20	55	59.49	-21.99	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
7220160_R	Rpg7_R32	GGCAGCATCGTCACCCTG	18	66.67	61.31	-21.68	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
7267119_F	Rpg7_F33	TGACCTTGGTGGGTATCGTG	20	55	59.9	-21.8	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
7267226_R	Rpg7_R33	ACGCGACCGATGACTTTGAG	20	55	61.23	-22.83	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
7454535_F	Rpg7_F34	CCTGCACACCGAGAAATCGA	20	55	60.89	-22.5	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
7454625_R	Rpg7_R34	GCCGAGCTCGATTGCATC	18	61.11	59.54	-21.09	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
7631112_F	Rpg7_F35	TTGGCCCCTTGATCACGATG	20	55	60.91	-22.27	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
7631204_R	Rpg7_R35	CTCCTTCCTACGGTGCATGT	20	55	59.97	-21.87	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
8049283_F	Rpg7_F36	GGTGCGTACCGTGTGTGG	18	66.67	61.52	-21.94	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
8049371_R	Rpg7_R36	CCTTCACCTATGTCAGCGGG	20	60	60.69	-22.31	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
8641986_F	Rpg7_F37	GGTCTTGGCACTGAGAGAGT	20	55	59.54	-21.61	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
8642094_R	Rpg7_R37	TCCTGCCACAGCTCACAATT	20	50	60.41	-21.92	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
8642384_F	Rpg7_F38	GTGCACGGACATCGAGAGTG	20	60	61.57	-23.11	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
8642470_R	Rpg7_R38	GCGTCGGTGCACATCTCAAT	20	55	61.86	-23.09	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
8643191_F	Rpg7_F39	GTGCAGACGGGTGGAGAC	18	66.67	60.53	-21.34	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
8643276_R	Rpg7_R39	ATTAGAACCAACCTGCCGCG	20	55	61.54	-22.74	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT

9077208_F	Rpg7_F40	GCCTTGCACTCGGATCTTGT	20	55	61.19	-22.54	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
9077304_R	Rpg7_R40	TGGGCGAGCATGTCCTAATC	20	55	60.4	-22.1	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
9153463_F	Rpg7_F41	TCAAGCTCCAAGAAGCCCAA	20	50	60.04	-21.64	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
9153525_R	Rpg7_R41	GGGGTGGTGGGAGTCTGTAA	20	60	61.37	-22.25	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
9280714_F	Rpg7_F42	CCGCAATATCAGCTCTTTGTG	21	47.62	58.16	-21.73	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
9280804_R	Rpg7_R42	CGAAGAGCGCAGATGTTGAA	20	50	59.42	-21.9	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
9281490_F	Rpg7_F43	CTGCTCCCTCGTTTGCAGTA	20	55	60.55	-22.23	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
9281586_R	Rpg7_R43	CGCGACCTGCTGACATGAA	19	57.89	61.23	-22.24	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
9295998_F	Rpg7_F44	GCTGAACCAATCGTACCTCG	20	55	59.21	-21.83	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
9296086_R	Rpg7_R44	ACGTGCAGCTCAAATGGTTC	20	50	59.91	-22.01	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
9524972_F	Rpg7_F45	GTTATGGTAGCTGCGTTGCC	20	55	60.12	-22.24	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
9525049_R	Rpg7_R45	ACCCAGCCTAATTTTGAGCC	20	50	58.65	-21.07	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
9797053_F	Rpg7_F46	GTTTTGGCCTTCCACACGTC	20	55	60.47	-22.32	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
9797153_R	Rpg7_R46	ACTTCTTTACTGTCTCACAAGGGT	24	41.67	60.13	-23.72	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
9797373_F	Rpg7_F47	AACCAGACCTCGATATGCCG	20	55	60.12	-22.02	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
9797482_R	Rpg7_R47	ACAATACCGAGATGCTACGAGT	22	45.45	59.57	-22.71	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
9797516_F	Rpg7_F48	CGTTCGTGTCAGCTGCTCTA	20	55	60.61	-22.51	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
9797586_R	Rpg7_R48	CCACTTTTCAGACATCACCGC	21	52.38	60.31	-22.79	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT
9798368_F	Rpg7_F49	CCTGTGAATTCAAGGCCTGC	20	55	59.97	-21.95	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
9798455_R	Rpg7_R49	TTGGGAGGACAACCACAGATG	21	52.38	60.45	-22.46	GACTGGAGTTCAGACGTGTGCTCTTCCGATCT

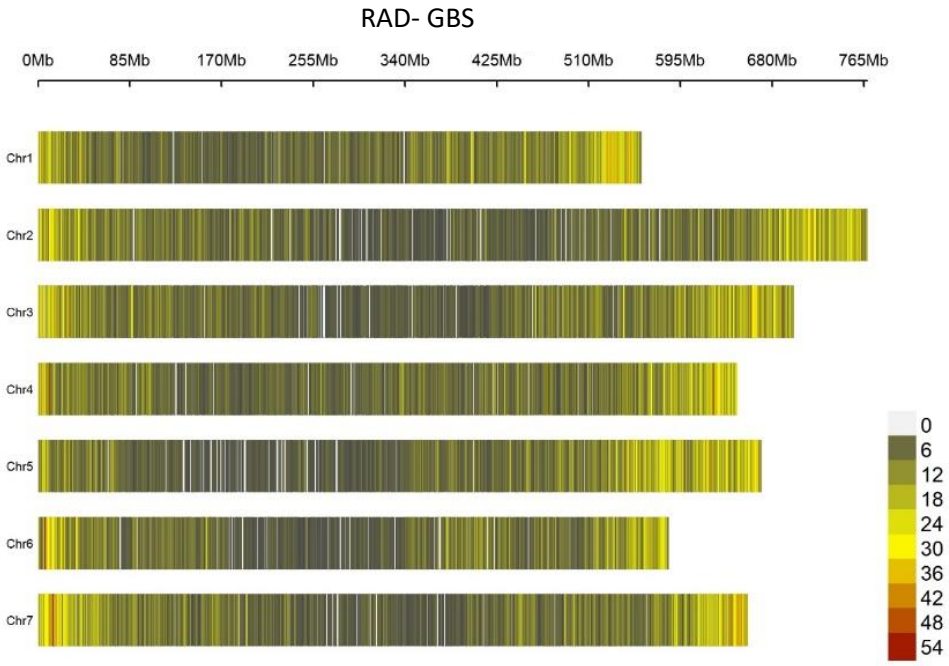
Supplementary Table S4: PACE Primers for F_3 Genotyping of Morex x WBDC-94 in the F_3.

Name	Primer	FluoroBinder
PACE1_F1	GAAGGTGACCAAGTTCATGCCAGAGATGCCGTTCGCGT	FAM
PACE1_F2	GAAGGTCGGAGTCAACGGATCAGAGATGCCGTTCGCGG	VIC/HEX
PACE1_CR	TGCCTCCGGTACTGCGA	
PACE2_F1	GAAGGTGACCAAGTTCATGCTTACTAGGGGCATACATACCC	FAM
PACE2_F2	GAAGGTCGGAGTCAACGGATTTACTAGGGGCATACATACCA	VIC/HEX
PACE2_CR	TGCCCCTAACTTAAAGATGGT	
PACE3_F1	GAAGGTGACCAAGTTCATGCTGGACACCGAGAAGGAGTTC	FAM
PACE3_F2	GAAGGTCGGAGTCAACGGATTGGACACCGAGAAGGAGTTT	VIC/HEX
PACE3_CR	CCTTACCAGGGACATGCATG	
PACE4_F1	GAAGGTGACCAAGTTCATGCAAAGCTAGAGCCATGCAGC	FAM
PACE4_F2	GAAGGTCGGAGTCAACGGATAAAGCTAGAGCCATGCAGA	VIC/HEX
PACE4_CR	TGAATGGAAGCGTAAGCTTTTC	
PACE5_F1	GAAGGTGACCAAGTTCATGCCAAAAGCATCGGCAAGGTG	FAM
PACE5_F2	GAAGGTCGGAGTCAACGGATCAAAAGCATCGGCAAGGTA	VIC/HEX
PACE5_CR	CCGTGGACAGTTCCCATA	
PACE6_F1	GAAGGTGACCAAGTTCATGCTTATGAGATGGTCCATTTGCAA	FAM
PACE6_F2	GAAGGTCGGAGTCAACGGATTTATGAGATGGTCCATTTGCAT	VIC/HEX
PACE6_CR	GTCAGCATTCAAGGGCTTA	
PACE7_F1	GAAGGTGACCAAGTTCATGCACGCATGCTTGTCAAAATGT	FAM
PACE7_F2	GAAGGTCGGAGTCAACGGATACGCATGCTTGTCAAAATGC	VIC/HEX
PACE7_CR	TGTCTCCCAGGGCCAAG	

