FROM BIOINFORMATICS TO IDENTIFYING *R*-GENES, ENHANCERS, SIGNALING PATHWAYS AND PATHOGEN ELICITORS IN THE BARLEY-STEM RUST PATHOSYSTEM

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Stem rust, caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*) is a threat to wheat and barley. *Rpg1* is the only deployed stem rust resistance gene in barley that provides resistance to the predominant races in North America, except to the local race QCCJB and the widely virulent race TTKSK (aka Ug99) and its lineages. The barley *rpg4*-mediated resistance locus (RMRL) confers resistance against the majority of *Pgt* races, including TTKSK and its lineage. With the goal of identifying *Pgt* effectors/suppressors that elicit/suppress RMRL resistance responses twenty-four *Pgt* isolates showing differential infection types were genotyped utilizing *in planta* RNAseq. The RNAseq experiment identified 114K SNPs within genes that resulted in predicted nonsynonymous amino acid changes and were utilized to identify genes associated with virulence/avirulence. Twenty-two genes were identified that were associated with RMRL virulence that represent candidate suppressors of resistance. Host differential gene expression analysis comparing virulent vs avirulent isolates identified virulent isolate specific down regulation of stress response genes, genes involved in chloroplastic ROS, and non-host resistance responses, suggesting that *Pgt* isolates may contain a conserved virulence factor that elicits RMRL responses and virulent isolates contain suppressors of virulence rather than dominant avirulence genes. The second chapter focused on the observations that introgression of RMRL into the elite malting variety Pinnacle (*Rpg1*+) resulted in susceptibility to *Pgt* race QCCJB (RMRL) and HKHJC (*Rpg1*) suggesting the presence of a gene required for *rpg4*/*Rpg5* and *Rpg1* resistance. Utilizing a Pinnacle RMRL-NIL X Q21861 derived RIL population and PCR-GBS genotyping, the required for *rpg4*- and *Rpg1*-mediated resistance 1, *Rrr1* gene was mapped ~5cM proximal to RMRL on barley chromosome 5H. A second gene required for *Rpg1*-mediated resistance 2, *Rrr2*, complimentary to *Rrr1* was mapped to the telomeric region of the

short arm of barley chromosome 7H. A novel *Pgt* race TTKSK resistance gene designated *RpgHv645* was identified in an unimproved swiss landrace Hv645. Utilizing a RIL population developed from a Hv645 X Harrington cross and *Pgt* race TTKSK phenotyping data generated at the adult plant stage in Njoro, Kenya, *RpgHv645* was mapped distal of RMRL and delimited to an ~11cM region.

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DEDICATION

This dissertation is dedicated to my beloved family.

Dad Kul Prasad Sharma, Mom Laxmi Kumari Dhakal, Brother Rahul Sharma Poudel,

Wife Subidhya Shrestha and Daughter Reya Sharma.

Thank you so much for you love, trust and guidance.

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CHAPTER 1. LITERATURE REVIEW

Barley

Barley (*Hordeum vulgare* L.) was one of the founder crops in ancient agriculture and one of the first crops domesticated during the Neolithic agriculture revolution (Zohary et al., 2012; Pourkheirandish et al., 2015). It was domesticated from its wild relative *Hordeum spontaneum*. The domestication of barley was hypothesized to have primarily started from the east fertile crescent, which is considered as a center of origin and diversity of wild barley and wheat. However, several studies have suggested multiple centers of barley domestication, with the Tibetan plateau being a recently proposed center of domestication (Dia et al., 2012).

Domesticated barley is the most widely grown cereal crop in the world (Horsley et al., 2009) as its genetic diversity and adaptive nature has allowed for its growth from the arctic circle to subtropical regions including the highest elevations of crop production. Barley varieties have been improved over time to meet the nutritional requirement of both animals and humans (Rogers et al., 2017). It is grown globally for animal feed and human consumption, with the majority grown for human consumption being used for malting, which is mainly utilized for brewing beer and the production of spirits. Currently, barley ranks fourth in terms of global crop production and acreage (FAO-STAT, 2014) with the United States ranking 7th in terms of global barley production, which mainly grows malting barley for beer production (USDA, 2018, Case et al., 2018). The Northern Great Plains of the US dominate US barley production, with the majority produced in the states of North Dakota and Montana (USDA, 2017).

Barley genome

Barley is a true diploid member of the Triticeae tribe that consist of seven chromosomes (2n=2x=14), designated from 1H to 7H (IBSC, 2012). It is the largest sequenced diploid genome

with an approximate size of 5.3 Gb (Mascher et al., 2017). A total of 83,105 gene model have been annotated in the current IBSC v1 genome sequence, among which 39,734 are of high-confidence.

Stem rust

Rust fungi (Pucciniales) are among the most common and economically important disease-causing fungi in cereals throughout the world (Broad Institute, 2010 http://www.broadinstitute.org/). Pucciniales is the largest order under the subphylum Basidiomycota and contains 160 genera and 7,000 species (Aime, 2006; Duplessis et al., 2011). The genus *Puccinia* in the *Puccinales* order contains 4,000 species, among which *P. triticina* (leaf rust), *P. graminis* (stem rust), and *P. striiformis* (stripe rust) are the most destructive species on the cereal crops barley and wheat. The species *P. graminis* has a much broader host range compared to other *Puccinia* species and is divided into various subspecies, varieties and formae speciales (f. sp.) based on the spore size and host range (Leonard and Szabo, 2005). The most common formae speciales of *P. graminis* that infect cereal crops are: *P. graminis* f. sp. *tritici* (*Pgt*), *P. graminis* f. sp. *secalis* (*Pgs*), and *P. graminis* f. sp. *avenae* (*Pga*). The wheat stem rust, *Pgt* has a broader host range and can infect 28 species belonging to eight genera, that includes wheat and barley (Leonard and Szabo, 2005). Other formae speciales of *P. graminis* have comparatively narrow host range. Stem rust in barley can be caused by *Pgt* and *Pgs*.

History of rust

Cereal rust is believed to be one of the earliest recorded diseases that caused disease epidemics in ancient agriculture (USDA-ARS, 2013; Wegulo, 2012; Marsalis and Goldberg, 2006). The evidence of its destruction can be found in ancient Aristotle's literature dating back to 384-322 BC (Roelfs et al., 1992). Aristotle and Theophrastus studied rust in 383 B.C. and

concluded that the rust epidemics were associated with the warm and wet weather (Chester, 1946). The ancient roman literatures mentioned wheat production losses from rust epidemics following a heavy rainfall. Epidemic of stem rust on wheat is considered as one of the major reason for the fall of Roman Empire. Even the wheat storage jars found in archaeological sites in Israel dating from 1300 B.C contained spores of rust, thus concurring with literature mentioning the havoc of what is believed to be stem rust in ancient days (Kislev, 1982).

The first detailed report on wheat stem rust was provided by Italian Fontana and Mozzetta in 1767 (Roelfs et al., 1992). Later in 1797, Christian Hendrik Persoon named it *Puccinia graminis*. Since the first report, several surveys and reports had been conducted and reported to increase the awareness of the potential threat of stem rust. In 1916 a stem rust epidemic was reported that caused the loss of almost 300 million bushels of wheat (USDA-ARS, 2013). In the 1920's and 1930's, stem rust epidemic in United States and Canada caused by *Pgt* race 56 (MCCFC) resulted in major losses in wheat yield, up to 50% in North Dakota and Minnesota. Barley losses during this epidemic were reported to be ~15% (Roelfs, 1978). In the 1950's another major stem rust epidemic caused by an outbreak of race 15B (TPMKC) was reported that caused significant yield losses of wheat, but barley remain resistance because of the deployment of barley stem rust resistance gene *Rpg1* (Roelfs et al., 1991; Steffenson, 1992). In 1989, a minor stem rust epidemic was reported in the USA and Canada that was caused by pathotype QCC (later designated as QCCJB) (Roelfs et al., 1991; Jin et al., 2008). By the early 1990's, QCCJB became the most common race in the Upper Midwestern rust population, but subsided rather quickly after Midwestern US QCCJB susceptible wheat varieties were replaced with resistant ones (Steffenson, 1992).

Life cycle of *Pgt*

Pgt is a heteroecious and macrocyclic fungus that required two phylogenetically separate hosts (primary host: wheat and/or barley; alternate host: *Berberis spp*. and *Mahonia spp)* and five distinct spore stages to complete its life cycle (Schumann and Leonard, 2000; Leonard and Szabo, 2005; Jin et al., 2014). However, disease epidemics on cereal crops are initiated by the landing of fresh aeciospores from the secondary host or urediniospores from other grass hosts as primary inoculum on the primary grass host. The type of spores depends upon the climate, season, and presence/absence of the alternate host. In the United States, the warmer climate region can have overwintering urediniospores on volunter wheat and also receive urediniospore inoculum from the Gulf Coast or southern Texas (Roelfs et al., 1989). While in temperate regions, aeciospores are disseminated from nearby barberries or urediniospores from regions with moderate winters (Schumann and Leonard, 2000; Sharma Poudel, 2015).

The disease cycle of *Pgt* depends upon the presence or absence of the alternate host. In the presence of the alternate host, the primary host (barley and/or wheat) is infected by a continuous source or asexual or sexual infesting spores. At the end of the growing season of the primary grass hosts, the pathogen overwinters in the grass or primary host as teliospores. The teliospores undergo meiosis to give four basidiospores that infect the neighboring alternate host if present. In barberry, the basidiospores produces haploid mycelia giving rise to a pycinia within leaves. Pycnia produce receptive hyphae and pycniospores that produce a sticky honeydew to attract insects. The movement of insect allows the cross-fertilization of receptive hyphae and pycnia that give rise to a dikaryotic mycelia. At this stage, the sexual recombination may give rise to diverse progeny with new combinations of virulence genes or avirulence genes. The dikaryotic mycelia grows to become aecia that release dikaryotic aeciospores. The aeciospores

can only infect the primary host, so it is primarily wind dispersed from barberries to neighboring susceptible grasses including wheat or barley fields. The aeciospores germinate to give rise to germ tubes that allows the fungus to penetrate the leaf and grow as dikaryotic mycelium. Each mycelium produces uredia filled with dikaryotic urediniospore after 1-2 weeks post penetration. Urediniospores erupt from the leaf and stem epidermis and can give rise to multiple cycles of infection if the environment is conducive to spore germination and stomatal invasion. Later at the end of the growing season the pathogen lifecycle shifts to form overwintering telia that if the environment is suitable will overwinter and repeat the cycle the following spring.

Due to the extensive barberry eradication program started in the US in the early 20th century, which effectively eliminated the secondary host in the Midwestern US, the *Pgt* life cycle in the Upper Midwestern US starts from urediniospores wind disseminated from wheat fields in Sothern regions. At the end of the growing season, the Upper Midwestern severe winter conditions of extended temperatures below -20°C prevents overwintering of the telia, thus, terminating the *Pgt* life cycle. In ND and the region, the only source of early season or primary inoculum is the wind disseminated urediniospores blown up through the Puccinia pathway from the south where milder winter conditions allow for the consistent growth of a winter wheat crop. This overwintering wheat provides a green island where uredinia can over winter and provide the inoculum for the next season.

Infection process of *Pgt*

Stem rust is a polycyclic disease, meaning it can have multiple disease cycles within the same growing season and these multiple cycles of infection can lead to destructive epidemics in small grain crops (Schumann and Leonard, 2000). The multiple infection in the same growing season is caused by air-borne urediniospores. Warm temperatures within the range of 18°C to

30°C with the presence of dew or rain moistened leaves is conducive for initiating *Pgt* infection (Schumann and Leonard, 2000; McMullen et al., 2008). After, the urediniospores land on moist leaf surfaces, they germinate and develop appressoria on the top of stomata which facilitates the penetration into the host by developing the infection peg (Figure 1.1).

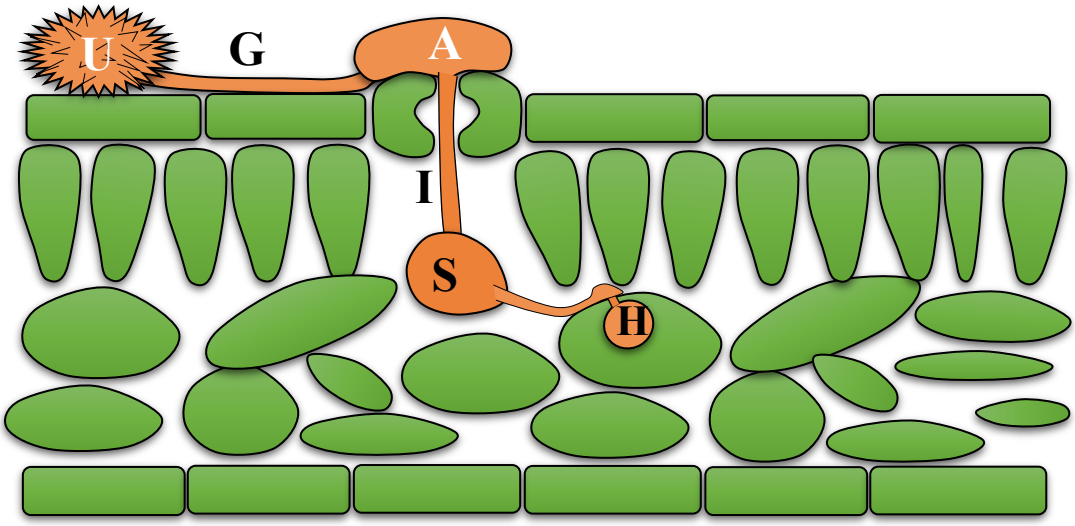

Figure 1.1. Infection process of *Puccinia graminis* f. sp. *tritici*. (Abbreviations: U- Urediniospore, G- Germ tube, A-Appressorium, I- Infection Peg, S-Sub Stomatal Vesicle, and H- Haustorium)

When the fungus reaches the substomatal cavity, it develops a substomatal vesicle which develop infection hypha that grow until they encounter mesophyll cells. The infectious hyphae develop haustorial mother cells when they encounter host mesophyll cells and invaginate the host plasma membrane to form a special enlarged feeding structure called the haustorium (Leonard and Szabo, 2005, Staples, 2001; Smith and Smith, 1990). The haustorium is encased by a thickened host derived membrane known as the extrahaustorial membrane (EHM). Haustoria play an important role in acquisition of nutrients and production of effector protein that are secreted into the EHM (Catanzariti et al., 2007). A subset of these effectors is transported from EHM into the host cytoplasm where they function to alter host metabolism and immunity

mechanisms/pathways. After successful trafficking of nutrients and proliferation, the fungal pustule breaks through the leaf epidermis of the host and erupts as reddish-brown pustules containing thousands of urediniospores (Schumann and Leonard, 2000). These spores act as the repeating secondary inoculum and get wind-dispersed and infect other healthy plants.

Nomenclature of *Pgt* race

The *Pgt* isolates were given four-letter race codes based on their disease response on a standard set of wheat differential lines containing 16 distinct *Pgt* resistance genes (Roelfs and Martens, 1998). Later, after the detection of the virulent isolate of TTKS on the resistance gene *Sr24*, a fifth differential set containing four wheat lines, each with one of the four stem rust resistance genes *Sr24*, *Sr31*, *Sr38*, or *SrMcN*, was added to provide *Pgt* races with five letter designations (Jin et al., 2008). The fifth set allowed the differentiation between race TTKSK and TTKST, that is avirulent and virulent on *Sr24*, respectively.

Genetic control of wheat stem rust

Genetic resistance is the most feasible and economic way of controlling rust diseases in wheat and barley (Roelfs, 1988; Kolmer et al., 1996). The idea of the genetic basis for wheat stem rust resistance was initiated in 1908 (Biffen, 1908; FAO, 2011). However, it gained effective momentum after the ground-breaking work by Flor in the 1940s and 1950s with the flax -flax rust pathosystem, which resulted in the gene-for-gene hypothesis (Flor 1971; McIntosh, 2009). Flor's gene-for-gene hypothesis genetically states that a dominant resistance (*R*) gene confers resistance in response to interaction with its corresponding dominant Avr gene in the pathogen. In breeding programs, the qualitative resistance genes following the gene-for-gene interaction are typically classified as 'major' resistances or 'race specific resistances' that are usually effective at the seedling stage and known as seedling resistances (Ellis et al., 2014).

In wheat stem rust resistance breeding, a considerable amount of efforts had been made to identify diverse sources of race-specific disease resistance that are introgressed into elite wheat backgrounds to develop resistant varieties (Yu et al., 2014; Singh et al., 2015). Currently, more than 70 stem rust resistance (*Sr*) genes, including the alleles of some of these genes showing race specificity have been catalogued in the International Wheat Genetics Symposium Gene Catalog; (McIntosh et al., 2011; Case et al., 2014).

In the United States wheat stem rust epidemics were common between the years 1900-1954, but were effectively controlled by the introduction and pyramiding of *Sr* gene/s in North American wheat cultivars (Vanegas et al., 2008). The first spring wheat cultivar that was primarily bred for stem rust resistance was Thatcher (Kolmer et al., 1999; Vanegas et al., 2008). Thatcher became the major source of stem rust resistance in the majority of Canadian and US breeding programs (Kolmer et al., 1991). Thatcher was the source of four race-specific *Sr* genes: *Sr5, Sr9g, Sr12* and *Sr16* that provided a moderate level of rust resistance for about 20 years, but later became susceptible to prevalent north American and Canadian *Pgt* races (Green et al., 1975; Green, 1981, Kolmer 1991). The major race specific seedling resistance genes present in Midwest and southeastern US hard red winter wheat cultivars are *Sr6, Sr24, Sr31, Sr36, SrTmp,* and the resistance gene transferred from the *1AL.1RS* translocation (Kolmer et al., 2007). S*r6, Sr9b, Sr11,* and *Sr17* are the most widely used *Sr* genes in spring wheat. However, the identification of the highly virulent race TTKSK and its derivatives makes these wheat varieties vulnerable if this race migrates to wheat growing regions of the US (Pretorius et al., 2000; Kolmer et al., 2007).

The deployment of single race-specific *R* genes exerts strong selection pressure on the pathogen population leading to the “boom-bust-cycle” where mutations in corresponding

pathogen avirulent genes allows the pathogen to overcome *R*-gene based resistances (Ellis et al., 2014; Delmotte et al., 2016). The most notable example of catastrophic losses by prioritizing a single *R* gene in wheat is the gain of virulence by *Pgt* race TTKSK over the widely deployed stem rust resistance gene *Sr31* (Pretorius et al., 2000). *Sr24* was another gene that was deployed to confer resistance to TTKSK, which was later found to be susceptible to a derivative of *Pgt* race TTKSK, designated race TTKST (Jin et al., 2008). To avoid the repeated boom-and-bust cycle, obtain durable resistance, and not waste valuable *R*-genes, the pyramiding of multiple genes with unique race specificity was institutionalized by cereal breeders.

Gene pyramiding schemes incorporating race specific resistance with adult plant resistance (APR) genes is considered ideal for effective durable resistance (Ellis et al., 2014; Singh et al., 2015). APR gene are another class of resistance genes that exhibit a partial resistance to a wider range of rust pathotypes and are generally expressed at the adult plant stage (Ellis et al., 2014). They are also referred to as 'slow rusting' genes based on their characteristic nature of suppressing or slowing pathogen growth that will eventually produce small pustules with little sporulation and the resistance mechanisms are not associated with prominent resistance responses that rely on programmed cell death or the hypersensitive response. *Sr2* is the best known and well studies APR gene in wheat (Ellis et al., 2014; Singh et al., 2015). For more than 100 years, *Sr2* has effectively provided a moderate level of resistance against the majority of the known *Pgt* races, and thus is considered an effective and durable source of stem rust resistance. The most interesting feature of *Sr2* is its ability to boost other *Sr* gene mediated resistances, apart from its ability to provide APR when used alone (Ellis et al., 2014). Because of this unique feature, *Sr2* has been utilized in all wheat stem rust resistance breeding programs.

Currently, *Sr55*, *Sr56*, *Sr57*, and *Sr58* are another four genes that have been characterized as *Sr2*-like slow rusting APR genes in wheat.

Genetic control of wheat stem rust in barley

Unlike wheat, only eight genes, designated *Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, *rpg6*, *rpgBH* and *rpgU*, have been identified in barley that confer resistance to *Pgt* or *Puccinia graminis* f. sp. *Secalis* (*Pgs*) (Sun and Steffenson, 2005; Fetch et al., 2009; Kleinhofs et al., 2009). *Rpg1* was the first stem rust resistance gene identified from a single selection of the cultivar Wisconsin 37 and two unimproved Swiss cultivars (Lejeune, 1951; Steffenson et al., 1992). *Rpg2* is an APR gene that was derived from 'Heitpas-5' (Patterson et al., 1957). *Rpg3*, was derived from PI 382313 (Jedel, 1990), *rpgBH* was derived from Black Hulless (Steffenson et al., 1984) and *rpgU was* derived from Peatland (Fox and Harder, 1995) and all provide low to moderate levels of seedling resistance in barley. The stem rust resistance gene *rpg4* and *Rpg5* were derived from the exceptional stem rust resistant line Q21861 which provides strong resistance to most of the known *Pgt* races and *Pgs* races as well (Jin et al., 1994; Brueggeman et al., 2008). High-resolution mapping confirmed *Rpg5* as an independent gene tightly linked to *rpg4* that were colocalized within a 70 Kb region on the long arm of barley chromosome 5H, thus, was given the designation as the *rpg4/Rpg5*-mediated resistance locus (RMRL). The *rpg6* gene was derived from a tertiary wild relative of barley, *Hordeum bulbosum*, and provides a recessive resistance to *Pgt* race QCCJB and MCCFC (Fetch et al., 2009). Among these genes, *Rpg1*, *rpg4*, and *Rpg5* are the only well characterized genes at the molecular level (Brueggeman et al., 2002; Nirmala et al., 2006; Brueggeman et al., 2008; Wang et al., 2013).

Rpg1 (Reaction to *Puccinia graminis*-1)

Historically, the northern Great Plains of the USA had suffered catastrophic yield losses from severe stem rust epidemics (Roelfs, 1992; Steffenson, 1992). During the 1935 severe stem rust epidemic, the North Dakota farmer Sam Lyken, identified a single rust-free barley plant standing in his field of 'Wisconsin 37' barley and increased 18 seed from that plant for six generations (Lejeune, 1951). Later in 1942, a progenitor of this particular selection was released by the North Dakota Agricultural Experiment Station as the commercial cultivar 'Kindred' and became the first stem rust resistant cultivar.

A presumably second source of *Rpg1* was derived from a Swiss landrace that was imported by the USDA from canton Lucerne, Switzerland. Two resistance sister line selections became the varieties 'Chevron' and 'Peatland'. A study by Power and Hines (1933) characterized a dominant gene *T* in 'Peatland' that was later name Reaction to *Puccinia graminis 1, Rpg1* (Søgaard and von Wettstein-Knowles, 1987). However, after the cloning of *Rpg1* and allele analysis of these three sources, it was shown that they contained identical *Rpg1* genes, and based on comparison of the *Rpg1* allele from the susceptible line Wisconsin 38 it was determined that the healthy plant identified by Sam Lyken in his field of 'Wisconsin 37' barley was not a mutation which reverted to a functional gene but most probably came from an admixture of Chevron or Peatland (Brueggeman et al., 2002).

Currently, most of the barley cultivar in the Midwestern USA and Canada contain *Rpg1* derived from either Kindred, Chevron, or Peatland (Steffenson et al., 1992). *Rpg1* has proven to be one of the most durable stem rust resistance genes providing effective resistance against the majority of the known *Pgt* races for over 80 years (Johnson, 1984; Steffenson et al., 1992). Since, *Rpg1* was the most important stem rust resistance gene in barley, significant amounts of

genetic and molecular studies were conducted to map and characterized this gene. The *Rpg1* gene was mapped to the telomeric region of the short arm of barley chromosome 7H (Jin et al., 1993; Kilian et al., 1994). A synteny based approach was used to initiate map-based cloning of *Rpg1* by identifying the syntenic region between the short arm of rice chromosome 6 and short arm of barley chromosome 1 (7H) (Kilian et al., 1995; Kilian et al., 1997). Despite the excellent synteny between rice and barley at this chromosome position there was not a rice ortholog of *Rpg1* identified in rice (Han et al., 1999). The cloning of *Rpg1* later became possible with the construction of the first bacterial artificial chromosome (BAC) library using the barley cultivar Morex, which contains the *Rpg1* gene (Yu et al., 2000; Brueggeman et al., 2002). Ayliffe et al. (1999) initially proposed pic20, a barley homologue of the maize rust-resistance Rp1-D as the barley stem rust resistance gene *Rpg1*, however, a high-resolution genetic map constructed from 8,518 gametes using the co-dominant flanking markers ABG704 and ABG077 identified seventeen critical recombinants showing that pic20 segregated away from *Rpg1*, thereby eliminating pic20 as an *Rpg1* candidate gene (Brueggeman et al., 2002). The high-resolution map delimited *Rpg1* to a 0.21 cM region spanned by a physical distance of 110 Kb (Brueggeman et al., 2002). Initially, three putative candidate genes were identified within the delimited physical region, but the susceptible Steptoe X Morex recombinant individual, ASM170, that contained a recombination within the *Rpg1* gene narrowed it to the only candidate gene in the region. Using genomic and cDNA sequences, *Rpg1* was predicted to contain 14 exons within a 4,466 bp genomic sequence and was predicted to encode an 837-aa protein (94.5kDa). *Rpg1* is a novel dual kinase protein that encodes two tandem kinase domains, a pseudokinase domain (pK1) and an active kinase domain (pK2), which are both required for *Rpg1*-mediated resistance (Brueggeman et al., 2002; Nirmala et la., 2006). A subcellular localization study of the RPG1

protein determined that it is primarily located in the cytosol but a small but significant amount appeared to be associated with the plasma and intracellular membranes (Nirmala et la., 2006). Phosphorylation and protein degradation assays showed that *Rpg1* phosphorylation occurs within 5 minutes of inoculation with avirulent isolates and the protein is degraded within 24 hours and both phosphorylation and degradation are required to confer *Rpg1*-mediated resistance (Nirmala et al., 2007; Nirmala et al., 2010)

For nearly eighty years, *Rpg1* provided durable resistance against most of the prevalent races in the US. However, the emergence of *Rpg1* virulent *Pgt* race QCCJB and TTKSK that pose a risk to the global barley production. It is now imperative for barley scientists throughout the world to identify new sources of durable resistance in barley to tackle the ever-evolving *Pgt* races.

***rpg4*-mediated resistance locus (RMRL)**

In 1989, a new race of *Pgt*, designated as QCC, later designated as QCCJB (Jin et al., 2008) was identified in North Dakota (ND) that was virulent on barley cultivar carrying *Rpg1* (Roelfs et al., 1991). The *Pgt* race QCCJB became the most prevalent race in the Upper Midwestern region of the US leaving the majority of commercial malting barley production vulnerable to potential stem rust epidemics (Roelfs et al., 1993). To address this threat, Jin et al. (1994a) screened 18,000 barley accessions from the USDA National Small Grains collection (Aberdeen, ID) and identified the unimproved barley line Q21861 as an outstanding source of resistance against QCCJB. Genetic characterization utilizing several biparental mapping populations derived from Q21861 and different susceptible barley cultivars identified a temperature sensitive and recessive gene designated *rpg4* as the gene conferring resistance to pathotype QCCJB (Jin et al., 1994b). The *rpg4* gene was mapped to the sub-telomeric region of

barley chromosome 5H (Borovkova et al., 1995). Q21861 also contains a partial dominant resistance gene that confers resistance to the rye stem rust pathogen *Puccinia graminis* f. sp. *secalis* (*Pgs*) isolate 92-MN-90 and was originally reported to co-segregate with *rpg4*, later designated as *Rpg5* (Sun et al., 1996; Brueggeman et al., 2008).

For map-based cloning of *Rpg5*, a total of 5,232 recombinant gametes derived from three crosses, Steptoe/Q21861 (SQ), Harrington/Q21861 (HQ), and MD2/Q21861 (MQ) were used to develop a high-resolution genetic map of the *Rpg5* regions. The high-resolution mapping delimited the *rpg4/Rpg5* locus between the two RFLP markers ARD5112 and ARD5016, and the same markers identified a 70-Kb region from two cv. Morex BACs (Druka et al., 2002; Brueggeman et al., 2008). The annotation of that 70-Kb region identified five candidate genes, among which, two encode NBS-LRR proteins (*HvRga1* and *HvRga2*), two actin depolymerizing-like factors (*HvAdf2* and *HvAdf3*) and a protein phosphatase 2C-like protein (*HvPP2C.1*) (Brueggeman et al., 2008). Since, Morex is a QCCJB susceptible line and *rpg4* and *Rpg5* were both identified from Q21861, a lambda phage library of Q21861 along with Morex sequence was used to generated Q21861 sequence of the delimited region. The sequence analysis revealed perfect homology between Q21861 and Morex for *HvRga1*, *HvAdf2* and *HvAdf3*, but Q21861 had an STPK coding gene in place of the Morex *HvPP2C.1* gene. The cDNA sequence of *Rpg5* sequence revealed seven exons within an 8,504bp genomic region that was predicted to encode a 1,378 aa NBS-LRR-S/TPK class of immune receptors. The post-transcriptional gene silencing of the candidate *Rpg5* gene utilizing barley stripe mosaic virus-virus induced gene silencing (BSMV-VIGS) shifted the resistance in Q21861 to susceptibility validating the NBS-LRR-S/TPK gene as *Rpg5* (Brueggeman et al., 2008).

To identify the *rpg4* gene, the 5,223 recombinant gametes developed by Brueggeman et al., (2008) to clone the *Rpg5* gene were further analyzed for SNP markers by genome sequence comparisons between the Q21861 and Morex barley lines. The SNP markers identified were used to saturate the high-resolution map of the region and were used to identify fourteen lines showing recombination between SNP marker Rsnp.4 and the RFLP marker ARD5112 (Wang et al., 2013). The selected 14 lines further delimited the *rpg4*-mediated resistance locus (RMRL) into two distinct but tightly linked loci, RMRL1 and RMRL2. Among three different recombination population; Steptoe/Q21861 (SQ), Harrington/ Q21861 (HQ), and MD2/Q21861(MD2Q) developed by Brueggeman et al. (2008), only the SQ population segregated for the RMRL2 locus, suggesting a second gene at RMRL2 is required for *rpg4*-mediated resistance. The second gene in RMRL2 was designated as *rpg4*-modifier element 1 (*Rme1*) and based on the genetic analysis it is suspected be functional in Q21861 but non-functional in Steptoe. However, our new analyses described in chapter three of this dissertation suggests that the *Rme1* gene previously described, may actually be the *Rrr1* gene characterized here. Analysis of resistant HQ and MD2Q recombinants and their susceptible parents showed that the resistant progeny lines carry a Q21861-like RMRL1 and susceptible RMRL2 like allele, suggesting that the susceptible lines Harrington and Steptoe carry functional *Rme1* but are susceptible due to absence of a functional gene at RMRL1. Post- transcriptional gene silencing utilizing BSMV-VIGS was used to silence all three genes present within the delimited *rpg4*-mediated resistance locus 1 (RMRL1), *HvRga1, HvRpg5* and *HvAdf3.* The specific silencing of any of the three genes, *HvRga1, HvRpg5* or *HvAdf3,* resulted in a shift from resistance to susceptibility showing that all three genes are required together to confer *rpg4*-mediated wheat stem rust resistance (Wang et al., 2013).

The *rpg4*-mediated resistance is recessive in nature and the genetic analysis suggested *HvPP2C.1* gene at the locus may act as dominant susceptibility factor that suppresses the *Rpg5*-STPK-mediated resistance response resulting in the recessive nature of *rpg4*-mediated resistance (Brueggeman et al., 2008; Wang et al., 2013; Arora et al., 2013). Arora et al. (2013) conducted allele analysis of three gene required for RMRL resistance; *HvRga1*, *HvAdf3* and *HvRpg5* from 14 domesticated and 8 wild barley accessions to characterize the diversity governing the resistance. The limited nucleotide sequence diversity identified did not result in any predicted amino acid substitutions in the *HvAdf3* gene. For *HvRga1,* very few amino acid substitutions were identified between *Pgt* race QCCJB resistant and susceptible lines, and these differences did not correlate with resistance or susceptibility. However, the allele analysis of *Rpg*5 correlated perfectly with resistance and susceptibility to the wheat stem rust resistance and the susceptible alleles were placed into four groups. The genotypes containing the group 2 and 3 *rpg5* susceptible alleles (Harrington, Morex, Steptoe, Hv672, WBDC019, and WBDC269) code for a predicted nonfunctional RPG5 protein that carries the PP2C1 domain in place of the RPG5-S/TPK domain as a result of a putative insertion-deletion event.

RMRL is not only effective against the domestic *Rpg1* virulent *Pgt* race QCCJB, but also provides effective resistance against the highly virulent *Pgt* race TTKSK (also known as Ug99) and its lineage (Steffenson et al., 2009). TTKSK and its derivatives poses an alarming threat to global wheat and barley production and food security (Singh et al., 2011; Steffenson et al., 2006; Steffenson et al., 2017). Race TTKSK was first reported in 1999 from wheat fields in Uganda, Africa (Pretorius et al., 2000). The extensive monitoring of this race showed a rapid spread of TTKSK and its evolving lineages throughout other wheat and barley growing countries of Africa (Singh et al., 2011). About 80 to 95% of the varieties grown on the worlds wheat acreage and

most of the breeding material being utilized across the globe were found to be moderately to highly susceptible to TTKSK and its derivatives (Singh et al., 2006; Singh et al., 2011).

Recently, Steffenson et al. (2017) reported that 96% of the 2,913 barley accessions examined to date, including those containing *Rpg1* were extremely susceptible to the evolving *Pgt* race TTKSK. The monoculture of *Rpg1* carrying barley and identification of races like QCCJB, TTKSK and their derivatives leaves barley production in the Midwestern US vulnerable to potential stem rust epidemics. In barley, RMRL is the only well characterized locus that provides resistance to QCCJB, TTKSK and its lineages (Steffenson et al., 2009). Thus, introgression of RMRL in commercially grown barley cultivars can potentially help to minimize the risk of stem rust infection in this important barley growing region.

Host parasite interaction

Plants are subjected to diverse array of invading pathogens that triggers a two-tiered immune response upon detection of the pathogen on its cell surface (Jones and Dangl, 2006). The first tier involves the recognition of pathogen conserved and exposed molecules known as pathogen-associated molecular patterns (PAMPs) or more accurately microbe-associated molecular patterns (MAMPs) by host membrane-localized pattern recognition receptors (PRRs) to trigger PAMP-triggered immunity (PTI) (Nürnberger and Brunner, 2002; Newman et al., 2013). Bacterial flagellin (Flg), elongation factor (EF-TU), fungal chitin, are some of the commonly known pathogen PAMPs or MAMPs. Likewise, extracellular plasma membrane-localized receptor-like kinase (RLKs) or receptor like proteins (RLPs) are some of the general pattern recognition receptors (PRRs) in plant that detects PAMPs/MAMPs. In *Arabidopsis*, an early PTI response is triggered by recognition of bacterial flagellin (flg22) by the flagellin receptor (FLS2), a leucine rich repeat (LRR)-RLK (Gomez-Gomez and Boller, 2002). A

specialized or adapted pathogen releases effector through their specialized structure to suppress PTI responses, manipulate the host physiology to secure nutrient to sustain pathogen growth and completion of its lifecycle (Thomma et al., 2011). However, the host counter evolves cytoplasmicly localized *R* genes that detect the presence of these effectors and trigger an effector triggered immunity (ETI) response. Pathogen effectors that are recognized by *R*-genes, triggering ETI response are known as avirulence effectors or avirulence (Avr) genes (Petre and Kamoun, 2014; Selin et al., 2016). Typically, ETI responses are stronger than PTI and activate disease response that leads to localized and pronounced programmed cell death known as the hypersensitive response (HR).

The major class of *R* genes encodes an intracellular receptor that belongs to nucleotide-binding leucine-rich-repeat (NB-LRR, also known as NLR) class of genes (Martin et al., 2003; Dangl and Jones, 2006; Dodds and Rathjen, 2010). Plant NLRs can be subdivided into two classes based their N-terminal motifs: Toll/interleukin-1 receptor (TIR) or coiled-coil (CC) (Dangl and Jones, 2006; McHale et al., 2006). The second class of *R* gene are the one that contain at least one serine/threonine protein kinase (STPK) domain (Martin et al., 2003, Brueggeman et al., 2009). The presence of STPK domain in *R*-genes have been reported in diverse plant species and are suggested to be involved in phosphorylation activation during an immune response (Hanks et al., 1988; Brueggeman et al., 2008; Brueggeman et al., 2009; Wang et al., 2013).