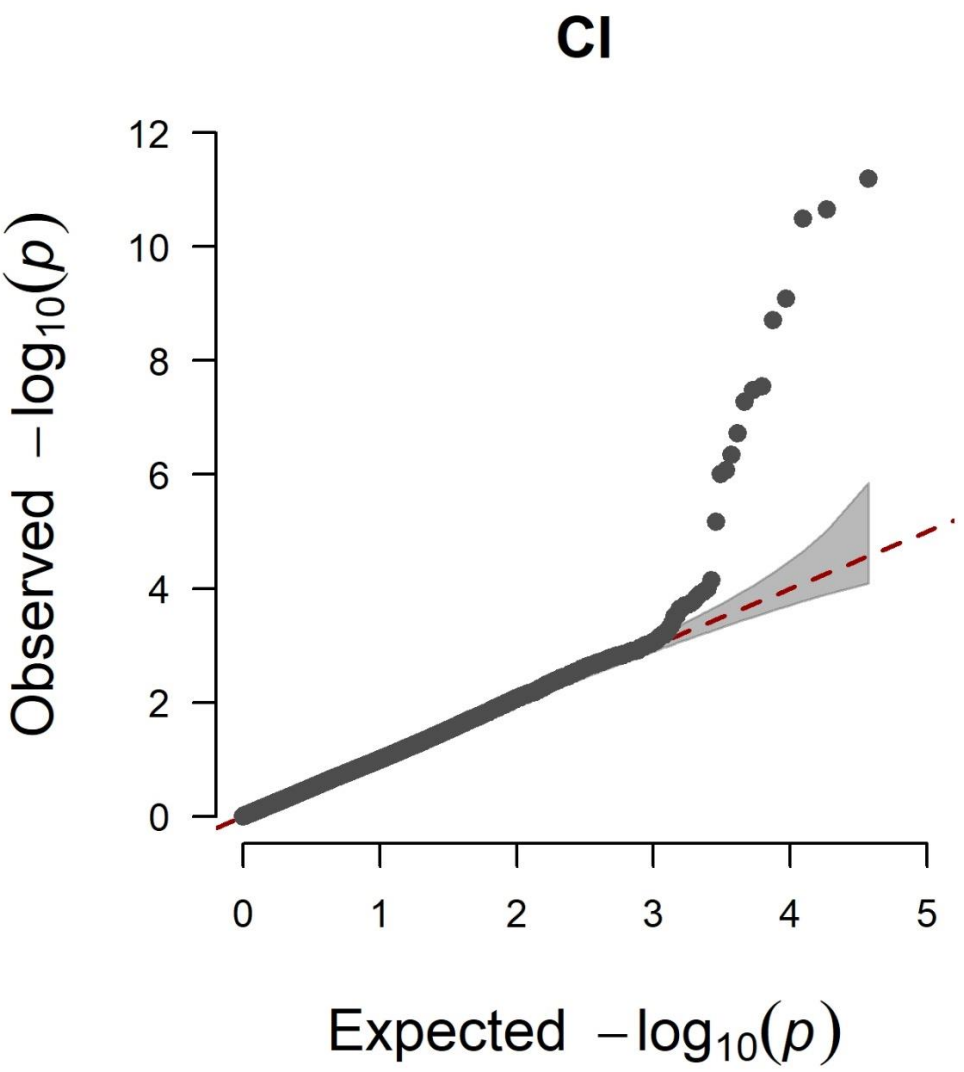
Supplementary Table S5: Plasmisouris Primers to sequence across candidate genes for *Rpg7.*

Name	Sequence	Length	Tm	% GC	Gene of interest
Plasidsouris_F1	AACAAACGCATAAACAAGTAGCGCA	25	62.7	40	RPM1
Palsmisouris_R1	GCTTGTGGTCTTGTAAGCAGTATTT	25	60	40	
Plasidsouris_F2	TTGGCCGTCAACCTATATAAGTAGG	25	60	44	RIN4
Plasidsouris_R2	GCCCCTCCATTTTTAGGTATTCAAC	25	60.2	44	

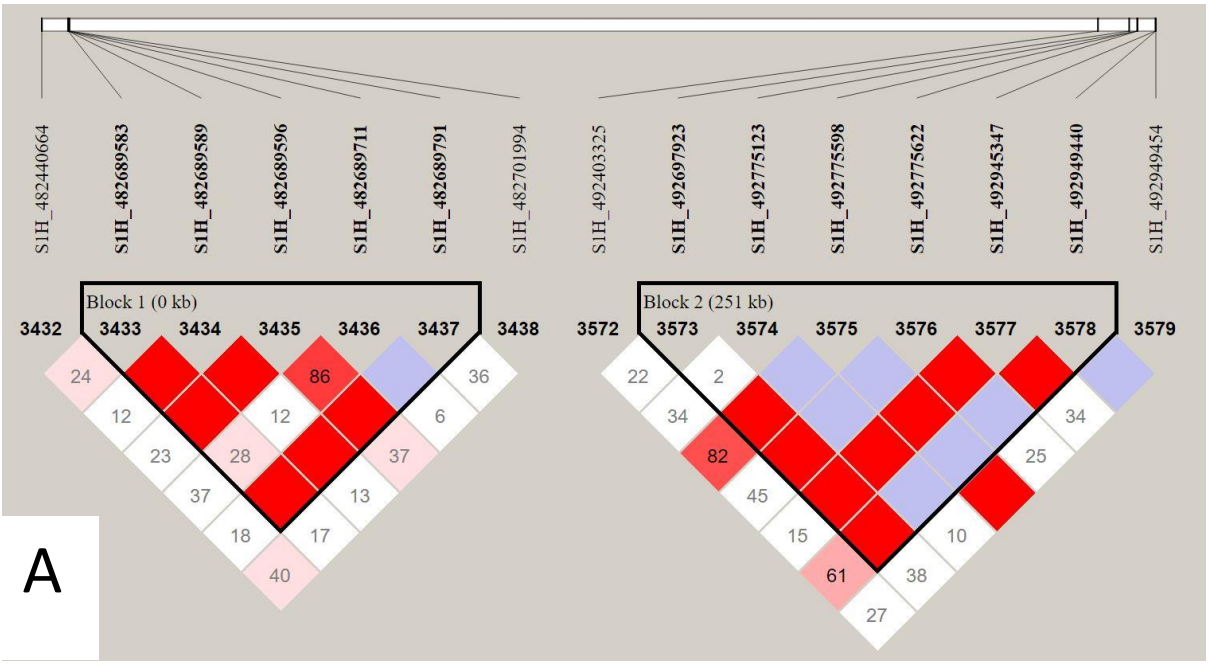
APPENDIX B: SUPPLEMENTARY FIGURES



Supplemental Figure S1: Heatmap of the RAD-GBS marker distribution across the genome of *H. vulgare* subsp. *Spontaneum*.



Supplementary Figure S2: QQ Plot generated GAPIT v3 showing the quality results of the WBDC GWAS against the PNW *Pgt* isolate Lsp21.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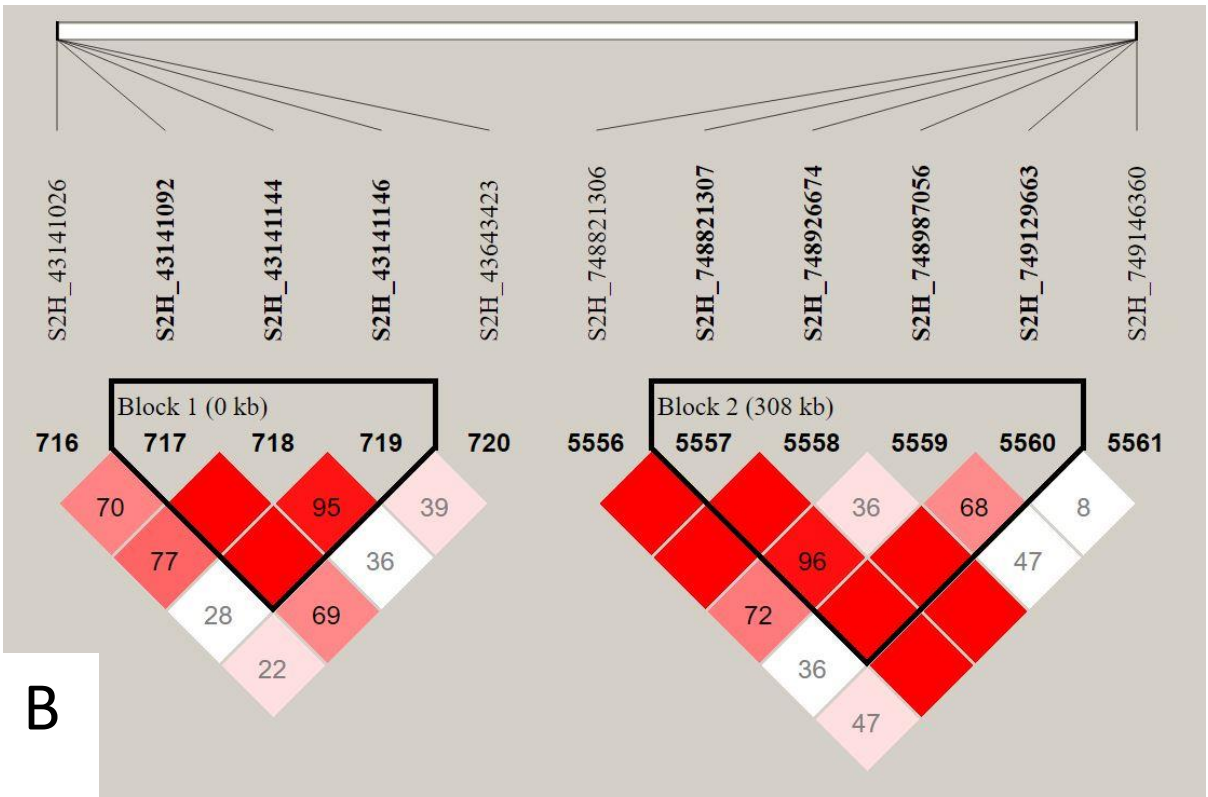
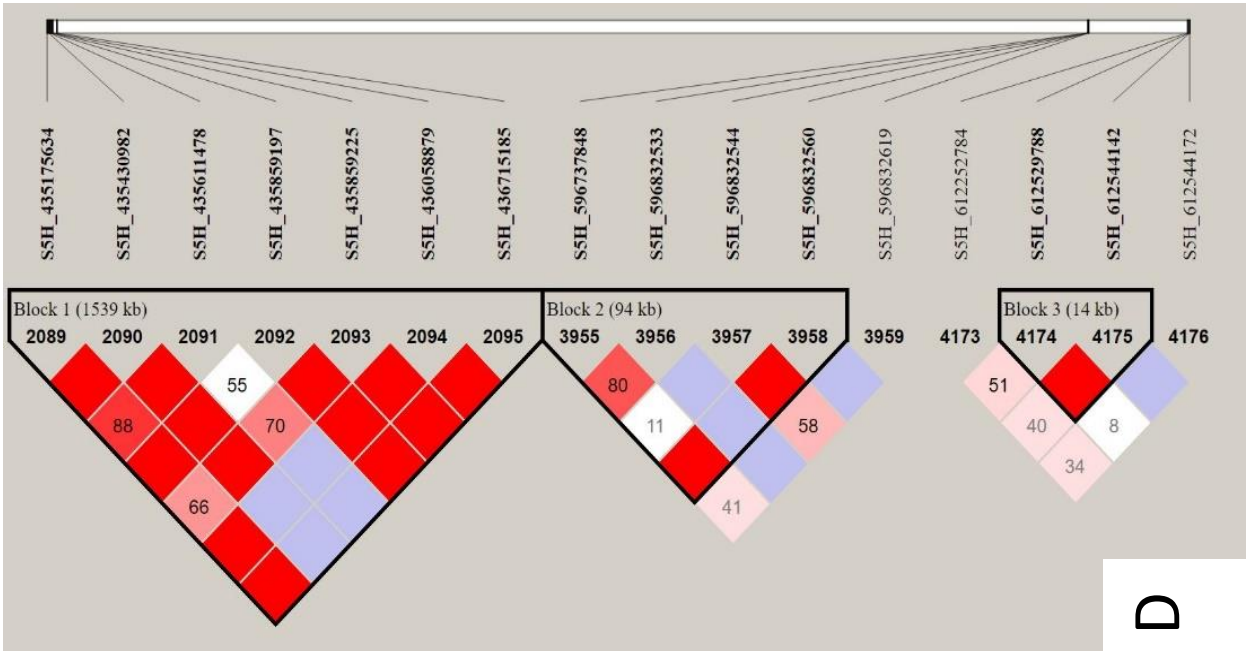
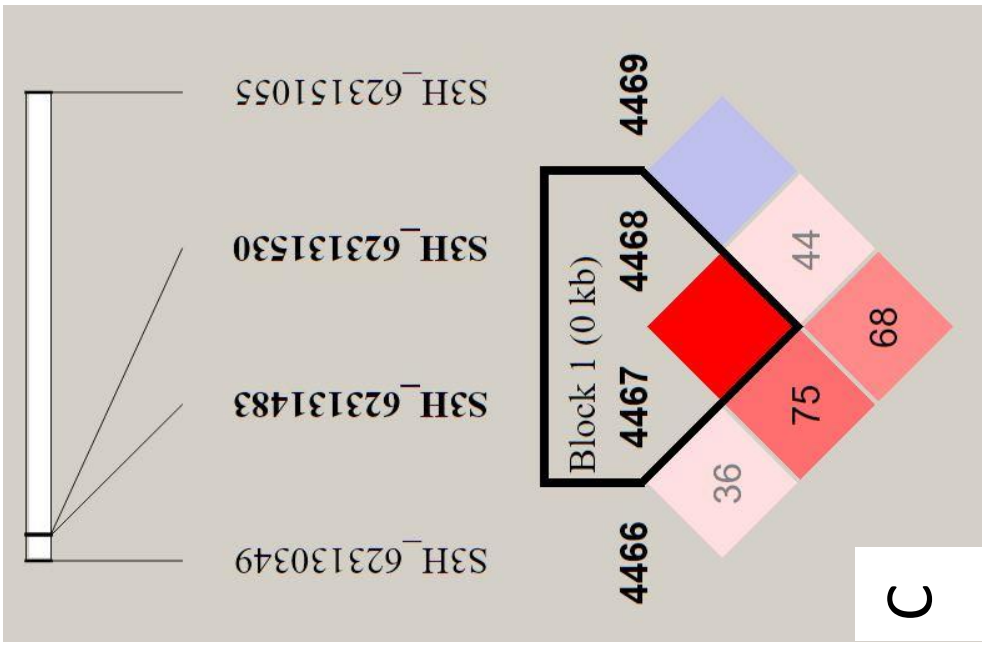
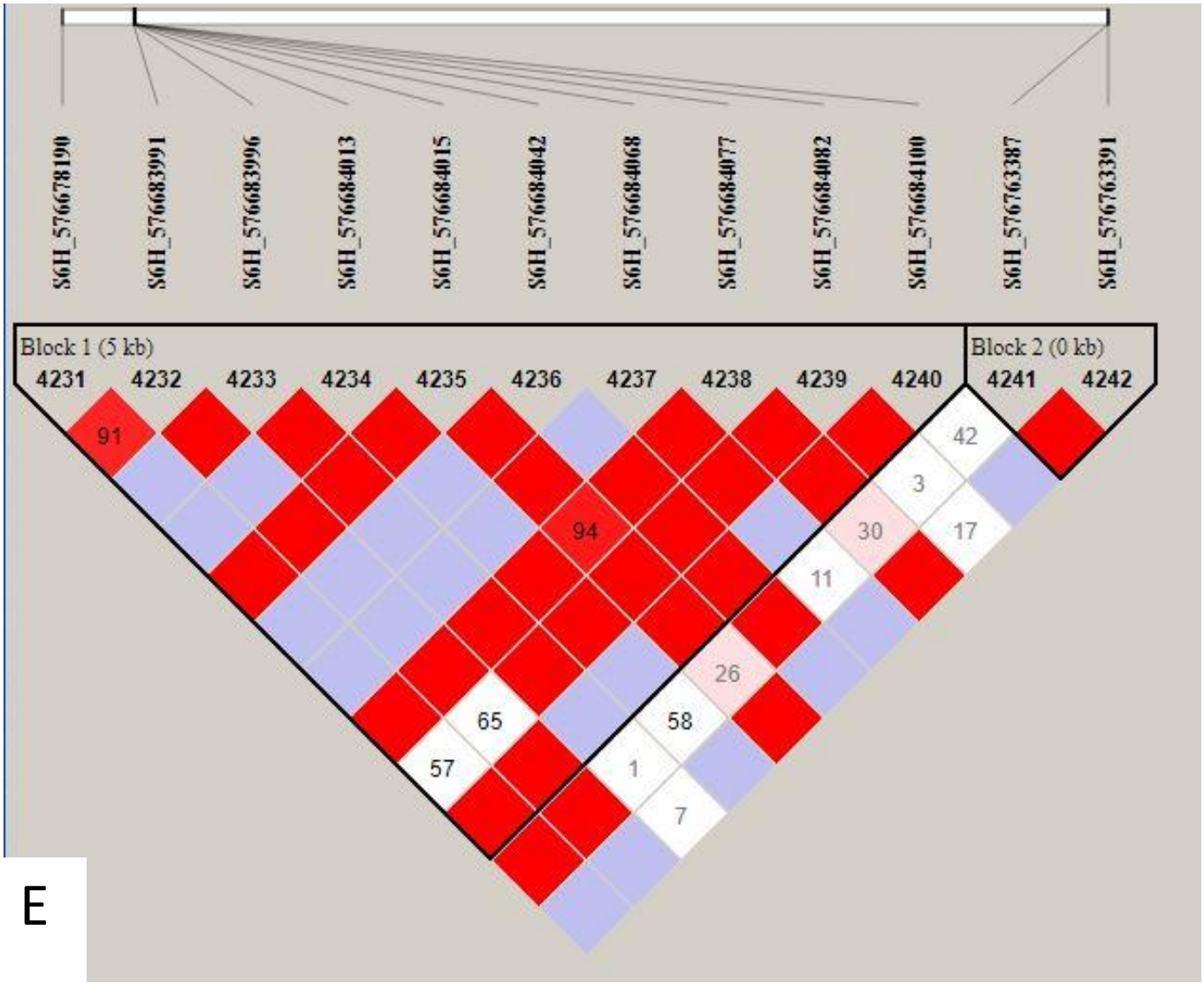
S1H_492949454
Block 1 (0 kb)
3432 3433 3434 3435 3436 3437 3438
24
86 36
12 12 6
23 28 37
37 13
18 17
40
Block 2 (251 kb)
3572 3573 3574 3575 3576 3577 3578 3579
22 2
34 34
82 25
45
15 10
61 38
27
A