R genes can interact with Avr effectors either directly or indirectly through "guard" or "decoy" proteins that are targeted by pathogen virulence effectors to facilitate colonization and proliferation on the host (Van der Biezen and Jones, 1995; Dangl and Jones, 2006; van der Hoorn and Kamoun, 2008). The *L5, L6* or *L7* flax rust resistance gene are known to directly

interact with the *Melampsora lini AvrL567* avirulence protein to confer resistance (Dodds et al., 2004). Indirect interaction is explained by the guard or decoy models that proposed *R* proteins act to monitor effector targets (guardee or decoy) and an ETI response in triggered by the R proteins upon detection of the effector protein's action on the guardee or decoy to facilitate virulence on the host (Van der Biezen and Jones, 1995; van der Hoorn and Kamoun, 2008). One of the classical examples of a presumed guardee is *avrPphB* susceptible 1 (PBS1) kinase that is guarded by a member of the NLR immune receptors, RPS5 (Resistance to Pseudomonas syringae 5) (Swiderski et al., 2001; van der Hoorn and Kamoun, 2009; Block and Alfano, 2011). The cysteine protease AvrPphB of *Pseudomonas syringae* cleaves its target PBS1 to inhibit PTI responses but upon cleavage the NLR RPS5 detects this perturbation and triggers ETI. However, later it was understood that PBS1 is in fact a mimic/decoy of the original effector target/guardee BIK1 (*Botrytis*-induced kinase 1) that interacts with the PAMP-recognition receptor FLS2.

An emerging model of the co-evolution of the pathogen virulence effectors and the plant immune system is the "integrated decoy model" or "integrated sensory domain model' that propose a role of complementary NLR genes to trigger immune response in a concerted fashion (Cesari et al., 2014; Wu et al., 2015). Recent genome analyses determined that these dual NLR genes are present in a head-to-head gene orientation and that this genome architecture somehow facilitates the evolution of effector targets as bait or integrated domains onto one of the corresponding NLRs. This model suggests that a possibly directed evolutionary process (Bailey et al., 2018) leads to the integration of the host virulence effector targets onto NLR immunity receptors, which, essentially act as "effector baits" to trap the pathogen in the act of utilizing its virulence effector repertoire. Interestingly, it has been shown that the NLR with the integrated sensory domain acts as the suppressor and upon manipulation of its integrated domain a

conformational change occurs which releases it from a heteroduplex with its cognate partner, the activator NLR which is then free to signal and elicits the ETI responses (Cesari et al., 2014). In *Arabidopsis*, the TIR-NLR pair RRS1 (Resistance to *Ralstonia solanacearum* 1) and RPS4 (Resistant to *Pseudomonas syringae 4*) were reported to be involved in ETI upon recognition of the avirulence protein/effector AvrRps4 from the bacterial pathogen *P. syringae*, PopP2 from *Ralstonia solanacearum* and an unknown effector from the fungal pathogen *Colletotrichum higginsianum*. RRS1 is an AVR-receptor NLR that represses its signaling NLR partner RPS4, thus, in the non-elicited state they are in an inactive heteroduplex state, representing a molecular switch or pathogen trap. Upon virulence effector secretion into the host cytoplasm, the WRKY domain in RRS1 recognizes AvrRps4 or PopP2 leading to the disruption of the hetero-dimerization between the NLR pair followed by a homo-dimerization of the signaling NLR RPS4 which activates signaling resulting in a strong ETI response.

Fungal effectors

Effectors are microbial molecules that manipulate host cellular structure and function, to facilitate host colonization (virulence factor or toxins) and if or when the host evolves to recognize their function or manipulation of the host, trigger immune responses and effectively become avirulence genes (Petre and Kamoun, 2014; Selin et al., 2016). The effectors are secreted either into the plant apoplast or cytoplasm using pathogen effector delivery systems. The delivery system of a fungal effector is dependent on whether a fungus is a biotroph (feeds on live tissue), necrotroph (feeds on dead tissue) or hemibiotrophic (have both biotrophic and necrotrophic phases). Biotrophic and hemibiotrophic fungi presumably secrete the majority of their effector into the apoplast or cytoplasm through specialized infection structures like appresoria or haustoria (Dodds et al., 2004; Catanzariti et al., 2006; Kleemann et al., 2012).

Biotrophic fungi, including the flax rust pathogen *Melampsora lini* form haustoria, a balloon-shaped feeding structure that facilitates nutrient uptake and effector delivery into the host cytoplasm (Dodds et al., 2004; Lo Presti et al., 2015). Unlike rust fungi, the biotrophic fungal pathogen *Ustilago maydis* does not form a bulbous structure, it rather penetrates the host plasma membrane and the host plasma membrane wraps the intracellular hyphae that acts as an extended area to mediate molecule exchange between host and the pathogen (Djamei and Kahmann, 2012; Selin et al., 2016). The hemibiotrophic fungus *Colletotrichum higginsianum* can release effectors immediately after appressoria formation through appressorium penetration pores to suppress early plant immune response (Kleemann et al., 2012). While, necrotrophic fungi like *Botrytis cinerea* grows subcuticularly and release toxins and effector proteins that kill epidermal cells and gradually colonizes the plant epidermis with their hyphae (Lo Presti et al., 2015).

The secretion of effectors from these delivery systems to interact with the host are presumably guided by the molecular structure of the effector proteins. Studies done in oomycetes host targeting (Selin et al., 2016). Different species of oomycetes share a host targeting N-terminal domain like the RxLR (Arg-x-Leu-Arg), LxLFLAK or CHxC and CRN (Crinkler motif) aa sequences that helps to define effector families (Jiang et al., 2008; Petre and Kamoun, 2014). Compared to bacteria and oomycetes, translocation of fungal effector is poorly understood and has only been partially characterized in a few pathosystem and still much controversy surrounds many of the findings reported (Sperschneider et al., 2016). Fungal effector appears to diversify rapidly, thus, lack conserved motifs impeding efficient effector prediction (Sperschneider et al., 2016; Selin et al., 2016). However, some exceptions were observed for a limited set of fungal effector families that share conserved motifs. The effector of *Blumeria graminis* f. sp. *hordei* was found to share an N-terminal [YFW]xC motif downstream of the signal peptides (Godfrey et al.,

2010). Two flax rust fungi effectors, AvrL567 and AvrM contains unique N-terminal signal peptide whose primary sequence are not conserved between these effectors but play a role in secretion into the host cytoplasm (Rafiqi et al., 2010). These N-terminal signal peptides were shown to facilitate the internalization of these effector proteins autonomously, even in the absence of the pathogen (Rafiqi et al., 2010) but, subsequent studies performed by another group questioned the validity of these findings (Ve et al., 2013).

Avirulence proteins in rust fungi

Effector proteins that are recognized by host R-proteins and betray the pathogen by triggering host defense responses which impedes pathogen growth are known as avirulence (Avr) proteins (Catanzariti et al., 2010). So far only six avirulence gene have been identified and cloned in the rust pathogens. Out of six Avr genes, four avirulence protein were identified in *M. lini*, *AvrL567* (Dodds et al., 2004), *AvrP123* (Catanzariti et al., 2006), *AvrP4*, and *AvrM* and two were identified in *Pgt*, *AvrSr35* (Salcedo et al., 2017) and *AvrSr50* (Chen et al., 2017).

Avr protein in *Melampsora lini*

Linum usitatissimum (flax) contains a minimum of 30 flax rust resistance specificities distributed among polymorphic loci of *K*, *L*, *M*, *N*, and *P* (Dodds et al., 2004). The *R*-genes at the L, *M*, *N*, and *P* loci encode Toll Interleukin-1 Receptor class (the TIR-NB-LRR class) *R*-genes (Dodds et al., 2004; Dodds et al., 2006; Catanzariti et al., 2010). The first identified *M. lini* avirulence gene *AvrL*567 was detected using a subtractive hybridization screen of rust genes from rust infected flax. The subsequent mapping of the genes identified in the subtractive hybridization screen, as restriction fragment length polymorphism markers on 74 F_2 individuals derived from crossing of C and H strains of *M. lini*, showed cosegregation of the genes with *Avr* specificities (Dodds et al., 2004; Dodds and Thrall, 2009). The cDNA probe identified as co-

segregating with the *AvrL5, AvrL6,* and *AvrL7* gene cluster was shown to cause a *R*-gene specific cell death by coexpression with the corresponding *L5, L6* or *L7* R-proteins, respectively. The *Avr* L567 gene encodes a 150 aa small secreted protein containing a 23aa N-terminal signal peptide that guides the translocation of the effector into the host cytoplasm after its cleavage (Dodds et al., 2006). The other three *Avr* genes, *AvrM, AvrP123,* and *AvrP4* were identified by thoroughly inspecting sequences of 822 haustoria enriched cDNA clones to identify candidate secreted proteins (Catanzariti et al., 2006; Dodds and Thrall, 2009). Out of 20 identified candidate proteins, one of them co segregated with *AvrM,* a second one with *AvrP4* and third one with the *AvrP, AvrP1, AvrP2,* and *AvrP3* (*AvrP123)* cluster of avirulence genes. *Agrobacterium* mediated transient expression showed a *R*-gene dependent HR in response to their corresponding flax *Avr* genes.

Avr protein in *Puccinia graminis* f. sp. *tritici*

Two *Pgt* Avr genes, *AvrSr35* and *AvrSr50* were recently identified using ethyl methane sulfonate (EMS) based chemical mutagenesis and naturally available spontaneous mutations, respectively (Salcedo et al., 2017; Chen et al., 2017; Moscou and van Esse, 2017). *AvrSr35* encodes a 578-aa protein that contains a N-terminal secretion signal to guide the translocation of this effector into host cytoplasm which presumably directly interacts with the *Pgt R* gene *Sr35* (Salcedo et al., 2017). *Sr35* is a CC-NLR gene that confers resistance to TTKSK and its lineage (Saintenac et al., 2013). *Sr35* detects the fungal pathogens growth at a very early stage and confers an early immune response exhibiting a very pinpoint, single cell, strong HR response (Saintenac et al., 2013; Salcedo et al., 2017). The identification of *AvrSr35* was initiated by identifying 15 mutants of *Pgt* race RKQQC (an *Sr35* avirulent race) that were virulent on *Sr35*. Time course based transcriptomic data coupled with microscopy showed no apparent difference

in pathogenicity of the wild-type (RKQQC) and mutant *Pgt* isolates. Genomic sequencing of these mutant revealed a single gene in each mutant with either a non-synonymous mutation (in 12 mutants), splice site disruption (in 1 mutant), and a non-synonymous mutation with a valine to isoleucine (V128I) substitution. This single gene was identified as *AvrSr35* and allele analysis was conducted on natural variants of *Pgt* isolates that were virulent and avirulent on *Sr35*. Allele analysis of the natural variants confirmed that the evasion of *Sr35* detection in virulent isolates were gained by the insertion of a miniature inverted transposable element (MITE) in *AvrSr35*.

Another independent study identified the avirulence gene *AvrSr50* by investigating a spontaneous mutant (*Pgt* isolate *Pgt632*) derived from the *Pgt* isolate *Pgt279* (*Sr50* avirulent isolate) that is virulent on *Sr50* (Chen et al., 2017). Comparison of genomic sequence of wild type *Pgt* isolate *Pgt279* and *Pgt* mutant isolate *Pgt632* revealed a loss of heterozygosity in a 2.5 MB region of *Pgt632* caused by somatic exchange between two haplotypes. A total of 24 annotated haustorial secreted proteins (HSPs) were identified in the region that had a single allele in *Pgt632* but were heterozygous in *Pgt279*. Out of the 24 HSPs, 21 had two allelic types in *Pgt632* and the remaining 3 belonged to multigene families, so a total of 20 variants representing these 3 genes were obtained after DNA amplification. A total of 41 different HSP, 21 representing *Pgt279* type allele and 20 variants obtained from DNA amplification of HSP belonging to multicluster families were co-expressed in *Nicotiana benthamiana* by cleaving their signal peptides. Only one HSP showed a HR symptom that was further validated using yeast two hybrid using *Sr50* and *Sr33* as bait, which gave a *Sr50* specific interaction, thus confirming it as *AvrSr50*. Allele analysis of diverse *Sr50* specific virulent and avirulent isolates showed that the virulent isolates evade *Sr50* detection by sequence divergence and DNA insertion in *AvrSr50*.

Suppressor of avirulence (*Svr)* gene

Typically, the recognition of *Avr* genes by their cognate *R*-genes, either directly or indirectly triggers an ETI mediated HR type resistance. However, some pathogens have evolved a suppressor/ inhibitor of avirulence (*Svr* or *I*) gene than can inhibit/surpass a R-Avr interaction mediated resistance response (Ellis et al., 2007; Bourras et la., 2015; Bourras et al., 2016). Several genetic studies in flax rust show evidence of the presence of a single or tightly linked cluster of dominant inhibitor/s (*I*) genes that inhibit the recognition of several *Avr* genes, (*AvrL1*, *AvrL567*, *AvrL8*, *AvrL10*, and *AvrM*) by their cognate *R* genes (Jones, 1998; Ellis et al., 2007). These *I* genes has been categorized into three forms; a) the first form inhibits resistance conferred by *L1*, *L7*, *L8*, *L10*, and *M1* resistance alleles, b) the second form only inhibits action on *AvrM1*, and c) the "null" form cannot inhibit any of these genes (Ellis et al., 2007). So far, molecular mechanism of how these inhibitors, *I* genes, inhibit the resistance responses is poorly understood. The expression studies on *M. lini* strains that are avirulent on *L6* but virulent on *L7* due to the presence of the *I* gene and *AvrL567* showed that the *I* gene does not suppress the level of transcription of Avr effectors. Based on this observation, it was speculated that the *I* gene might modify *AvrL567* before its secretion into the host cytoplasm, thus, altering the recognition by *L7*. Alternatively, it was speculated that the *I* gene might be secret along with *Avr L567* into host cytoplasm and inhibit the AvrL567-L7 interaction. Since, *L6* and *L7* differ only in their TIR domain and the *I* gene specifically inhibits *L7* containing flax, the *I* gene was hypothesized to be involved in altering the downstream signals of the TIR domain rather than interfering with the AvrL567-L7 interaction. However, some studies using a chimeric protein in the TIR region showed that the *I* gene based susceptibility may not be entirely governed by the TIR sequences, but may also involve other region in the *R*-gene that interacts with the TIR. Thus, sequence

variation in both region alter the *I* gene recognition. Despite all these studies, the exact nature of *I* gene based inhibition of resistance in flax is unknown, thus, making *I* genes a major target in future map based cloning efforts (Ellis et al., 2007)

A much-detailed study of a suppressor of avirulence (*Svr*) was done in the powdery mildew-wheat pathosystem where the causal agent of disease is the biotrophic fungal pathogen *Blumeria graminis* (Bourras et al., 2015). Bourras et al. (2015) genetically dissected avirulence on six allelic series of the wheat powdery mildew resistance gene *Pm3* (*Pm3a-f*) that confers different resistance specificity to the powdery mildew causing pathogen *Blumeria graminis.* A F_1 haploid population consisting of 167 individuals was derived by crossing *B. graminis* isolate 96624 that is avirulent on six alleles of the *Pm3* resistance gene (*Pm3a-f*) and the isolate 94202 that is virulent on all six alleles. This study lead to the positional cloning of the *Pm3a/*f specific effector gene $AvrPm3^{a2/f2}$ from the avirulent isolate; and identification of the candidate suppressor of the Pm3a/f-mediated resistance ($SvrPm3^{a1/f1}$) from the virulent isolates. The gene expression study of the parental isolates and segregating F_1 confirmed that *Pm3f* specific resistance was observed only in the isolates carrying $AvrPm3^{a2/f2}$ but lacking $SvrPm3^{a1/f1}$. Based on these observation, a *Avr-R-Svr* model for race-specific resistance was proposed that suggests that in a host-pathogen interaction involving; *R*-gene, *Avr* gene and suppressor of R-Avr interaction (*Svr*), resistance is observed only when an *Avr* gene is recognized by its cognate *R* gene in the absence of *Svr*.

Conclusion

A complete understanding of a host pathogen interaction requires information from both host and pathogen perspective. In the barley stem rust resistance system, two sources of resistance, *Rpg1* and RMRL have been cloned and functionally validated but no studies have

ever been conducted to identify *Pgt* effectors that elicit or suppress barley immune response. To gather a complete picture of this pathosystem, this doctoral dissertation focused on identifying putative effector/elicitors that target these barley stem rust resistance genes and utilized host genetics to identify novel components required for the function of these resistance mechanisms.

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CHAPTER 2. COMPARATIVE TRANSCRIPTOMICS TO IDENTIFY PUTATIVE ELICITORS/SUPPRESSORS OF BARLEY *RPG4*-MEDIATED STEM RUST RESISTANCE

Abstract

The barley *rpg4*-mediated resistance locus (RMRL) confers resistance to many *Puccinia graminis* f. sp. *tritici* (*Pgt*) races, including QCCJB and TTKSK. *Rpg5* is a resistance gene at this locus encoding a typical NLR with a C-terminal serine threonine protein kinase (STPK) integrated sensory domain (ISD). To identify *Pgt* effectors/suppressors that elicit or suppress RMRL, 24 *Pgt* isolates with differential reactions were genotyped utilizing *in-planta* RNAseq to identify 114K SNPs that result in predicted nonsynonymous amino acid changes. Comparative analysis identified 22 genes associated with dominant RMRL virulence representing candidate suppressors of resistance. Host transcript analysis of a single barley line inoculated with the 24-virulent –vs- avirulent isolates showed virulent isolate specific down regulation of biotic stress responses suggesting all *Pgt* isolates may contain a conserved effector that elicits RMRL responses by interacting with the RPG5 ISD, yet virulent isolates harbor suppressors of resistance rather than the lack of a functional Avr protein.

Introduction

Plants are subjected to a diverse array of microbes that trigger a two-tiered immune response with the first level activated upon detection of potential pathogens at the cell surface (Jones and Dangl, 2006). The first tier involves the recognition of conserved and exposed microbe derived molecules known as pathogen-associated molecular patterns (PAMPs) or more appropriately as microbe-associated molecular patterns (MAMPs). These conserved molecules across diverse microbe genera are essential for their survival, thus, cannot be shed and are under purifying selection. Well characterized examples of MAMPs are the bacterial flagellin which is

required for motility and the structural molecule chitin found in fungal cell walls. The conserved bacterial flagellin subunits, flg22, is recognized by the host membrane-localized pattern recognition receptor (PRR) FLS2, which contains a leucine rich repeat (LRR) extracellular receptor domain, a transmembrane domain and an intracellular serine threonine protein kinase (STPK) cytosol signaling domain. This broad class of cell surface receptors are known as receptor like kinases (RLKs) and are known to trigger PAMP-triggered immunity (PTI) responses (Nürnberger and Brunner, 2002; Gomez-Gomez and Boller, 2002; Newman et al., 2013). The conserved fungal cell wall component chitin is recognized by another host PRR receptor, CERK1, that encodes a LysM extracellular receptor domain, transmembrane domain and the intracellular STPK domain, which triggers PTI responses upon chitin subunit detection by the LysM extracellular receptor domain (Petutschnig et al., 2010). For a microbe to become specialized on a host it must overcome these PTI or non-host resistance responses, which requires the evolution of effectors that can suppress PTI responses and manipulate the host for nutrient acquisition, pathogen proliferation and ultimately reproduction. However, plants coevolved a second tier of defense responses that rely on race specific resistance (R)-genes that recognize the action of these virulence effectors and elicit a higher amplitude of defense responses known as effector triggered immunity (ETI). Pathogen effector or their action on the host are recognized by *R*-genes, triggering ETI response effectively transforming them into avirulence genes (Avr) (Petre and Kamoun, 2014; Selin et al., 2016). Typically, ETI response are stronger than PTI and the defense responses activated result in localized and pronounced programmed cell death known as the hypersensitive response (HR).

Puccinia graminis f. sp. *tritici* (*Pgt*) is an obligate biotrophic fungal pathogen that causes the economically important disease stem rust in wheat (*Triticum aestivum*) and barley (*Hordeum*

vulgare) (Roelfs, 1982). Like any obligate biotroph, *Pgt* proliferates on living host tissue by hijacking normal cellular physiological processes in the cells to facilitate the extraction of nutrients to power their own growth and eventual sporulation (Glazebrook, 2005). During the infection process, *Pgt* develops an appressoria over the top of the stomata and penetrates the host by developing infection peg that breaches the guard cell barrier and allows for substomatal intercellular growth. Once the intracellular infection hyphae encounter mesophyll cells they breach the cell wall, invaginate the host plasma membrane and form a specialized feeding structure called the haustoria (Schumann and Leonard, 2000; Dodds et al., 2009). The haustorium remain separated from the host cell cytoplasm by the extrahaustorial membrane and extrahaustorial matrix (EHMs) that is derived from the invaginated host plasma membrane. The EHMs acts as the focal point of host-pathogen interaction through which haustorially expressed fungal effector are delivered into the host cytoplasm that function to manipulate the host machinery to acquire nutrient to sustain pathogen growth and eventual proliferation (Dodds et al., 2009; Eckardt, 2009). During this process of effector delivery and function within the host cytoplasm, the cytoplasmicaly localized R-proteins in host cell detects the action of these secreted effectors to trigger the resistance response. The understanding of these haustorial secreted effectors and their role in differential regulation of host gene expression is imperative in devising a durable resistance mechanism in the cereal-rust pathosystems.

Transcriptomics/RNAseq has proven to be an instrumental molecular tool to fill knowledge gaps in the understanding of the underlying molecular mechanisms dictating the outcome of virulence effector manipulation, *R*-gene detection and the resulting compatible and incompatible interactions. As both the host and pathogen interact in this closely orchestrated battle for supremacy, the underlying transcriptional regulation of gene expression in the plant

and pathogen provide clues to their reactions and counter reactions (Dobon et al., 2016; Rutter et al., 2017, Xia et al., 2017). In past research focused on characterizing cereal-rust pathosystems, RNAseq has been extensively used to characterize the transcriptional changes in both host and pathogen at different stages of infection (Fofana et al., 2007; Duplessis et al., 2011; Chen and Cao et al., 2015; Dobon et al., 2016; Rutter et al., 2017; Xia et al., 2017). In addition, to utilizing the transcriptomics data to identify specific genes that are differentially regulated during compatible and incompatible interactions, the data can also be mined for variation including single nucleotide polymorphism (SNPs) and/or insertion/deletions INDELs. These polymorphic markers can be used to perform transcriptome-wide association analyses to identify variants that are associated with virulence/avirulence in the pathogen (Lu et al., 2016). Lu et al. (2016) conducted a transcriptome-wide association mapping study using data from 17 *Blumeria graminis* f. sp. *hordei* (*Bgh*) isolates and identified AVR_{a1} and AVR_{a13} as an avirulence effector recognized by the *Mla1* and *Mla13* alleles, respectively, based on non-synonymous SNPs in these effector genes.

The previous gene expression studies performed during the cereal-rust interactions were focused on hexaploid wheat as the host (Fofana et al., 2007; Manickavelu et al., 2010; Bruce et al., 2014; Dobon et al., 2016; Rutter et al., 2017; Xia et al., 2017; Salcedo et al., 2017; Chen et al., 2017) and no *in-planta* transcriptomic studies have been conducted during barley-rust interactions. Despite, barley being an economically important cereal crop worldwide and equally vulnerable to rust in the absence of effective stem rust resistance genes, the majority of research has focused on wheat due to its importance concerning world food security. The wheat stem rust resistance gene *Rpg1* is the only source of stem rust resistance deployed in Midwestern US barley varieties (Steffenson et al., 1992). Thus, barley production is vulnerable to future stem

rust epidemics because of the emergence of the domestic *Pgt* race QCCJB (Roelfs et al., 1989; Jin et al., 2008) and race TTKSK (aka Ug99) and its lineage in Africa (Pretorius et al., 2000), that are virulent on barley containing *Rpg1*. The only well characterized resistance to *Pgt* races QCCJB and TTKSK in barley is the *rpg4*-mediated resistance locus (RMRL) that required the concerted action of three tightly linked genes: two NBS-LRR (NLR) resistance-like genes, *Rpg5* and *HvRga1*, and the actin depolymerization factor *HvAdf3* that are all required together for resistance (Jin et al., 1994; Brueggeman et al., 2008; Steffenson et al., 2009; Wang et al., 2013).

Allele analysis from a diverse set of *Pgt* race QCCJB resistant and susceptible barley lines determined that *HvRga1* and *HvAdf3,* although required for resistance, are conserved genes with no functionally relevant primary sequence polymorphism that explain RMRL function in resistance or susceptibility (Wang et al., 2013). Despite the different recessive -vs- dominant nature of resistance between the wheat stem rust *R*-gene *rpg4* and the rye stem rust *R*-gene *Rpg5* it appeared that the functional polymorphism in *Rpg5,* primarily the STPK to protein phosphatase 2C domain insertion/deletion, showed that it is the polymorphic gene that explains *rpg4*-mediated stem rust resistance in barley (Arora et al., 2013).

Although a combination of the *Rpg1* and RMRL would confer resistance to all currently characterized rust races the presence of races that are virulent on *Rpg1* or RMRL just in the ND *Pgt* population suggests that isolates with both virulences may exist or could possibly emerge from other regions of the globe where the sexual stage of the pathogen still occurs. The possibility of this combination of genes occurring in North America has been greatly diminished by stabilizing the *Pgt* population by the effective removal of the secondary host barberry through the barberry irradiation program (Roelfs, 1982). However, continued surveillance of diverse *Pgt* races to detect virulence pattern on both barley stem rust resistance gene is important as few *Pgt*

resistances have been identified to date. It is also important to focus basic research effort to understand the molecular mechanism underlying the broad *Rpg1* and *rpg4*-mediated resistance mechanisms to get a better evolutionary understanding of the barley-*Pgt* pathosystem as it appears that barley is a near non-host or recent host of *Pgt* as little co-evolution of race specific resistances have evolved.

In the present study, a total of 37 *Pgt* isolates were initially utilized to assay their virulence patterns on *Rpg1* and RMRL and to assess their diversity. Twenty-four diverse isolates were then selected to conduct an *in planta* transcriptomic analysis during the infection cycle on the susceptible barley variety Harrington. Since, previous studies reported a direct interaction between the avirulence effector of *M. lini* (Dodds et al., 2004), and *Pgt* (Salcedo et al., 2017; Chen et al., 2017) with their cognate *R*-genes, this study also aimed to identify avirulence effectors in *Pgt* isolates that are specifically recognized by *Rpg1* and *Rpg5*-STPK. The overall objectives of this study were to: a) identify *Pgt* isolates that are virulent/avirulent on *Rpg1* and RMRL, b) utilize *in planta* transcriptomics data to identify differentially expressed host and pathogen genes, and c) utilize *Pgt* gene expression data to conduct a transcriptome wide association mapping to identify variants associated with virulence/avirulence specific to *Rpg1* and RMRL

Materials and methods

Plants, pathogens and inoculation

Five different barley varieties, accessions or recombinant lines, Q21861, HQ1, Harrington, Morex and Steptoe, were used in this study. Q21861 is an unimproved barley line that contains two wheat stem rust resistance genes, *Rpg1* and *rpg4* (Jin et al., 1994). After genetic characterization of the genes required for *rpg4*-mediated resistance the genes or more

appropriately the locus containing the three genes required for wheat stem rust resistance is now referred to as the *rpg4*-mediated resistance locus, *RMRL* (Wang et al., 2013). HQ1 is a near isogenic line with the Q21861 *RMRL* introgressed into the susceptible cultivar Harrington background (Brueggeman et al., 2008) that was developed through backcrossing and marker assisted selection. Morex is the source of *Rpg1* from which the gene was identified via a positional cloning approach (Brueggeman et al., 2002). Both Harrington and Steptoe are wheat stem rust susceptible lines which do not harbor any known stem rust resistance genes (Brueggeman et al., 2008).

Thirty-Seven *Pgt* isolates collected in North Dakota from 1977 to 1999 were randomly selected from a large collection housed at the Red River Agriculture Research Center USDA-ARS, kindly provided by Dr. Timothy Friesen. The virulence of most of these isolates on barley lines containing the wheat stem rust resistance genes *Rpg1* and RMRL were not known and were characterized in this study using the aforementioned barley accessions.

Seven individual barley seedlings of each line (Q21861, HQ1, Harrington, Morex and Steptoe) grown in separate cone containers were assayed for each replication. The seedlings were grown in a growth chamber (Model 7301-75-2; Caron, Marietta, OH, USA) set at a 16/8-hour light/dark cycle and day/night temperatures of 21/18°C. Seven days after planting, the seedlings were inoculated using the previously established protocol described by Steffenson et al., 2009. Immediately following inoculation, the seedlings were placed in the humidity chambers with intense light provided by a high-pressure sodium lamp for 18 hrs. Then the seedlings were returned to the growth chamber set at the previously described condition for 12-14 days. The infection types (ITs) were assessed 12-14 days post inoculation (DPI) using a modified 0-4 scale as previously described (Stakman et al., 1962; Steffenson et al., 2009). The scale was slightly

modified from that developed for (Stakman et al., 1962) as barley exhibits slightly deferent responses including more mesothetic reaction types (Miller and Lambert, 1955).

DNA extraction and RAD-GBS

Fungal genomic DNA was extracted directly from rust spores using the PowerPlant® Pro DNA (MO BIO Laboratories, Inc.) extraction kit with slight modification to the manufacturer's protocol to meet the fungal genomic DNA extraction needs. Approximately 30mg of fungal spores were transferred to 2ml PowerPlant® Bead Tubes provided with the kit and pre-crushed in a Mixer Miller Type 301 tissue grinder (Retsch Gmbh & Co. KG, Germany) set at a frequency of 30/sec for 2 min. The samples were mixed with 410 µl of solution PD1 and 60 µl of Phenolic Separation Solution to disassociated phenolics from the DNA that was later removed by the Inhibitor Removal Technology® step. About 3 µl of RNase was added to the mix and heated for 15 min at 65°C. The heated samples were again crushed in Mixer Miller Type 301 tissue grinder set at a frequency of 30/sec for 4 min and centrifuged at 13,000 x g for 2 minutes. The supernatant was transferred to a clean 2 ml collection tube provided in the kit and 210 µl of PD3 solution was added and vortexed. The mix was incubated at 4°C for 5 minutes and the remaining steps were followed as recommended in the kit's manual. DNA concentration were measured using the Qubit® High Sensitivity DNA kit with the Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). A total of 200 ng of DNA from each isolate was used to construct a Restriction Site Associated DNA-Genotyping by Sequencing (RAD-GBS) library as described by Leboldus et al. (2015). The 37-isolate specific barcoded libraries were size selected for 200 bp, 240 bp, 275 bp and 300 bp by loading 30 µl; per lane from the 120 µl total library using separate 2% agarose cassettes with the Pippin Prep (Sage Science, Beverly, MA, USA) size selection system set with the narrow selection option for each fraction collected. Four different

sized libraries were loaded separately on Ion Torrent 318™ Chips v2 and sequenced using the Ion Torrent Personal Genome Machine® (PGM™) System.

RAD-GBS data analysis

For each *Pgt* isolate, four different FASTQ files sequestered by barcodes representing each different size fraction library were combined for analysis. The sequence reads were trimmed for quality using default settings in CLC Genomics Workbench 8 (QIAGEN). The quality reads from each isolate were aligned to the *Pgt* race SCCL reference genome sequence v2.0 (www.broadinstitute.com; Duplessis et al., 2011) using the Burrows–Wheeler Aligner maximal exact match (BWA-MEM) algorithm (Li, 2013). The variant calling was done using GATK UnifiedGenotyper with default setting for multi-sample Single Nucleotide Polymorphism (SNP) calling (Van der Auwera et al., 2013). VCFtools was used to remove individual calls with a read depth less than six and genotype quality less than ten (Danecek et al., 2011). Variants, SNPs and Insertions/Deletions (INDELs) with LowQual FLAG were removed from the dataset. A minor allele frequency cutoff of > 1% and missing data cutoff of < 50% was used to select variants for association mapping (AM).

RNAseq library preparation and Sequencing

Twenty-four comparatively diverse *Pgt* isolates were utilized to inoculate the susceptible barley cv. Harrington for the *in-planta* RNAseq analysis. Nine seven-day old cv Harrington seedlings growing in separate plastic containers were swab inoculated using a cotton bud soaked with soltrol containing 20 mg of freshly collected urediniospore/ml of each isolate. The inoculated seedlings were incubated in humidity chambers at 100% relative humidity for 18 hrs following the previously established protocol described in Steffenson et al. (2009). The inoculated seedlings were moved to isolation chamber in the greenhouse to allow the

colonization process to proceed. At five days post inoculation (DPI) six primary leaves were collected per isolate and immediately frozen in liquid nitrogen and placed in a -80°C freezer until RNA isolation was conducted for *in-planta* RNAseq. The remaining seedlings were allowed to grow in the isolation chambers until fourteen DPI to evaluate infection and efficiency of inoculations. Three replications of uninoculated cv Harrington were collected to obtain uninoculated control RNAseq data.

Total RNA was extracted from the inoculated leaves using the RNeasy mini kit (Qiagen, Chatsworth, CA). RNA concentrations were measure using the Qubit® Broad Range RNA kit on a Qubit® 2.0 fluorometer. The quality of the RNA was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Three inoculated leaves of equal size (~2 cm long) per isolate were combined in a single tube and used for total RNA extraction. About 1µg of total RNA was used for RNAseq library construction using the TruSeq RNA Library Prep Kit v2 (Illumina, San Diego, CA) following the manufactures standard protocol. The final library was validated and quantified on the Agilent 2100 Bioanalyzer. Two library pools, each representing 12 different cDNA libraries were prepared and normalized according to the manufacturer's protocol. Each of the library pools were diluted to a concentration of 1.8pm and sequenced on the Illumina NextSeq 500 sequencer on a single flow cell at the USDA Cereal Genotyping Centre, Fargo, ND, USA. To generate 150 bp single end sequencing reads, the NextSeq® 500/550 High Output Kit v2 (150 cycles) was used. The raw sequencing reads were demultiplexed and converted into individual fastq files using bcl2fastq software v2.17.1.14 (Illumina, San Diego, CA). The fastq reads were quality trimmed in CLC Genomics Workbench v8.0.3 (CLC bio, Aarhus, Denmark) using default settings.

Host and pathogen specific expression analysis

The high quality trimmed sequencing reads were mapped to the *Pgt* race SCCL reference genome sequence (www.broadinstitute.com; Duplessis et al., 2011) and barley RefSeq v1.0 (http://webblast.ipk-gatersleben.de/barley_ibsc/) in CLC Genomics Workbench v8.0.3 to obtain *Pgt* specific and barley specific genes for expression analyses, respectively. In both cases, the reference gene along with both gene track and mRNA tract information was provided to obtain both gene specific and transcript specific reads. Also, this analysis pipeline allows reads to align to both intronic and intergenic regions, which enabled us to assess the quality of the publicly available gene models of barley and *Pgt*. The reads that were less than 90% identical for 90% of the read length and mapped to more than 10 positions were discarded. The total reads mapped for each gene model were normalized to obtain reads per kilobase of exon model per million mapped reads (RPKM) expression values for each sample (Mortazavi et al., 2008). The expression values were used to make comparisons between virulent and avirulent *rpg4/5* isolates as well as virulent and avirulent *Rpg1* isolates. The Exact Test in the EdgeR bioconductor package (Robinson et al., 2010) embedded in CLC genomics was used to calculate the fold change and False Discovery Rate (FDR) corrected p-value in all the comparisons. Genes with fold change > 3 and FDR corrected p-value < 0.05 were considered as differentially expressed genes (DEGs).