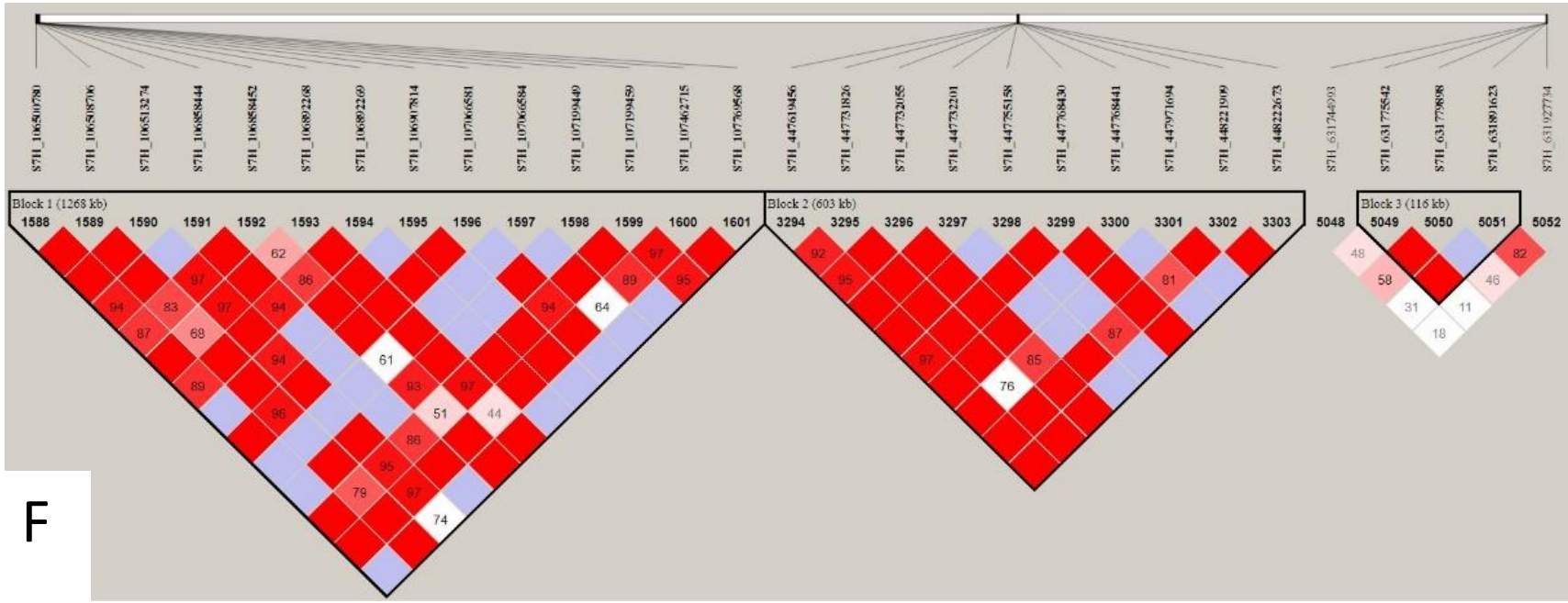

S2H_43141026
S2H_43141092
S2H_43141144
S2H_43141146
S2H_43643423
S2H_748821306
S2H_748821307
S2H_748926674
S2H_748987056
S2H_749129663
S2H_749146360
Block 1 (0 kb)
716 717 718 719 720
70 95 39
77 36
28 69
22
Block 2 (308 kb)
5556 5557 5558 5559 5560 5561
36 68 8
96 47
72
36
47
B


S5H_435175634
S5H_435430982
S5H_435611478
S5H_435859197
S5H_435859225
S5H_436058879
S5H_436715185
S5H_596737848
S5H_596832533
S5H_596832544
S5H_596832560
S5H_596832619
S5H_612252784
S5H_612529788
S5H_612544142
S5H_612544172
Block 1 (1539 kb)
2089 2090 2091 2092 2093 2094 2095
55
88 70
66
Block 2 (94 kb)
3955 3956 3957 3958 3959
80
11 58
41
Block 3 (14 kb)
4173 4174 4175 4176
51
40 8
34
D

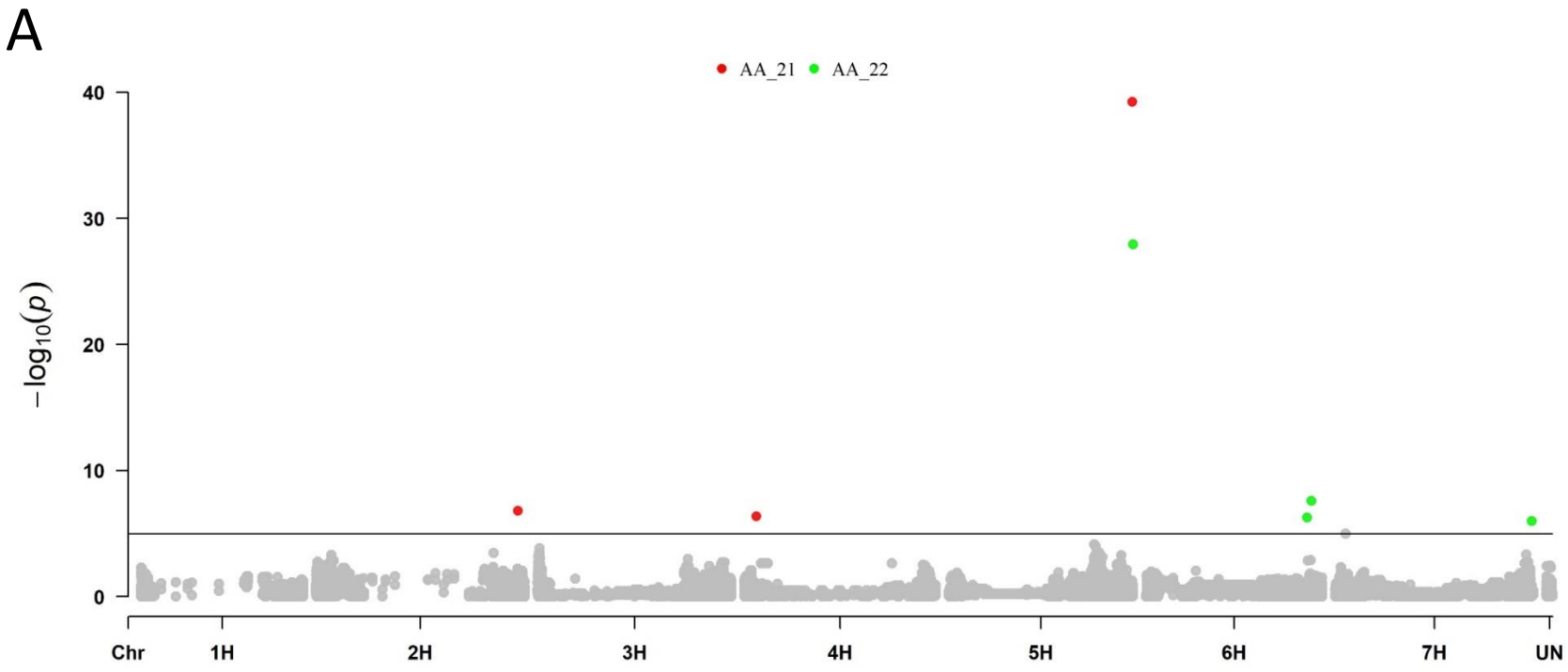

S3H_623151055
S3H_623131530
S3H_623131483
S3H_623130349
Block 1 (0 kb)
4469
4468
4467
4466
44
68
75
36
C

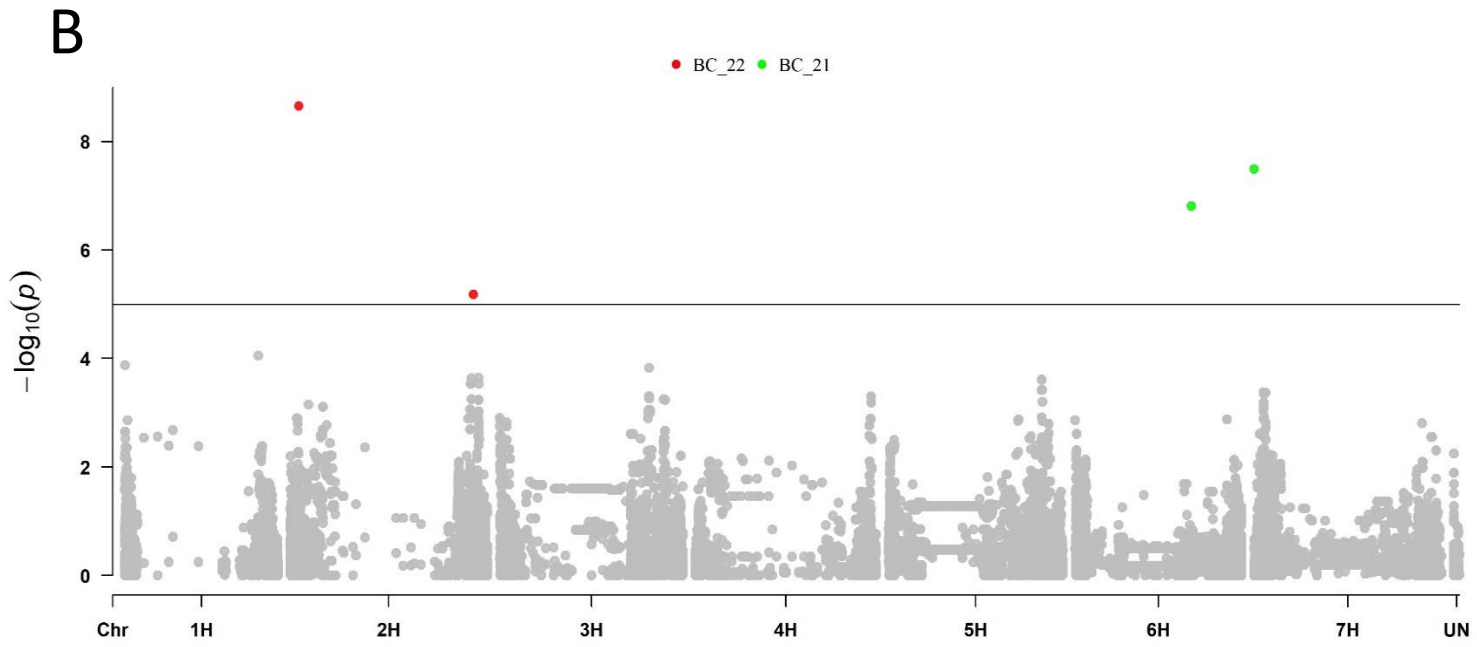

S6H_576678190
S6H_576683991
S6H_576683996
S6H_576684013
S6H_576684015
S6H_576684042
S6H_576684068
S6H_576684077
S6H_576684082
S6H_576684100
S6H_576763387
S6H_576763391
Block 1 (5 kb)
Block 2 (0 kb)
4231
4232
4233
4234
4235
4236
4237
4238
4239
4240
4241
4242
91
94
65
57
26
58
1
7
11
30
3
17
42

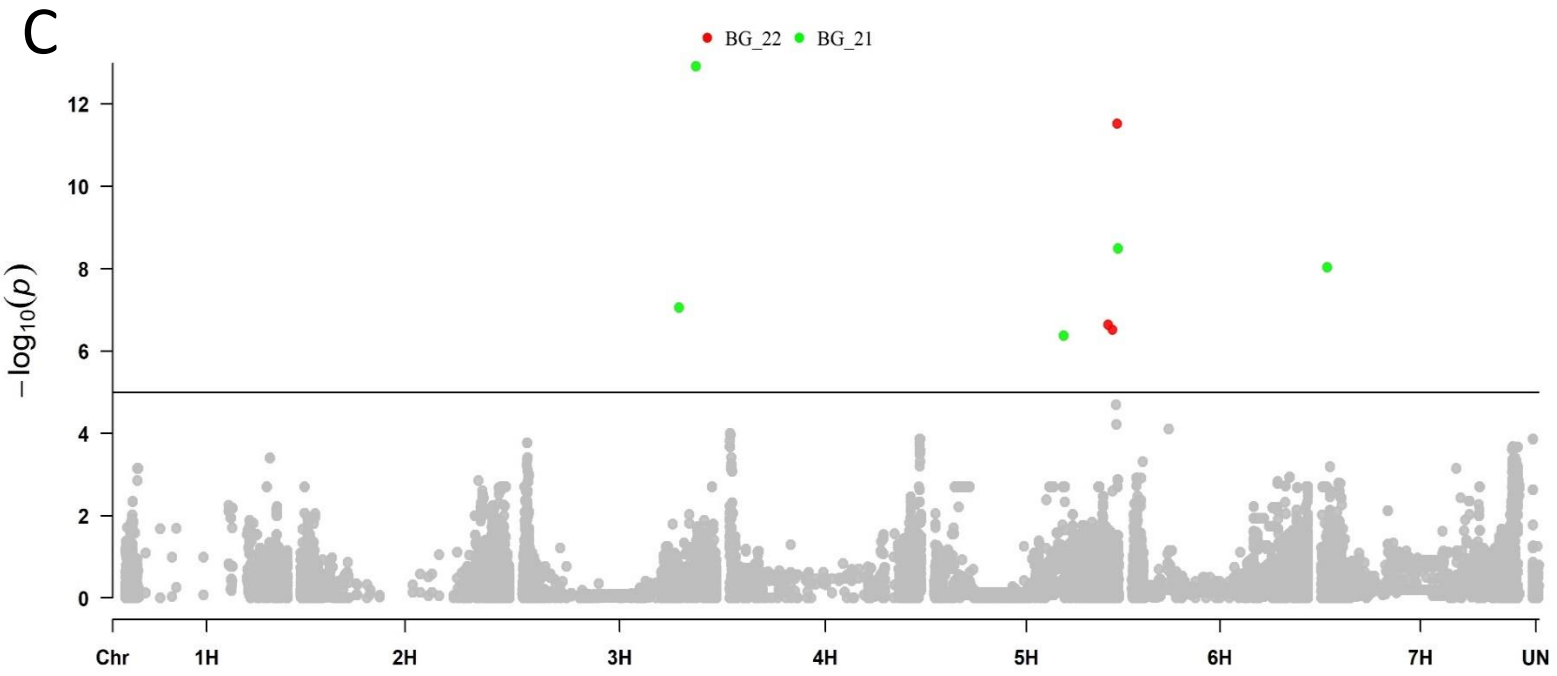
E



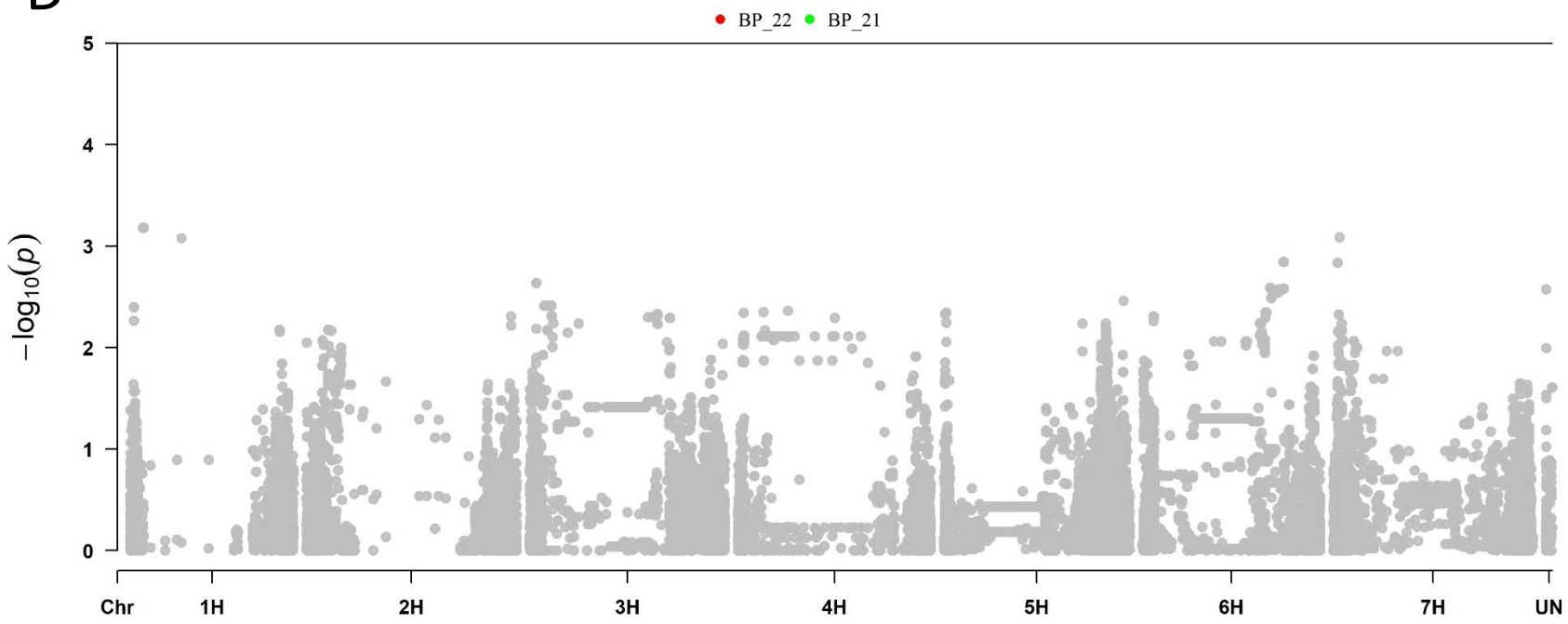
Supplementary Figure S3: Delimited significant LD blocks associated with resistance to the PNW *Pgt* isolate Lsp21 found in the WBDC. Section **A** shows the two significant estimated LD blocks on chromosome 1H. Section **B** is the two significant estimated LD blocks on chromosome 2H; Section **C** is the significant estimated LD block on chromosome 3H; Section **D** is the three significant estimated LD blocks on chromosome 5H; Section **E** is the significant estimated LD block on chromosome 6H; Section **F** are three significant estimated LD block on chromosome 7H.