De novo assembly of unmapped reads and expression analysis

RNA sequencing reads from *Pgt* inoculated samples and controls that did not map to either the barley or *Pgt* reference genome were extracted for *de novo* transcript assembly using Trinity v2.4.0 (Grabherr et al., 2013). The unmapped reads from all 24 samples were concatenated to generate a single input fastq file. Since the input files contained >300M reads,

trinity's *in silico* normalization was done to reduce the amount of reads for final assembly. The normalized reads were used as the input to generate a *de novo* transcript assembly. Trinity was run with the parameter '--min_kmer_cov 2' to reduce the total RAM requirement. The *de novo* transcripts obtained from Trinity were used as a query to detect sequence similarity within the 2017 NCBI nr database (Benson et al., 2005), using a BLASTX functionality in fast and sensitive open source program DIAMOND (double index alignment of next-generation sequencing data) (Buchfink et al., 2015). A minimum e-value of 1e-10 was set as a threshold for reporting a sequence similarity.

These *de novo* assembled transcripts were also used as reference sequences to realign the unmapped reads from each of the 24 samples. The RPKM value of total reads mapped to each *de novo* assembled transcripts was used to make a comparison between virulent and avirulent *rpg4/5* isolate groups in order to identify differentially expressed transcripts (DETs) that were not present in the avirulent isolate SCCL genome assembly. The filtering parameters applied to obtain DETs were similar as explained above in the host and pathogen specific expression analysis section. The top blast hit of each DET was extracted from the DIAMOND BLASTX search to retrieve a subject id for species assignment to the *de novo* assembled transcripts. Since genome sequences of the isolates used in this study are not available, the *de novo* transcripts without any hits against Nr-database could not be utilized to identify if they result due to the isolate specific sequences or due to miss-alignment in *de novo* assembly. So, only the DETs belonging to fungi and plantae kingdom were filtered for further analysis. DETs were then aligned against publicly available barley RefSeq v1.0 predicted protein sequence and DEGs belonging to fungi were against predicted protein sequences of *Pgt* race SCCL.

Expression profiling of *Pgt* genes

Apart from the differential gene expression, average RPKM expression values were calculated over the 24 samples using the gene models for both *Pgt* and barley. The average expression levels of each gene were categorized by RPKM values into 5 groups: Extremely low (<0-10 RPKM), Low (11-50 RPKM), Moderate (51-100 RPKM); High (100-500 RPKM) and Extremely high (>500 RPKM).

Expression analysis was also performed on a subset of *Pgt* genes that were predicted to encode candidate secreted effector proteins (CSEPs). Genes were considered a CSEP based on the presence or absence of three predicted protein domain features: i) the presence of an N-terminal signal peptide; ii) the absence of a mitochondrial signal peptide; and iii) the absence of a transmembrane domain after 60 amino acids (aa) (Saunders et al., 2012; Petre et al., 2014; Sperschneider et al., 2016). SignalP 4.1 was used to predict the presence of putative N-terminal secretion signals (Petersen et al., 2011). Putative transmembrane helices were predicted using TMHMM v. 2.0 (Krogh et al., 2001). The subcellular localization of the proteins was predicted using TargetP v.1.1 (Emanuelsson et al., 2000) and LOCALIZER (Sperschneider et al., 2017). The average RPKM expression values of the proteins fulfilling the above criteria were calculated to determine the levels of CSEP expressed at the 5 DPI time point used for tissue collection. These expression analyses data were also used to determine if differential expression of the CSEPs or variants in the CSEPs were associated with virulence or avirulence specificity on either the *rpg4/5* or *Rpg1* wheat stem rust resistance genes in barley.

Functional annotation and gene enrichment analysis

An automated annotation of the predicted proteins expressed from the *Pgt* genome (CRL 75-36-700-3) was performed with BlastP search in Blast2Go using the default parameters.

(Conesa et al., 2008). The annotations from Blast2Go were supplemented with publicly available annotations for the *Pgt* genome (https://genome.jgi.doe.gov). For the gene enrichment analysis of *Pgt* DEGs, the publicly available gene ontology (GO) term mapping was used for *Pgt* race SCCL genes. For barley, the majority of the high confidence genes are annotated in the publicly available IBSC RefSeq v1.0 (http://webblast.ipk-gatersleben.de/barley_ibsc/downloads/). We supplemented the annotations by performing a local BlastX of the whole set of predicted barley proteins to the reannotated *Arabidopsis* Col-o genome (Araport11) (https://www.araport.org/data/araport11) (Madden et al., 2013). The top hits of high confidence barley genes with predicted amino acid homologies greater than 30% and alignment lengths greater than 50% with *Arabidopsis* annotated genes were used to assign *Arabidopsis* gene IDs to the barley genes. The GO term mapping for the best *Arabidopsis* gene hits for the barley DEGs were used for gene enrichment analysis.

In both cases, the GO term enrichment analysis was done in the bioconductor R package TopGO version 2.28.0 (Alexa et al., 2006; Alexa and Rahnenfuhrer, 2010). A GO term was considered significantly enriched if more than 5 genes were annotated for that term with classic Fisher p-value less than 0.001 using the Fisher's exact test performed in the TopGO package. Significantly enriched GO terms were observed only for the differentially expressed barley gene in a comparison between RNAseq libraries from samples inoculated with virulent *rpg4/5* versus avirulent *rpg4/5* isolates. The enrichment analysis was done to identify significantly enriched GO terms specific to subontology molecular function (MF), biological processes (BP) and cellular component (CC) to provide a better understanding of the molecular activity, biological role and cellular location of the DEGs.

Variant calling from RNAseq data

The quality RNAseq reads were mapped to the *Pgt* race SCCL reference genome sequence in Spliced Transcripts Alignment to a Reference (STAR) software using a two-pass alignment step (Dobin et al., 2013, Van der Auwera et al., 2013). The two-pass step utilized the splice junction loci identified in the first mapping to guide the second mapping. The mapped data were sorted and the PCR duplicate reads were tagged using Picard Mark Duplicates (http://broadinstitute. github.io/picard). The SplitNCigarReads tools in GATK was used to split reads into exons and hard-clip overhanging intronic sequence. This command was supplemented with ReassignOneMappingQuality read filter to convert the alignment quality assigned by STAR to a GATK compatible quality score. Base recalibration was done using the already known variant sites in *Pgt* (CRL 75-36-700-3) genome (http://fungi.ensembl.org/Puccinia_graminis). The variants were called individually for each sample using GATK HaplotypeCaller tools in ERC GVCF mode with the parameters suggested for RNAseq data (Van der Auwera et al., 2013). The individual variants were combined using GATK GentoypeGVCFs tool to obtain VCF files containing variant calls for all the samples. Variants with genotype quality greater than 10 and read depth greater than 6 were selected using Vcftools for association mapping (AM) (Danecek et al., 2011). All the variants, including multiallelic sites present in filtered VCF files were used as input in the Ensembl Variant Effect Predictor (VEP) program to identify non-synonymous variants (Aken et al., 2016). The multiallelic sites and variants with synonymous mutation were removed from the analysis. Only, the biallelic sites that have predicted non-synonymous mutation and contains genotypic data for more than 50% of the isolates with a minor allele frequency (maf) > 0.01 were selected for association mapping.

Association mapping

Association mapping (AM) was done separately using the high quality polymorphic variants identified from the RAD-GBS and RNAseq genotyping to identify variants that were significantly associated with virulence on barley containing the RMRL or *Rpg1* stem rust resistance genes. The phenotypic data were generated by transforming the stem rust infection types to quantitative data using the conversion formula provided by Zhou et al., 2014. The infection type of HQ1 and Morex were used to identify variants associated with virulence on RMRL and *Rpg1,* respectively. The phenotyping data was combined with the genotypic data containing filtered variants. To correct for the population structure in the isolates, principal component analysis (PCA) was performed in JMP® Genomics v8.0 using the default setting. Three PCA explained more than 25% of the variation in both the RAD-GBS and RNAseq data, thus, three principal components were used in the AM to correct for population structure in the Q and QK models. The familial relatedness (kinship matrix) between the isolates was assessed by computing identity by state (IBS) and hierarchical clustering of the isolates with the fast ward method JMP® Genomics v8.0. The output from the kinship matrix analysis was used to correct for familial relatedness in the QK model for AM analysis. Along with the Q and QK models, AM was also performed with the naive model (no correction for population structure and familial relatedness) to identify effectors/suppressors associated with phenotypes on RMRL and *Rpg1* containing barley lines. All the significant variants were manually inspected to eliminate false positives. Variants potentially associated with virulence/avirulence for *Rpg1* and RMRL, but not detected in AM analyses using the Q and/or QK models were also manually inspected to avoid false negative calls for each variant. If a variant had an alternate call, either heterozygous or homozygous for the alternate allele, for more than 75 % of virulent isolates (maximum of 2

outlier calls) and homozygous reference call for more than 80% of the avirulent isolates (maximum of 2 outlier calls), then the variant was selected as significantly associated with virulence/avirulence for the specific stem rust resistance, RMRL or *Rpg1*.

Results

The *Pgt* isolates used in this study were placed into four groups based on their virulence pattern on the only effective wheat stem rust resistance genes in barley, *Rpg1* and RMRL. Based on these limited differential genes, the group 1 isolates were virulent on barley lines containing *Rpg1* and RMRL*;* group 2 isolates were virulent on barley lines with *RMRL* only; group 3 isolates were virulent on barley lines with *Rpg1* only; and group 4 isolates were not virulent on barley carrying either *Rpg1* or RMRL. Only four of the 37 isolates selected, R29JA and R29JB (group 2), QCC-2 (group 3) and A-5 (group 4) had been previously assayed for seedling resistance on barley lines containing these two differential resistance genes. Though, R29JA and R29JB were both race typed as HKHJ, they were obtained from different sources and both isolates were included in this study. The phenotypic assays identified 9 group 2, 8 group 3 and 20 group 4 *Pgt* isolates (Appendix Table A1). No isolates belonging to group 1 were identified in this study. The infection types of the *Pgt* isolates belonging to the three groups are shown in supplementary Appendix Table A2-A4. For comparison in RNAseq and AM, isolates belonging to group 2 and group 4 are designated as isolates avirulent on *Rpg1* (*AvrRpg1*) and group 3 and group 4 are designated isolates avirulent on RMRL or *rpg4* (*Avrrpg4*) (Appendix Table A1.).

Diversity assay using RAD-GBS to select isolates for RNAseq

Sequencing data from 4 different size selected (200 bp, 240 bp, 275 bp and 300 bp) RAD-GBS libraries were combined to obtain a single FASTQ file of each *Pgt* isolate for alignment with the *Pgt* SCCL reference genome sequence. Five samples were removed from the

analysis due to poor quality as a result of bad sequencing data and poor alignment. On average 545.9K (S.D. ±290.5K) reads were obtained from the remaining 32 sample (Appendix Table A5). The percentage of reads aligned to the *Pgt* reference genome ranged from 55.48% to 87.28% with average alignment of 78.70% (S.D. ±9.05%). Variant calls followed by several filtering parameters (M&M) resulted in 11,423 markers for AM analysis. The AM did not result in significant association between the different groups of *Pgt* and specific virulence on barley lines containing the resistance genes *rpg4* or *Rpg1*. However, a relation matrix constructed to obtain identity by state for running the Q-K model for AM was used to assess the diversity in group 4 *Pgt* isolates (Figure 2.1).

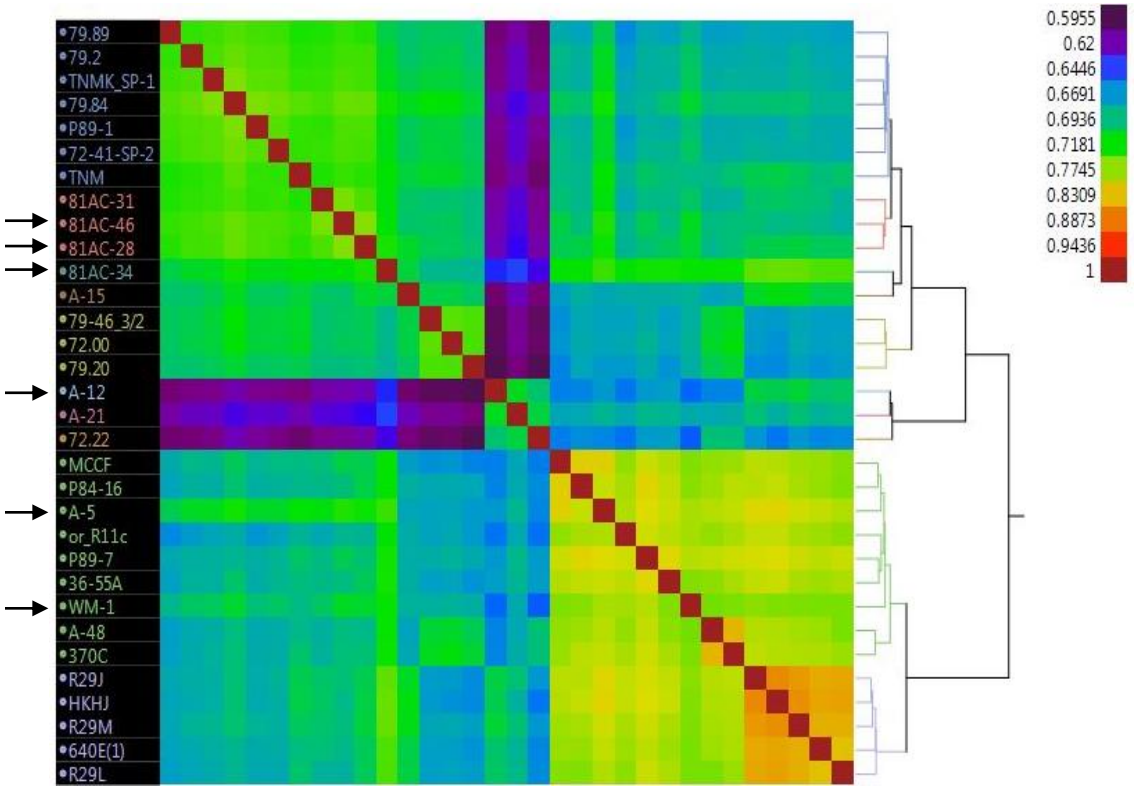

Figure 2.1. Heatmap of 32 *Pgt* isolates with differential virulence on barley lines with and without the stem rust resistance genes *rpg4* and/or *Rpg1*. The colors represent different identity by state value (shown on top right corner) that explain the degree of relatedness between the isolates. IBS with a value of 1 represent a perfect relationship and are dark red. The isolates with an arrow on the left are the *Rpg1* and *rpg4* avirulent *Pgt* isolates selected for RNAseq analysis based on this diversity assay, along with 9 *rpg4* virulent *and* 8 *Rpg1* virulent isolates.

Seven group 4 *Pgt* isolates, that were comparatively diverse compared to each other and other isolates in group 2 and group 3, were selected for the *in planta* RNAseq experiment.

RNAseq reads aligned satisfactorily with barley and *Pgt* reference genome

A total of 1.2 billion single end reads ranging from 34 million (M) to 82M per samples were generated from two different runs on an Illumina NextSeq 500. After demultiplexing and quality trimming, a total of 1.12 billion reads yielding an average of 46.7M (S.D.±14.4M) reads per sample. The average percentage of reads that mapped to the *Pgt* SCCL gene models were 34.62% (S.D.±12%), among which 33.71% (S.D.±11.69%) uniquely mapped and 0.91% (S.D.±0.32%) mapped to multiple locations in the genome (Appendix Table A6; Figure 2.2).

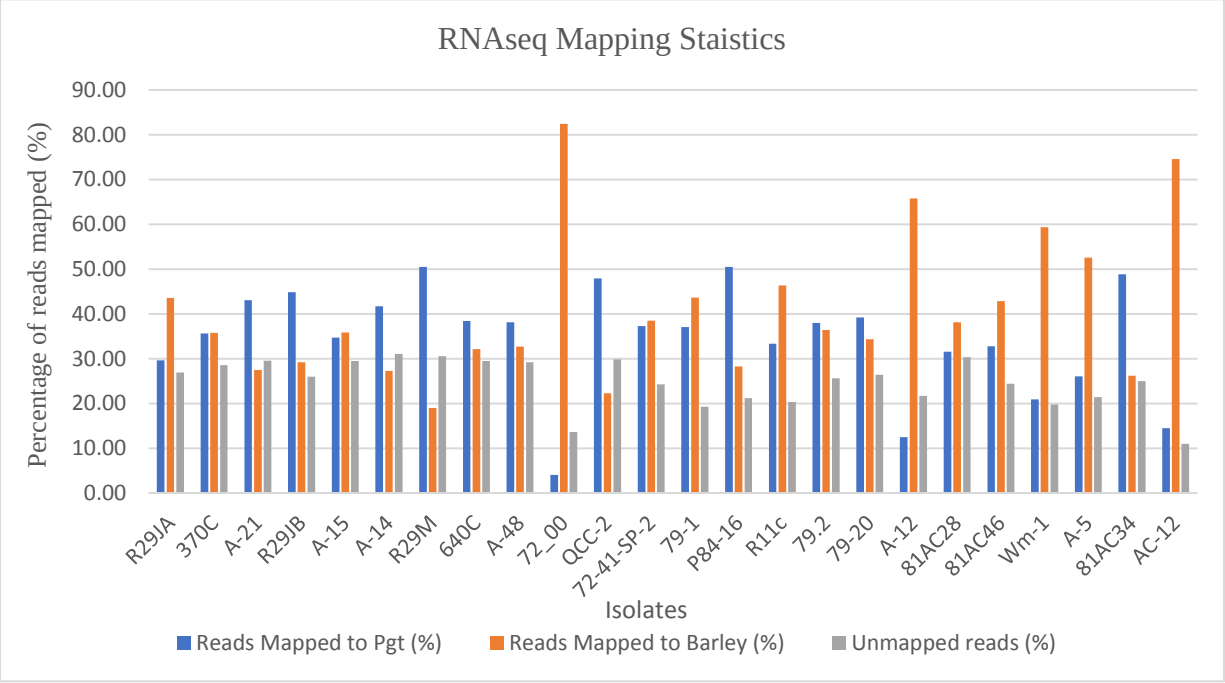

Figure 2.2. Bar graph showing the mapping statistics for the RNAseq reads on the *P. graminis* f. sp. *tritici* and barley reference gene models. The data represented in this bar graph is provided in Appendix Table A6

Likewise, 40% (S.D.±16.12%) of the reads mapped to the barley RefSeq v1.0 gene models (Appendix Table A6; Figure 2.2). On average, 34.33% (S.D.±13.73%) of the total

mapped reads aligned to a single unique locus in the barley genome and 6.27% (S.D.± 2.43%) aligned to multiple positions (Appendix Table A6). After alignment to the *Pgt* and barley reference sequences, 31.96% (S.D.±3.86%) of the sequencing reads were extracted as unmapped. In the non-inoculated control samples, 46.4M (S.D.±2.9M) reads were obtained from three replicates. On average, 81.94% (S.D.±1.24%) of the reads mapped to the barley genome, of which 64.71% (S.D.±0.65%) mapped to unique positions in the genome. Approximately 35.29% (S.D.±0.65%) did not map to the barley reference genome sequence (Appendix Table A7).

Differentially expressed genes (DEGs) and gene enrichment analysis of DEGs

The comparative analysis utilizing RPKM expression values from reads mapped to the *Pgt* and barley reference genome sequences yielded several DEGs in both comparisons representing virulent -vs- avirulent *rpg4* isolates and virulent -vs- avirulent *Rpg1* isolates. In the comparisons using reads that mapped to *Pgt*, we found 87 DEGs (69 upregulated and 18 downregulated) between virulent -vs- avirulent *Rpg1* isolates and 114 DEGs (44 upregulated and 70 downregulated) between virulent -vs- avirulent *rpg4* isolates. Despite showing nearly 200 fungal DEGs, no significantly enriched GO terms were found from the gene enrichment analysis. This problem generally arises when dealing with biotrophic fungal pathogens that are known to have many effector genes that are unique to their own species with very few showing homology to previously characterized genes (Saunders et al., 2013; Lorrain et al., 2015; Sonah et al., 2016). Out of all the annotated gene models in *Pgt* race SCCL genome sequence, only 37% of the genes were assigned with a GO term. These 37% of the *Pgt* genes with known GO terms only represented 24% and 15% of the DEG observed in a comparison between *Rpg1* virulent and avirulent isolates and *rpg4* virulent and avirulent isolates, respectively. Due to this small number

of gene model with GO terms in the DEG sets, gene enrichment analysis for *Pgt* DEGs was not successful.

The analysis using host specific expression data compared between samples inoculated with *Rpg1* virulent vs avirulent isolates resulted in five upregulated DEGs. Because of this small number of DEGs, we could not perform a gene enrichment analysis for this comparison. However, very interestingly 115 high confidence DEGs (58 upregulated and 57 downregulated) were identified when using the host expression data set from comparison between samples inoculated with virulent *rpg4* vs avirulent *rpg4 Pgt* isolates (Appendix Table A8).

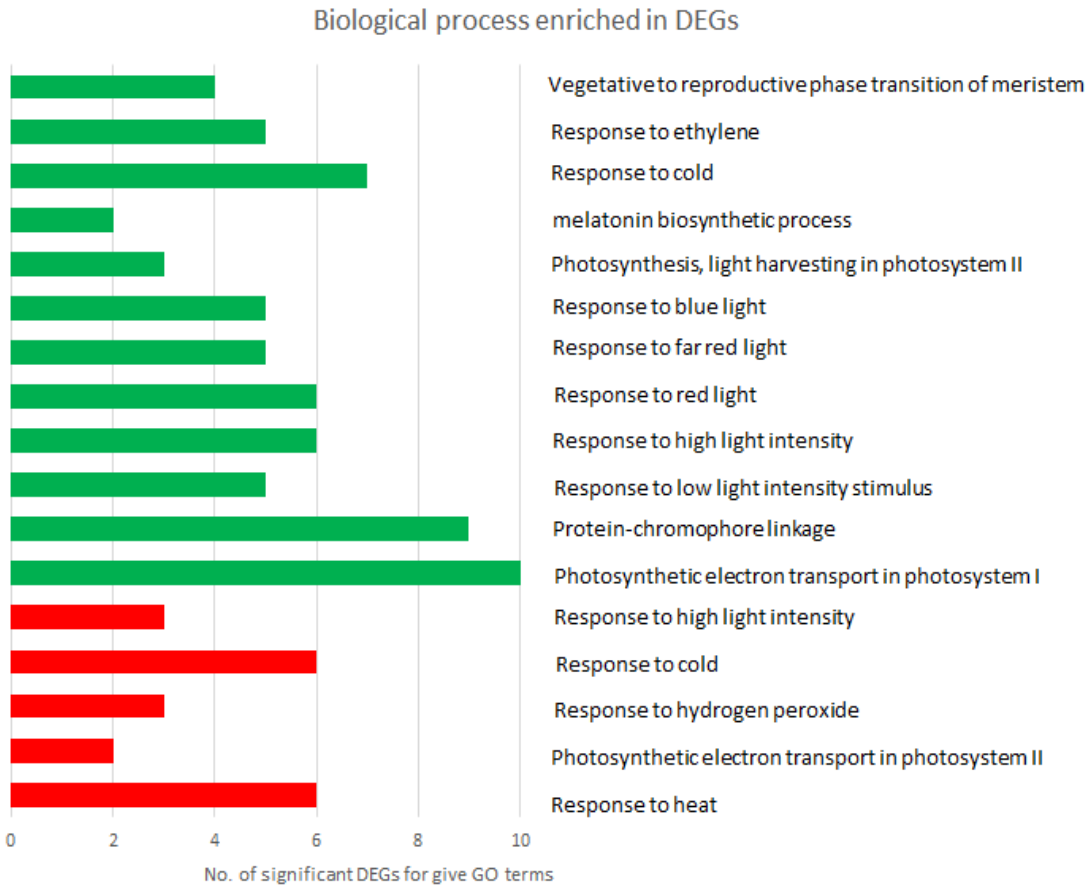

Figure 2.3. Bar graph showing the number of differentially expressed barley genes identified utilizing gene ontology (GO) enrichment analysis for involvement in specific biological processes. Red color represents downregulated and green color represents upregulated sets of genes between comparisons of samples inoculated with virulent *rpg4* vs avirulent *rpg4 Pgt* isolates.

All 115 DEGs were subdivided into two groups, upregulated and downregulated genes, to conduct enrichment analysis on each set separately. For each set of up and downregulated genes we identified GO terms that were significantly enriched for subontology BP, MF and CC. The set of downregulated genes were involved in response to heat, electron transport of photosystem II, cold, and high light intensity (Appendix Table A9; Figure 2.3). The upregulated genes contain the genes enriched for reaction to different light spectrum and light intensity, components of the photosystems I and II, and responses to ethylene and cold stress. Enrichment for cellular components suggests the majority of the DEGs acts or are involved in the activity in chloroplasts (Appendix Table A9). Only the upregulated set of genes were enriched for GO term belonging to subontology MF (Molecular Function). Chlorophyll binding genes were highly enriched followed by pigment binding, protein binding and metal ion binding.

De novo assembled transcripts did not add information to the analysis

A total of approximately 333M reads from all 24 samples and three replicates of control samples did not map to either the *Pgt* or *H. vulgare* genomes. In silico normalization of the unmapped reads resulted in 70.4M reads to process for *de novo* assembly. The *de novo* Trinity assembly process resulted in a total of 192,130 contigs of size >300bp. Mapping of the unmapped reads to the *de novo* transcripts gave an average total mapping of 44.13% (S.D.±5.64%), among which 28.73% (S.D.±5.01%) were uniquely assembled to transcripts (Appendix Table A6). The assembly still resulted in an average of 55.86% (S.D.±5.64%) unmapped read. Since, the low-quality reads were trimmed in early fastq processing steps, the quality parameters of the reads may not affect the mapping of the reads back to *de novo* assemble transcripts. This would suggest that those mRNA reads might be originated from repeat rich

regions, genes with multiple copies and/or from domains shared across multiple genes thus generating highly similar reads that map to multiple location in a genome (Dozmorov et al., 2015). In the cases where sequencing reads can map to more than a maximum number of allowable multi-mapping sites, reads were allowed to map to only 10 different regions in this analysis, the reads were discarded from the mapping steps and considered as unmapped reads. To assess if increasing the number multi-mapping sites will affect the total % of mapped reads, few samples were mapped to the *de novo* assembled transcripts by allowing the reads to map up to 30 different location in the genome, as 30 is the maximum allowable multiple mapping in CLC. This resulted in a reduction of total unmapped reads from ~55% to ~50% (results not shown). Though the percentage of unmapped reads were reduced only by 5%, this analysis partially confirms that the majority of unmapped reads might represent highly repetitive regions. Such reads that can map to numerous sites in a genome are generally recommended to be ignored (Dozmorov et al., 2015) and thus were not pursued further in the present study.

The RPKM value obtained from reads mapped to *de novo* transcripts were utilized to identify 879 significant differentially expressed transcripts (DETs) between the group of *rpg4* virulent-vs-avirulent *Pgt* inoculated samples. The top blast hit of DETs were extracted from DIAMOND BLASTX search done against NCBI-nr. Out of 879 DETs, only 457 (80 belonging to plantae and 377 belonging to fungal kingdom) had hits to either plantae or fungal kingdom, and rest had no significant hits in any sequence in NCBI-nr database (Appendix Table A10-A11). The transcripts belonging to the 80 DETs identified from the *de novo* assembly of unmapped transcripts hit the barley RefSeq v1.0 sequence and remaining 377 DETs had hits on *Pgt* race SCCL reference genome. The differential gene expressions from the unmapped reads in the original analyses compared to the original differential gene expression obtained from the

original read mapping showed similar expression differences or no significant difference between the analyses. It is not completely understood why the algorithms did not map these reads in the original analysis but these additional analyses of the unmapped reads revealed that the majority of the transcripts followed similar pattern of differential suggesting that no additional or novel output were obtained utilizing the *de novo* assembled transcript (Appendix Table A11-A12).

Association mapping using RNAseq reads

A total of 600K variants (biallelic and multiallelic) in the *Pgt* gene models were obtained after combining all the observed variants from the 24 RNAseq samples. VEP predicted about 215K variants as non-synonymous mutations. All the multiallelic variants were removed and the remaining biallelic variants missing more than 50% of their calls and having a minor allele frequency of less than 1% were filtered out leaving a select 104K biallelic variants for association mapping. These 104K variants were distributed across 9122 *Pgt* gene models, with an average of about 11 (S.D.±11) variants per gene. The AM analyses were performed using the naive, Q model, and QK model, along with Q model using 3 PCA that explained about 25% of the variation gave a comparatively better association. Upon manually inspecting the significant variants, the majority of the significant association using these models turned out to be a false positive. However, the manual screening allowed for the positive identification of 22 variants distributed across 20 gene models that were significantly associated with *rpg4* specific virulence (Appendix Table A12).

Expression profiling

Out of the 15,979 genes 1858 gene had no expression at 5DPI and were removed from the analysis. Among the remaining 14,121 genes, 51.9% were expressed at extremely low levels,

29.9% were expressed at low levels, 9.3% were expressed at moderate levels, 7.29% were expressed at high levels and 1.6% were expressed at extremely high levels. Among the 1,672 genes categorized as CSEPs, only 22 had no expression at the given point of time analysis. For the remaining CSEPS, 61.4%, 18.8%, 5.9%, 3.1% and 2.8% were expressed at low, moderate, high and extremely high level.

Discussion

In this study, we originally set out to identify genes hypothesized to represent dominant avirulence factors, as this is the central dogma hypothesized for gene-for-gene interactions. However, our results suggest that virulence on the RMRL may actually be conferred by dominant suppressors of resistance rather than the lack of a functional avirulence protein. The data generated in the present study resulted in the new hypothesis that our search might need to shift towards identifying effectors (elicitors/suppressors of resistance) that function as dominant virulence genes that suppress defense mechanisms in barley that are elicited by the barley stem rust resistance gene *Rpg5* in response to different isolates of *Pgt*.

For the analysis utilized in this study, we identified a group of isolates collected in the Upper Midwestern United States that exhibited differential virulence on a barley line containing the effective and broad-spectrum resistance conferred by RMRL and *Rpg1*. This was achieved by screening differential barley lines with thirty-seven *Pgt* isolates that were collected from various fields in North Dakota since 1970. This was the second study conducted to systematically evaluate seedling reaction of barley line with different stem rust resistance gene, yet this study looked into unknown rust isolates and surprisingly determined that 22% and 25% of the isolates tested were virulent on *Rpg1* and *rpg4*/RMRL, respectively. This proportion of isolates containing virulence on these two major resistances was surprising as only a single race had

previously been identified in the US virulent on *Rpg1* (Roelfs et al., 1991; Steffenson et al., 1992) and a single isolate reported that was virulent on *rpg4* or RMRL. Since barberry eradication effectively removed the sexual cycle from the US, it has been postulated that the population has been stabilized in the upper Midwestern US (Roelfs, 1982). Thus, it was previously posited that only a few isolates would contain virulence on these two major genes in barley, yet we identified several isolates with virulence to both *Rpg1* and RMRL that contained a high level of genome diversity as determined by SNP analysis.

The RAD-GBS and *in planta* RNAseq analyses allowed us to identify several avirulent and virulent isolates for both *Rpg1* and RMRL with a high level of polymorphism/genetic diversity that could be utilized for significant comparison and association analyses. The analyses also required knowledge of a single time point and compatible host variety that can provide RNAseq libraries with a balance of both host and fungal transcripts. To obtain samples enriched for fungal transcripts from all the *Pgt* isolates utilized, a susceptible barley variety Harrington and a time point of 5DPI was selected for this study. Based on extensive phenotypic observations across the stem rust pathogen's colonization process in barley, the first macroscopic sign of successful infection by *Pgt* are seen at 5DPI which allowed for the collection of leaf samples displaying multiple infection sites. Likewise, a time-course transcriptomics study done on stripe rust inoculated wheat showed an increasing trend of fungal reads starting at 5DPI, suggesting 5DPI as a suitable time point for this study (Dobon et al., 2014). As expected, we saw a nice balanced number of reads representing both the fungal pathogen as well as the host, facilitating transcript analyses of both at this time points. The expression profiling of CSEPs showed that about 99% of the predicted CSEPs were expressed at some level at this time point as well, thus, supporting the use of this single time point in this study to achieve our objective of

characterizing the genetic diversity in the pathogen as well as studying differential gene expression in *Rpg1* and RMRLspecific virulent and avirulent interactions from both the pathogen and host perspectives.

Despite having a group of isolates with balanced avirulence-virulence profile for both *rpg4* (9 out of 24 isolates) and *Rpg1* (8 out of 24 isolates) and samples collected at a time point that contained a high proportion of fungal transcripts, the AM analyses utilizing the expression data did not produce informative marker trait associations between the phenotypic and genotypic variation. The AM using the disease reaction of the 24 *Pgt* isolates on HQ1 (*rpg4/5* +) virulent phenotype produced MTA with low significance spread throughout the *Pgt* genome representative of background noise with no highly significant marker trait associations for RMRL virulence/avirulence. Similarly, the AM analysis using infection types on cv Morex (*Rpg1*+; *rpg4/5*-), showed no MTA with *Rpg1* virulence/avirulence. A study similar to the present research was conducted to identify avirulence effectors of *P. striiformis* f. sp. *tritici* (*Pst*) using 14 diverse *Pst* isolates with different virulence/avirulence profiles for 18 yellow rust resistance (*Yr*) genes (Xia et la., 2017). That study identified candidate *Avr* effector specific to six *Yr* genes, but failed to obtain significant effector associated with five other *Yr* genes (*Yr7*, *Yr27*, *Yr43*, *Yr44*, and *YrExp2*), despite having a balanced virulence-avirulence profiles similar to that observed in this study. In the present study, the absence of MTA can probably be attributed to the small population size as well as the lack of diversity in the isolates since they were collected within a small geographical region from a presumably asexual population. *Pgt* populations in the Great Plains of United States are mostly clonal asexual populations that resulted due to the eradication of the alternate host barberry (Roelfs, 1982; Roelfs et al., 1997; Peterson et al., 2001). The clonality in the population can have a big effect on the accuracy of

determining linkage disequilibrium as the individuals within the clonal population retain a high level of heterozygosity that can reduce the power of AM, leading to the detection of no significant MTA or conversely multiple false positives (de Meeûs and Ballous, 2004; Xia et la., 2017). A transcriptome wide association study utilizing 17 geographically diverse powdery mildew fungal isolates identified two candidate effectors, Avr_{a1} and Avr_{a13} that are recognized by the cognate powdery mildew resistance gene alleles *Mla1* and *Mla13*, respectively (Lu et al., 2016). The noisy association observed in the AM in this study was also attributed to the presence of multiple effectors that have specificity for a given *R*-gene (Xia et al., 2017). The interaction between different isolates adds complexity to the association leading to several false positives as well as false negative results. Also, if diverse isolates contain effectors that are recognized by a single *R*-gene, then a MTA for a single effector may be insignificant as the power of the single gene interaction would be lost due to the variant allele and associated MTA not being shared or supported by other avirulent isolates especially when a small population was utilized for the analyses.

Due to the lack of apparent association between virulence and genotype, the genotypic data were manually inspected to find some false negative variants associated with virulence/ avirulence for *rpg4*. The reference genome used in this study was generated from *Pgt* race SCCL (Duplessis et al., 2011) which is avirulent to *Rpg1* (Nirmala et al., 2007) and to *rpg4* (Pers. Comm. Brian Steffenson, University of Minnesota, MN). This would suggest that the reference genome contains avirulent alleles specific to *Rpg1* and *rpg4*. So, a virulent isolate should carry alternate allele (allele different from SCCL) for genes encoding *Avr* effectors and/or suppressor of resistance. If the virulence is governed by dominant suppressor of resistance then the virulent isolate can have the suppressor allele in either a homozygous or heterozygous state. Thus, a

manual screening of each variants was done to identify variant/s carrying an alternate allele, either in the heterozygous or homozygous state for more than 75 % of virulent isolates (maximum of 2 outlier calls) and homozygous reference allele for more than 80% of the avirulent isolates (maximum of 2 outlier calls). No significant variants associated with *Rpg1* specific virulence were found even after manual screening of variants. However, twenty-two variants were identified that were associated, 78% to 100% with virulence on RMRL. These 22 variants were within 20 different gene models, among which three fulfilled the criteria to be considered a CSEP. Interestingly, most of the variants had a heterozygous genotype for the *rpg4* virulent isolates (Appendix Table A12). The heterozygosity in a pathogen, especially at avirulence loci is suggested to result from a positive selection that favors adaptive fitness (Xia et al., 2017) and plays a role in progressive virulence (Roelfs, 1952). The presence of heterozygosity at the putative virulence loci in isolates with specific virulence on *rpg4* suggests that the avirulent genes present in these isolates may have an essential virulence function that cannot be disposed of representing a PAMP. Thus, the pathogen evolved a dominant virulence gene that suppresses the *rpg4*-mediated resistance response which manifests itself genetically as a dominant virulence gene.