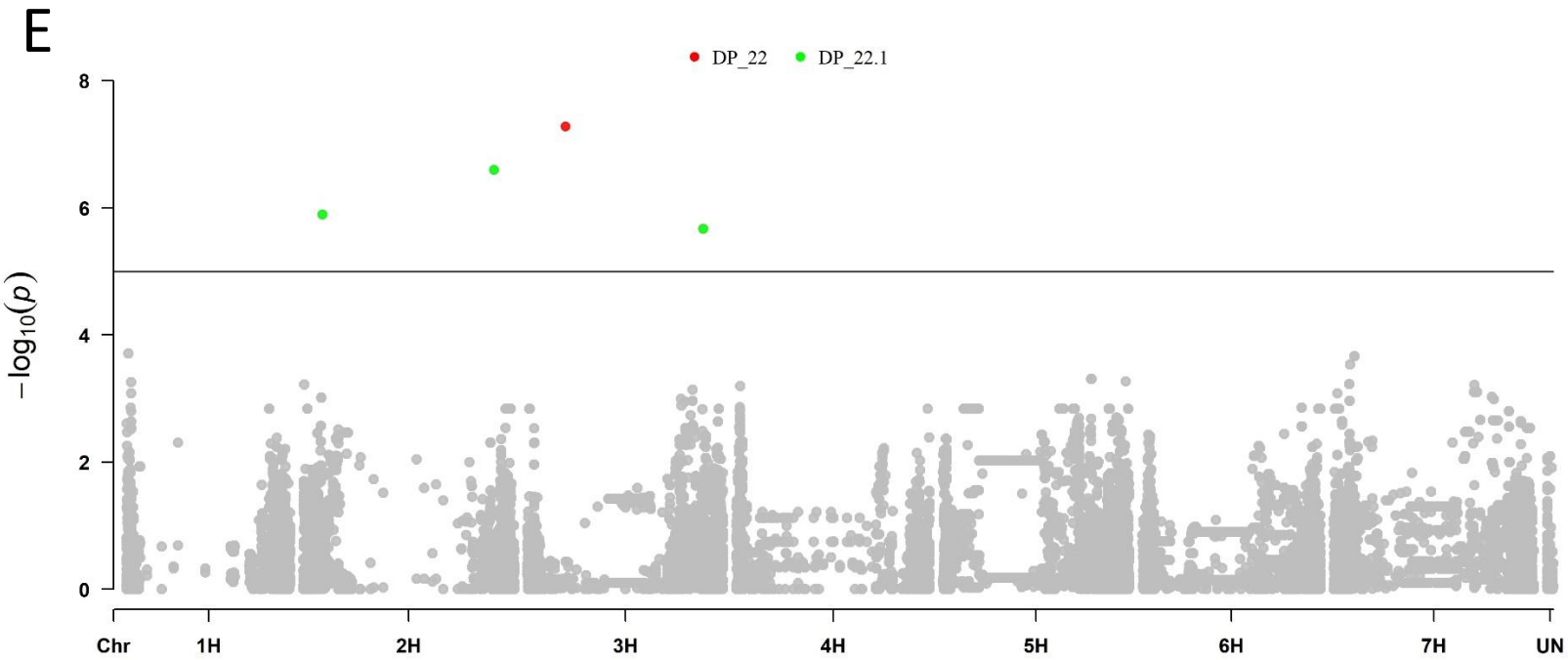

A
AA_21
AA_22
40
30
20
10
0
$-\log_{10}(p)$
Chr
1H
2H
3H
4H
5H
6H
7H
UN

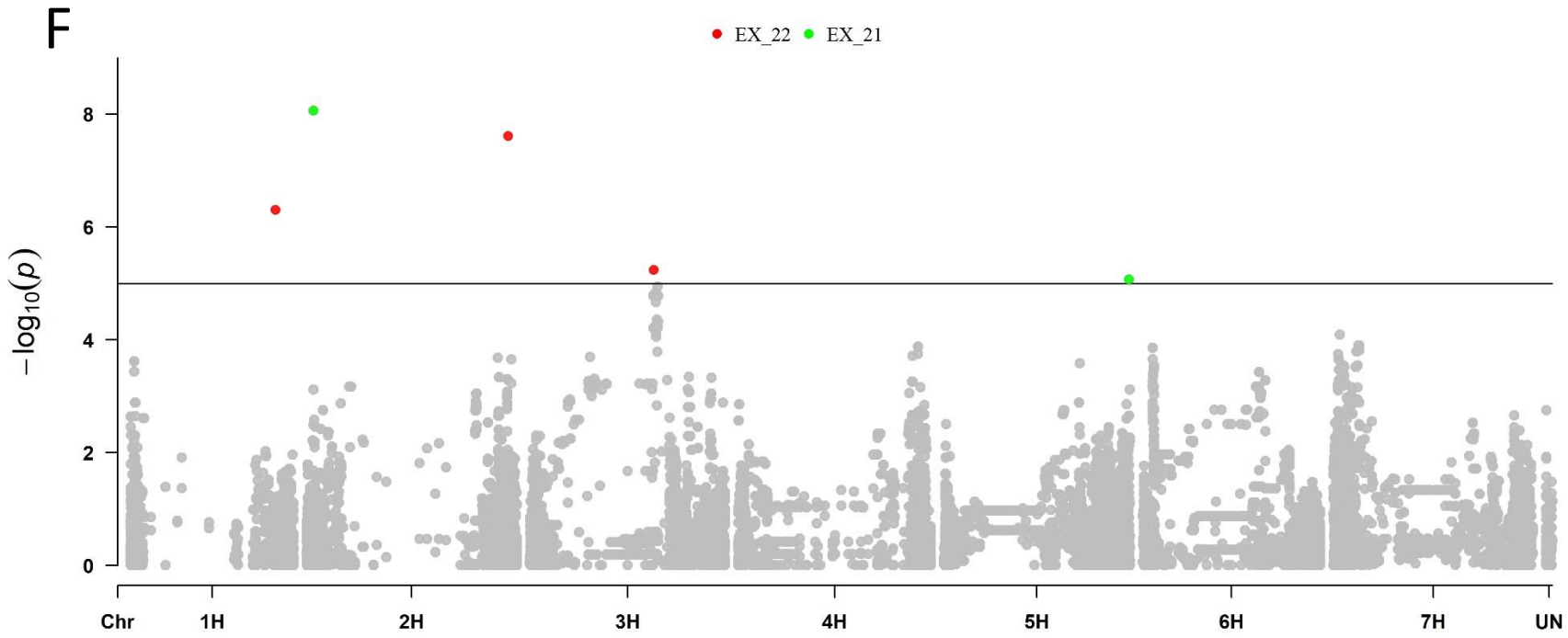

B
BC_22
BC_21
−log10(p)
8
6
4
2
0
Chr
1H
2H
3H
4H
5H
6H
7H
UN


C
BG_22
BG_21
12
10
8
6
4
2
0
$-\log_{10}(p)$
Chr
1H
2H
3H
4H
5H
6H
7H
UN

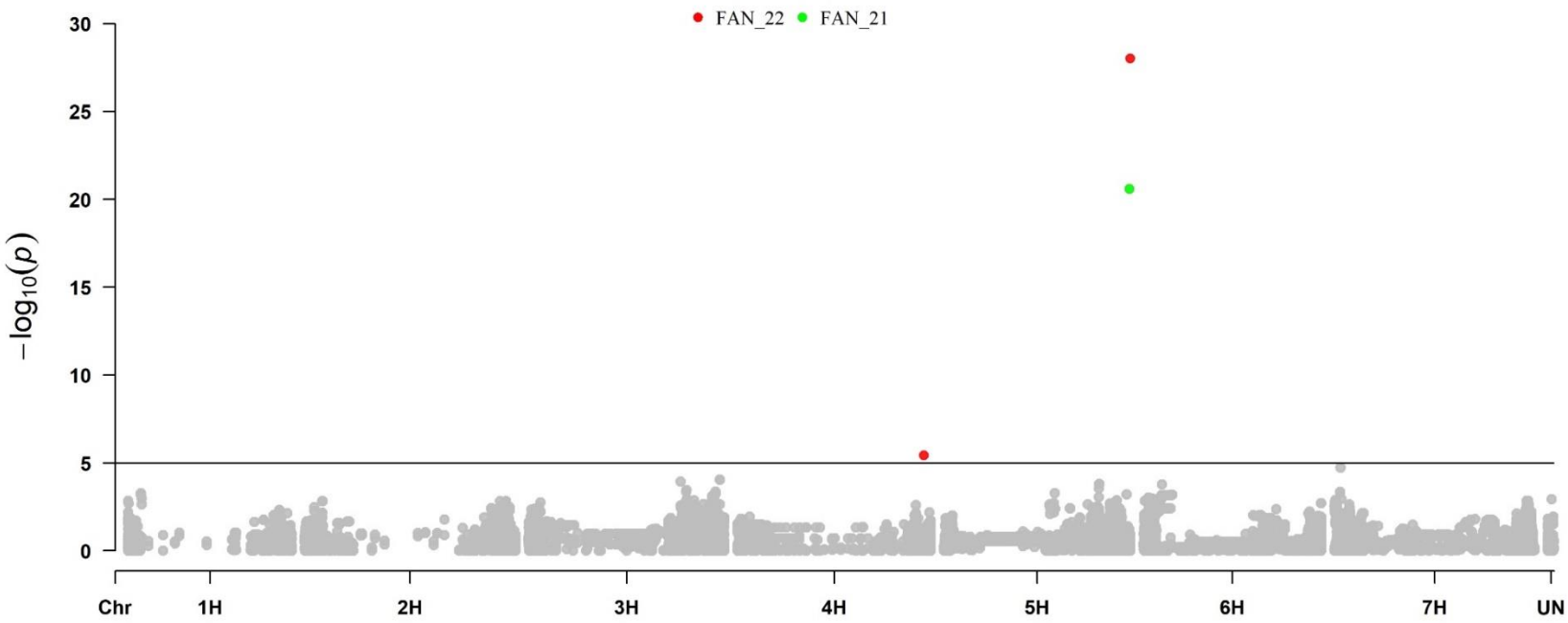
D



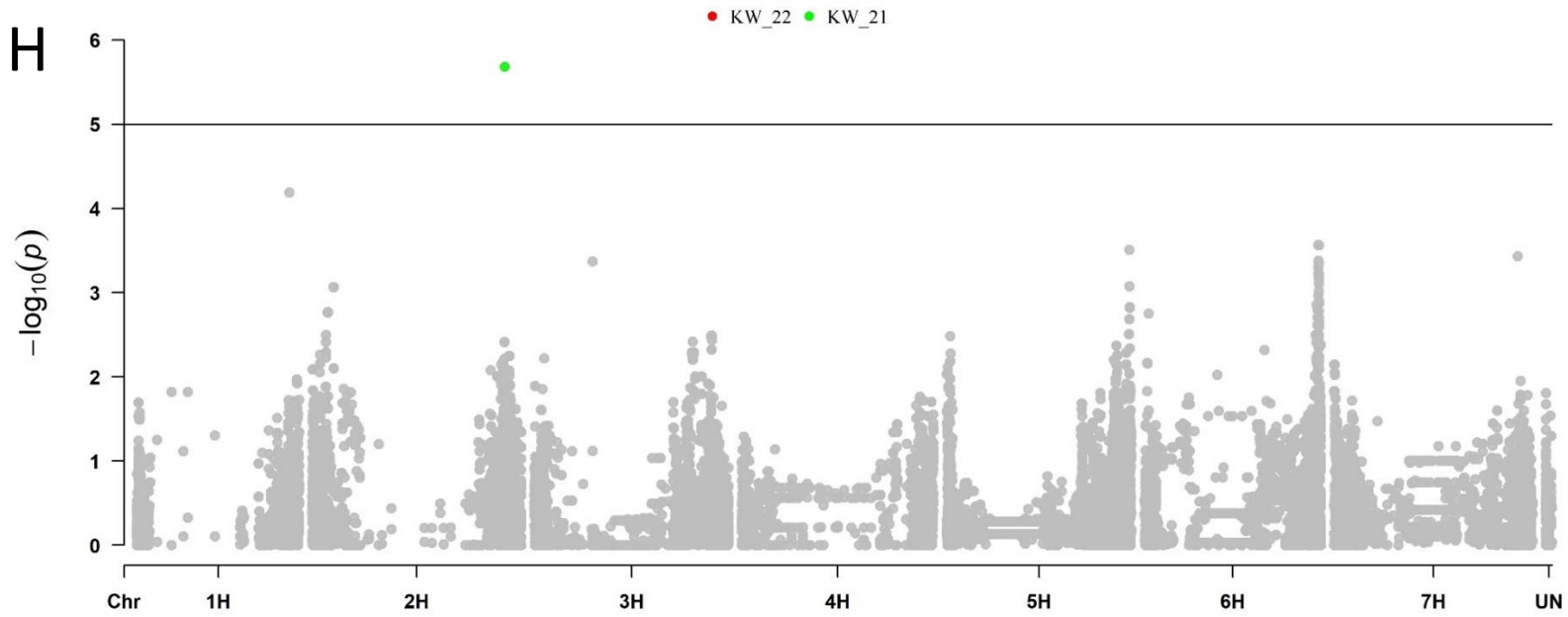

E
DP_22
DP_22.1
8
6
4
2
0
−log10(p)
Chr
1H
2H
3H
4H
5H
6H
7H
UN

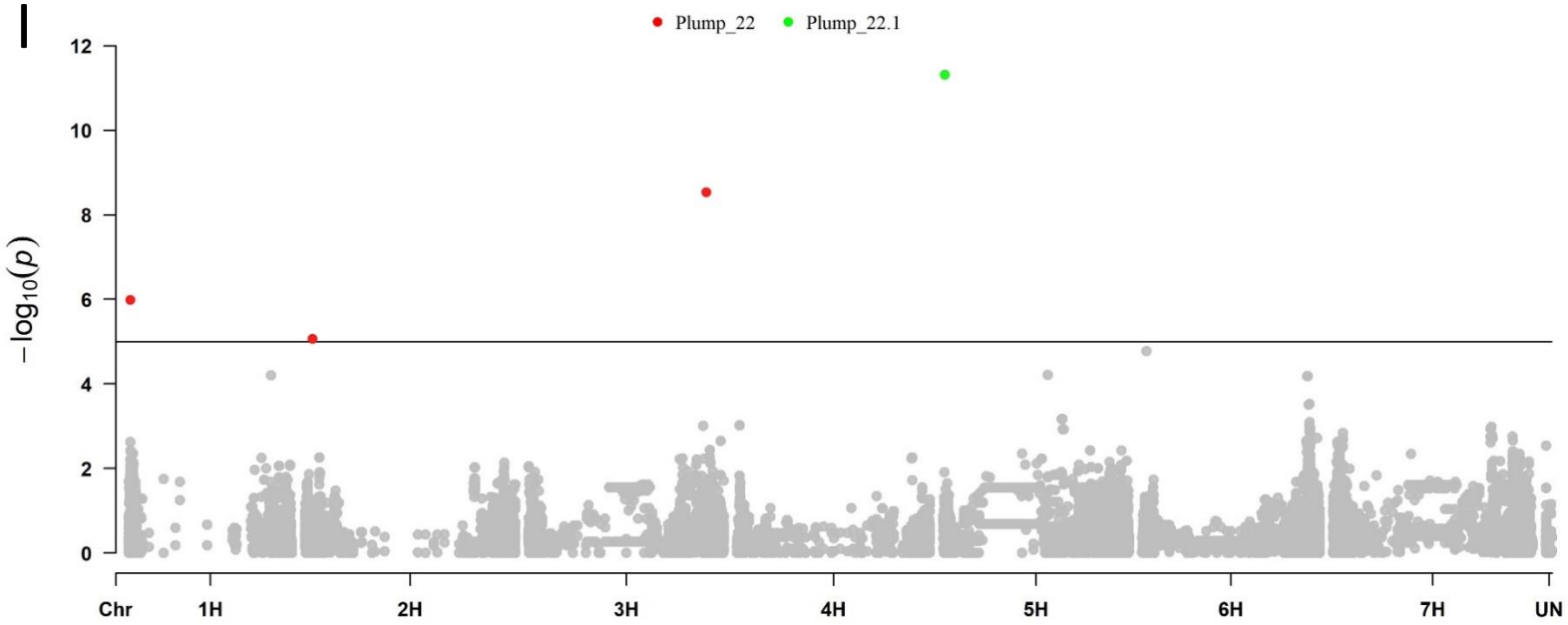

F
EX_22
EX_21
$-\log_{10}(p)$
8
6
4
2
0
Chr
1H
2H
3H
4H
5H
6H
7H
UN

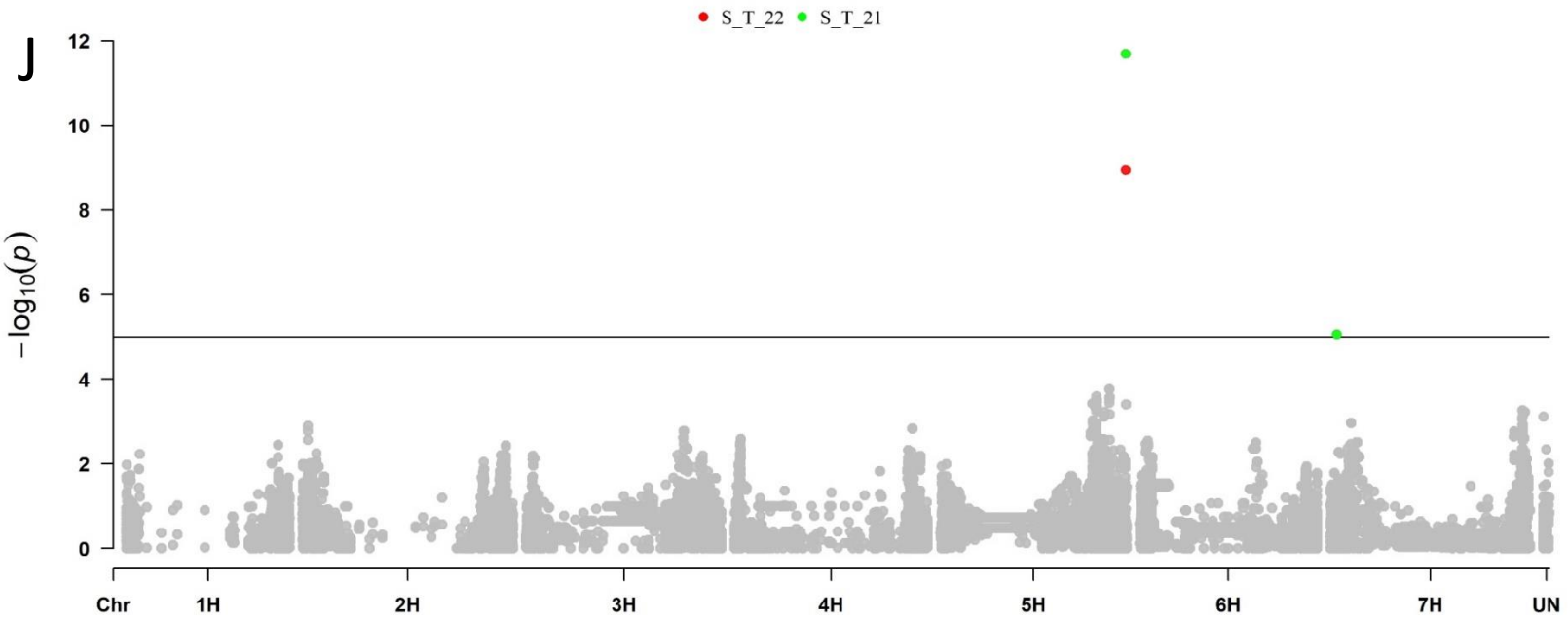
G



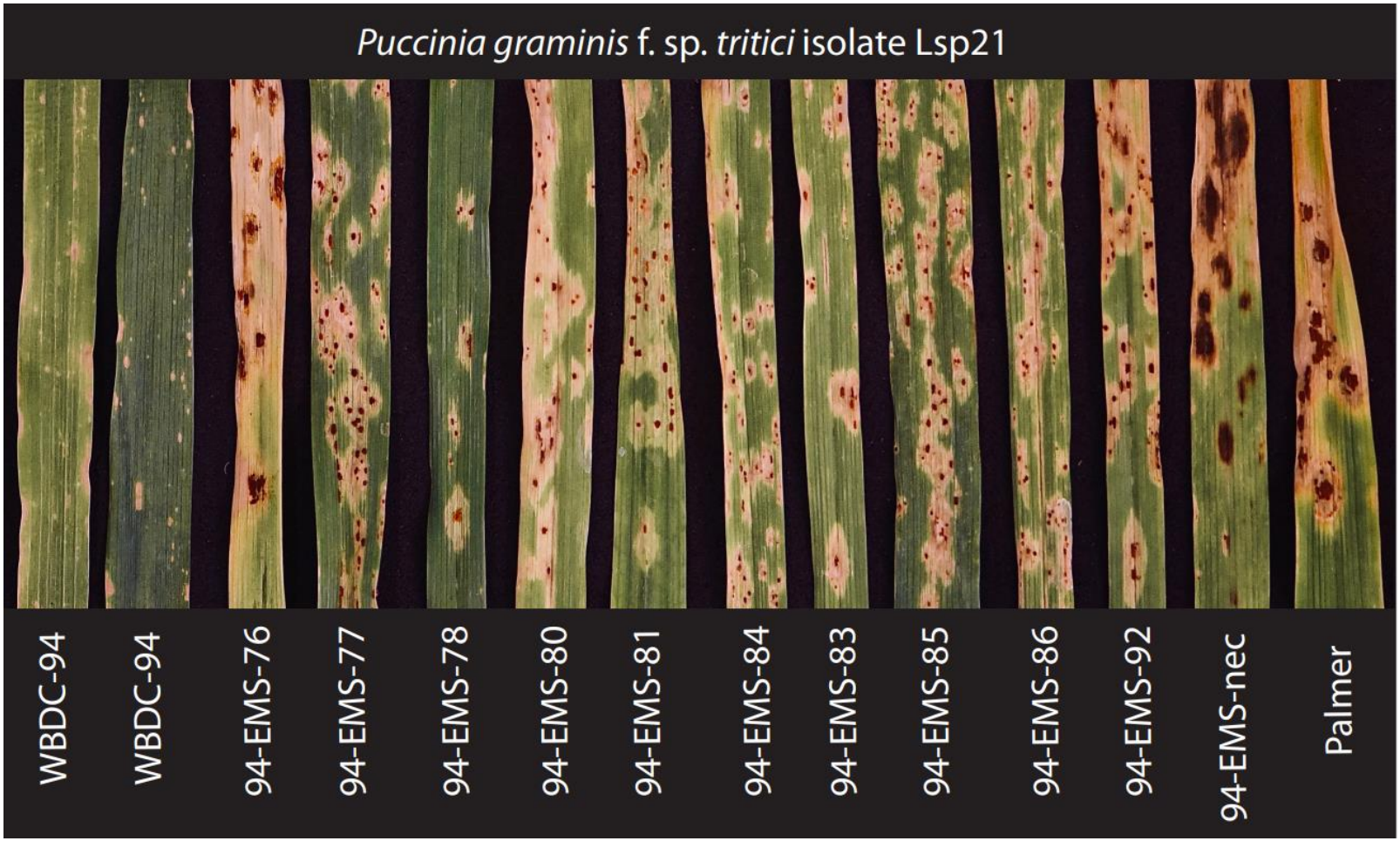
H




Plump_22
Plump_22.1
12
10
8
6
4
2
0
$-\log_{10}(p)$
Chr
1H
2H
3H
4H
5H
6H
7H
UN



Supplementary Figure S4: 13 Manhattan plots for the 13 malting quality traits for single replicated individuals between 2021 and 2022. The seven chromosomes of barley are labeled on the x-axis. SNPs are shown distributed across the chromosome based on positions on the cv Morex v1 genome assembly as grey dots. Significant SNPs in 2021 are shown in yellow, and significant SNPs in 2022 are shown in red. Manhattan plot (**A**) for α-amylase (AA), (**B**) barley color (BC), (**C)** β-glucan (BG), (**D)** barley protein (BP), (**E**) diastatic power (DP), (**F**) extract (EX), (***G***) free amino nitrogen (FAN), (**H**) Kernel weight (KW), (**I**) kernel plumpness (Plump), (**J**) soluble/total ratio (S_T).



Supplementary Figure S5: Seedling stage stem rust assay on barley EMS mutant population of WBDC 94. from the Wild Barley Diversity Collection (WBDC) against *P. graminis* f. sp. *tritici* at 14 days after inoculation. The panel shows a typical disease assay with the virulent *Pgt* isolate, Lsp21.