Effector proteins that function to suppress *R*-gene-mediated resistance upon recognition of the cognate avirulence protein have been identified for the biotrophic fungal pathogen *Blumeria graminis* the causal pathogen of powdery mildew (Bourras et al., 2015; Bourras et al., 2016). This study with the wheat-powdery mildew pathosystem identified two *B. graminis* genes, $AvrPm3^{a2/f2}$ an avirulence gene and $SvrPm3^{a1}$ suppressor of avirulence, that specifically interact with the wheat powdery mildew resistance gene *Pm3f* (Bourras et al., 2015). *Pm3f* based resistance was observed only in the isolates containing functional $AvrPm3^{a2/f2}$ and a non-

functional *SvrPm3*^{a1}. In rust pathosystem it has been shown, genetically, that some strains of *Melampsora lini,* the causal pathogen of flax rust (Ellis et al., 2007) are known to carry an inhibitor of avirulence gene designated the *I* gene. The *I* gene inhibits the recognition of the avirulence genes, *AvrL1*, *AvrL567*, *AvrL8*, *AvrL10*, and *AvrM* by their corresponding *R*-gene alleles, *L1*, *L7*, *L8*, *L10*, and *M1*, respectively (Ellis et al., 2007). A recent study of the wheat stripe rust pathosystem conducted to characterize the host-pathogen genetic interactions that determined *P. striiformis* f. sp. *tritici* virulence on wheat, the causal agent of stripe rust (Yuan et al., 2017) identified virulence loci in *Pst* isolate 08-220 corresponding to the yellow rust resistance gene *Yr1, Yr6, Yr7, Yr8, Yr9, Yr21, Yr25, Yr27, Yr28, Yr35, Yr8, Yr35, Yr41, Yr43, Yr44, Yr76, YrA or Yr74* (Yuan et al., 2017). Interestingly, going into the study to characterize these interactions with dominant *Yr* resistance genes it was expected that they would represent dominant *R*-gene-Avr gene interactions, however to their surprise almost all the interactions were explained by dominant virulences or recessive avirulence gene interactions suggesting the presence of dominant suppressors of resistance. All of these finding within biotrophic pathogen-cereal host pathosystems suggest the presence of suppressors/inhibitors of avirulence (*Svr*/*I*) thus, supporting the hypothesis that in our barley-stem rust pathosystem, avirulence may be determined by the absence of a dominant virulence effector or suppressor of immunity responses similar to what has been recently reported for the wheat-stripe rust pathosystem.

Among the listed genes with variants associated with virulence (Appendix Table A12), the gene model *PGT*G_06872 was particularly interested because it is predicted to encode a protein phosphatase 2C (PP2C) protein. Barley lines harboring the *rpg5* allele containing the PP2C integrated sensory domain, *rpg5*-PP2C, are susceptible to *Pgt* races. The functional *Rpg5* alleles contain a serine threonine protein kinase (STPK) integrated sensory domain and this allele

is required to provide broad resistance against the majority of *Pgt* races, including the North American *Rpg1* virulent race QCCJB and the highly virulent African race TTKSK and its lineages (Brueggeman et al., 2008; Steffenson et al., 2009; Wang et al., 2013). However, when the *Rpg5-STPK* and *rpg5-PP2C* alleles are present together in the heterozygous state it was determined that the *Rpg5-PP2C* allele functions as a dominant suppressor of the otherwise dominant *Rpg5-STPK* resistance response suggesting that the suppressor action explains the previously reported recessive nature of *rpg4*-mediated wheat stem rust resistance (Solanki et al., unpublished). Therefore, although the putative fungal effector containing the PP2C domain does not have significant homology to the *rpg5* PP2C domain it is an attractive candidate for a suppressor of RMRL resistance. It could be speculated that *PGT*G_06872 PP2C could antagonize the *Rpg5 STPK*-mediated phosphorylation events underlying *rpg4*-mediated resistance. Thus, the variation or mutation in *PGT*G_06872 may have allowed this allele to function as a dominant suppressor of RMRL resistance in virulent isolates. However, *PGT*G_06872 does not contain a signal peptide or transmembrane domain suggesting that it may not represent a secreted effector.

Another objective of this study was to conduct comparative analysis of barley gene expression in response to virulent and avirulent *Pgt* isolates. For the host specific comparative analysis, the universal stem rust susceptible barley line Harrington was selected for this study. Harrington is susceptible to all the isolates used in this study because it does not carry either *Rpg1* or *RMRL*. Although, inoculating the resistance line with virulent and avirulent isolates for comparison of host transcripts during compatible and incompatible interactions may have been more informative, the major objective of this study was to identify fungal effectors/elicitors by studying the expressed fungal transcripts from virulent and avirulent isolates. To this end the use

of a resistant line would have affected the recovery of fungal transcripts from the avirulent isolates. It was also informative to inoculate these isolates on a susceptible genotype independent of the strong resistance responses elicited by the resistance genes as it allowed for the determination of differential pathogen genes elicited between the *Rpg1* and RMRL virulent and avirulent isolates. Thus, to accommodate our first objective this study was conducted by using the susceptible barley line only.

A successful pathogen that is able to surpass early PTI responses release effectors into the host cells to manipulate host physiology genes for acquisition of nutrient facilitating growth and ultimately reproduction (Jones and Dangl, 2006). Avirulent pathogens carry an effector that is specifically identified by a host R-protein, while virulent pathogens lack the avirulence effector and/or carries suppressor of resistance to negate the *R*-gene based recognition (Ellis et al., 2007; Petre and Kamoun, 2014). In this analysis the use of the susceptible line Harrington does not significantly impede the infection process of any of the *Pgt* isolates used, thus, all isolates should be able to release their effectors/elicitor repertoire to manipulate the host. The differential expression of host genes during their interaction with diverse sets of virulent and avirulent isolates in respect to *Rpg1* or RMRL in the absence of the corresponding resistance genes implies that the differential host gene expression is in response to specific polymorphic effector/s between virulent and avirulent isolates. Thus, we hypothesize that the differences in the effector profile in the sets of diverse RMRL virulent and avirulent isolates induced or suppressed the differential expression levels of basal defense related gene in the susceptible line Harrington.

Interestingly, the comparative analysis between samples inoculated with *RMRL* virulent and avirulent isolates showed a set of 115 high confidence DEGs with differential gene expression specific to interactions with *RMRL* virulent *Pgt* isolates, with an evenly split 58

upregulated and 57 downregulated genes (Appendix Table A8). In contrast when the analysis was performed by separating the groups into *Rpg1* virulent -vs- avirulent isolates, only five high confidence DEGs were detected and all were upregulated. Due, to this significant number of DEGs being induced specifically by the RMRL virulent isolates and very few DEGs observed in the comparison between *Rpg1* virulent and avirulent isolates and no association identified for *Rpg1* virulence, the remaining discussion will focus on RMRL virulence and avirulence. To validate that the 57 genes were truly downregulated during the interaction with RMRL virulent isolates a comparative assay of barley gene expression was performed between *RMRL* virulent *Pgt* inoculated-vs-non- inoculated controls and *RMRL* avirulent *Pgt* inoculated-vs-non-inoculated controls. The analysis confirmed that these downregulated genes were in fact expressed at higher levels in the *RMRL* avirulent inoculated samples compared to the non-inoculated samples; while the expression levels were not significantly different between the *RMRL* virulent inoculated and non-inoculated control samples. Thus, these expression analyses support the hypothesis of a virulence factor or factors in *RMRL* virulent isolates potentially suppressing the expression of multiple genes during their host-pathogen interaction (Appendix Table A8).

The host genes downregulated in response to the *RMRL* virulent isolates were enriched for heat shock proteins (HSPs), with the majority in the class of small HSPs (sHSPs) and HSP70. Previously it had been shown by Maimbo et al. (2007) that sHSPs are involved in HR-independent non-host type basal immune response by studying the pathogenic *Ralstonia solanacearum* OE1-1 and the non-pathogenic *R. solanacearum* 8107 strains when infecting tobacco. Similar studies were also performed with the bacterial pathogens *Xanthomonas axonopodis* pv. *citri* and *Xanthomonas campestris* pv. *vesicatoria* that cause citrus canker and

bacterial spot of pepper, respectively. These studies showed that the induced expression of the sHSP was a PAMP triggered immunity (PTI) response (Garofalo et al., 2009). Several HSP70 studies have also shown that they play an important role in HR-mediated ETI resistance responses and are targeted by several pathogenic effectors to gain virulence (Kanzaki et al., 2003; Jelenska et al., 2010; Lee et al., 2012; Park et al., 2015; Song et al., 2015). The bacterial pathogen *Pseudomonas syringae* is also able to induce virulence by utilizing a virulence effector HopI1 to target an *Arabidopsis* HSP70 protein (Jelenska et al., 2010). The oomycetes pathogen *Phytophthora sojae* utilizes the effector PsCRN 108 (Crinkler or crinkling- and necrosis-inducing protein) to target a HSP promoter to suppress its expression (Song et al., 2015).

In mammalian pathosystems there is a parallel function of suppression of HSP proteins to induce virulence. The transcriptional regulation of *Hsp70* is dependent upon the binding of the heat shock factor (HSF) to specific DNA component in heat shock element (HSE) (Kanei-Ishii et al., 1994). The expression of a heat shock factor -3 (HSF3) was found to be regulated by direct binding to the c-myb proto-oncogene product (c-MYB) suggesting a role of c-MYB in transcriptional activation of *Hsp70* (Kanei-Ishii et al., 1997). A study has shown that a tumor suppressor protein p53 can modulate the expression of *Hsp70* by binding to HSF3 and disrupting the c-MYB/HSF3 association (Tanikawa et al., 1997), suggesting that suppressors can act on either component of the c-MYB/HSF3 complex to inhibit HSP70 expression. These findings show that HSPs can be suppressed at different levels of transcriptional regulation possibly presenting different targets of effector manipulated to suppress HSP proteins and immunity responses.

In plants, the R2R3 MYB-protein are the classical MYB factors that are close homologs to c-MYb in mammals (Stracke et al., 2001). Interestingly, the *RMRL* virulent *Pgt* isolates

significantly downregulates a host gene encoding a myb domain that could partially explain the observed downregulation of *Hsp* family of genes in the barley line Harrington (Appendix Table A8).

Another interesting aspect of the DEGs was the predicted subcellular locations where their protein products function. Gene enrichment analysis for cellular function showed that the majority of both up and downregulated genes were chloroplast localized proteins (Appendix Table A9). The chloroplasts play a major role in plant defense response by producing several reactive oxygen species (ROS) which are involved in both signal transduction and HR-mediate resistance (de Torres Zabala et al., 2015). Thus, evolutionary it may be beneficial for pathogens to evolve effectors that can subvert chloroplast function and suppress chloroplast ROS production. The downregulation of genes encoding ferredoxin, ribulose bisphosphate carboxylase (rubisco), NAD(P)H dehydrogenase subunit H, Photosystem II protein D1 (Appendix Table A8) (Telfer et al., 1970; Rumeau et al., 2007) suggest that virulent *RMRL* isolates may have evolved suppressors that target defense responses that once activated provide induction of chloroplast function.

During the molecular arms race between rust pathogens and their host the main hypothesis concerning the pathogens ability to gain virulence once detected by a cognate specific *R*- gene is to evolve avirulence gene diversity such that recognition is lost following Flor's classic gene-for-gene model (Flor, 1947). However, it is also possible that the *Avr* gene recognition could be lost via gene deletion through unequal recombination or mutations that result in loss of expression. To determine if *Pgt* virulence was possibly due to the loss of an avirulence gene or transcriptional silencing, transcriptome analysis was done to identify differentially expressed CSEPs. Though 29 differentially expressed CSEPs were identified, they

were expressed at lower levels in virulent isolates ruling them out as potential RMRL avirulence genes.

Understanding and developing hypotheses explaining the complex host-pathogen genetic interactions occurring in this pathosystem requires information from both the host and pathogen perspective. Thus, information recently discovered on the putative function of the genes underlying the RMRL-mediated resistance mechanisms suggest the "integrated sensory domain (ISD) hypothesis" is responsible for the evolution of this resistance mechanism. Genome analysis of several plant species (Sarris et al., 2016, Kroj et al., 2016) suggests that the two NLR genes present at the RMRL fit the role of dual plant NLR immunity receptors found in the tightly linked head-to-head genome architecture with one NLR containing a non-canonical domain that represents a pathogen virulence effector target. Recent, functional analyses of the RGA4/RGA5 proteins that confer resistance to *Magnaportha oryzae* determined that one NLR contains an ISD that represent a virulence effector target that was translocated to the immunity receptor (Cesari et al., 2015), possibly via a targeted mechanism mediated by the dual NLR architecture (Bailey et al., 2018). The barley NLRs at the RMRL, *Rpg5* and *HvRga1*, are both required for resistance and are present in the head-to head genome architecture suggesting that the STPK domain that was putatively translocated to the *Rpg5* NLR immunity receptor possibly had an original function in normal physiological processes that the stem rust pathogen hijacked to facilitate virulence. Thus, the STPK ISD now may functions as a pathogen "bait" that is targeted by a virulent effector that initiates RMRL-mediated defense responses. *Rpg5* contains the STPK ISD whose progenitor, designated the guard cell associated kinase 1 (*Gak1*), is a paralog of the *Arabidopsis* AT5G15080 and *AtApk1b* guard cell proteins that are required for stomatal opening in response to light. We hypothesize that *Pgt* manipulation of Gak1 during infection, possibly

facilitates entry through closed stomata at night. Experiments using confocal laser microscopy showed pathogen entry through stomata in the dark and LASER microdissection and qPCR on isolated stomata show high levels of *Gak1* transcript. A 48h post inoculation Y2H library was screened with Rpg5-PK as bait identifying the transcription factor, HvVOZ1. In *Arabidopsis* voz1 mutants reduced fungal pathogen immunity and stomatal opening. We posit interplay between Gak1-HvVoz1 and a *Pgt* effector during early infection facilitating pathogen entry which could represent a conserved effector across both the virulent and avirulent isolates. Counter evolution directed by duplication and translocation of the Gak1 target protein, resulted in a functional Rpg5-Gak1 ISD immunity receptor, thus, the pathogen's incognito entry betrays itself resulting in recognition and activation of host defense responses. In our model (Figure 2.4) although both virulent and virulent isolates contain *Avrrpg4/5* the virulent isolates possibly evolved an effector that suppresses to inhibits the RMRL resistance mechanisms leading to virulent isolates.

With this information and hypothesis in mind revisiting the expression profile of the CSEPs from both the virulent and avirulent isolates showed that that 99% of CSEPs in the *Pgt* genome were expressed at some level at the time point used for analysis, suggesting that an RMRL avirulence effector, Avr*rpg4/5*, may be conserved across *Pgt* isolates as proposed by the one that targets the STPK ISD (Figure 2.4). Thus, the gain of virulence specific for *RMRL* may be guided by the presence of virulent effectors/suppressors in the RMRL virulent isolates. The association mapping indeed did identify putative effectors that have gene action supporting the dominant virulence model. This model is also supported by the fact that a significant number of host genes involved in resistance responses are suppressed specifically by the RMRL virulent isolates. Most interestingly, one of the putative effectors that could be involved in suppression is

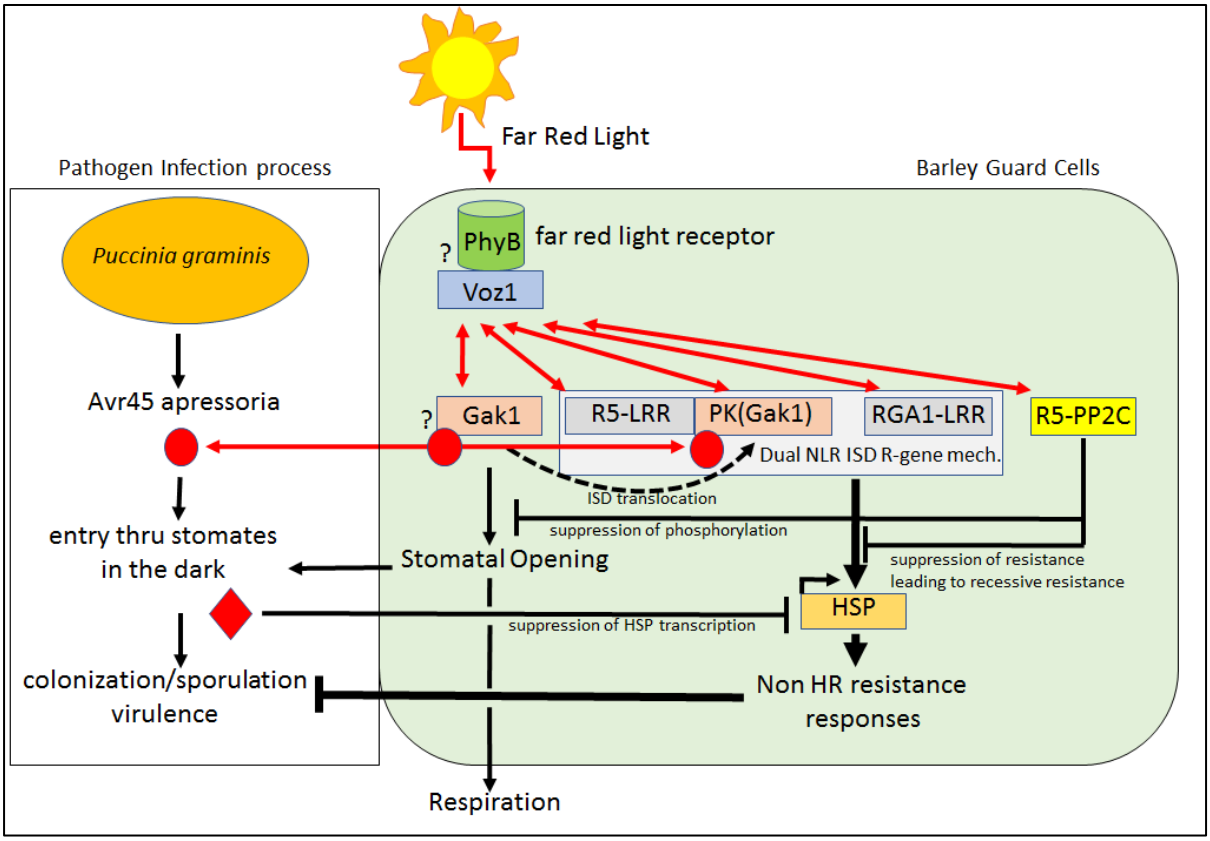

Figure 2.4. A model showing the hypothetical evolution and mechanisms of *Puccinia graminis* f. sp. *tritici* (*Pgt*) effector interactions and function with the barley RMRL-mediated resistance mechanisms. The hypothetical model based on information from the genes involved in RMRL-mediated resistance (Solanki, 2018) suggests that the barley Phytochrome B receptor may interact with *HvVoz1* upon far red-light sensing, and this interaction activates the *HvGak1* protein kinase to facilitate the induction of stomatal pore opening, a vital plant physiological process involved in light induced respiration. Thus, *Pgt* evolved an effector to manipulate HvGak1 to facilitate stomata opening in the night when *Pgt* spores evolved to germinate. Barley, counter evolved a HvGak1 NLR immunity receptor fusion, following the integrated sensory domain hypothesis, as the Rpg5 protein kinase domain is a fusion paralog of HvGak1. Thus, the Rpg5 STPK domain acts as an integrated sensory domain (ISD) bait to trap the Avr4/5 elicitor to initiate the downstream defense signaling via non-hypersensitive resistance responses. In the susceptible barley varieties (Group-2 susceptible) with PP2C ISD, rpg5-PP2C suppress the Rpg5-PK mediated resistance response and act as a dominant susceptibility factor. Thus, Rpg5 represents a unique NLR (NBS-LRR) protein with two different diverse ISDs. Upon infection with the virulent races of *Pgt* on the RMRL containing barley lines, a virulence effector efficiently inhibiting the downstream components of RMRL resistance, the non-HR defense signaling gets suppressed, enabling successful pathogen establishment and sporulation.

a PP2C.This may be significant due to our recent finding that the barley *rpg5-PP2C* allele is able to suppress the otherwise dominant *Rpg5-STPK* allele leading to susceptibility against the

majority of *Pgt* isolates. Thus, this putative effector's predicted gene function suggests that it could utilize a similar mechanism to block RMRL-mediated resistance. However, there still remains major knowledge gaps in the mechanisms underlying this pathosystem as well as in the understanding of suppressor or inhibitor of avirulence (*Svr* or *I)* and we still have a long way to go before we validate our proposed model. The recent genetic evidences of inhibitor genes in flax rust (Ellis et la., 2007), virulence loci in stripe rust (Yuan et al., 2017) and suppressor of avirulence in powdery mildew (Bourras et al., 2015) suggest that the gain of virulence by acquiring a functional *Svr/I*, instead of losing an *Avr* effector could be much more common in pathosystems than previously thought. The transcript analysis presented in this chapter filled some gaps in our knowledge that allowed us to construct a model (Figure 2.4) to move our hypothesis driven research on this complex stem rust resistance mechanism forward from both the host and pathogen perspectives.

Conclusions

The understanding of the original virulence function of effectors and their eventual transformation to an avirulence protein due to host evolution of *R*-genes that specifically recognize the effector or its function is of utmost important in designing a breeding program for the deployment of durable resistance genes. Advanced sequencing technology coupled with powerful bioinformatics tools has made it possible to dissect the host pathogen interactions from both perspectives. In this study we were able to utilize several bioinformatics tools with RAD-GBS and RNA sequencing data to understand and predict the possible mechanisms of *Pgt* virulence in barley. We hypothesize that the pathogen has evolved to gain virulence by suppressing effector elicited resistance responses and the virulence factors are possibly able to impede downstream signaling of resistance responses after early pathogen recognition.

Alternatively, the virulence factors could possibly inhibit the interaction between resistant and avirulence genes to suppress the *R*-gene mediated responses. The next step for this study will be to functionally validate these genes. For the host, we aim to utilize virus induced gene silencing to knock down the downregulated genes to observe if silencing induces a reduction in disease resistance (Hein et al., 2005; Wang et al., 2013). However, there exist a challenge in validating *Pgt* genes because of the lack of molecular tools like transformation or gene knock-out (Rafiqi et al., 2012; Figueroa et al., 2016). However, we can possibly utilize host induced gene silencing (Yin et al., 2015) or bacterial type III secretion system to deliver the effectors for future validation and analyses (Upadhaya et al., 2014).

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CHAPTER 3. GENETIC MAPPING OF THE GENES, *RRR1* AND *RRR2* REQUIRED FOR *RPG4* AND *RPG1*-MEDIATED STEM RUST RESISTANCE IN BARLEY

Abstract

Stem rust, caused by *Puccinia graminis* f. sp. *tritici* (*Pgt*) is an economically important disease in wheat and barley. *Rpg1* is the only deployed stem rust resistance gene in Midwestern US barley varieties and provides resistance to the predominant races in North America, except local *Pgt* race QCCJB and the highly virulent race TTKSK and its lineages. The barley *rpg4*-mediated resistance locus (RMRL) confers resistance to QCCJB and TTKSK (and its variants). To deploy QCCJB and TTKSK resistance, RMRL was introgressed in two elite malting cultivars, Pinnacle and Conlon (both contains *Rpg1*) resulting in the near isogenic lines (NILs), Pinnacle RMRL-NIL and Conlon RMRL-NIL, respectively. The Conlon RMRL-NILs exhibited a resistance response to TTKSK in field trails and QCCJB and HKHJC at the seedling stage in growth chamber assays. In contrast, the Pinnacle RMRL-NILs were susceptible to TTKSK in the field and QCCJB and HKHJC in growth chamber assays. This suggested the presence of other gene/s required for RMRL and *Rpg1*-mediated resistance in the primary barley germplasm pool that were missing in Pinnacle. Utilizing a Pinnacle RMRL-NIL/Q21861 derived RIL population and PCR-GBS genotyping, *Rrr1* (Required *rpg4/Rpg5*- and *Rpg1*-mediated resistance 1) was mapped ~5cM proximal to RMRL on barley chromosome 5H. A second gene, *Rrr2* (Required for *Rpg1*- mediated resistance 2), complimentary to *Rrr1* was mapped to the telomeric region of the short arm of barley chromosome 7H spanning a 63.28cM genetic distance. This study also suggested that *Rrr1* or *Rrr2* is required for *Rpg1*-mediated resistance when RMRL is introgressed into a genetic background.

Introduction

The obligate biotrophic fungus *Puccinia graminis* f. sp. *tritci* Eriks. and E. Henn. (*Pgt*) is the causal agent of stem rust (or black rust) in bread wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). Historically, stem rust epidemics caused devastating yield losses of wheat and barley in several parts of the world, including the northern Great Plains of the USA (Roelfs, 1992; Steffenson, 1992). The northern Great Plains is a stem-rust prone area that regularly suffered barley stem rust epidemics prior to the 1940 (Steffenson, 1992). In 1942, barley cv. Kindred was released as the first commercial barley variety containing the stem rust resistance gene *Rpg1* (Resistance to *Puccinia graminis 1*) (Steffenson, 1992). Since then, barley-breeding programs in the upper Midwest of the United States have fixed the *Rpg1* gene in their lines, which originally came from either cv. Peatland (CIho 5267), Chevron (Ciho 111) or Kindred (Ciho 6969) (Steffenson, 1992; Jin et al., 1994a, Zhou et al., 2014). For nearly 80 years, *Rpg1* has been the only source of durable resistance in barley and has maintained its effectiveness against the majority of isolates in North American *Pgt* populations.

In 1989, a new race of *Pgt*, designated as QCC (later designated as QCCJB (Jin et al., 2008)) was identified in North Dakota (ND) that was virulent on barley cultivars carrying *Rpg1* (Roelfs et al., 1991). The *Pgt* race QCCJB became the most prevalent race in the northern US, threatening all commercial barley cultivars with potential stem rust epidemics that had not been experienced for nearly 50 years (Roelfs et al., 1993). To tackle this looming threat, Jin et al. (1994a) screened 18,000 barley accessions from the USDA National Small Grains collection (Aberdeen, ID) and identified the unimproved barley line Q21861 as an outstanding source of resistance against QCCJB. Genetic studies utilizing several biparental populations derived from Q21861 and different susceptible barley cultivars characterized a temperature sensitive and

recessive gene designated *rpg4* as the gene in line Q21861 conferring resistance to *Pgt* race QCCJB (Jin et al., 1994b). The *rpg4* gene was mapped to the sub-telomeric region of barley chromosome 5H (Borovkova et al., 1995). Q21861 also contains a partial dominant resistance gene that confers resistance to the rye stem rust pathogen *Puccinia graminis* f. sp. *secalis* (*Pgs*) isolate 92-MN-90 that was originally reported to co-segregate with *rpg4*, and was designated as *Rpg5* (Sun et al., 1996; Brueggeman et al., 2008). A map-based cloning approach identified the dominant rye stem rust resistance gene *Rpg5* as an NBS-LRR-Serine/Threonine Protein Kinase (NBS-LRR-STPK) domain protein (Brueggeman et al., 2008). This high-resolution map also determined that *Rpg5* is an independent gene tightly linked to *rpg4* that are colocalized within a 70Kb region on the long arm of barley chromosome 5H (Brueggeman et al., 2008). Utilizing this high-resolution mapping population and additional SNP markers, the *rpg4*-mediated resistance locus (RMRL) was better defined leaving three tightly linked genes delimited within the region; the nucleotide-binding site leucine-rich repeat (NBS-LRR) gene *HvRga1*, the rye stem rust resistance gene *HvRpg5*, and an actin depolymerizing factor-like (Adf) gene, *HvAdf3* (Wang et al., 2013). Post-transcriptional gene silencing utilizing barley stripe mosaic virus (BSMV) virus-induced gene silencing (VIGS) of each of the genes delimited to the RMRL determined that all three genes, *HvRga1*, *HvRpg5* and *HvAdf3* are required together to confer *rpg4* -mediated stem rust resistance. Thus, the successful introgression of RMRL to mediate stem rust resistance requires *HvRga1*, a functional *Rpg5* and *HvAdf3*. However, a study of multiple alleles of *HvRga1* and *HvAdf3* determined that they do not carry polymorphisms that explain susceptibility, therefore *Rpg5* appears to be the genetic determinant with functional polymorphism at the locus, yet *HvRga1* and *HvAdf3* are required for resistance (Arora et al., 2013). Interestingly, the presence of a PP2C domain in place of the Rpg5-STPK domain in most *Pgt* race QCCJB

susceptible barley lines results in the loss of RMRL-mediated resistance, thus markers based on this polymorphism are used to track RMRL introgression via marker assisted selection.

Currently, the highly virulent *Pgt* race TTKSK (also known as Ug99) and its lineage pose an alarming threat to global wheat and barley production and world food security (Singh et al., 2011; Steffenson et al., 2006; Steffenson et al., 2017). Race TTKSK was first reported in 1999 from wheat fields in Uganda, Africa (Pretorius et al., 2000). The extensive monitoring of this race showed a rapid spread of TTKSK and its evolving lineage throughout other countries in Africa and jumped the red sea into Asia (Singh et al., 2011). The varieties grown on approximately 80 to 95% of wheat acreage and the majority of breeding materials were found to be moderately to highly susceptible to TTKSK and its lineage (Singh et al., 2006; Singh et al., 2011). Recently, Steffenson et al. (2017) reported 96% of 2913 barley accessions, including those containing *Rpg1* were extremely vulnerable to this evolving *Pgt* race. The monoculture of *Rpg1* carrying barley and identification of North American race QCCJB, and African race TTKSK and its lineage leaves barley production in the mid-western US vulnerable to potential stem rust epidemics. In barley, RMRL is the only well characterized locus that provides resistance to QCCJB, TTKSK and its lineage (Steffenson et al., 2009). Thus, introgression of RMRL into commercially grown barley cultivars is the logical step towards minimizing the risk of stem rust races that have overcome the remarkably durable *Rpg1*-mediated resistance.

The primary goal of barley breeding programs is to release barley cultivars that are high yielding, have good malting quality, are stable across dynamic environments and have resistance to important pathogens or pests (Horsley et al., 2009). In 2006, the North Dakota State University (NDSU) barley breeding program released a two-row barley cultivar Conlon that had improved yield, early maturity and higher stress tolerance than the existing 2-rowed malting

barley standard cv. Bowman (http://www.ndsuresearch foundation.org/conlon). Conlon was also powdery mildew and net blotch resistant and was better adapted to western ND and its neighboring states, but was prone to lodging and moderately susceptible to spot blotch. Later, in 1999, the two-row variety Pinnacle was released by NDSU that showed promising yield, better straw strength and early maturity compared to Conlon (http://www.ndsuresearchfoundation. org/pinnacle). It also exhibited excellent malting quality that was on par with Conlon. Conlon and Pinnacle became highly recommended malting barley cultivars in the major growing region of the Upper Midwestern United States (USDA, 2012). Like all Midwestern barley cultivars, Conlon and Pinnacle contain *Rpg1*-mediated stem rust resistance thus, are prone to potential stem rust epidemics from *Pgt* races like QCCJB or TTKSK and its lineage if or when it was to arrive in the US. Thus, a breeding scheme was initiated to introgress RMRL in these lines (breeding schemes explained in *Materials and methods*). The Conlon RMRL- near isogenic line (NIL) and Pinnacle RMRL-NIL carrying both *Rpg1* and full length RMRL were selected via marker assisted selection. Field trials in Kenya using *Pgt* race TTKSK (explained under *Rust assay* section in *Materials and methods*) showed that only Conlon RMRL-NILs gained resistant to *Pgt* race TTKSK at the adult plant stage in the field but the Pinnacle RMRL-NILs did not gain *Pgt* race TTKSK adult plant resistance. Corresponding with the results seen in the rust assay at the adult plant stage in the field, the Conlon RMRL-NIL exhibited resistance to *Pgt* race QCCJB while Pinnacle RMRL-NIL was susceptible to *Pgt* race QCCJB in growth chamber seedling resistance assays. Intriguingly, a later growth chamber assay of the Pinnacle RMRL-NIL using the *Rpg1* avirulent *Pgt* race HKHJC showed that the Pinnacle RMRL-NIL not only lacked RMRL-mediated resistance but also lost *Rpg1*-mediated stem rust resistance, even though the *Rpg1* gene was fixed.

Arora et al. (2013) sequenced *Rpg5* alleles from a diverse set of barley accession and categorized them into four resistant and four susceptible groups based on polymorphisms present in the functional *Rpg5* and nonfunctional *rpg5* alleles. Pinnacle and Conlon both carry a *rpg5-PP2C* integrated domain allele, thus, belongs to either group 2 or group 3 susceptible genotype. So, a combination of Rpg5-LRR/STPK and Rpg5-LRR/PP2C specific sequence tag site PCR markers were used for marker assisted selection to confirm the presence of full-length Q21861 like *Rpg5-STPK* alleles in both the Conlon and Pinnacle RMRL-NILs. The single *Rpg5* marker was considered sufficient to track the entire RMRL containing *HvRga1*, *Rpg5* and *HvAdf3* as genotyping of over 5,000 recombinant gametes did not identify a single recombinant separating (Wang et al., 2013; Brueggeman et al., 2008) the three genes and the *HvRga1* and *HvAdf3* genes are highly conserved across resistant and susceptible genotypes (Arora et al., 2013). Also, the *Rpg1* gene in all resistance sources contributing to resistance in Midwestern breeding programs including the NDSU breeding program that produced Conlon and Pinnacle are identical to the *Rpg1* gene found in Q21861 thus the identical *Rpg1* gene is fixed in all lines utilized in the pyramiding schemes. However, despite the presence of the Q21861 like RMRL, and intact *Rpg1* in the Pinnacle RMRL-NIL, the loss of resistance to both *Rpg1* and RMRL avirulent *Pgt* races HKHJC and QCCJB at the seedling stage and TTKSK at the adult stage in the field should be due to a gene/s that are required for resistance but are nonfunctional in the line Pinnacle. A similar case of failed resistance, despite the introgression of full length functional RMRL, was also observed in winter barley lines from the Oregon State University barley breeding program (Pers. comm. Prof. Dr. Pat Hayes, Oregon State University). These results of failed effectiveness of the RMRL in different barley backgrounds suggests the existence of genes required for resistance with natural polymorphism in the primary barley germplasm pool.

The aim of this study was to identify the region of the barley genome that segregates for genes associated with RMRL and *Rpg1* resistance. To this end a $F_{4:5}$ recombinant inbred line (RIL) mapping population was developed from the cross Pinnacle RMRL-NIL/Q21861 using single seed descent to map this region/s. Utilizing this biparental mapping population, two genes, *Rrr1* (Required *rpg4/Rpg5*- and *Rpg1*-mediated resistance 1) and *Rrr2* (Required for *Rpg1*-mediated resistance 2) were mapped in barley chromosome 5H and 7H, respectively. The genetic and phenotypic analysis revealed that a functional *Rrr1* is required for RMRL resistance yet neither gene is required for *Rpg1*-mediated resistance in the absence of the RMRL. However, when RMRL is added to the genetic background then *Rrr1* or *Rrr2* are required and complimentary for the *Rpg1* mediated resistance to function. Thus, based on results from this study, we hypothesize that the introgression of RMRL in *Rpg1* carrying line necessitates a functional *Rrr1* or *Rrr2* to confer *Rpg1*-mediated resistance, and the failure to incorporate either functional allele renders *Rpg1* non-functional.

Materials and methods

Development of Pinnacle and Conlon RMRL-NIL

Pinnacle and Conlon RMRL-NIL introgression lines were developed via a backcrossing and marker assisted selection scheme. The 2-row malting barley variety Pinnacle was developed by the NDSU barley breeding program and released by the North Dakota Agricultural Experiment Station in 2006. Pinnacle was crossed with the unimproved 2-row barley line Q21861 as the RMRL donor. Conlon is also a 2-row malting barley variety developed by the NDSU barley breeding program that was an earlier release by North Dakota Agricultural Experiment Station in 1996. Conlon was crossed with the Harrington x Q21861 BC_3F_2 line retained the original F_2 recombinant designation HQ1 as it was originally identified as a critical

recombinant utilized to delimit the *Rpg5* and *rpg4* genes (Brueggeman et al., 2008; Wang et al., 2013). The HQ1 F_2 recombinant line was backcrossed to Harrington to BC_3 using *rpg5-PP2C* allele specific STS markers (primers R5iN-F2 and PP2C-R3) for MAS to insure introgression of RMRL (Brueggeman et al., 2008). The susceptible variety Harrington used to develop the HQ1 line is a 2-row malting variety that contains no known stem rust resistance genes and was developed at the University of Saskatchewan (Harvey and Rossanagel, 1994). The BC_1F_1 derived individuals at each generation derived from backcrosses to the recurrent elite malting background were selfed to generate F_2 progeny. Approximately 12-15 F_2 individuals from each cross were genotyped using the RMRL specific markers (described below in *Marker assisted selection for RMRL*) and progeny homozygous for RMRL were selected. The homozygous *rpg5*-STPK/RMRL selected F_2 individuals from the Pinnacle and Conlon backcrosses were again crossed to the recurrent parents. The backcrossing, selfing and marker assisted selection for RMRL was repeated twice for Pinnacle and thrice for Conlon to develop, BC_3F_1 Pinnacle and BC_4F_1 Conlon, individuals respectively. The final backcrossed individual was selfed to developed BC_3F_3 Pinnacle (Q21861/4*Pinnacle F_3) and BC_4F_3 Conlon (Q21861/4*Conlon F_3) NILs containing both *Rpg1* and RMRL.

Marker assisted selection for RMRL

Genomic DNA (gDNA) was extracted from leaf samples using the protocol described in Richards et al. (2016). Two previously designed dominant markers, Rpg5-LRK and Rpg5-LRK/PP2C were used to detect the presence of functional *Rpg5-STPK* allele and absence of the *rpg5-PP2C* allele, respectively (Derevnina et al., 2014). Polymerase chain reaction (PCRs) were performed using a Mastercycler Pro programmable thermocycler (Eppendorf, Hauppauge, NY, U.S.A.) programmed for 95°C for 4 min; followed by 35 cycles of 95°C for 30 s, 62°C for 1 min,

and 72°C for 1 min; followed by 72°C for 5 min. PCR amplifications were performed in 20 µl reaction volume containing 10 ng of template gDNA, 1X GoTaq® Flexi Buffer (Promega, USA), 1 mM $MgCl_2$, 0.15 mM dNTP mix, 0.4 µM of each forward and reverse primer, 1.25u GoTaq® DNA polymerase. BC_nF_2 lines were selected for further backcrossing if they amplified a 1,046-bp fragment from *Rpg5-STPK* alleles utilizing Rpg5-LRK markers but had no amplification for the *rpg5-PP2C* allele as indicated by an 840 bp amplicon when utilizing the Rpg5-LRK/PP2C marker.

Development of RIL mapping population

The Pinnacle RMRL-NIL (FAR14-1-1) at the BC_3F_3 generation was crossed with barley accession Q21861 to develop the Pinnacle RMRL-NIL/Q21861 RIL mapping population. The F_1 generated from the Pinnacle RMRL-NIL/Q21861 cross was self-fertilized and F_2 individuals were advanced to the $F_{4:5}$ generation by single-seed descent (Brim, 1966) to generate a 120 individual RIL population. At the F_2 stage, 94 seeds (selected out of the 120 seeds that were advanced to make RILs) were selected to conduct a phenotypic assay using *Pgt* race QCCJB to study the mode of inheritance of *Rrr1* (rust inoculation explained in rust assay section). These individuals were assayed at 14 days post inoculations and later transplanted from cones to 6-inch pots to grow along with other remaining F_2 individuals to self-fertilize.

Stem rust disease phenotypic assays

Field assay

Four F_2 derived Pinnacle RMRL-NIL families and 12 F_2 derived Conlon RMRL-NIL families were evaluated in the International Stem Rust Nursery at the Kenyan Agricultural Research Institute in Njoro, Kenya during the 2015 field season (Appendix Table A13). The parental lines (Q21861, Pinnacle and Conlon) and susceptible control (Steptoe) and the spreader

rows for rust inoculum were planted as described in Zurn et al., (2014). The entries were planted in two replications and the screening was done at the adult plant stage. The modified cobb scale was used to record the disease severity of stem rust (Peterson et al., 1948, McIntosh et al., 1995). Based on the size and type of the uredinia, the infection response (IR) were categorized into resistant (R; small uredinia surrounded by chlorosis or necrosis), moderately resistant (MR; medium sized uredinia surrounded by chlorosis or necrosis), moderately susceptible (MS; medium to large size uredinia without chlorosis or necrosis), and susceptible (S; large compatible uredinia without chlorosis or necrosis), or intermediate of any two categories (MRMS, MSS, SMS) (Roelfs et al., 1992, McIntosh et al., 1995, Yu et al., 2011). The weighted score of modified cobb scale were taken as coefficient of infection as explained by Yu et al. (2011) (conversion of modified cobb explained in Appendix Table A13).

Growth chamber assays

After planting, the rack containing cones with seeds were transferred to a growth chamber (Model 7301-75-2; Caron, Marietta, OH, USA) set at 16/8 hours light/dark periods and a day/night temperature of 21/18°C. When the primary leaves were fully expanded and the secondary leaf was still at the whorl stage (7-8 days post sowing), the seedlings were inoculated with *Pgt* race QCCJB using the protocol previously described by Steffenson et al. (2009) and moved to a humidity chamber. After 18 hours in a dark humidity chamber at 100% relative humidity, the inoculated plants were moved back to the growth chamber set at the previously described growing condition for 14 days. At 14 days post inoculation (DPI), the infection type was assessed using a modified 0-4 rating scale established for barley (Stakman et al., 1962; Steffenson et al., 2009) (Fig 1). The scores were converted to a quantitative score using a conversion scale devised by Zhou et al (2014) (conversion explained in Appendix Table A14).

One of the resistant Conlon RMRL-NIL families (FAR14-94A-1) and one susceptible Pinnacle RMRL-NIL families (FAR14-1-1) were selected for seedling assays using *Pgt* race QCCJB and HKHJC in the growth chamber. *Pgt* isolate QCCJB is virulent on *Rpg1* but avirulent on RMRL*;* and HKHJC is virulent on RMRL but avirulent on *Rpg1*. Any NIL line resistant to both isolates suggests the presence of a functional *Rpg1* and *rpg4/Rpg5* gene. The parental lines, Q21861, HQ1, Pinnacle wildtype, Conlon type and the susceptible checks, Harrington and Steptoe were planted for each disease reaction assay. Each entry was planted in seven cones and randomly distributed in a 12 X 7 rack.

Seedling assay was also conducted on F_2 progeny lines and the Pinnacle RMRL-NIL/Q21861 $F_{4:5}$ RIL using *Pgt* race QCCJB and the further advanced $F_{5:6}$ RIL population was screened using the two *Pgt* races QCCJB (avirulent on RMRL and virulent on *Rpg1*) and HKHJC (virulent on RMRL and avirulent on *Rpg1*). The barley seedlings were grown in six-inch plastic cone with one seed per cone for they assay of F_2 progenies and two seeds per cone for the RIL assays. For each rep of the 120 RIL population the parental lines Q21861 and Pinnacle RMRL-NIL, as well as Pinnacle, Conlon RMRL-NIL, Conlon, Morex, Steptoe and Harrington were assayed as controls. Morex is a six-row malting barley cultivar release from University of Minnesota that carries *Rpg1* (Kleinhofs et al., 1993). Harrington and Steptoe are wheat stem rust susceptible lines which do not harbor any known stem rust resistance genes (Brueggeman et al., 2008). The $F_{4:5}$ RILs were screened with *Pgt* race QCCJB in two replications arranged in a randomized complete block design (RCBD). For *Pgt* race HKHJC, the data was obtained from a single replication, however another experimental replication will be completed on the further advanced $F_{5:6}$ RIL population.

Statistical analysis

The quantitative scores of infections type obtained using the conversion scale of Zhou et al (2014) was used to classify the individual as resistant and susceptible. Any individual with score greater than 3 was classified as susceptible. The Pearson's chi-square ($\chi 2$) goodness of fit test statistics was used to evaluate the independent segregation of resistance to susceptible in the F_2 population and $F_{4:5}$ RIL, for single or two gene segregations. The test result in the F_2 population was used to determine the nature of inheritance of the gene. Since, only *Pgt* race QCCJB was used to assay the F_2 population, we were only able to interpret the nature of inheritance of *Rrr1,* the single gene required for RMRL-mediated resistance. The p-value associated with Pearson's $\chi 2$ statistics was obtained using the CHISQ.TEST function in Microsoft Excel.

Genomic DNA extraction for PCR-GBS

Polymerase chain reaction-genotyping by sequencing (PCR-GBS) was used to genotype the Pinnacle RMRL-NIL/Q12861 $F_{4:5}$ RIL population and the parental lines, along with Pinnacle. The genomic DNA from the samples for PCR-GBS was isolated using a modified version of 96-well plate extraction methods described by Ivanova et al. (2008) (protocol obtained from Dr. Xuehui Li's lab, Department of Plant Sciences, NDSU)., Roughly, 3 cm long leaf tissue from the individual sample were collected in 96-well 2.2 ml deep well plates (VWR, PA, USA) and lyophilized for 24 hours at -40°C. Two 4 mm stainless steel grinding balls (VWR, PA, USA) were added to each well and the samples were powdered using a Mixer Miller Type 301 tissue grinder (Retsch Gmbh & Co. KG, Germany) set at a frequency of 20/sec for 3 to 6 min. The powdered tissue samples were homogenized in 500 µl extraction buffer (100 mM Tris base pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 1.25% SDS) and incubated at 65°C for 30 min with brief vortexing every 10 min. The plates were incubated at -20°C for 10 min, after which 166 µl

of precipitation solution (5M ammonium acetate) was added to each sample. Samples were briefly vortexed and transferred back to -20°C for 10 min. The plates were centrifuged at room temperature at 4,000 RPM (Revolution Per Minute) for 25 min. About 400 µl of supernatant was transferred to a fresh 96-well 2.2 ml deep well plate containing 600 µl DNA binding solution (6M Guanidine-HCL, 63% alcohol) and mixed by pipetting 3-5 times. The mixed samples were transferred to a AcroPrep^{TM} DNA filter plate stack (part# 8132, Pall Corporation, Port Washington, NY) set over 96-well 2.2 ml deep well plates and centrifuged (3000 RPM) for 10 min at room temperature. The eluents were discarded after centrifugation followed by two rounds of filter wash using 800 µl of wash solution (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 50 mM NaCl, 67% ethanol). The remnant wash solution after the final wash was removed by centrifugation for 10 min at 3000 RPM. The glass plates were placed over a 1.2 ml deep well plate and 200 µl of elution buffer (10 mM Tris pH 8.0, 20 µg/ml RNase) was added to each well. Finally, 200 µl of eluted DNA was collected in 1.2 ml deep well plate by centrifugation at 3000 RPM for 5 min. About 10-15 samples were randomly selected to check for concentration using Qubit® HS DNA kit in Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA).

PCR-GBS library preparation and Ion Torrent sequencing

A PCR-GBS single nucleotide polymorphism (SNP) marker panel containing 365 barley SNP markers designed by Tamang (2018) was used in this study. The marker panels were divided into six pools as described by Tamang (2018). Six different PCR amplification reactions per sample, each representing one primer pool were run for library preparation. Each reaction volume of 5 µl contained 1.5 µl of genomic DNA (DNA concentration ranging for 10 ng/µl – 50 ng/µl), 1 µl of 500 nM primer pool and 2.5 µl of 2X Platinum® Multiplex PCR Master Mix (Life Technologies, CA, USA). The primary PCR amplifications were done in a Mastercycler Pro

programmable thermocycler (Eppendorf, Hauppauge, NY, U.S.A.) with conditions set at: initial denaturation of 94°C for 10 min; followed by a touchdown step of 10 cycles of denaturation at 94°C for 20s and annealing at 62°C for 1 min, where temperature was decreased by 0.8 °C each cycle; followed by 20 cycles of denaturation at 94°C for 20s, annealing at 57°C for 1 min, and extension at 72°C for 1 min; ending with final template extension at 72°C for 3 min. The PCR reactions were diluted to a volume of 20 µl using nuclease-free H_2O. After brief vortexing and centrifugation, 5 µl of diluted PCR product from each well was aliquoted to a new 96-well plate; each well represented a single sample with a total volume of 30 µl per sample (5 µl X 6 primer pools). A barcoding PCR mix of 20 µl volume was prepared using 2 µl of pooled single genotype amplicons, 1 µl of 5 µM unique barcode primers, 150 µM of dNTPs, 250 nM of universal reverse primer, 1X GoTaq® Buffer (Promega, USA) and 1U of GoTaq® DNA polymerase. The PCR amplification parameters were the same as the primary PCR. After completion of secondary PCR cycles, 15 µl of nuclease-free water was added to each well followed by a quick vortex and spin. From each well, 5 µl of samples are aliquoted and combined in a clean 1.5 mL tube (total volume = 5 µl X total number of samples) and purified using a E.Z.N.A Cycle Pure Kit (Omega Bio-tek, Inc., GA, USA). An aliquot of 2 µl from the purified pooled PCR amplicons was used for PCR-GBS library amplification in a 30 µl reaction containing 1X GoTaq® Buffer, 333 µM ABC1 and P1 primer, 166 µM dNTPs and 1 U GoTaq® DNA polymerase. A negative control reaction was set up without GoTaq® DNA polymerase. Both reactions were run in a PCR thermocycler with parameters set at: initial denaturation of 95°C for 5 min; followed by 8 cycles of denaturation at 95°C for 30s, annealing at 62°C for 30s, and extension at 72°C for 30s; ending with final template extension at 72°C for 7 min. After PCR, the concentration of the Taq and No-Taq reactions were measured using the Qubit® HS

DNA kit with the Qubit® 2.0 fluorometer. About 2 µl of reaction were run on a 1% agarose gel pre-stained with Gelred (Biotium, Hayward, CA, USA) to visualize the amplicons. No spurious band (< 100bp) were observed in the reaction with Taq and thus, processed for enrichment using Ion PI™ Hi-Q™ OT2 200 Kit (Life Technologies) without any further purification. The enrichment was done in Ion OneTouch™ 2 System and finally, the samples were sequenced on Ion Torrent Personal Genome Machine (PGM) using Ion 318™ Chips following the manufacturers standard protocol.

SNP calling and genotyping

The sequencing reads generated with the Ion Torrent PGM were uploaded to CLC genomics workbench v8.0 software (CLC bio, Aarhus, Denmark) to perform the quality trimming (using default parameters) and end reads trimming. The 5' and 3' end of each sequencing reads were trimmed by 22 nucleotides to remove the PCR adapters (Richards et al., 2016). The trimmed reads were used for alignment using the Burrows–Wheeler Aligner maximal exact match (BWA-MEM) algorithm (Li, 2013). The sequence for each marker were obtained from the barley T3 database (https://triticeaetoolbox.org/barley/) and used to design a fasta reference file for mapping. The SNP calling was done on the aligned BAM (Binary Alignment Map) file using the Genome Analysis Toolkit (GATK) Unified Genotyper tool with default setting for multi- sample SNP calling (Van der Auwera et al., 2013). VCFtools was used to remove individual calls with read depth of less than six and genotype quality less than ten (Danecek et al., 2011). Only the SNPs reported in the iSelect 9K SNP array for the selected markers were used for mapping the genes.

An in-house visual basic script was utilized to calculate the frequency of reference and alternate SNP allele called for each marker in each sample. The genotype of a sample for a given

marker called as homozygous for reference allele or alternate allele, if the allele frequency of either of the allele is greater than 70%. Sample with allele frequency within 30-70% for either of the allele for a given SNP were scored as heterozygous

Genetic mapping

The iSelect consensus genetic map developed by Muñoz-Amatriaín et al. (2014) was used as a reference to assign the markers to their respective barley chromosome and chromosomal position. The genotypic and phenotypic data were used to manually construct a .qdf file containing a standard format for importing marker, map and trait data into the publicly available QTL mapping software QGene v.4.3.10 (Joehanes and Nelson, 2008). The imported data were then analyzed by a composite interval mapping (CIM, Zeng 1994; Jansen and Stam, 1994) in QGene v.4.3.10. To control the background variation, a forward cofactor selection method was used to select marker as cofactors with options, 'Maximum number of cofactor' and 'F to add' set at auto. Permutations of 1000 were used to obtain a LOD threshold for an experiment-wide significance level of 0.01.

Marker saturation

Genotypic data on Q21861 and Pinnacle were obtained from different genotypic experiments that are publicly available in the Barley T3 database (https://triticeaetoolbox.org/barley). A total of 24 iSelect markers located between the two barley iSelect markers 11_21247 and 12_30162 that were identified as polymorphic between Q21861 and Pinnacle were selected to saturate the region harboring *Rrr1*. For each marker, the forward and reverse primers were designed to amplify the region containing the diagnostic SNP (Appendix Table A15).

Adaptor sequences CS1 and CS2 were attached to the forward and reverse primers, respectively, for Ion Torrent sequencing compatibility (Richards et al., 2016). The primers were multiplexed into two pools, each containing 12 markers, as recommended by Richards et al. (2016). These new pools were used to re-genotype the Pinnacle RMRL-NIL/Q21861 $F_{4:5}$ RILs and parental lines. The PCR-GBS library preparation, Ion Torrent sequencing, SNP calling and genetic mapping was performed as previously described. This genotypic data from the new set of markers on barley chromosome 5H was combined with the 5H marker data from 365 marker panel.

The phenotypic score obtained from screening the $F_{4:5}$ RILs using *Pgt* race QCCJB was converted to a binary scale to match the genotypic calls (score <=3 = A (Q21861 like allele), score > 3 = B (Pinnacle like allele). A genetic map was created using MapDisto v1.7.7.0.1.1 with default minimum LOD of 3.0, rmax of 0.3 and Kosambi mapping function (Kosambi, 1943). In order to compute a physical distance between RMRL and the iSelect marker used in this mapping, their physical positions were obtained by conducting a blast search against IBSC (International Barley Sequencing Consortium) v1 Morex genome using Viroblast in the IBSC blast server containing IBSC (Deng et al., 2007; Mascher et al., 2017; http://webblast.ipk-gatersleben.de).

Results

Phenotypic evaluation of NILs

Field assay using Pgt race TTKSK

In the Njoro, Kenya TTKSK inoculated field disease nursery, the RMRL donor parent Q21861 for the Pinnacle RMRL-NIL, showed consistent resistance responses with a median infection response of trace (T) for disease severity and moderately resistant for infection type

(TMR; Appendix Table A13). The HQ1 introgression line was the donor of the Q21861 RMRL for the Conlon RMRL-NIL, however HQ1 data was not produced in this disease nursery location. However, previous site year screening of HQ1 in the same disease nursery showed a median infection response of 5RMR. The infection response of Pinnacle ranged from 15MSS to 30MSS with a median response of 25MSS. Likewise, Conlon gave a clear susceptible reaction with an infection response ranging from 5MS to 25MSS and median score of 20MS. Interestingly, a segregating disease response was observed for the 12 Conlon RMRL-NIL families tested. However, three of the Conlon RMRL-NIL families (FAR14-94A-1, FAR14-94A-2, FAR14-95A-2) were clearly resistant compared to the susceptible recurrent parent Conlon. FAR14-94A-1 gave a consistent disease response of 5MSMR across both replication, while FAR14-94A-2 had data from replication 2 only with a score of 5MR and FAR14-95A-2 gave a consistent disease score of 5MS across all replications (Appendix Table A13). However, all four families of Pinnacle RMRL-NIL (FAR14-1-1, FAR14-1-2, FAR14-1-3 and FAR14-1-4) were susceptible with scores comparable to its susceptible parent Pinnacle (Appendix Table A13). The highest disease score of 30MSS was observed in FAR14-1-2 and the lowest score of 10MSS was observed in FAR14-1-4.

Seedling assay using Pgt race QCCJB

Inoculation of resistance parental lines of, Q21861 and HQ1 with *Pgt* race QCCJB exhibited an extremely low infection types (Its) (Appendix Table A14). The susceptible parental lines, Pinnacle and Conlon were both susceptible to QCCJB with median score of 3-2 and 3,3- respectively. Conlon RMRL-NIL (FAR14-94A-1) inoculated with QCCJB exhibited a resistance response with median infection response of 1; while the Pinnacle RMRL-NIL (FAR14-1-1) was as susceptible as Pinnacle wild type with a median score of 3,3- (Appendix Table A14).

Seedling assay using Pgt race HKHJC

The RMRL donor of Pinnacle RMRL-NILs, Q21861 was also highly resistant to HKHJC and exhibited a median score of 0;1 (Table A14). Likewise, the RMRL donor of Conlon RMRL-NILs, HQ1 exhibited a high ITs of 3-2. Pinnacle and Conlon were also resistant to HKHJC. The Conlon RMRL-NIL (FAR14-94A-1) was not significantly different from Q21861 and exhibited a median IT of 1; However, Pinnacle RMRL-NIL (FAR14-1-1) was significantly different from both of its parent and exhibited a highly susceptible reaction of 3-3.

Seedling assay of Pinnacle RMRL-NIL/Q21861 progenies

For the Pinnacle RMRL-NIL/Q21861 F_2 individuals, only the *Pgt* race QCCJB isolate was used for the rust assay. The parental types, Pinnacle RMRL-NIL and Q21861 exhibited an infection response of 3-2 and 0;1, respectively (Appendix Table A16). Among the F_2 individual progeny, the lowest infection response was of 0; and highest infection response of 3+ (Appendix Table A16). Likewise, the lowest observed infection response in the $F_{4:5}$ RILs inoculated with QCCJB was 0; and the highest infection response was 3,3+ (Appendix Table A16, Figure 3.1A). The screening of $F_{5:6}$ RILs with race HKHJC resulted in lowest infection response of 0; and highest infection response of 3+ (Appendix Table A4, Figure 3.1B).

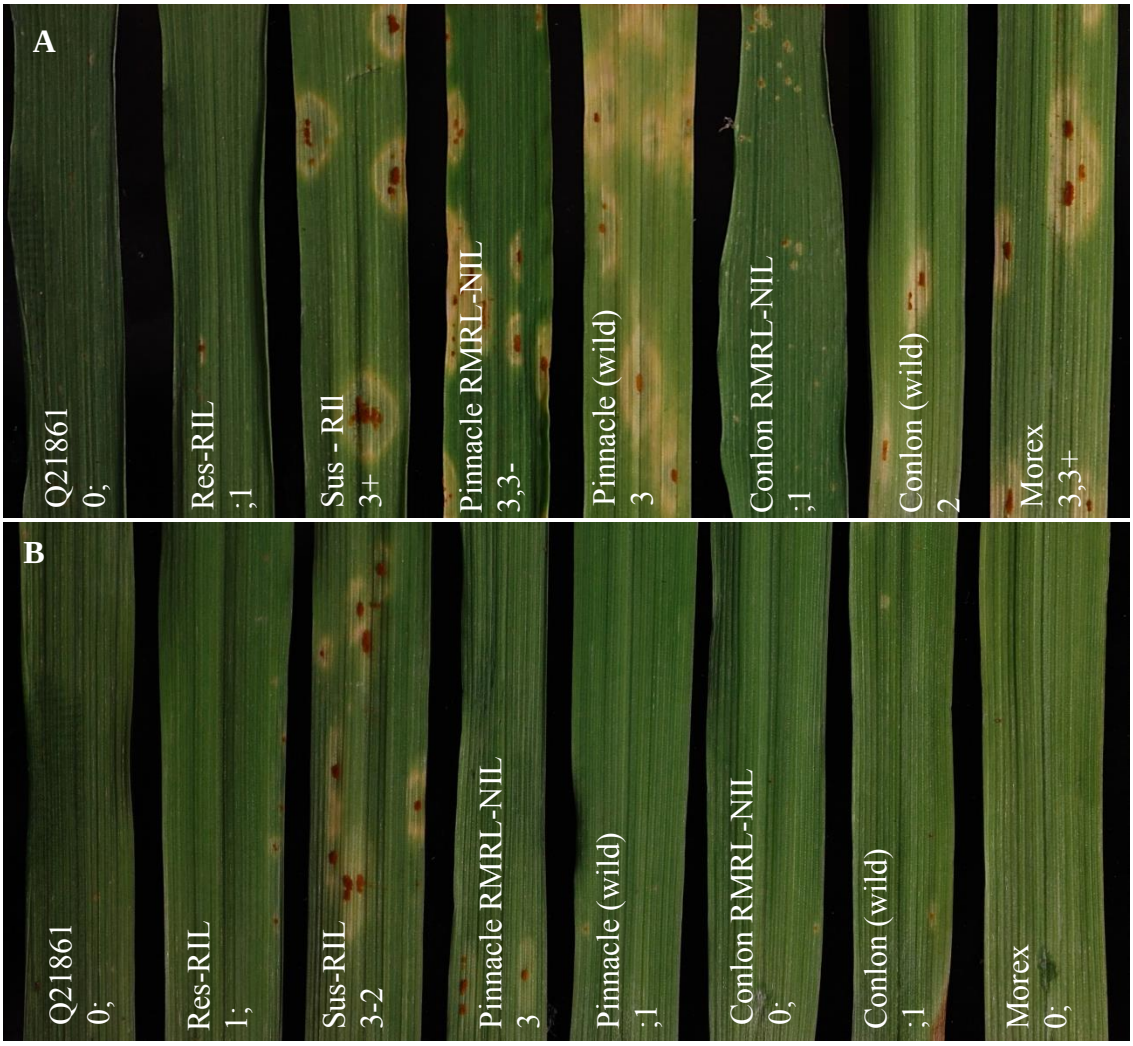

Figure 3.1. Seedling assay using *Pgt* race (A) QCCJB and (B) HKHJC. QCCJB is virulent of barley lines containing *Rpg1* and HKHJC is virulent on line containing RMRL. From left: Q21861 (RMRL+, *Rpg1*+), Res-RIL (Resistant $F_{4:5}$ Pinnacle RMRL-NIL/Q21861 RIL (RMRL+, *Rpg1*+)), Sus-RIL (Susceptible $F_{4:5}$ Pinnacle RMRL-NIL/Q21861 RIL (RMRL+, *Rpg1*+)), Pinnacle NIL (RMRL+, *Rpg1*+), Pinnacle wild (RMRL-, *Rpg1*+), Conlon NIL (RMRL+, *Rpg1*+), Conlon wild (RMRL-, *Rpg1*+), Morex (RMRL-, *Rpg1*+). A 0-4 modified scale for barley was used to score the disease response (Stakman et al., 1962; Steffenson et al., 2009).

Statistical analysis

The Pearson's chi-square ($\chi 2$) goodness of fit test confirmed that the F_2 progeny derived from the Pinnacle RMRL-NIL/Q21861 cross segregated as 3 resistant: 1 susceptible, when inoculated with *Pgt* race QCCJB, suggesting a monogenic inheritance of a single dominant gene

required for *rpg4* -mediated stem rust resistance (Table 3.1). This result was further validated using the $F_{4:5}$ RILs inoculated with QCCJB that did not significantly deviate from 1 resistant:1 susceptible ratio as expected for single gene segregation in the RIL population (Figure 3.2). segregated for a 1 resistance: 1 susceptible ratio (chi-square ($\chi 2$) p-value > 0.05, Table 3.1)

Table 3.1. Chi-square goodness-of-fit test to assess the segregation for resistance to susceptibility in different generation of progenies generated from Pinnacle RMRL-NIL/Q21861

***Pgt* race**	**Generation[a]**	**Replication**	**No. of individuals[b]**		**Expected segregation ratio[c]**	**P-value[d]**
			Resistant	**Susceptible**		
QCCJB	F_2	1	63	31	3:1	0.074
	$F_{4:5}$	1	66	54	1:1	0.27
		2	63	52		0.31
HKHJC	$F_{5:6}$	1	75	39	3:1	0.028*

[a]The generation of progenies derived from Pinnacle RMRL-NIL/ Q21861 cross that was screened with given *Pgt* race

[b]The number of individuals that gave resistance or susceptible response when inoculated with given *Pgt* race

[c]The expected Mendelian segregation ratio for resistance to susceptible in given assay. A trait governed by a single trait will segregate for 3:1 and 1:1 ratio in F_2 and RIL, respectively. In a RIL, two genes that are complementary for a single phenotype will give a phenotypic segregation 3:1 for resistance to susceptibility.

[d]P-value > 0.05 represents that dataset that fit the expected Mendelian segregation ratio for resistance to susceptibility (*-segregation distortion possibly due to small sample size).

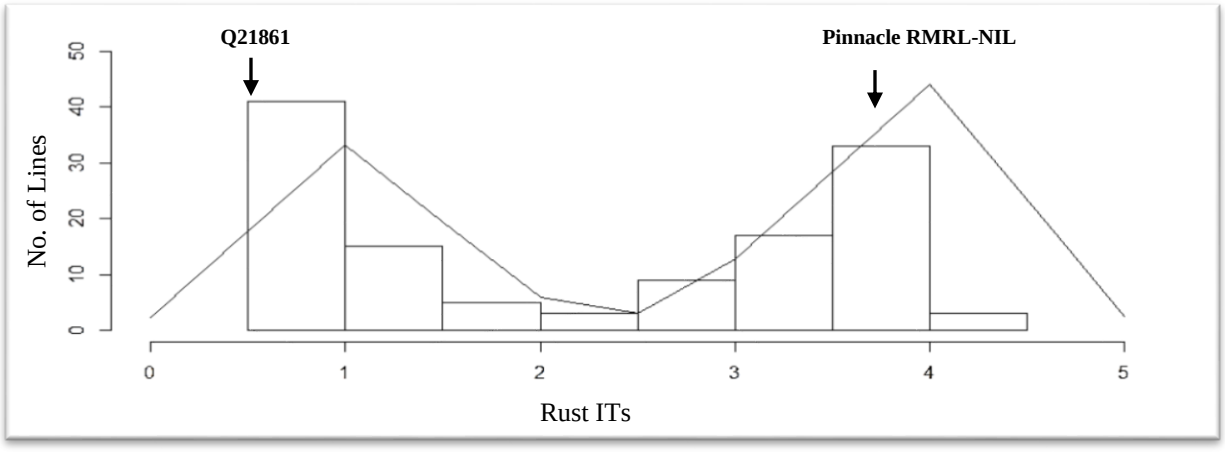

Figure 3.2. Phenotypic distribution of RIL mapping population inoculated using with *Pgt* race QCCJB. The infection types were converted into a quantitative score using the conversion scale provided by Zhou et al. (2013) (conversion formula in Appendix Table A14). The RIL

However, the segregation ratio in the first experiment of QCCJB assay $F_{4:5}$ RIL were significantly different from expected 1:1 ratio for resistance to susceptibility. It was mainly due to the poor inoculation that was corrected in second experiment by using fresh QCCJB isolates with a better germination. The segregation ratio of infection response of $F_{5:6}$ RILs inoculated with HKHJC did not comply with 1:1 (resistance: susceptibility) but was inclined towards a 3:1 ratio (resistance: susceptibility). A 3:1 segregation ratio for resistance to susceptibility in RIL suggest that two genes with complementary gene function are involved in *Rpg1*-mediated resistance. However, a slight distortion from a 3 resistant: 1 susceptible ratio ($P > 0.05$ but $P < 0.01$) was observed which could be due to the small number of RILs used for screening (Table 3.1).

Genetic mapping of *Rrr1*

As expected from the Pearson's chi-square ($\chi 2$) goodness of fit test, a single gene required for *rpg4/Rpg5*-mediated resistance, designated *Rrr1*, was mapped to the telomeric region of barley chromosome 5H, ~5 cM proximal to RMRL (Figure 3.3; Table 3.2). The genotypic data obtained from the 365 PCR-GBS SNP marker panel was utilized to map the *Rrr1* gene to a region of barley chromosome 5H, flanked by the iSelect markers 11_20551 (130.93 cM iSelect consensus map position (Muñoz-Amatriaín et al., 2014)) and 12_30162 (156.7 cM iSelect consensus map position). After saturating the delimited *Rrr1* region using a custom PCR-GBS SNP panel designed from SNPs mined from the recently released barley 50k iSelect markers the *Rrr1* region was further delimited to an ~ 1.4 cM region distal to iSelect marker 11_21018 (153.74 iSelect consensus map position) (Figure 3.3).

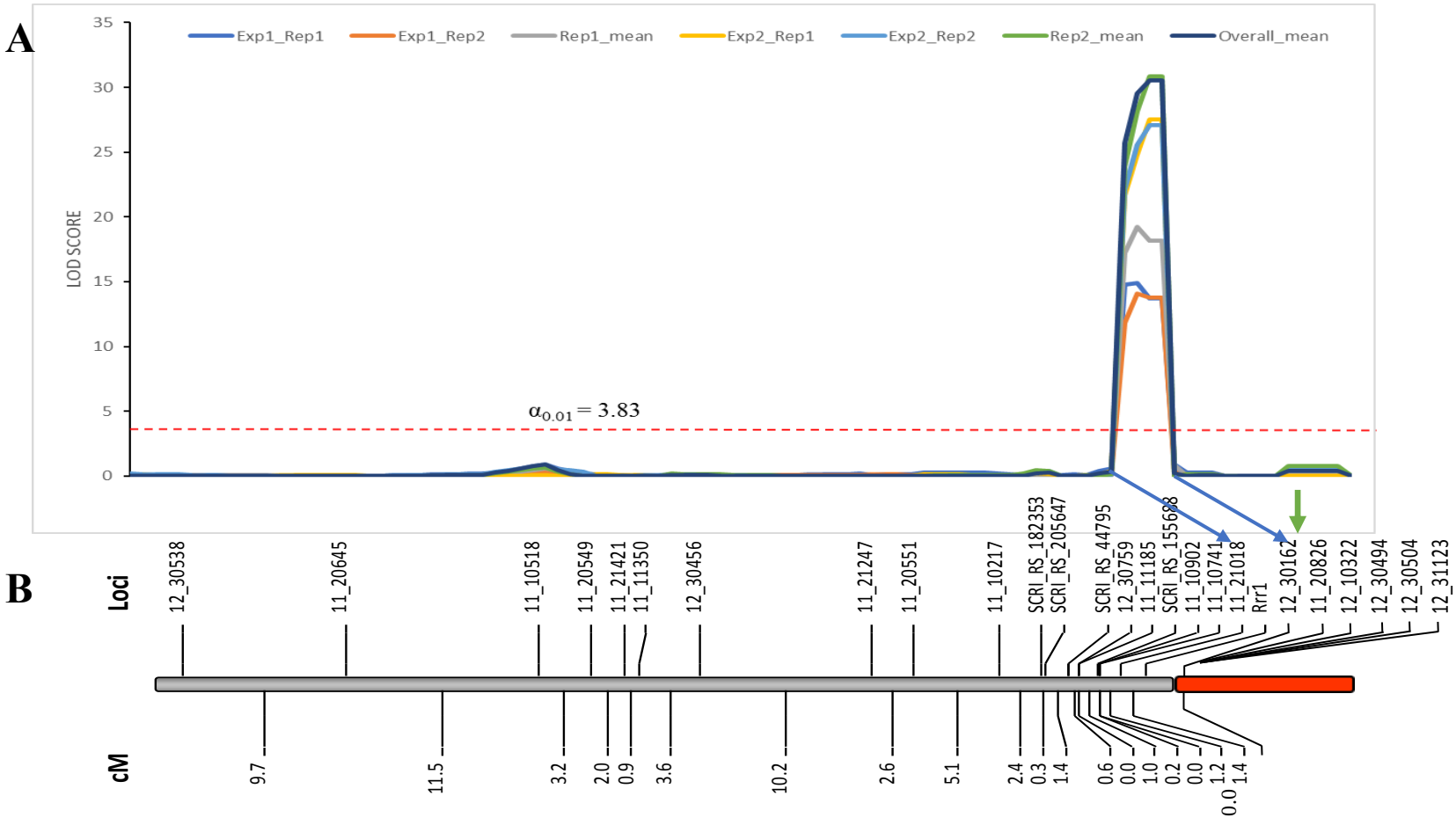

Figure 3.3. *A*) Genetic mapping of *Rrr1* using the composite interval mapping in Qgene v4.3.0. on barley chromosome 5H. The dotted line represents a LOD threshold obtained at experiment-wide significance level of 0.01 after 1000 permutations test (B) The genetic map of barley chromosome 5H developed using the polymorphic markers available in our dataset. A default minimum LOD score of 3.0, rmax of 0.3 and Kosambi mapping function was used in MapDisto v1.7.7.0.1.1 to generate this map. The cM score does not correspond to the iSelect consensus map position provided by Muñoz-Amatriaín et al., 2014. The red color represents the Q21861 introgression region containing RMRL. The green arrow represents the position of RMRL (obtained using physical position (Table 3.2)). All the markers within the red colored region are monomorphic for Q2186 like allele.

Table 3.2. Genetic and physical location of markers associated with *Rrr1* and *Rrr2*

iSelect Marker	Chr[a]	cM[b]	cM[c]	Bp Start[d]	Bp End[d]
11_20551	5H	130.93	N/A	608536936	608536936
11_10217	5H	144.86	139.24	623995670	623995670
11_10741	5H	149.41	144.65	631586711	631586711
11_21018	5H	153.74	N/A	636679883	636679883
12_30162	5H	156.7	N/A	638951179	638951179
RMRL[h]	5H	-	-	640813520	640817077
11_20826	5H	161.41	N/A	642764921	642764921
11_21419	7H	0	N/A	737146	737146
12_30880	7H	63.28	54.39	67729137	67729137

[a]Chromosome assignment based on iSelect consensus map (Muñoz-Amatriaín et al., 2014) and POPSEQ map (Mascher et al., 2013) and the IBSC v2 barley genome sequence (Mascher et al., 2017)
[b]Chromosomal position based on iSelect consensus map developed by Muñoz-Amatriaín et al. (2014)
[c]Chromosomal position based on POPSEQ map developed by Mascher et al. (2013)
[d]Physical position in IBSC v1 genome sequence of barley cultivar Morex (Mascher et al., 2017)

Even though all the markers used to construct linkage map were from the chromosome 5H, MapDisto generated two different linkage group due to the region distal of *Rrr1* being monomorphic or fixed for Q21861 like haplotypes because of the RMRL introgression into the Pinnacle RMRL-NIL.

Genetic mapping of *Rrr2*

The genetic mapping of gene segregating for HKHJC resistance in Pinnacle RMRL-NIL/Q21861 RIL identified two regions associated with *Rpg1* resistance pathway. One of the region was mapped at the same region carrying *Rrr1* (Figure 3.4A), while another region was mapped in telomeric region of barley chromosome 7H, flanked by markers 11_21419 (0 cM iSelect consensus map position) and 12_30880 (63.28 cM iSelect consensus map position) (Figure 3.4B). This result confirmed that *Rrr1* is not only involved in *rpg4/Rpg5* mediated resistance, but also in *Rpg1* mediated resistance.

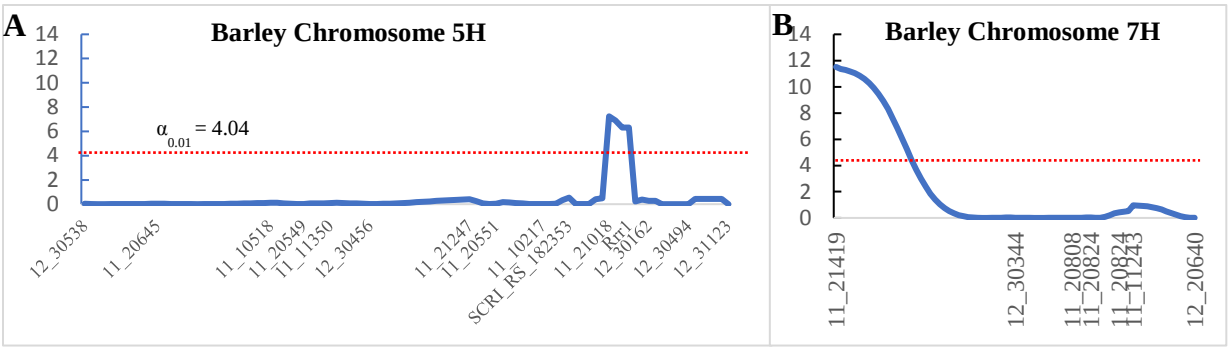

Figure 3.4. Genetic mapping of *Rrr1* and *Rrr2* using the composite interval mapping in Qgene v4.3.0. Two different regions were mapped as a region containing gene required for *Rpg1* mediated resistance pathway. (A) The genetic mapping showing that one of the gene required for *Rpg1* mediated resistance maps to same region as *Rrr1* (Figure 3.3). (B) A second gene involved in *Rpg1* mediated resistance mapped to the telomeric region of barley chromosome 7H, designated *Rrr2* delimited by iSelect marker 11_21419 and 12_30344 spanning 63.28 cM. The presence of two gene involved in *Rpg1* mediated resistance was validated by a Pearson's chi-square ($\chi 2$) goodness of fit test (Table 3.1).

Discussion

The emergence of the highly virulent *Pgt* race TTKSK and subsequent reports of significant barley and wheat yield losses due to disease epidemics caused by this race and its lineage on two continents leaves North American barley production vulnerable if it is introduced (Singh et al., 2011; Steffenson et al., 2017). The only well characterized *Pgt* race TTKSK resistance in barley that provides effective resistance to this virulent race is the *rpg4*-mediated resistance locus (RMRL) which requires the concerted action of three tightly linked genes (Brueggeman et al., 2008; Steffenson et al., 2009). The availability of *Rpg5*-specific diagnostic markers has made it possible to efficiently introgress RMRL, which contains the functional *Rpg5* allele, into elite barley backgrounds (Derevnina et al., 2014). In this study, a combination of two dominant markers, Rpg5-LRK and Rpg5-LRK/PP2C, which differentially amplify 840 bp and 1046-bp of genomic DNA from *Rpg5-STPK* and *rpg5-PP2C* alleles, respectively. The region amplified by Rpg5-LRK spans the LRR to STPK domain from the functional *Rpg5-STPK* alleles

and Rpg5-LRK/PP2C amplifies the region from the LRR to PP2C domain of the non-functional *rpg5*-PP2C alleles to detect the presence/absence of RMRL for MAS. Since, the elite 2-row malting varieties Conlon and Pinnacle have *rpg5* alleles that belong to either the group 2 or group 3 susceptible genotype that carry the *rpg5* PP2C domain (Arora et al., 2013), these primer combinations (Rpg5-LRK and Rpg5-LRK/PP2C) allowed us to successfully develop Pinnacle and Conlon RMRL-NILs containing the functional Q21861 RMRL. The Conlon RMRL-NIL exhibited resistance response comparable to Q21861, while the Pinnacle RMRL-NIL remained as susceptible as Pinnacle suggesting the presence of an additional functionally polymorphic gene between the resistant barley accession Q21861 and the susceptible barley variety Pinnacle that is required in addition to RMRL for stem rust resistance.

To identify the additional gene required for *rpg4*/*Rpg5*-mediated resistance, designated Required for *rpg4/Rpg5*-mediated resistance 1, present in Q21861, the Pinnacle RMRL-NIL was crossed with Q21861 to develop a RIL mapping population that was fixed for both RMRL as well as the *Rpg1* stem rust resistance gene. Since, Q21861 and the Pinnacle RMRL-NIL share identical *Rpg1* and *Rpg5* alleles, the loci segregating in the RIL mapping population will only be associated with the additional gene/s required for stem rust resistance, other than *Rpg1* and *Rpg5*.

The disease response for *Pgt* race QCCJB on the Pinnacle RMRL-NIL/Q21861 RIL mapping population segregated as a single dominant gene that mapped to the sub telomeric region of the long arm of barley chromosome 5H, ~5 cM proximal to RMRL (Figure 3.3). Similar to the need for *Rrr1* to mediate RMRL resistance in the Pinnacle background, the segregating disease response for *Pgt* race HKHJB, *Rpg1* specificity, also mapped to the *Rrr1* region. However, an additional highly significant region was also detected on the telomeric region of the short arm of barley chromosome 7H, that was designated as *Rrr2*. These

observations confirmed that *Rrr1* is required for both RMRL- and *Rpg1*-mediated resistance, while *Rrr2* is involved only in *Rpg1* resistance pathway (Figure 3.5).

	Haplotype	QCCJB	HKHJC
Q21861	RMRL+, *Rrr1+*, *Rpg1+*, *Rrr2+*	Resistant 	Resistant 
Pinnacle	rmrl-, *rrr1-*, *Rpg1+*, *rrr2-*	Susceptible 	Resistant 
Pinnacle NIL	RMRL+, *rrr1-*, *Rpg1+*, *rrr2-*	Susceptible 	Susceptible 
RIL-Type1	RMRL+, *Rrr1+*, *Rpg1+*, *Rrr2+*	Resistant 	Resistant 
RIL-Type2	RMRL+, *rrr1-*, *Rpg1+*, *Rrr2+*	Susceptible 	Resistant 
RIL-Type3	RMRL+, *Rrr1+*, *Rpg1+*, *rrr2-*	Resistant 	Resistant 
RIL-Type4	RMRL+, *rrr1-*, *Rpg1+*, *rrr2-*	Susceptible 	Susceptible 

Figure 3.5. Seedling assay using *Pgt* race QCCJB and HKHJC showing disease response on barley containing different combination of RMRL, *Rpg1*, *Rrr1* and *Rrr2* alleles. QCCJB is virulent on *Rpg1* and HKHJC is virulent on RMRL. Pinnacle is the recurrent parent and Q21861 is the RMRL donor of the Pinnacle RMRL-NIL. Four different allelic combinations of the two additional genes required for stem rust resistance, *Rrr1* and *Rrr2*, occur in the Pinnacle RMRL-NIL/Q21861 progeny. *Rrr1* is required to confer RMRL mediated QCCJB resistance (Q21861, RIL-Type1 and RIL-Type2). *Rpg1* mediated resistance is independent of *Rrr1* and *Rrr2* in absence of RMRL (Pinnacle). The introgression of RMRL into the Pinnacle background necessitates the presence of either a functional *Rrr1* or *Rrr2* to confer *Rpg1* mediated HKHJC resistance (Q21861, RIL-Type1, RIL-Type2, RIL-Type3). *Rpg1* mediated HKHJC resistance in RIL-Type1 and RIL-Type1 shows that *Rrr1* and *Rrr2* have a complementary gene function in the *Rpg1*-mediated resistance pathway.

However, the segregation ratio for resistance to susceptibility in response to HKHJB suggested that *Rrr2* is complementary to *Rrr1,* and either *Rrr1* or *Rrr2* is enough for *Rpg1* resistance (Table 3.1). Based on these observation, it can be speculated that the Pinnacle RMRL-NIL recovered both the non-functional allele of *Rrr1* and *Rrr2*, thus failed to confer RMRL- or *Rpg1*-mediated stem rust resistance. Since the Pinnacle RMRL-NIL obtained, non-functional *Rrr1* and *Rrr2* alleles, it is apparent that Pinnacle and Q21861 have functionally polymorphic *Rrr1* and *Rrr2* alleles (Figure 3.5). Thus, the *Pgt* race HKHJC and QCCJB resistance segregate in the Pinnacle RMRL- NIL/Q21861 RIL mapping population.

It was established that the Pinnacle RMRL-NIL contains non-functional *Rrr1* and *Rrr2* alleles thus Q21861 carries dominant functional *Rrr1* and *Rrr2* alleles, and Pinnacle contains both non-functional allele of these genes. Interestingly, Pinnacle confers *Rpg1*-mediate resistance despite lacking functional *Rrr1* or *Rrr2* suggesting that *Rpg1*-mediated resistance is independent of *Rrr1* and *Rrr2*, in the absence of RMRL. Thus, the introgression of RMRL in barley genomes necessitates the need for *Rrr1* or *Rrr2* to function suggesting that the two resistance mechanisms certainly have some level of interaction. This has also been exemplified by irradiation induced mutants that disrupt both RMRL and *Rpg1*-mediated resistance as well (Solanki et al., unpublished).

Typically, a plant exhibits a two-tiered immune response, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) in response to an invading pathogen (Jones and Dangl, 2006; Thomma et al., 2011). The PTI responses are activated by pattern recognition receptors (PRRs), through the recognition of conserved microbial structural molecules, like bacterial flagellin, or fungal chitin. ETI response generally involve nucleotide binding–leucine-rich repeat (NLR) type R-proteins that recognize pathogen

proteinaceous effectors and avirulence gene products that trigger a resistance response. The ETI response triggered by the recognition of a pathogen avirulence gene by its cognate host *R* gene also activates a signal transduction network that involves numerous signaling genes to differentially regulate signaling molecules for the execution of resistance response, typically a hypersensitive response (HR). Genetics and mutational analysis in different plant pathosystem of barley, tomato and Arabidopsis have deciphered the existence of such signaling genes that are quintessential for activating a *R*-gene mediated defense response (Torp and Jørgensen, 1986; Freialdenhoven et al., 1994; Aarts et al., 1998; Feys and Parker, 2000; Glazebrook, 2001). The mutational studies utilizing fast neutron irradiated Arabidopsis ecotype Col-1 produced mutations in the *nonrace-specific disease resistance*, *NDR1*, and *enhanced disease susceptibility*, *EDS1* genes which showed their imperative roles in conferring disease resistance to biotrophic oomycetes and bacterial pathogens conferred by distinct classes of *R* genes (Aarts et al., 1998). Another study using barley mutants showed a role of two genes *Rar1* and *Rar2* in executing a race specific resistance by the gene *Mla12* against the fungal pathogen *Erysiphe graminis* f. sp. *hordei* (Torp and Jørgensen, 1986; Freialdenhoven et al., 1994). A mutagenesis study in tomato identified a gene *Rcr3* that is required for *Cf-2* mediated leaf mold resistance caused by *Cladosporium fulvum* (Dixon et al., 2000).

Previously, two studies utilized fast neutron irradiation of barley variety Morex produced a mutant, Required for *P. graminis* resistance, Rpr1, that disrupted *Rpg1*-mediated resistance. Morex is a six-row malting barley cultivar release from University of Minnesota that carries *Rpg1* (Kleinhofs et al., 1993). Zhang et al. (2006) characterized *Rpr1* utilizing a Q21861/*Rpr1* mutant mapping population and mapped the gene to a region of barley chromosome 4H that placed *Rpr1* between the flanking markers Adh4 and ABA003. A microarray analysis of the

mutants and wild type identified three deleted genes in that region, and a putative serine/ threonine protein kinase-like protein was considered as a strong candidate for *Rpr1* gene.

Another study by Gill et al. (2016) used gamma-irradiation to develop six independent mutants in the cv Morex background designated *Rpr2-7* that also compromised *Rpg1*-mediated resistance. Gill et al. (2016) utilized a Q21861 X *rpr2* mutant F_2 population to map a region of barley chromosome 6H that contained the *rpr2* mutated gene. However, none of these studies reported the regions on chromosome 5H and 7H containing the *Rrr1* and *Rrr2* genes reported here thus these are novel genes that function in stem rust resistance pathway/s. Also, these studies utilized irradiation to produce artificial functional polymorphism suggesting that these genes involved in the *Rpg1*-mediated resistance pathway are probably are conserved signaling components in the barley genomes. However, *Rrr1* and *Rrr2* are functionally polymorphic in the primary barley germplasm pool and more importantly in the Midwestern breeding programs thus our study is important to address a critical issue that could deem the pyramiding schemes of *Rpg1* and RMRL futile. Several cases were reported from the pacific north-west barley breeding program where the introgression of RMRL led to a loss of resistance in barley cultivars (Pers. Comm Pat Hayes, Senior Barley Breeder, OSU). This suggests that the finding from our study might lead us to identify some diagnostic markers that could be used in combination with *Rpg5* specific markers for effective marker assisted selection.

To identify candidate genes residing within the mapped regions that represent candidate *Rrr1* and *Rrr2* genes, we can mine the gene models available from the recently released IBSC (International Barley Genome Sequencing Consortium) barley RefSeq v1(http://webblast.ipk-gatersleben.de/barley_ibsc/). A search of the Rrr1 region delimited by the iSelect markers 11_10741 and 12_30162 identified a total of 155 high confidence genes Likewise, 1064 high

confidence gene were identified at the 63cM region delimiting *Rrr2*. In order to narrow the region delimiting these gene and delimit candidate genes further, the RIL population will be genotyped with the barley 50K iSelect SNP array in near future (Bayer et al., 2017). Also, the saturation of the regions harboring *Rrr1* and *Rrr2* will allow us to identify a diagnostic marker for the *Rrr1* and *Rrr2* regions that could be used in MAS.

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CHAPTER 4. GENETIC MAPPING OF UG99 ADULT PLANT RESISTANCE CONFERRED BY SWISS LANDRACE HV645 AND ITS PYRAMIDING WITH MALTING BARLEY CULTIVARS

Abstract

Stem rust caused by *Puccinia graminis* f. sp. *tritici* is an economically important disease in wheat and barley. *Rpg1* is the only deployed stem rust resistance gene in north American barley that provides resistance to majority of north American *Pgt* races. However, the detection of the *Rpg1* virulent race QCCJB race TTKSK and its lineages threatens the entire north American barley production. The *rpg4*-mediated resistance locus (RMRL) only known and well-characterized source that confers resistance to *Rpg1* virulent races. It is imperative in a resistant breeding program to identify novel sources of resistance to avoid a directed selection pressure on *Pgt* that would cause to evolve virulence against a deployed single resistance gene. A preliminary seedling assay identified a swiss barley landrace Hv645, that carries a non-functional *Rpg5* allele to confer a seedling resistance to TTKSK. To map the gene associated with *Hv645* mediated TTKSK resistance a recombinant inbred line (RIL) mapping population was developed from a Swiss 645/Harrington cross. The RILs were evaluated in Njoro, Kenya during the 2015 and 2016 field season. A TTKSK adult plant resistance gene, provisionally designated *RpgHv645* was mapped distal of RMRL and delimited to an 11cM region in the sub-telomeric region of the long arm of barley chromosome 5H. Two previous independent studies in past, one utilizing the world barley core collection and another utilizing a biparental mapping population from Swiss landrace Hv602/Steptoe identified significant markers 11_10236 and11_20536, respectively that were associated with TTKSK and mapped to the region in barley chromosome 5H containing *RpgHv645*.

Introduction

Barley (*Hordeum vulgare L.*) is the most widely adapted cereal crop in the world (Horsley et al., 2009). It is grown worldwide and is primarily used as malt to produce beer and spirits and for livestock feed. The United States ranks 7th in global barley production and mainly grows malting barley for beer production (USDA, 2018, Case et al., 2018). The northern Great Plains of the US dominates barley production, with the majority produced in the states of North Dakota and Montana (USDA, 2017). Historically, the environment in the northern Great Plains was conducive to the development of stem rust epidemics prior to the deployment of genetic resistances in both barley and wheat. The northern great plains had suffered severed stem rust epidemics in barley prior to the 1940s (Steffenson, 1992) with barley yield losses of 15-20% reported due to stem rust epidemics in North America during the 1920s and 1930s, (Roelfs 1978; Steffenson 1992).

Wheat stem rust (or black rust) is a fungal disease adapted to both wheat and barley and is caused by the obligate biotrophic fungal pathogen *Puccinia graminis* f. sp. *tritci* Eriks. and E. Henn. (*Pgt*) (Roelfs, 1982). In barley, stem rust is also caused by the rye stem rust pathogen *Puccinia graminis* Pers.: Pers. f. sp. *secalis* Eriks. & E. Henn. (*Pgs*) and some isolates of *Puccinia graminis* f. sp. *avenae* the oat stem rust pathogen. Different races of stem rust can cause significant yield loss of both wheat and barley, which can be averted by deploying resistances in wheat and barley breeding programs (Roelfs, 1988; Singh et al., 2006; Singh et al., 2011). In barley, the effective control of wheat stem rust was achieved by the introduction of the Resistance to *Puccinia graminis 1, Rpg1,* resistance gene, which is now fixed in all Upper Midwest major barley cultivars (Steffenson, 1992). For 70 years, *Rpg1* was the only source of resistance deployed against stem rust races in North America, and proved effective resistance

against diverse races over a long period of time demonstrating remarkable durability. Later in 1989, a minor stem rust epidemic was reported in the USA and Canada that was caused by pathotype QCC (later designated as QCCJB) (Roelfs et al., 1991; Jin et al., 2008). The *Pgt* race QCCJB became the most widespread stem rust race posing a threat to North American barley production (Roelfs et al., 1993). In an attempt to identify a source of QCCJB resistance, 18,000 barley accession from the USDA National Small Grains collection (Aberdeen, ID) were screened (Jin et al., 1994a). The unimproved barley line Q21861 was identified as an excellent source of *Pgt* race QCCJB resistance providing effective protection at both the seedling and adult stages. The recessive and temperature sensitive gene conferring QCCJB resistance in line Q21861was designated as *rpg4*. A biparental mapping approach localized *rpg4* to the subtelomeric region of barley chromosome 5H (Jin et al., 1994b). The incomplete dominant rye stem rust resistance gene *Rpg5,* which confers resistance to *Puccinia graminis* f. s. *secalis* isolate 92-MN-90 cosegregated with *rpg4* in a low resolution genetic map on chromosome 5H (Sun et al., 1996). Later, a high-resolution map was utilized to clone the *Rpg5* rye stem rust resistance gene which encodes a typical nucleotide binding-leucine rich repeat (NLR) *R*-gene, but also contains an atypical serine threonine protein kinase (S/TPK) integrated domain at its C-terminus (Brueggeman et al., 2008). Interestingly, the high-resolution mapping of two populations, Q21861xHarrington and Q21861xMD2 delimited the *rpg4* and *Rpg5* genes within a 70Kb physical region (Brueggeman et al., 2008), which was later designated the *rpg4*-mediated resistance locus (RMRL) by Wang et al., (2013). RMRL also confers resistance to the rapidly evolving highly virulent *Pgt* race TTKSK (also known as Ug99) and its lineage (Steffenson et al., 2009, Singh et al., 2011).

The *Pgt* race TTKSK was first reported from a wheat field in Uganda, Africa in year 1999 (Pretorius et al., 2000). Since its detection, extensive monitoring across the African continent and wheat and barley growing regions across the Red Sea in the middle eastern region of Asia showed a rapid spread of this race and its evolving virulent lineages across the region, thus, *Pgt* race TTKSK poses a threat to wheat and barley production globally that have environments conducive to stem rust disease epidemics (Singh et al., 2011). In wheat, about 85 to 95% of breeding materials shows moderate to high susceptibility to this destructive pathotype (Singh et al., 2006; Singh et al., 2011). Likewise, about 93% of 2913 barley accession that were screened using the African pathotypes or TTKSK and its lineage races were moderately to highly susceptible to these races, including those lines containing *Rpg1* (Steffenson et al., 2017). The virulence of these evolving races on barley lines harboring the *Rpg1* gene warranted concerns that barley production was facing an imminent threat across North American, because commercial lines with *Pgt* race QCCJB and TTKSK resistance are not available. To face the threat imposed by these new races, breeders need to prioritize the pyramiding of novel effective *Pgt* race QCCJB and TTKSK resistances with *Rpg1* in their new barley varieties. However, currently in the primary barley germplasm pool the only well characterized source of QCCJB and TTKSK resistance is the RMRL.

The introgression of RMRL into *Rpg1* containing elite malting barley cultivars should provide effective resistance against the majority of North American stem rust races and rye stem rust races (Sun et al., 2005). However, growing monoculture varieties over vast acreages that contain a single stem rust resistance gene will impose selection pressure on the pathogen population which pressures the pathogen to eventually overcome the resistance, which usually occurs in a relatively short period of time (Moscou and van Esse, 2017). The monoculture of

barley varieties containing *Rpg1* and wheat varieties containing *Sr31* are classic examples of single races within diverse pathogen populations, QCCJB (virulent on *Rpg1*) and TTKSK (virulent on *Sr31*), that overcame important single resistances to endanger barley and wheat production on a large scale (Roelfs et al., 1991; Pretorius et al., 2000).

Rpg1 is still the only stem rust resistance gene deployed in barley cultivars in the Upper Midwestern US but the remarkable durability of this single resistance gene was in part achieved by the removal of the stem rust alternative host barberry (*Berberis spp.*). The barberry eradication program effectively eliminated the sexual stage of the pathogen in the US removing much of its potential to recombine new gene combinations and evolve new virulent isolates, effectively stabilizing the wheat stem rust population (Roelfs, 1982). The contribution of effectively reducing inoculum coming from wheat, which covers much more acreage in the *Puccinia* pathway, by pyramiding resistances in this major crop was also important for managing stem rust in barley. However, to stay vigilant in efforts to deploy broad resistance to known isolates and races within the US stem rust population pyramiding RMRL and *Rpg1* is an important first step. This combination should provide effective resistance since *Pgt* races virulent on both genes have yet to be identified in the US (Sun et al., 2005). However, it is not unlikely to face a scenario where *Pgt* races can evolve to overcome both *Rpg1* and RMRL. Also, since *rpg4*-mediated resistance *is* temperature sensitive, the current trend of rising temperature due to global warming might render this gene ineffective. During years with elevated temperatures the African races like TTKSK and its lineage could evoke stem rust epidemics if they reached the US (Jin et al., 1994b; Rosenzweig et al., 2001). However, unlike wheat, where approximately 39 stem rust resistance genes have been characterized that are effective against *Pgt* race TTKSK and its lineages, RMRL is the only effective source of resistance to these African isolates in barley.

Rpg1, Rpg2, Rpg3, rpg6, rpgBH, and *RpgU* are other available wheat stem rust resistance genes in barley, but none of these genes confer resistance to *Pgt* race TTKSK (Case et al., 2018). This situation leaves cultivated barley vulnerable to these virulent wheat stem rust races warranting focused efforts on the identification of new source of *Ug99* resistance in barley.

Several attempts were made to identify new sources of *Ug99* resistance in barley effectiveness like that provided by RMRL but concluded with limited success (Zhou et al., 2014; Turuspekov et al. 2016, Case et al., 2018). Zhou et al (2014) identified two resistance QTL, the seedling resistance QTL *Rpg-qtl-7H-12_30528* on chromosome 7H and an adult plant resistance (APR) QTL designated *Rpg-qtl-5H-11_11355* present on chromosome 5H. In their studies, even the most significant QTL, *Rpg-qtl-5H-11_11355,* only contributed 21.1% of the phenotypic variability, under high disease pressure, to 55% under low disease pressure. Another genome wide association study done by Case et al (2018) utilizing a USDA barley core collection identified the highly significant APR QTL *Rpg-qtl-5H-11_11355* and seedling resistance QTL *Rpg-qtl-5H-11_10236* on chromosome 5H at position 71-75cM and ~172cM (chromosome assignment and map position based on POPSEQ map of Mascher et al. (2013), respectively. APR QTL *Rpg-qtl-5H-11_11355* explained about 32-42% of QCCJB and TTKSK phenotypic variability, while the seedling resistance QTL *Rpg-qtl-5H-11_11355* only explained 10.8%. The RMRL was positioned at 152-168cm on barley chromosome 5H suggesting that these QTL are distinct from RMRL with Rpg-*qtl-5H-11_11355* located ~ 90 cM proximal and *Rpg-qtl-5H-11_10236 ~ 5* cM distal to RMRL. None of the QTLs identified in these studies confer a high level of resistance like the Q21861 RMRL seedling and APR, but rather confer a lower amplitude of stem rust resistance. However, these slow rusting or more basal level resistances in

barley could be utilized to pyramid with *Rpg1* and RMRL which could contribute to the effectiveness and durability of stem rust resistance in barley cultivars (Steffenson et al., 2017).

In the quest for novel resistance sources, we often times search the diversity pools beyond elite cultivated barley and explore landraces and wild grass relatives of the cultivated cereals which have proven to be invaluable sources of genetic variability and novel resistance genes (Marais et al., 2014; Steffenson et al., 2016). Adversity due to extreme geography and climates including broad spectra of biotic and abiotic stresses have exerted diverse selection pressure on wild and landrace barley populations that favor diverse superior performing individuals at each environment contributing to the diversifying selection (Steffenson et al., 2016). Landraces are the superior performing individuals that are selected by farmers to grow for future generations. Switzerland is among the oldest sites where farmers grew and selected diverse landraces of barley where about 700 barley landraces have been documented and preserved in the Agrospace genebank in Changins. Among these Swiss landraces, some have served as the original source of *Rpg1*-mediated stem rust resistance in North American cultivated barley. 'Chevron' and 'Peatland' are two selections from the Swiss landrace collection that were utilized as *Rpg1*sources and used to transfer this effective and durable resistant gene into malting barley cultivars in the Upper Midwestern United States and Prairie provinces of Canada, (Steffenson et al., 1992; Steffenson et al., 2016). To identify potential new sources of stem rust resistance, about 73 Swiss lines were subjected to screening for seedling resistance using *Pgt* races QCCJB, TTKSK and HKHJC (Steffenson et al., 2016). About 43% of the landraces showed resistance to *Pgt* races QCCJB and TTKSK, however most of the resistance was conferred by the well characterized *rpg4/Rpg5* locus. In the study by Steffenson et al (2016), the Swiss line Hv645 showed a susceptible reaction to *Pgt races* QCCJB, TTKSK and HKHJC.

However, preliminary assay of Swiss Hv645 for seedling resistance using TTKSK showed resistance response in some of the trials (Brian Steffenson, unpublished data).

Swiss landrace Hv645 belongs to a group 1 susceptible genotypes (groupings were based on RPG5 amino acid sequences (Arora et al., 2013) that contains a non-functional RPG5 protein resulting from a frameshift caused by the insertion of single cytosine in first exon. Thus, the observation of Ug99 resistance in Swiss Hv645 in some of the trials suggested a resistance response from this line was independent of RMRL. Based on this preliminary observation, we utilized Swiss Hv645 to develop a bi-parental recombinant inbred lines (RIL) mapping population by crossing with barley cultivar Harrington. The objectives of this study were, a) utilize this Swiss Hv645/Harrington RIL mapping population to map the adult plant resistance (APR) for Ug99 conferred by Swiss Hv645, provisionally designated *RpgHv645*, and b) exploit the marker closely linked to *RpgHv645* to initiate a gene pyramiding scheme in north American malting barley.

Materials and methods

Development of RIL mapping population

The RIL mapping population was developed by crossing a moderately resistance swiss landrace Hv645 with the universally susceptible barley cultivar Harrington which is susceptible to *Pgt* race TTKSK. Hv645 is a two-rowed swiss barley landrace that was originally collected from a small village named Scuol located in the canton Graubünden (Steffenson et al., 2016). Though, Hv645 has been reported as a TTKSK susceptible landrace (Steffenson et al., 2016), a preliminary seedling assay of Hv645 for seedling resistance using TTSKK gave a resistance response. The seed increased from that Hv645 source with a resistance response was selected for crossing in the present study. The susceptible parent Harrington is a two-rowed malting barley

cultivar released by University of Saskatchewan in 1981 (Harvey and Rossnagel, 1984). Harrington is a group 3 susceptible genotype as described by Arora et al., (2013) that contains a non-functional *rpg5* allele that is predicted to encode a nonfunctional RPG5 protein with the C-terminal PP2C integrated domain in place of the Rpg5-STPK domain (Arora et al., 2013). The F_1 generated from the Swiss Hv645/Harrington cross was self-fertilized to generate 88 $F_{6:7}$ RILs, that were obtained from single F_2 individual by single-seed descent (Brim, 1966).

Field screening using TTKSK

The mapping population, parental lines Hv645 and Harrington, the *Pgt* race TTKSK resistant control Q21861 and susceptible control cv. Steptoe were planted in the International Stem Rust Nursery at the Kenyan Agricultural Research Institute in Njoro, Kenya in the years 2012 and 2013. Each year, about 10-15 seed of each individual from the RIL population, parents and controls were planted in two replicated plots in a randomized complete block design (RCBD). The parental lines and controls along with the Ug99 susceptible spreader rows were planted in the plots as explained by Zurn et al., 2014. The nursery was inoculated with *Pgt* race TTKSK and susceptible wheat spreader rows acted as an inoculum source to continue the polycyclic infection cycle on the experimental lines. The screening was conducted at the adult plant stage to assess adult plant resistance to *Pgt* race TTKSK. The stem rust disease severity data were recorded using a modified cobb scale (Peterson et al., 1948, McIntosh et al., 1995). The infection response (IR) were based on the size and type of the uredinia and categorized into resistant (R), moderately resistant (MR), intermediate (M), moderately susceptible (MS), and susceptible (S), or intermediate of any two categories (Roelfs et al., 1992, McIntosh et al., 1995). The weighted score of 22 modified cobb scale were taken as coefficient of infection as explained by Yu et al. (2011) and use for mapping the regions associated with Hv645 APR.

Statistical test

The Hv645/Harrington RILs were categorized into resistance and susceptible based on the weighted score of coefficient of infection. Only the individuals with weighted scores of coefficients of infection less than 4 were considered resistant. The Pearson's chi-square (χ^2) goodness-of-fit test was used to evaluate independence of segregation of resistance to susceptible phenotype (1 res: 1 sus) for the RIL population. The p-value associated with Pearson's χ^2 statistics was obtained using the CHISQ.TEST function in Microsoft Excel. The correlation between the weighted score of coefficient of infection for the individuals within the RIL population in any two different trails were also assessed using the CORREL function in Microsoft Excel.

Genotyping using PCR-GBS

The genotyping of the RILs and parental lines was conducted using a polymerase chain reaction-genotyping by sequencing (PCR-GBS) procedure. First, leaf samples were collected for DNA extraction. DNA isolation was performed using the procedure described by Richards et al. (2016). A marker panel consisting of 365 markers evenly distributed across the seven barley chromosomes (1 marker/~5cM) were utilized for PCR-GBS. The 365-marker panel was designed by Tamang (2017) based on the polymorphism between barley lines Tradition and Pinnacle. The library preparation for PCR-GBS was performed using the protocol provided by Richards et al. (2017). The library was sequenced using the Ion Torrent Personal Genome Machine instrument using an Ion 314 Chip (Life Technologies) using the manufacturers standard sequencing protocol.

SNP calling and genotyping

The low-quality reads and the 22-mer PCR adapter sequence flanking each SNP source sequence from each read was trimmed using CLC genomics workbench 8.0 (Richards et al., 2017). The quality reads were mapped to a custom reference fasta file containing the source sequences of each of the 365 marker panels which were downloaded from the Triticeae toolbox (T3) database (https://triticeaetoolbox.org/barley/) using the Burrows–Wheeler Aligner maximal exact match (BWA-MEM) algorithm (Li, 2013). The Genome Analysis Toolkit (GATK) Unified Genotyper tool was used with default setting for multi- sample Single Nucleotide PolymorphismS (SNPs) to identify the SNP in each sample. Only the SNP position reported in T3 database (https://triticeaetoolbox.org/barley/) for a given markers was used for further analysis. The allele frequency of each SNP for a given sample was calculated using an in house visual basic script as described by Sharma Poudel et al (unpublished) to determine the genotype of a sample at that marker position. The POPSEQ chromosomal position of the markers flanking *RpgHv645* were obtained using the POPSEQ map described by Mascher et al. (2013). The sequences of these markers and *Rpg5* gene was used to conduct a blast using Viroblast in the IPK Blast server to obtain their physical position on the IBSC v2 Morex barley whole genome sequence (Deng et al., 2007; Mascher et al., 2017; http://webblast.ipk-gatersleben.de).

Genetic mapping

The SNP markers in the genotyping data was assigned to a chromosome and a chromosomal position based on the iSelect consensus genetic map developed by Muñoz-Amatriaín et al. (2014). The genotypic data was manually modified to match the standard format for importing marker, map and trait data into a publicly available QTL mapping software QGene v.4.3.10 (Joehanes and Nelson, 2008). Individual replication data from both years along with the

overall mean of the replicated data were evaluated using the generalized linear model of multiple interval mapping (MIM-GLZ) for single trait in QGene v.4.3.10. A 1000 permutation test was conducted to obtain a LOD threshold for an experiment-wise significance level of 0.05.

Gene pyramiding

The markers flanking the Swiss Hv645 APR gene, *RpgHv645* was utilized to introgress *RpgHv645* into a Conlon near isogenic line (NIL) containing the *rpg4*-mediated resistance locus (RMRL) from the *Pgt* race TTKSK resistant line Q21861. Conlon is a two-row malting barley cultivar that contains *Rpg1* and was released in 1996 by the barley breeding program in North Dakota State university (NDSU) (http://www.ndsuresearch foundation.org/conlon). The Conlon RMRL NIL was developed as part of pyramiding scheme to develop a line containing both *Rpg1* and *rpg4/Rpg5* by crossing it with a Harrington/Q21861 NIL (HQ1) (Sharma Poudel, unpublished). HQ1 is a NIL containing RMRL that was identified from a high-resolution mapping population developed by Brueggeman et al. (2008).

An F_1 individual obtained from the Swiss Hv645/Conlon RMRLNIL cross was selfed to generate F_2 progeny. A PCR-GBS sequencing was done on 96 F_2 individuals, along with Q21861, HQ1, Harrington, Swiss Hv645 and the Conlon RMRL NIL were sequenced using five iSelect markers (11_20826, 11_10600, 12_11010, 12_11450 and 11_10310) that were distal to RMRL and flank the *RpgHv645 QTL*. The sequencing and genotyping of these individuals were done as explained above. The genotypic data of F_2 progenies were analyzed to identify individuals that contain recombination between the Q21861-like RMRL and Swiss Hv645 region carrying *RpgHv645* to identify gametes with RMRL and *RpgHv645* in coupling. RMRL specific amplicons were generated from genomic DNA of the selected F_2 using PCRGBS-C-insert F (5'-ACACTGACGACATGGTTCTACAGCAGTCCTGATTCCGCTTC-3') and PCRGBS-C-insert

R (5'-TACGGTAGCAGAGACTTGGTCTCCCTGGAAGACGGTGGTC-5') primer pairs. The primers were initially designed with an aim to utilize them in for genotyping individuals for presence/absence of c-insertion using PCR-GBS. However, in the present study these primers were used to generate amplicon for a few individuals using regular thermocycler. Polymerase chain reaction (PCRs) were performed using a Mastercycler Pro programmable thermocycler (Eppendorf, Hauppauge, NY, U.S.A.) with the parameters set at initial denaturation of 94°C for 10 min; followed by a touchdown step of 10 cycles of denaturation at 94°C for 20s and annealing at 62°C for 1 min, where temperature was decreased by 0.8 °C each cycle; followed by 20 cycles of denaturation at 94°C for 20s, annealing at 57°C for 1 min, and extension at 72°C for 1 min; ending with final template extension at 72°C for 3 min. The PCR reaction consisted of containing 10 ng of template gDNA, 1X GoTaq® Flexi Buffer (Promega, USA), 1mM $MgCl_2$, 0.15mM dNTP mix, 0.4 µM of each forward and reverse primer, 1.25u GoTaq® DNA polymerase in 20 µl reaction volume. This primer pair produces a 172 bp amplicon from the 1st exon of the nonfunctional *rpg5-STPK* allele designated as the group 1 susceptible *rpg5*. The group 1 susceptible alleles still contain the STPK integrated domain similar to the functional *Rpg5* alleles however contain a single C-insertion which results in a predicted frame shift and 217 amino acid truncated protein. The 172 bp amplicon produced with the PCRGBS-C-insert F/ PCRGBS-C-insert R primer pair allows for the detection of the presence/absence of the Swiss Hv645 like C-insertion in *Rpg5* allele that causes it to be non-functional. The C-insertion in the first exon of *Rpg5* was detected by sequencing the PCR amplicons using standard dideoxy Sanger sequencing (GenScript, Piscataway, NJ). The chromatograms for the *Rpg5* and *rpg5* C-insertion alleles were visualized in Finch TV ® v1.4.0. (http://www.geospiza.com/Products/

finchtv.shtml) and genotypes manually called to assess the quality of the sequencing data and base calls.

Result

Phenotypic evaluation

Swiss Hv645 was moderately resistant to TTKSK with an average weighted coefficient of infection score of 1.76 (S.D. ±1.17) (Table 4.1, Figure 4.1). Even though Swiss Hv645 showed an inconsistency on seedling test for Ug99, it consistently exhibited a moderately resistant response across all six trails performed in the *Pgt* race TTKSK nursery in Kenya for years 2013 and 2014. The susceptible parent Harrington, was only moderately susceptible with an average weighted coefficient of infection score of 5 (S.D. ±0.87) (Table 4.1, Figure 4.1). Q21861 was highly resistance exhibiting an average weighted coefficient of infection score of 0.44 (S.D. ±0.33) and Steptoe was highly susceptible with an average weighted coefficient of infection score of 15 (S.D. ±7.06) (Table 4.1, Figure 4.1).

Table 4.1. Reaction of parental lines and control to *Pgt* race TTKSK in field in Kenya at the adult plant stages across multiple replication and years

Line[a]	2012				2013			
	Rep 1		Rep 2		Rep 1		Rep 3	
	Rating[b]	CI[c]	Rating	CI	Rating	CI	Rating	CI
Q21861	TRMR	0.2	1MRMS	0.6	TRMR	0.2	5RMR	1
Steptoe	10S	10	10S	10	20MSS	18	30MSS	27
Hv645	1MR	0.4	5MR	2	5MSMR	3	5MSMR	3
Harrington	-	-	10MSMR	6	5MSS	4.5	5MSS	4.5

[a]- Q21861 (resistant control), Steptoe (susceptible control), Hv645 (resistant parent), Harrington (susceptible parent)
[b]-The modified cobb scale was used to record the disease severity of stem rust (Peterson et al., 1948, McIntosh et al., 1995). Base on the size and type of the uredinia, the infection response (IR) were categorized into resistant (R), moderately resistant (MR), intermediate (M), moderately susceptible (MS), and susceptible (S), or intermediate of any two categories (Roelfs et al., 1992, McIntosh et al., 1995).
[c]-The conversion used for calculating the coefficient of infection as recommended by Yu et al. (2011).

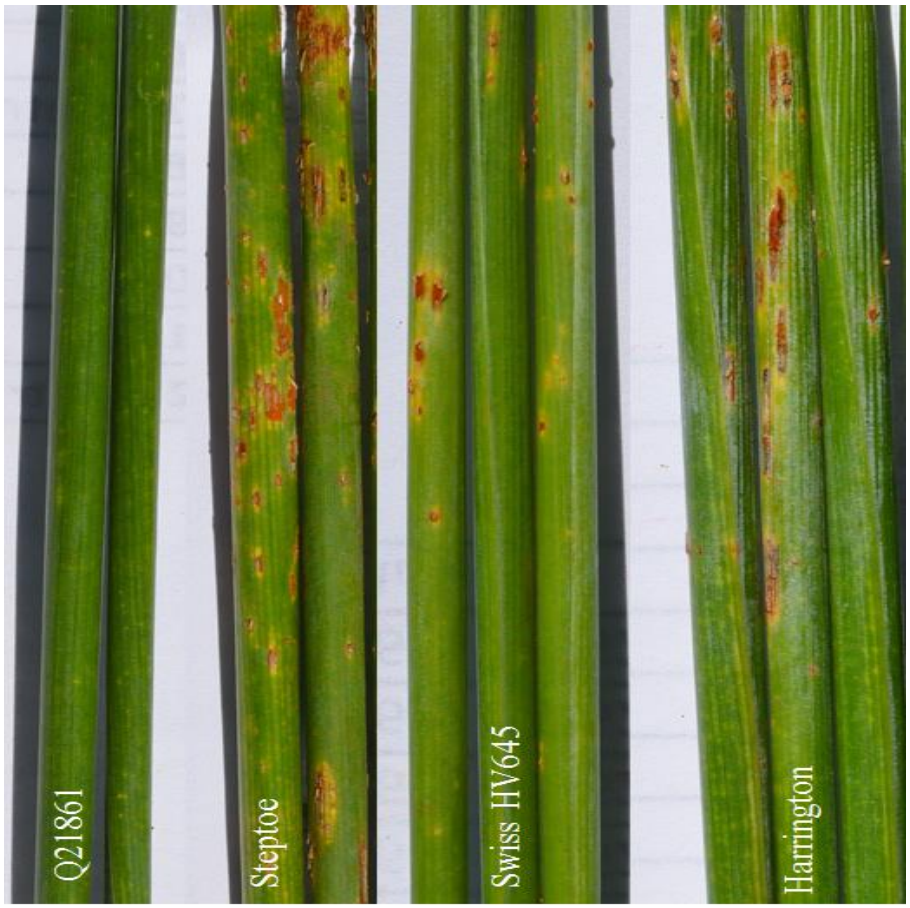

Figure 4.1. Representative stem rust disease phenotypes of Q21861 (resistant control), Steptoe (susceptible control), Hv645 (resistant parent), and Harrington (susceptible parent) to *Pgt* race TTKSK from stems collected from the stem rust disease nursery Njoro, Kenya. Q21861 was highly resistant, Steptoe was susceptible; while the parental lines, Swiss Hv645 was moderately resistant and Harrington was moderately susceptible

Table 4. 2. Correlation coefficients between the response of RILs in each trail to the *Pgt* race TTKSK

		2012		**2013**	
		Rep1	**Rep2**	**Rep1**	**Rep2**
2012	**Rep1**	1	0.13	0.15	-0.02
	Rep2	0.13	1	0.12	0.1
2013	**Rep1**	0.15	0.12	1	0.33
	Rep2	-0.02	0.1	0.33	1

Unlike the parental lines and the controls, the responses of the RILs varied across the trails (Appendix Table A19). The CORREL function in Microsoft Excel gave correlation

coefficient values for the weighted score of coefficient of infection between two trails (Table 4.2). None of them were strongly correlated, with ranges from Moderate (55%) to weak (-0.2239) correlation between two given trails (Table 4.2).

Mapping of *RpgHv645*

The Hv645/Harrington population segregated for 1 resistance: 1 susceptible ratio (χ^2 and P-value in Table 4.3) in all trials (except Rep 2 of year 2013), as expected for a monogenic inheritance in a RIL population.

Table 4.3. Chi-square goodness-of-fit test to assess the segregation of RIL for resistance to susceptibility reaction in each trial

Disease Response	No. of individuals			
	2012		2013	
	Rep1	Rep2	Rep1	Rep2
Resistance	41	37	22	32
Susceptible	39	35	54	39
Missing	6	14	10	14
χ^2 value	0.05	0	13.47	0.69
P-value[a]	0.82	0.81	0.0002	0.41

a- P-value > 0.05 represents that dataset that fit the Mendelian segregation ratio of 1:1 for resistance to susceptibility in RILs segregating for a single gene

However, only two trials (Rep 1 and 3 of year 2012) mapped *RpgHv645* and delimited it within an interval of 11cM in the telomeric region of barley chromosome 5H (Figure 4.2A). However, 2013 Rep2 also showed the QTL peak nearly at the significance level (Fig 4.2A). This QTL was given the provisional designation *RpgHv645* and is flanked by the iSelect SNP marker 11_10600 and 12_31352 at POPSEQ position 155.55cM and 166.67cm, respectively (POPSEQ positions are obtained from Mascher et al., 2013). The marker 12_11010 at POPSEQ position 158.89cM is the most significant *RpgHv645* SNP marker (Table 4.4).

Table 4.4. Genetic and physical location of markers associated with Hv645 APR in response to *Pgt* race TTKSK

Marker	Chr[a]	cM[b]	cM	Bp Start[d]	Bp End[d]	Year[e]	Rep1		Rep2	
							Add[f]	LOD[g]	Add	LOD
RMRL[h]	5H	-	-	640813520	640817077	-	-	-	-	-
11_20826	5H	161.41	N/A	642764921	642764921	2012	0.11	0.01	-0.43	0.20
						2013	0.84	0.22	-0.24	0.03
11_10600	5H	165.57	155.55	649232960	649232960	2012	0.11	0.01	-0.43	0.20
						2013	0.84	0.22	-0.24	0.03
12_11010	5H	168.72	158.89	651484482	651484482	2012	1.32	5.3*	-0.39	0.23
						2013	1.31	0.60	0.01	0.00
12_31352	5H	176.52	166.67	659736826	659736826	2012	-0.84	0.56	-0.43	0.36
						2013	1.55	0.88	-0.21	0.02
11_10310	5H	177.5	N/A	661443694	661443694	2012	-0.84	0.56	-0.43	0.36
						2013	1.55	0.88	-0.21	0.02

[a]- Chromosome assignment based on iSelect consensus map (Muñoz-Amatriaín et al., 2014) and POPSEQ map (Mascher et al., 2013) and the IBSC v2 barley genome sequence (Mascher et al., 2017)
[b]- Chromosomal position based on iSelect consensus map developed by Muñoz-Amatriaín et al. (2014)
[c]- Chromosomal position based on POPSEQ map developed by Mascher et al. (2013)
[d]- Physical position in IBSC v1 genome sequence of barley cultivar Morex (Mascher et al., 2017)
[e]- Two different years on which the RILs were screened in the wheat field of Kenya with *Pgt* race TTKSK
[f]- Additive effects for given marker in give replication and trails. Positive value means the susceptibly is due to the Harrington like allele and resistance is from Swiss Hv645 like allele
[g]- LOD score for the association between given genotype and phenotype at the give trail. A
[h]- The physical location of RMRL
*-Trails with LOD score > 2.44 (2.44 is LOD threshold obtained from 1000 permutation test for an experiment-wise error rate of 0.05) that mapped *RpgHv645* at the give marker location

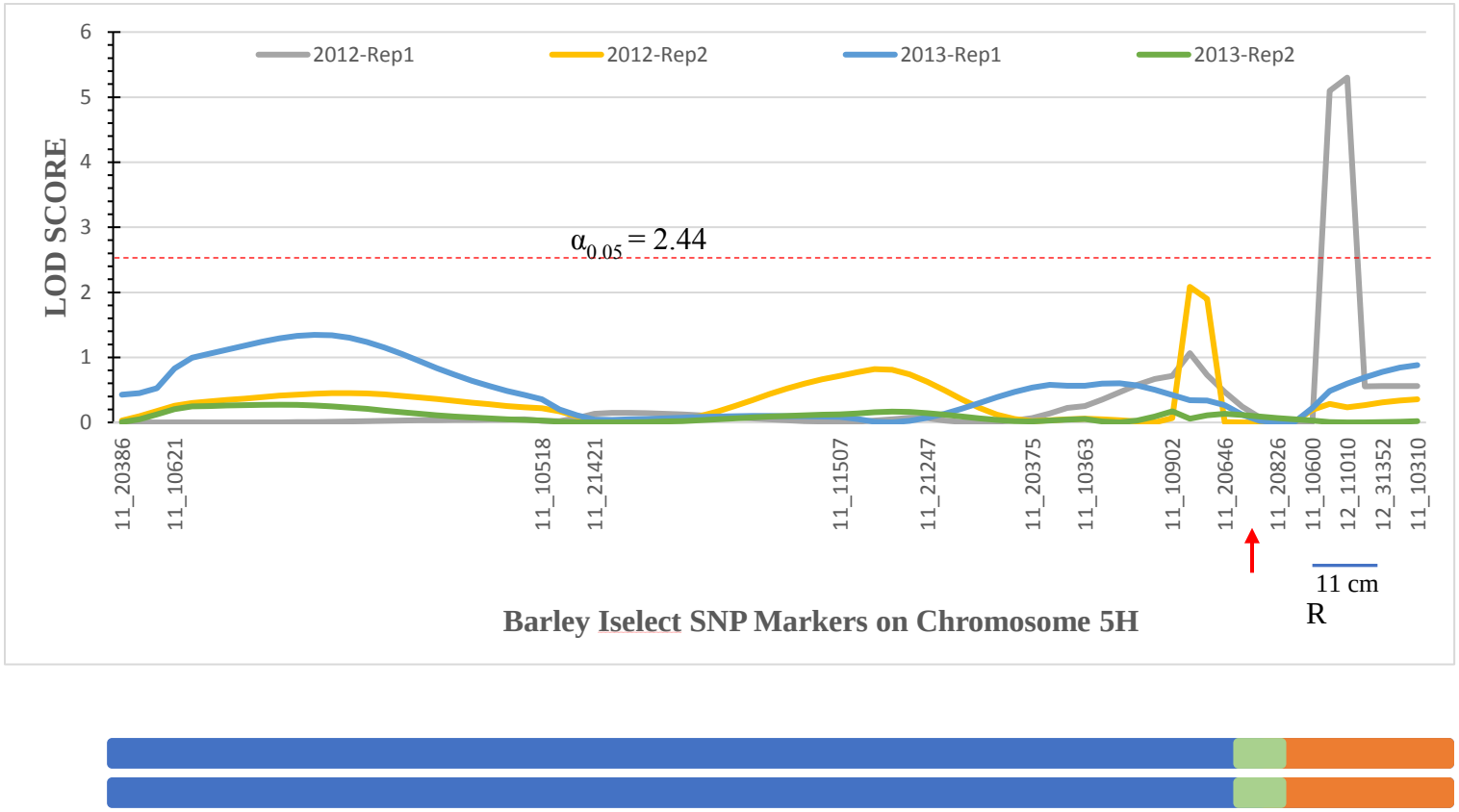

Figure 4.2. (A) Genetic mapping of Swiss Hv645 APR, *RpgHv645* using the generalized linear model of multiple interval mapping (MIM-GLZ) for a single trait. The SNP markers shown in the figure are polymorphic between Hv645 and Harrington and are located on chromosome 5H. *RpgHv645* is delimited within an 11cM interval, flanked by markers 11_10600 and 12_31352. The red arrow represents the approximate position of RMRL, showing that *RpgHv645* is distal to this locus. The red dotted line represents the experiment-wise significance level of 0.05 obtained from a 1000 permutation test. (B) Diagrammatic representation of chromosome 5H of the selected $F_{2:3}$ individual obtained from Hv645/Conlon RMRL NIL that was confirmed to have homozygous Q21861 like *Rpg5* allele and Hv645 region carrying *RpgHv645*. Orange color represent the Hv645 like region confirmed using PCR-GBS and the green color represent regions containing Q21861 like *Rpg5* confirmed using sequences of amplicon produced with PCRGBS-C-insert F/ PCRGBS-C-insert R primer pairs.

The additive effect value was used to predict the allele conferring resistance in Hv645/Harrington RIL mapping population. The positive value of the additive effects suggests that the increasing value (susceptible reaction) is contributed by Harrington like allele, and thereby confirming the resistance to TTKSK at adult plant stage in the RILs is contributed by the Hv645 like allele.

Gene pyramiding

The PCR-GBS data of 96 F_2 progeny obtained from the Hv645/Conlon RMRL NIL cross identified 5 F_2 individual with a swiss like allele for all five-iSelect marker spanning *RpgHv645* containing region (Table 4.5).

Table 4.5. Genotype of F_2 individuals from the Hv645/Conlon RMRL NIL cross selected for further screening in the RMRL-RpgHv645 pyramiding scheme

iSelect Marker	Hv645/Conlon NIL Selected F_2 individual # [a]					Conlon	Swiss 645	HQ1	Harrington
	17	46*	48*	59	62				
11_20826	AC	AA	AA	AC	AC	CC	AA	CC	AA
11_10600	GG	GG	GG	GG	GG	AA	GG	AA	GG
12_11010	GG	GG	GG	GG	GG	GG	GG	GG	GG
12_11450	TT	TT	TT	TT	TT	TT	TT	TT	TT
11_10310	GG	GG	GG	GG	GG	GG	GG	GG	CC

a-The F_2 individual were selected based on their genotype being either homozygous for Swiss Hv645 like allele or heterozygous at marker 11_20826 and distal to it. RMRL lies proximal to 11_20826 (Fig 2A). F_2 individuals, #46 and #48 (designated by an asterisk *) were selfed to obtain $F_{2:3}$ progeny from which $F_{2:3}$ individuals homozygous for the recombinant haplotype with the functional *Rpg5* and *RpgHv645* genes in coupling were selected.

The sequencing of amplicon produced using PCRGBS-C-insert F/ PCRGBS-C-insert R primer pairs confirmed a presence of functional *Rpg5* at heterozygous state in these 5 individuals (chromatograms of sequencing result shown in Appendix Figure B1). Two F_2 individuals, #46 (later designated 78.2 based on position in crossing block) and #48 (later designated 79.1) that were homozygous at iSelect marker 11_20826 were selfed to obtain $F_{2:3}$ progeny. Two $F_{2:3}$ progenies, 78.2.3 and 78.2.6 that were homozygous for functional *Rpg5* were identified by

sequencing PCR amplicon obtained using PCRGBS-C-insert F/ PCRGBS-C-insert R primer pairs. We thereby developed recombinant lines, 78.2.3 and 78.2.3that contains both functional *Rpg5* and *RpgHv645* gene in coupling phase (Figure 4.2B), and thus can be used in future breeding project to pyramid both genes in elite Midwestern malt barley cultivars

Discussion

The Northern Great Plains of the United States is a hub for malt barley production and North Dakota is one of the top three barley producing state in the US (USDA, 2017). This region has the environmental conditions making production vulnerable to stem rust epidemics, yet this historically devastating disease has been effectively controlled by the deployment of the stem rust resistance gene *Rpg1* in all commercial malting barley varieties released in the region (Roelfs, 1978; Steffenson, 1992). Apart from *Rpg1*, the *rpg4*-mediated resistance locus (RMRL) is the only other well characterized gene/s contributing resistance to the majority of know *Pgt* races, including the virulent *Pgt* race TTKSK (Steffenson et al., 1992; Steffenson et al., 2009) that emerged nearly two decades ago in Uganda. Currently there have been no commercial varieties released containing the RMRL *Pgt* race TTKSK resistance, but multiple breeding program are initiating the introgression of *rpg4/Rpg5* into their barley breeding programs thus barley varieties with TTKSK resistance should be available if race or its lineage did arrive in the US. The combination of *Rpg1* and *rpg4/Rpg5* will provide effective resistance against all known north American *Pgt* races and the migrating race of TTKSK and its evolving lineages. However, various reports of new lineage of TTKSK and other virulent *Pgt* races insinuates a scenario where a novel race could overcome both *Rpg1* and *rpg4/Rpg5* (Singe et al., 2011). Thus, the best strategy would be to pyramid other *Rpg* genes that provides novel resistance to *Pgt* isolates including Ug99, with *Rpg1* and *rpg4/Rpg5* to provide more durability (Steffenson et al., 2017).

In this study, we were able to identify a source of resistance in the Swiss landrace Hv645 that provide some level of resistance at adult plant stage against *Pgt* race TTKSK. However, despite a consistent disease response from the parental lines (Hv645 and Harrington) and the resistant and susceptible controls, Q21861 and Steptoe, respectively, we observe significant variation in the response of the RIL population individuals screened in a *Pgt* race TTKSK field nursey in Njoro, Kenya. Yet, variation is to be expected when field assays are conducted, especially when dealing with a resistance QTL. This variability is most probably due to the genotype by environment interaction for APR conferred by a minor QTL. Hv645 is only moderately resistance, and interestingly the susceptible parent Harrington was only moderately susceptible, compared to susceptible control Steptoe. This suggest that slight variations in inoculum load and other environmental condition could shift the disease response of RILs in multiple trails. Due to these cause of variability, we could map we could map *RpgHv645* only from one out of four.

The region associated with Hv645 APR is closely linked to RMRL. However, both RIL population parental lines, Hv645 and Harrington, carry different non-functional r*pg5* alleles (Arora et al., 2013), with Hv645 carrying the group 1 susceptible allele with a C insertion in exon 1 and Harrington containing the group three susceptible allele that encodes a full length NBS-LRR-PP2C gene but is susceptible due to the PP2C integrated domain in place of the STPK domain which is required for resistance. Thus, the segregation for *Pgt* race TTKSK resistance in this population was independent of RMRL. Since, no markers associated with RMRL were available in the 365 markers panel used to genotype the population, we utilized the recently available Morex barley genome sequence to pinpoint the physical position of RMRL and markers from the 50k SNP array to develop makers flanking and within the *RpgHv645* region

(Table 4.4). We confirmed that RMRL is at least 8.5MB proximal to the region carrying *RpgHv645.* A recent association mapping study reported by Case et al. (2018) mapped a RMRL independent *Pgt* race TTKSK seedling resistance QTL, *Rpg-qtl-5H-11_10236,* to chromosome 5H at the same region containing *RpgHv645.* Two markers, 11_10236 and SCRI_RS_167103 (both at POPSEQ position 160.48cM) that represent the *Rpg-qtl-5H-11_10236* is only 1.59 cM distal to the marker 12_11010, which is closely associated with *RpgHv645*. In terms of physical position, *Rpg-qtl-5H-11_10236* is only ~5MB away from iSelect marker 12_11010, suggesting that the *RpgHv645* and *Rpg-qtl-5H-11_10236* QTL may have the same underlying resistance gene. Another study was done to characterize the inheritance of TTSKS resistance in four swiss landraces (Hv501, Hv545, Hv602 and Hv612) by utilizing a biparental mapping population developed by crossing these landraces with susceptible cv. Steptoe (Mamo et al., 2015). The segregating F_3 derived from these crosses showed a monogenic inheritance of resistance for Hv501, Hv545 and Hv612, while the segregation Hv602/Steptoe derived F_3 suggested involvement of more than one gene. Alleles test confirmed the resistance confirmed by these swill line was derived from gene residing at or near RMRL (Mamo et al., 2015). However, for Hv602, another source of resistance is suspected to be derived from the QTL near iSelect marker 11_21061 (99.39cM, cM position based on iSelect position provided by Muñoz-Amatriaín et al., 2014) and/or 11_20536 (168.44cM) on chromosome 5H. Interestingly, 11_20536 is ~0.28cM proximal to 12_11010, a marker closely associated with *RpgHv645*. The world barley core collection utilized by Case et al., (2018) contained 39 barley cultivars and landraces from Switzerland the same region that Swiss Hv645 originated suggesting that this same source of resistance could be in the AM panel from Swiss barley as well as other geographically diverse regions. Thus, the AM mapping (Case et al., 2018) and bi-parental mapping (Mamo et al., 2015)

adds further support to the *RpgHv645*mapping. *RpgHv645* is an APR and *Rpg-qtl-5H-11_10236* is seedling resistance, suggesting that *RpgHv645* might be possibly involved in both APR and seedling resistance. However, Seedling testing of Sw645 at the CDL BL3 facility showed two early seedling assays with *Pgt* race TTKSK having nice seedling resistance and the later assays showing susceptibility. One explanation for this shift is that the original lines tested earlier where heterozygous for RpgHv645 and our single seed decent to clean up the background resulted in the homozygous RpgHv645 whereas the single seed descent at the University of Minnesota resulted in the selection of lines homozygous for rpgHv645. However, since the RIL population was not screened for seedling *Pgt* race TTKSK resistance, we are not able to confirm the hypothesis that RpgHv645 is effective at both the seedling and adult plant stages. However, *Rpg-qtl-5H-11_10236* identified by Case et al. (2018) and a QTL at iSelect marker 11_20536 identified in the Steptoe/Hv602 bi-parental mapping validates the presence of a novel source of seedling *Pgt* race TTKSK resistance at the same position as our APR resistance in the Swiss landrace Hv645 which in earlier assays showed seedling *Pgt* race TTKSK resistance as well.

The second objective of this study was to utilize the markers closely linked and flanking the *RpgHv645* QTL/gene to pyramid this APR gene with the stem rust resistance genes *Rpg1* and *rpg4* in elite Upper Midwest malting barley lines. The Conlon RMRL NIL was a suitable recipient as it was already developed as a part of pyramiding schemes to combine *Rpg1* and *rpg4/Rpg5* in a single malting cultivar background and was shown to be resistant to majority of known North American *Pgt* races including *Pgt* race QCCJB which overcame *Rpg1*-mediated resistance. The Conlon RMRL NIL was also shown to be resistant to *Pgt* race TTKSK in field studies conducted in Njoro, Kenya (Sharma Poudel, Thesis). In this study, we showed that the five markers (11_20826, 11_10600, 12_11010, 12_11450 and 11_10310) can be effectively

utilized to select for backcross individuals carrying the *RpgHv645* QTL. We also developed a donor line that carries both homozygous Q21861 like *RMRL* and Swiss Hv645 *RpgHv645* in the coupling phase, along with *Rpg1*. We are already utilizing this line to pyramid RMRL, *RpgHv645* and *Rpg1* into elite malting barley cultivars, like Pinnacle and ND-Genesis.

In summary, Swiss landrace Hv645 confers a moderate resistance to *Pgt* race TTKSK at adult plant stage. The gene governing this APR was mapped to the telomeric region of barley chromosome 5H, just proximal to RMRL. We identified diagnostic markers flanking *RpgHv645* that can be used to introgress this APR resistance QTL into other commercial barley cultivars. Pyramiding of *RMRL, Rpg1* and *RpgHv645* could increase the durability and effectiveness of stem rust resistance across wide range of *Pgt* isolates.

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CHAPTER 5. SUMMARY AND FUTURE DIRECTIONS

In summary, the studies in this doctoral dissertation was able to decipher the underlying genetic factors, in both host (barley) and pathogen (*Pgt*) that modulate the only available and well-characterized source of TTKSK resistance, *rpg4*-mediated resistance locus (RMRL). Prior to this study, RMRL based resistance in barley was based on two hypotheses; a) the presence/absence of an avirulent effector Avr*rpg4/5* would determine *Pgt* virulence/ avirulence in RMRL containing barley lines; and b) the introgression of full length RMRL would be adequate to gain *rpg4*-mediated stem rust resistance. However, the data generated from the study in chapter 1 suggests that *Pgt* evolved a virulent effector to gain virulence on *rpg4*-mediated resistance, and the avirulence gene may represent a conserved effector that is essential for the virulence function. Based on this study, the future direction could be shifted toward characterizing the putative virulence effectors and utilize molecular tools like Yeast-two-hybrid to go after an avirulent effector.

The second chapter addresses a problem that existed with the introgression of RMRL to gain an effective *rpg4*-mediated resistance. Since, RMRL is the only known source of TTKSK and QCCJB resistance and TTKSK is a global threat to food security, attempts were made to introgress the full-length RMRL into elite malting barley cultivars. However, the gain of resistance after the integration of RMRL in different barley background were only partially successful. With the genetic mapping approach utilized in this doctoral dissertation, a gene *Rrr1* was mapped in an elite barley genetic background that is linked to but distinct from RMRL but is required for RMRL-mediated resistance and *Rpg1*-mediated resistance in the present of RMRL. A second gene, *Rrr2* was also mapped on barley chromosome 7H that has complementary gene function of *Rrr1* but is only involved in *Rpg1*-mediated resistance.

The third chapter of this dissertation addressed the problem that could occur upon relying on a single resistance gene to manage a pathogen. A classic boom-bust-cycle suggest that the deployment of single *R*-gene could direct a strong selection pressure on the pathogen population to evolve a new race with novel effector that can overcome the deployed gene. Since RMRL is the only available source to combat the current global threat of TTKSK, the primary barley germplasm pool needs to be further explored to identify novel sources of resistance. The third chapter of this thesis identified a new source of adult plant resistance, *RpgHv645* that was derived from a swiss barley landrace Hv645.

Overall, this dissertation addresses the existing gap in understanding of *rpg4/Rpg5* and *Pgt* interaction. Stem rust is historically a major problem in barley production and with the detection of local virulent race QCCJB and highly virulent race TTKSK and its lineages, these new understanding will offer novel opportunity to view barley-*Pgt* interaction and help to devise effective tools to combat against pathogens that never sleep.

APPENDIX A. SUPPLEMENTARY TABLES

Table A1. Group of *Pgt* isolates based on their virulence on barley lines containing stem rust resistance gene *rpg4/5* and/or *Rpg1*

Isolates	Race	Group	Phenotype on	
			rpg4/5	*Rpg1*
R29JA	HKHJ	Group2	Virulent	Avirulent
A-14	HPHJ	Group2	Virulent	Avirulent
R29JB	HKHJ	Group2	Virulent	Avirulent
370C	QFMQ	Group2	Virulent	Avirulent
A-21	TCMJC	Group2	Virulent	Avirulent
64E(1)-1	QTHJ	Group2	Virulent	Avirulent
A-15	HPLG	Group2	Virulent	Avirulent
R29M	HKHJ	Group2	Virulent	Avirulent
A-48	QFCQ	Group2	Virulent	Avirulent
P84-16	N/A	Group3	Avirulent	Virulent
79-1	N/A	Group3	Avirulent	Virulent
QCC-2	QCCJ	Group3	Avirulent	Virulent
79_20	N/A	Group3	Avirulent	Virulent
72-41-SP-2	TMLK	Group3	Avirulent	Virulent
79.2	N/A	Group3	Avirulent	Virulent
R11c	RCBJC	Group3	Avirulent	Virulent
72_00	RTQQC	Group3	Avirulent	Virulent
A-12	THTS	Group4	Avirulent	Avirulent
81AC28	N/A	Group4	Avirulent	Avirulent
81AC46	TPPKC	Group4	Avirulent	Avirulent
81AC34	N/A	Group4	Avirulent	Avirulent
WM-1	RCBDC	Group4	Avirulent	Avirulent
A-5	MCCF	Group4	Avirulent	Avirulent
AC-12	N/A	Group4	Avirulent	Avirulent

Table A2. Infection type of group2 *Pgt* isolates on barley differential lines

Isolates	Q21861		HQ1		Harrington	
	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
HKHJ	0;1(0;/0;1)	0;1(0;1/0;1)	3-2(3-2/3)	3-2(3-2/3-3)	2,3-(2/3-2)	3-3(3-2/3,3-)
A-14	0;1(0;/1;)	0;(0;/0;1)	3-3+(3,2/3,3+)	3(3-2/3+)	3-2(3-2/3-)	3-3(3-2/3,3+)
A-15	2,3(2,3-/3,2)	3,2(2,1/3+)	2,3(2,3-/3-2)	3-2(3-2/3-3)	3,3-(2,3-/3)	3-(3-2/3+)
370-C	0;1(0;/0;1)	0;(0;/1;)	3-2(2,3-/3-3)	3-2(2,3-/3-2)	3-(2,3-/3,3-)	3-2(3-2/3,3-)
64E(1)-1	0;1(0;1/1;)	0;(0;/0;1)	3-2(2,1;/3,3+)	3-2(2,3-/3-2)	2,3-(2,1/2,3-)	2,1(2,1;/3-2)
A-48	1;(0;1/1,2;)	0;1(0;/1;)	3,3-(2,3-1/3+3)	3-3(3-2/3,3+)	3,3-(3-3/3,3-)	3,3-(3-2/3,3+)
R29M	1;(0;1/1;2)	0;(0;/0;1)	3-3(3,3-2/3,3-)	3-3(3-2/3,3-)	3(3-3/3,3+)	2,3-(2,3-1/3,3-)
R29J	0;1(0;1/0;1)	0;1(0;/0;1)	3-2(2;3-/3-)	3-2(2,3-/3-3)	3-2(2,3-/3-3)	2,1(2,1/2,3-)
A-21	0;1(0;/3)	0;1(0;/0;1)	2,3-(1;/3)	3(3-/3,3+)	3-2(2,3-/3)	3,3-(3-3/3+3)
	Steptoe		Chevron		Morex	
	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
HKHJ	NA	3-2(2,3-/3-)	NA	0;1(0;/2,1)	NA	1,2(1;2/2,1)
A-14	NA	3-2(2,3-/3-3)	NA	0;1(0;/1;)	0;1(0;/1;)	0;1(0;/1;)
A-15	3-2(3-2/3-3)	3-3(2/2)	3-2(3-2/3,3-)	2,1(1;2/2,3-)	2,3-(2,3-/3-2)	3(3-2/3,3+)
370-C	NA	NA	NA	NA	1,2;(1;2/2,1)	1;2(1;/2,1)
64E(1)-1	NA	1;(0;/1,2;)	NA	3-2(2,3-/3,3-)	1;2(1;/2,1;)	0;1(0;/0;1)
A-48	NA	3-2(3-2/3,3+)	NA	0;1(0;/1;2)	2,1(2;/2,3-)	2;3(1;/2,1)
R29M	3-2(3-2/3)	3-2(2,3-/3,3-)	0;1(0;1/1,2;)	0;(0;/2,3-)	NA	0;1(0;/0;1)
R29J	3-2(3-2/3,3-)	3-2(3-2/3-3)	0;1(0;/0;1)	0;1(0;/0;1)	NA	0;1(0;/1,2)
A-21	2,3-(2,3-/3-2)	3-2(3-2/3,3+)	0;(0;/3-2)	0;1(0;/3-2)	NA	0;1(0;1/1;)

The rating of the infection types (ITs) was done using modified Stakamn scale of 0-4 (Stakman et al., 1962) for rust scoring. The Its score is shown in order of median score followed by range of score in the parenthesis (Adapted from Sun and Steffenson, 2005).

Table A3. Infection types of group3 *Pgt* isolates on barley differential lines

Isolates	Q21861		HQ1		Harrington	
	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
QCCJ	0;(0;/0;1)	;1,2(0;1/2,1;)	3-3(3-2/3,3-)	2,1(;1,2/2,3-)	2,3-(2,3-1/3,3-)	2,3(2,3-/3,3+)
R11c	1;(0;/2,1;)	0;(0;/0;1)	0;1(0;1/;1,2)	0;1(0;/0;1)	2,3-(2,3-/3-)	2,3-(2,3-/2,3-)
72.00	1;(0;1/1;)	0;(0;/1,2)	1;(0;1/1;)	0;(0;/;1,2)	2,1(2,1;/2)	2,1(2,1/3,3-)
72-41-SP-2	0;1(0;/0;1)	0;1(0;/2,1;)	1;(0;1/1;2)	0;1(0;/1;)	2,3-1(2;3/3,2)	3,3-(3-2/3)
79.2	1;(0;1/1,2)	0;(0;/0;1)	1;(0;1/1,2)	1;(0;/2;1)	3-3(2,3-/3,3-)	3-2(3-2/3,3-)
P84-16	0;1(0;/2,1;)	0;1(0;/0;1)	2,1(1,2/3-2,1)	0;1(0;1/1;)	3(3-2/3)	3-(3-2/3-3)
79.1	0;(0;/1;)	0;(0;/0;1)	2;1(0;/2,1;)	1,2;(0;1/2,1)	3-2(3-2/3,3+)	3(3/3+)
79.20	0;1(0;/1;)	0;(0;/0;1)	0;1(0;/0;1)	0;(0;/0;1)	2,3-(2,1/3-3)	3-3(3-2/3+3)
	Steptoe		Chevron		Morex	
	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
QCCJ	2,3-(2,3-/3-2)	3-3(3-2/3,3-)	0;(0;/0;)	2,1;(1,2/2,3-)	0;1(0;/0;1)	3-2(2,3-/3-3)
R11c	NA	2,3-(2/3-2)	NA	2,1(2,1/2)	2,3-(2,1;/3,3-)	2,1;(1;/2,3-)
72.00	NA	3(3-2/3)	NA	1,2(1,2;/2,1)	3-(2,3/3,2)	1,2(1,2;/2,1)
72-41-SP-2	NA	3-2(2,1/3,3-)	NA	3-2(2,1/3,3+)	3,2(2,3/3,2)	3-2(2/3-2)
79.2	NA	NA	NA	NA	3-2(2/3,3-)	3,3-(3-/3,3+)
P84-16	3,3+(3-2/3,3+)	3-(3-2/3-3)	3-2(3-2/3,3+)	2,3-(2/3-2)	NA	3-2(2,3-/3-3)
79.1	3-2(3-2/3,3-)	2,3-(2,1/3-3)	3-2(2,1/3-2)	3,3-(3-2/3+)	3-2(3-2/3,3+)	3,3-(2,3-/3)
79.20	NA	3-3(3-2/3)	2,3(2,1/3-2)	1,2;(1,2;/2,1)	3-2(2/3-3)	2,3-(2,1/3-3)

The rating of the infection types (ITs) was done using modified Stakamn scale of 0-4 (Stakman et al., 1962) for rust scoring. The Its score is shown in order of median score followed by range of score in the parenthesis (Adapted from Sun and Steffenson, 2005).

Table A4. Infection type of group4 *Pgt* isolates on barley differential lines

Isolates	Q21861		HQ1		Harrington	
	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
81AC-46	0;(0;/0;1)	0;(0;/0;1)	1;(1;/1;)	0;1(0;/0;1)	3-2,1(2,1;/3-2)	2(2/3,3-)
WM-1	1;(0;1/1;2)	0;(0;/0;)	1;2(0;1/1,2;)	0;(0;/0;)	1;(1;/1;2)	2,1(2,1;/2,3-)
A-5	0;1(0;/2,1;)	0;1(0;1/0;1)	1,2;(;1,2/1,2;)	1,2;(0;1/1,2)	2(1,2/2,3-)	2,1(2,1/2,3-)
81AC-34	0;1(0,1;/0;1)	0;1(0;1/2)	2,3-(2,1/3,3-)	1;(0;1/3,2)	3,3-(2,3-/3+3)	3-2(2,3-/3,3-)
AC-12	0;(0;/1;)	0;(0;/0;)	0;1(0;1/0;1)	1;(0;/1;)	2,1(1;/2,1)	2(1,2/3-2)
A-12	0;(0;/0;1)	1;(0;1/1;)	1,2(0;1/2)	1;(0;1/1;)	3-2(2/3)	3(3-2/3)
81AC-28	0;1(0;/1;)	0;1(0;/1;)	;1,2(0;1/3-2)	0;1(0;1/1,2;)	3-3(1;/3,3-)	3-3(3-2/3,3-)
36-55A	0;(0;/0;1)	NA	;1(0;1/2;1)	NA	3-2(3-2/3-3)	NA
R29L	0;(0;/0;1)	NA	1,2;(1,2;/2,1)	NA	3-2(2/3,3-)	NA
72.22	0;1(0;1/0;1)	NA	2,1(1;2/2,1,3)	NA	3,2(3,2/3)	NA
TNMK SP-1	0;1(0;/1;2)	NA	1,2(0;1/2,1;)	NA	2,3-(2,1/2,3-)	NA
81AC-31	0;1(0;/0;1)	NA	1;(;1/1;2)	NA	2,3-(2,1/2,3-)	NA
P89-7	0;1(0;/0;1)	NA	1;2(1;2/2)	NA	3,3-(3-2/3,3+)	NA
79.89	;1,2(0;1/1,2;)	NA	1;2(;1/1,2;)	NA	3-2(2,3-/3-)	NA
79-46_3/2	0;(0;/2;1)	NA	2,1;(2,1;/2,3-1)	NA	2,3-(2,1/2,3-)	NA
R29H	0;(0;/1;2)	NA	1,2(1,2;/2,1,3-)	NA	3-2(2,3-/3)	NA
TNM	0;1(0;/2,1;)	NA	0;1,2(0;1/2)	NA	2,3-(2,3-/3-)	NA
79.84	1;(1;/1;)	NA	1;2(1;/2,3-1)	NA	3,3+(2,3-/3+3)	NA
R111	0;1(0;/1/2)	NA	1,2;(0;/2,1;3)	NA	2;3(2/2,3-)	NA
P89-1	0;1(0;1/;1,2)	NA	1;2(;1/2,1)	NA	3;2(2;3/3,2)	NA

(continued)

Table A4. Infection type of group4 *Pgt* isolates on barley differential lines (continued)

Isolates	Steptoe		Chevron		Morex	
	Exp1	Exp2	Exp1	Exp2	Exp1	Exp2
81AC-46	NA	1,2(1/2)	NA	1;(0;1/2,1)	;1,2(0;1/1;)	2(1;/2,3-)
WM-1	NA	1,2(1;/2,1)	NA	1,2(1;/2)	1;(1;/1;2)	1,2(1;/2)
A-5	NA	2,3-(2,3-/2,3-)	NA	0;(0;/1;)	1;2(1;/2,1;)	NA
81AC-34	NA	2,3(2,3-/3-2)	NA	1;2(1;/1,2)	1,2(1,2/2,1)	2,1(2,1;/2)
AC-12	1,2(1;/2,1)	2(1,2/2,3-)	1;2(1;/1)	1,2(1/2)	NA	2;3(1,2/2)
A-12	3-2(2,3/3)	3(3-2/3,3+)	0;(0;/0;)	1;(0;1/1;)	NA	2(2/2,3-)
81AC-28	3(2,3-/3+)	3(2,3-/3+)	1,2(1;/2,1;)	1,2(1;/2,1;)	2,1(2,1;/2,3-)	2,1(1,2/2,3-1)
36-55A	NA	NA	NA	NA	2(0;1/2,3)	NA
R29L	NA	NA	NA	NA	1;(0;1/1,2)	NA
72.22	NA	NA	NA	NA	1,2;(;1/2,1)	NA
TNMK SP-1	NA	NA	NA	NA	2,1;(2,1/2,3-)	NA
81AC-31	2,3-(2,1/3-2)	NA	1;(1;/1;2)	NA	NA	NA
P89-7	NA	NA	NA	NA	2,1(1,2;/2,3-)	NA
79.89	3-3(3-/3,3-)	NA	2(2/2,3-)	NA	NA	NA
79-46_3/2	3-2(2,3-/3)	NA	0;(0;/1;)	NA	NA	NA
R29H	2(2/3-2)	NA	0;1(0;/0;1)	NA	NA	NA
TNM	2,3-(2,1/2,3-)	NA	2,1(1,2/2,1,3-)	NA	NA	NA
79.84	2,3-(2/3-2)	NA	NA	NA	NA	NA
R111	NA	NA	NA	NA	2,3-(2/3-2)	NA
P89-1	2,1(2,1/2,3-)	NA	NA	NA	NA	NA

The rating of the infection types (ITs) was done using modified Stakamn scale of 0-4 (Stakman et al., 1962) for rust scoring. The Its score is shown in order of median score followed by range of score in the parenthesis (Adapted from Sun and Steffenson, 2005).

Table A5. Read and mapping statistics of RAD-GBS data of *Pgt* Samples

Isolates	# of reads	# of reads after trimming	Avg. length after trim	% mapped[a]
R29J	225,360	225,360	161.3	87.28
R29M	246,283	246,283	162.5	86.73
R29L	269,036	269,036	158.7	81.71
HKHJ	212,770	212,770	160.9	72.57
A-5	235,353	235,353	162.9	87.19
370-C	318,239	318,239	164.1	67.16
640E(1)	414,867	414,867	164.9	67.57
R11c	653,856	653,856	164.3	61.77
MCCF	710,944	710,944	164.6	76.22
A-15	547,851	547,851	160.6	86.27
36-55A	262,185	262,185	163.7	79.78
R29H*	105,177	105,177	160.4	76.4
R111*	45,693	45,693	165	58.71
QCCJ*	87,821	87,821	167.4	59.5
A-14*	36,386	36,386	164.7	30.39
AC-12*	77,671	77,671	163.7	76.81
A-48	644,808	644,808	163.8	64.22
WM-1	601,385	601,385	163.6	84.87
72.22	1,048,132	1,048,132	159.2	76.55
72.00	589,183	589,183	163.4	83.09
A-12	909,063	909,063	162.5	86.5
A-21	494,788	494,788	164.2	86.2
72-41-SP-2	569,513	569,513	163.7	82.41
81AC-31	1,231,338	1,231,338	161.4	85.78
TNM	309,945	309,945	164.9	55.48
P89-7	251,872	251,872	164.1	86.63
79.2	754,627	754,627	165.4	67.94
P84-16	515,582	515,582	161.9	62.3
TNMK SP-1	579,489	579,489	164.3	81.55
79.84	153,735	153,735	164.1	84.83
79-46_3/2	753,717	753,717	161.5	87.06
P89-1	639,511	639,511	163	80.23
81AC-28	239,826	239,826	161.3	78.21
81AC-46	304,951	304,951	155.1	86.77
79.89	1,149,540	1,149,540	156.9	81.6
79.20	925,050	925,050	162.3	76.88

[a]- Percentage of reads that mapped to reference genome of *Pgt* race SCCL

* - Isolates discarded from AM analysis due to poor read quality and read mapping to reference genome of *Pgt* race SCCL

Table A6. RNAseq read mapping statistics of the *Pgt* inoculated Harrington samples

Isolates	Total reads	Percentage reads mapped to *Pgt (%)*		
		Total Mapping	Unique mapping	Multiple mapping[a]
72.00	33926507	4.01	3.94	0.07
QCCJ	50379404	47.89	46.64	1.25
72-41-SP-2	39461242	37.30	36.32	0.98
79.1	34865876	37.08	36.11	0.97
P84-16	54792085	50.48	49.16	1.32
R11c	33056488	33.35	32.53	0.82
79.2	42171550	37.97	36.92	1.05
79.20	56420024	39.23	38.18	1.05
R29J	46077493	29.59	28.82	0.77
370C	43175619	35.66	34.70	0.96
A-21	77764164	43.02	41.76	1.26
HKHJ	38939003	44.84	43.67	1.17
A-15	37431077	34.69	33.76	0.93
A-14	80641470	41.67	40.65	1.02
R29M	37412371	50.47	49.19	1.28
64E(1)-1	34213515	38.41	37.40	1.01
A-48	54020962	38.13	37.11	1.02
A-12	40825001	12.48	12.11	0.37
81AC-28	81518797	31.57	30.74	0.83
81AC-46	39106468	32.79	31.92	0.87
WM-1	39492058	20.92	20.36	0.56
A-5	44481150	26.02	25.32	0.70
81AC-34	41699689	48.85	47.57	1.28
AC-12	38521363	14.48	14.13	0.35
Average	46,683,057	34.62	33.71	0.91
Standard Deviation	14,387,160	12.00	11.69	0.32

(continued)

Table A6. RNAseq read mapping statistics of the *Pgt* inoculated Harrington samples (continued)

Sample	Percentage reads mapped to barley (%)			Unmapped reads (%)[b]
	Total Mapping	Unique mapping	Multiple mapping[a]	
72.00	82.4	70.05	12.35	13.59
QCCJ	22.26	18.96	3.3	29.85
72-41-SP-2	38.46	32.38	6.08	24.24
79.1	43.65	36.75	6.9	19.27
P84-16	28.3	24	4.3	21.22
R11c	46.32	39.14	7.18	20.33
79.2	36.38	31.1	5.28	25.65
79.20	34.35	28.96	5.39	26.42
R29J	43.52	36.74	6.78	26.89
370C	35.78	30.39	5.39	28.56
A-21	27.45	23.39	4.06	29.53
HKHJ	29.19	24.61	4.58	25.97
A-15	35.82	30.39	5.43	29.49
A-14	27.25	23	4.25	31.08
R29M	19	16.08	2.92	30.53
64E(1)-1	32.14	27.21	4.93	29.45
A-48	32.66	27.72	4.94	29.21
A-12	65.81	55.68	10.13	21.71
81AC-28	38.12	30.51	7.61	30.31
81AC-46	42.82	36.42	6.4	24.39
WM-1	59.32	50.45	8.87	19.76
A-5	52.55	44.58	7.97	21.43
81AC-34	26.16	22.03	4.13	24.99
AC-12	74.57	63.38	11.19	10.95
Average	40.60	34.33	6.27	24.78
Standard Deviation	16.12	13.73	2.43	5.37

(continued)

Table A6. RNAseq read mapping statistics of the *Pgt* inoculated Harrington samples (continued)

Sample	Reads mapped to *de novo* Assembled Transcripts (%)c			
	Total Mapping	Unique mapping	Multiple mapping[a]	Unmapped reads
72.00	48.04	30.92	17.12	51.96
QCCJ	40.28	26.58	13.7	59.72
72-41-SP-2	44.39	28.03	16.36	55.61
79.1	41.66	27.55	14.11	58.34
P84-16	53.27	35.61	17.66	46.73
R11c	54.05	38.14	15.91	45.95
79.2	43.07	29.59	13.48	56.93
79.20	45.27	26.66	18.61	54.73
R29J	43.06	28.59	14.47	56.94
370C	41.66	27.55	14.11	58.34
A-21	40.75	27.26	13.49	59.25
HKHJ	46.01	32.86	13.15	53.99
A-15	42.26	28.31	13.95	57.74
A-14	43.69	30.68	13.01	56.31
R29M	41.95	28.14	13.81	58.05
64E(1)-1	43.59	29.72	13.87	56.41
A-48	42.14	27.82	14.32	57.86
A-12	40.91	25.41	15.5	59.09
81AC-28	48.13	32.29	15.84	51.87
81AC-46	41.93	26.23	15.7	58.07
WM-1	44.75	28.38	16.37	55.25
A-5	43.12	26.96	16.16	56.88
81AC-34	55.22	36.6	18.62	44.78
AC-12	47.25	30.43	16.82	52.75
Average	44.85208	29.59625	15.25583	55.14792
Standard Deviation	4.21369	3.35567	1.701406	4.21369

[a]The percentage of reads that mapped to 2 to 10 different position in reference genome
[b]Percentage of Reads that did not map to either of the barley or *Pgt* reference gene models
[c]Mapping statistics of unmapped reads that mapped to *de novo* assembled transcripts

Table A7. RNAseq read mapping statistics for the non-inoculated Harrington samples

		Percentage reads mapped to barley *(%)*			Reads mapped to *de novo* Assembled Transcripts (%)[c]				
Sample	Total Reads	Total mapping	Unique mapping	Multiple mapping[a]	Unmapped reads (%)[b]	Total mapping	Unique mapping	Multiple mapping[a]	Unmapped reads
Harrington_non-inoculated_Rep1	41313362	83.12	65.24	17.87	34.76	35.21	19.38	15.83	64.79
Harrington_non-inoculated_Rep1	44973475	82.06	64.90	17.16	35.10	33.64	18.13	15.51	66.36
Harrington_non-inoculated_Rep1	47195423	80.65	63.98	16.66	36.02	33.25	17.20	16.05	66.75
Average	46439838.4	81.94	64.71	17.23	35.29	34.03	18.24	15.80	65.97
Standard Deviation	2970188.5	1.24	0.65	0.61	0.65	1.04	1.09	0.27	1.04

[a]The percentage of reads that mapped to 2 to 10 different position in reference genome
[b]The percentage of Reads that did not map to either of the barley or *Pgt* reference gene models
[c]Mapping statistics of unmapped reads that mapped to *de novo* assembled transcripts

Table A8. Differentially expressed gene in between virulent *rpg4/5* and avirulent *rpg4/5* inoculated sampled and its comparison with the non-inoculated controls

Feature ID	Barley Annotation[a]	Arabidopsis Homology[b]	Vir *rpg4/5* vs Avr *rpg4/5*	
			Fold change[c]	Corrected P-value[d]
HORVU5Hr1G092120	Dehydrin7	AT5G66400.1	-73.25	1.9E-04
HORVU7Hr1G042790	ADP	AT4G28390.2	-37.06	3.3E-07
HORVU4Hr1G063350	heatshockprotein21	AT4G27670.1	-24.86	3.6E-07
HORVU5Hr1G092160	Dehydrin7	AT5G66400.1	-20.01	4.9E-03
HORVU3Hr1G007500	16.9kDaclassIheatshockprotein1	AT1G53540.1	-14.46	3.6E-09
HORVU2Hr1G077710	22kDaclassIVheatshockprotein	AT4G10250.1	-11.94	4.6E-03
HORVU7Hr1G034630	mybdomainprotein5	AT2G16720.1	-11.40	1.5E-03

(continued)

Table A8. Differentially expressed gene in between virulent *rpg4/5* and avirulent *rpg4/5* inoculated sampled and its comparison with the non-inoculated controls (continued)

Feature ID	Barley Annotation[a]	Arabidopsis Homology[b]	Vir *rpg4/5* vs Avr *rpg4/5*	
			Fold change[c]	Corrected P-value[d]
HORVU5Hr1G010330	Outerenvelopeporeprotein16-2	AT4G16160.2	-10.55	1.4E-03
HORVU4Hr1G060760	17.9kDaclassIheatshockprotein	AT1G53540.1	-10.42	2.4E-04
HORVU4Hr1G000150	MethylobacteriumaquaticumDNA	AT1G78700.1	-9.45	3.2E-02
HORVU3Hr1G006530	16.9kDaclassIheatshockprotein1	AT1G53540.1	-8.83	1.6E-06
HORVU4Hr1G069260	DNAreplicationlicensingfactormcm4	AT1G52380.4	-8.68	1.1E-05
HORVU4Hr1G051010	Phosphoglyceratemutase	AT1G09780.1	-8.43	3.3E-07
HORVU3Hr1G084210	ferredoxin3	AT2G27510.1	-7.80	3.3E-07
HORVU0Hr1G037040	Orf108a	ATCG01110.1	-6.73	7.3E-07
HORVU4Hr1G059260	Heatshock70kDaprotein3	AT5G02500.1	-6.01	1.2E-03
HORVU2Hr1G116730	Wound-responsivefamilyprotein	AT4G10270.1	-5.48	6.9E-03
HORVU5Hr1G076970	Senescence-associatedprotein	AT5G39830.1	-5.40	3.9E-02
HORVU2Hr1G121820	PhotosystemIIproteinD1	ATCG00020.1	-5.33	3.6E-07
HORVU5Hr1G012990	ProteinTAR1	AT5G24850.1	-5.23	1.7E-02
HORVU4Hr1G067430	Heatshock70kDaprotein8	AT2G32120.2	-5.22	1.5E-02
HORVU2Hr1G038570	Ribosome-recyclingfactor	AT3G63190.1	-4.75	6.6E-04
HORVU5Hr1G064800	Cellwall-associatedhydrolase	AT4G22180.1	-4.73	1.6E-02
HORVU6Hr1G049260	Ribulosebisphosphatecarboxylaselargechain	ATCG00490.1	-4.62	1.1E-04
HORVU3Hr1G073860	chlororespiratoryreduction7	AT5G39210.1	-4.47	4.2E-03
HORVU4Hr1G054110	MitochondrialimportinnermembranetranslocasesubunitTim 17/Tim22/Tim23familyprotein	AT4G16160.1	-4.38	5.0E-02
HORVU7Hr1G082830	1,4-alpha-glucan branching enzyme GlgB	AT3G20440.2	-4.36	4.5E-05
HORVU0Hr1G007340	Progesterone5betareductase	AT4G24220.2	-4.23	1.3E-02
HORVU3Hr1G018810	FASCICLIN-likearabinogalactan-protein11	AT5G03170.1	-4.16	4.7E-02
HORVU4Hr1G042710	MATEeffluxfamilyprotein	AT2G27660.1	-4.75	1.8E-02
HORVU4Hr1G015170	17.6kDaclassIIheatshockprotein	AT4G10250.1	-4.15	4.4E-02

(continued)

Table A8. Differentially expressed gene in between virulent *rpg4/5* and avirulent *rpg4/5* inoculated sampled and its comparison with the non-inoculated controls (continued)

Feature ID	Barley Annotation[a]	Arabidopsis Homology[b]	Vir *rpg4/5* vs Avr *rpg4/5*	
			Fold change[c]	Corrected P-value[d]
HORVU0Hr1G024560	Ribulosebisphosphatecarboxylaselargechain	ATCG00490.1	-4.02	2.8E-03
HORVU3Hr1G078940	tubulinbetachain2	AT5G12250.1	-4.02	1.6E-04
HORVU7Hr1G038770	dehydroascorbatereductase1	AT5G16710.1	-3.98	3.3E-07
HORVU3Hr1G088970	Nucleicacid-binding	AT3G48500.1	-3.97	1.9E-07
HORVU5Hr1G016020	RRNAintron-encodedhomingendonuclease	AT1G22270.1	-3.96	2.1E-02
HORVU3Hr1G007380	16.9kDaclassIheatshockprotein2	AT1G53540.1	-3.95	6.4E-04
HORVU2Hr1G067250	TranscriptionfactorbHLH62	AT3G07340.2	-3.93	3.8E-02
HORVU3Hr1G060060	Mannanendo-1	AT5G66460.1	-3.90	3.0E-02
HORVU3Hr1G089910	Triggerfactor	AT2G30695.3	-3.67	1.5E-03
HORVU7Hr1G098280	xyloglucanendotransglucosylase/hydrolase25	AT4G25810.1	-3.65	3.1E-03
HORVU7Hr1G081510	DnaJ/Hsp40cysteine-richdomainsuperfamilyprotein	AT3G47650.1	-3.63	2.0E-06
HORVU4Hr1G009190	Sec14p-likephosphatidylinositoltransferfamilyprotein	AT1G75170.2	-3.53	1.5E-03
HORVU7Hr1G098410	Inositolpolyphosphatemultikinase	AT5G61760.2	-3.40	4.5E-04
HORVU4Hr1G052450	Hexosyltransferase	AT2G47180.1	-3.39	4.0E-02
HORVU2Hr1G056440	Valine--tRNAligase	AT5G16715.1	-3.31	1.5E-04
HORVU7Hr1G082850	N/A	AT3G20440.1	-3.53	2.6E-02
HORVU2Hr1G065060	DiseaseresistanceproteinRPP13	AT3G46530.1	-3.22	4.6E-04
HORVU5Hr1G053480	ProteinFLUORESCENTINBLUELIGHT	AT3G14110.2	-3.19	3.6E-06
HORVU2Hr1G102190	WD-40repeatfamilyprotein	AT4G33270.1	-3.19	1.3E-04
HORVU3Hr1G074250	Pentatricopeptiderepeat-containingprotein	AT5G56310.1	-3.17	2.2E-04
HORVU7Hr1G038200	Hexosyltransferase	AT3G25140.1	-3.16	4.1E-02
HORVU5Hr1G068360	Leucine-richrepeatproteinkinasefamilyprotein	AT3G19230.1	-3.13	3.9E-02
HORVU2Hr1G079840	Lipase/lipooxygenase	AT4G39730.1	-3.12	1.5E-04
HORVU4Hr1G010160	serinecarboxypeptidase-like19	AT1G33540.1	-3.30	1.2E-03

(continued)

Table A8. Differentially expressed gene in between virulent *rpg4/5* and avirulent *rpg4/5* inoculated sampled and its comparison with the non-inoculated controls (continued)

Feature ID	Barley Annotation[a]	Arabidopsis Homology[b]	Vir *rpg4/5* vs Avr *rpg4/5*	
			Fold change[c]	Corrected P-value[d]
HORVU1Hr1G030060	S-typeanionchannelSLAH3	AT5G24030.1	-3.10	2.9E-02
HORVU2Hr1G004720	UDP-Glycosyltransferasesuperfamilyprotein	AT1G22400.1	-3.10	1.1E-02
HORVU7Hr1G116760	mybdomainproteinr1	AT3G50060.1	3.02	4.4E-02
HORVU4Hr1G007420	ZincfingerproteinCONSTANS-LIKE16	AT1G25440.1	3.04	1.9E-03
HORVU5Hr1G038630	ribulosebisphosphatecarboxylasesmallchain1A	AT1G67090.1	3.04	2.0E-03
HORVU7Hr1G046050	Carboxyl-terminal-processingprotease	AT3G57680.1	3.07	3.1E-02
HORVU4Hr1G060940	RNA-bindingprotein1	AT4G35785.2	3.11	3.3E-02
HORVU1Hr1G007800	Alcoholdehydrogenase	AT4G37970.1	3.17	2.4E-03
HORVU3Hr1G115420	CytochromeP450superfamilyprotein	AT3G26170.1	3.25	3.1E-02
HORVU5Hr1G071920	PhotosystemIreactioncentersubunitV	AT1G55670.1	3.25	1.2E-02
HORVU7Hr1G116960	ChaperoneproteinDnaJ	AT2G17880.1	3.29	1.5E-02
HORVU5Hr1G081190	B-boxzincfingerfamilyprotein	AT4G38960.6	3.31	7.6E-03
HORVU2Hr1G060480	PhotosystemIreactioncentersubunitpsaK	AT1G30380.1	3.31	2.6E-02
HORVU6Hr1G060370	CBSdomain-containingproteinCBSX5	AT4G27460.1	3.32	7.8E-03
HORVU5Hr1G084140	SKP1-like4	AT5G42190.1	3.34	1.8E-04
HORVU3Hr1G075870	PhotosystemIIreactioncenterWprotein	AT2G30570.1	3.35	7.6E-03
HORVU7Hr1G040740	Cysteine-richvenomprotein	AT5G57625.1	3.35	2.8E-03
HORVU4Hr1G015260	Chlorophylla-bbindingprotein2	AT4G10340.1	3.38	3.3E-02
HORVU5Hr1G011730	E3ubiquitin-proteinligaseRMA1H1	AT4G03510.4	3.41	3.6E-02
HORVU0Hr1G010880	CytochromeP450superfamilyprotein	AT3G26210.1	3.42	2.1E-03
HORVU1Hr1G089700	O-methyltransferase1	AT5G54160.1	3.46	2.0E-03
HORVU0Hr1G002870	RING/U-boxsuperfamilyprotein	AT1G26800.1	3.54	2.2E-02
HORVU3Hr1G005500	TranscriptionfactorbHLH35	AT5G57150.5	3.59	8.2E-04
HORVU6Hr1G039840	thionin2.1	AT1G66100.1	3.62	2.4E-02
HORVU2Hr1G098110	nudixhydrolasehomolog8	AT5G47240.1	3.28	5.3E-03

(continued)

Table A8. Differentially expressed gene in between virulent *rpg4/5* and avirulent *rpg4/5* inoculated sampled and its comparison with the non-inoculated controls (continued)

Feature ID	Barley Annotation[a]	Arabidopsis Homology[b]	Vir *rpg4/5* vs Avr *rpg4/5*	
			Fold change[c]	Corrected P-value[d]
HORVU6Hr1G033160	chlorophyllA/Bbindingprotein3	AT1G61520.3	3.69	3.5E-02
HORVU2Hr1G073370	photosystemIsubunitO	AT1G08380.1	3.79	2.5E-02
HORVU2Hr1G040780	Chlorophylla-bbindingprotein13	AT5G54270.1	3.81	2.1E-02
HORVU2Hr1G036960	Chlorophylla-bbindingprotein7	AT3G61470.1	3.83	3.9E-04
HORVU2Hr1G065450	floweringpromotingfactor1	AT5G24860.1	3.84	3.3E-05
HORVU7Hr1G116750	mybdomainproteinr1	AT5G67300.1	3.86	2.7E-02
HORVU6Hr1G090510	O-methyltransferasefamilyprotein	AT4G35160.1	3.86	3.6E-05
HORVU2Hr1G041280	Chlorophylla-bbindingproteinCP29	AT3G08940.1	3.91	4.7E-03
HORVU3Hr1G096910	Beta-glucosidase3	AT3G60140.2	3.95	2.2E-04
HORVU2Hr1G082330	undescribedprotein	AT2G24430.2	3.95	2.1E-03
HORVU0Hr1G019640	basichelix-loop-helix(bHLH)DNA-bindingsuperfamilyprotein	AT3G21330.1	4.11	2.3E-02
HORVU7Hr1G046320	Chlorophylla-bbindingprotein1B-21	AT3G54890.1	4.21	2.0E-02
HORVU5Hr1G066280	Chlorophylla-bbindingprotein1B-20	AT3G47470.1	4.24	1.8E-02
HORVU3Hr1G055260	mybdomainprotein66	AT4G01060.1	4.27	2.5E-02
HORVU7Hr1G070870	circadianclockassociated1	AT1G01060.4	4.30	2.8E-03
HORVU3Hr1G033870	CytochromeP450superfamilyprotein	AT3G26330.1	4.38	1.4E-02
HORVU3Hr1G027970	TRICHOMEBIREFRINGENCE-LIKE38	AT1G29050.1	4.39	2.3E-02
HORVU1Hr1G071500	undescribedprotein	AT3G59100.1	4.52	1.7E-02
HORVU6Hr1G009360	Peroxidasesuperfamilyprotein	AT5G05340.1	4.57	3.5E-03
HORVU6Hr1G026340	earlynodulin-likeprotein9	AT5G53870.1	4.59	3.4E-06
HORVU2Hr1G098160	Oleosin	AT5G51210.1	4.60	2.5E-03
HORVU2Hr1G041080	sugartransporter1	AT1G11260.1	4.72	2.7E-02

(continued)

Table A8. Differentially expressed gene in between virulent *rpg4/5* and avirulent *rpg4/5* inoculated sampled and its comparison with the non-inoculated controls (continued)

Feature ID	Barley Annotation[a]	Arabidopsis Homology[b]	Vir *rpg4/5* vs Avr *rpg4/5*	
			Fold change[c]	Corrected P-value[d]
HORVU5Hr1G095580	CathepsinB-likecysteineproteinase	AT5G45890.1	3.92	1.8E-02
HORVU2Hr1G090730	GRAMdomain-containingprotein/ABA-responsiveprotein-related	AT5G23370.1	4.81	1.4E-03
HORVU6Hr1G093520	Orphanstranscriptionfactor	AT1G12060.1	5.05	2.8E-03
HORVU5Hr1G082420	Chlorophylla-bbindingprotein	AT3G27690.2	5.12	2.3E-02
HORVU7Hr1G027560	CONSTANS-like5	AT2G24790.1	5.20	3.9E-05
HORVU5Hr1G124160	Chlorophylla-bbindingprotein2	AT2G34420.1	5.28	2.5E-02
HORVU1Hr1G070190	Nodulin-like/MajorFacilitatorSuperfamilyprotein	AT2G30300.1	5.34	1.5E-06
HORVU2Hr1G065000	Encodesaproteininvolvedinsalttolerance	AT5G02020.1	5.64	6.6E-04
HORVU3Hr1G096910	Beta-glucosidase3	AT3G60140.2	3.95	2.2E-04
HORVU7Hr1G096040	Non-specificlipid-transferprotein2	AT3G18280.2	6.46	4.0E-03
HORVU3Hr1G055740	S-typeanionchannelSLAH2	AT5G24030.1	7.65	4.9E-06
HORVU1Hr1G089540	Chalconesynthase2	AT5G13930.1	9.28	2.3E-05
HORVU1Hr1G089620	O-methyltransferase1	AT5G54160.1	10.01	2.0E-03
HORVU2Hr1G038940	PhotosystemII10kDapolypeptide	AT1G79040.1	5.68	1.4E-06

(continued)

Table A8. Differentially expressed gene in between virulent *rpg4/5* and avirulent *rpg4/5* inoculated sampled and its comparison with the non-inoculated controls (continued)

Feature ID	Non-inoculated vs Vir *rpg4/5* inoculated		Non-inoculated vs avr *rpg4/5* inoculated	
	Fold change[c]	Corrected P-value[d]	Fold change[c]	Corrected P-value[d]
HORVU5Hr1G092120	2.45	1.00	183.06	0.05
HORVU7Hr1G042790	4.56	1.00	176.97	0.00
HORVU4Hr1G063350	3.88	0.88	94.97	0.00
HORVU5Hr1G092160	2.88	1.00	59.49	0.08
HORVU3Hr1G007500	20.43	0.05	307.44	0.00
HORVU2Hr1G077710	2.31	1.00	27.93	0.10
HORVU7Hr1G034630	1.73	1.00	19.68	0.34
HORVU5Hr1G010330	7.63	1.00	85.13	0.01
HORVU4Hr1G060760	10.82	0.77	118.52	0.00
HORVU4Hr1G000150	-1.89	1.00	4.76	1.00
HORVU3Hr1G006530	6.46	1.00	59.68	0.00
HORVU4Hr1G069260	5.50	1.00	48.69	0.00
HORVU4Hr1G051010	14.33	0.24	127.01	0.00
HORVU3Hr1G084210	44.50	0.00	366.51	0.00
HORVU0Hr1G037040	-2.98	0.00	2.23	0.80
HORVU4Hr1G059260	14.11	0.04	85.49	0.00
HORVU2Hr1G116730	1.37	1.00	7.39	0.19
HORVU5Hr1G076970	-5.39	0.00	-1.00	1.00
HORVU2Hr1G121820	-12.46	0.00	-2.36	0.08
HORVU5Hr1G012990	-12.30	0.00	-2.35	0.83
HORVU4Hr1G067430	4.38	1.00	23.50	0.19
HORVU4Hr1G042710	2.08	1.00	9.84	0.18
HORVU2Hr1G038570	-20.37	0.00	-4.37	0.00
HORVU5Hr1G064800	-14.45	0.00	-3.06	0.26
HORVU6Hr1G049260	-26.77	0.00	-5.92	0.00
HORVU3Hr1G073860	-130.37	0.00	-29.98	0.00

(continued)

Table A8. Differentially expressed gene in between virulent *rpg4/5* and avirulent *rpg4/5* inoculated sampled and its comparison with the non-inoculated controls (continued)

Feature ID	Non-inoculated vs Vir *rpg4/5* inoculated		Non-inoculated vs avr *rpg4/5* inoculated	
	Fold change[c]	Corrected P-value[d]	Fold change[c]	Corrected P-value[d]
HORVU4Hr1G054110	8.09	1.00	37.33	0.07
HORVU7Hr1G082830	6.57	1.00	29.29	0.00
HORVU0Hr1G007340	21.45	0.12	95.07	0.00
HORVU3Hr1G018810	-1.50	1.00	2.71	1.00
HORVU4Hr1G015170	10.86	0.77	47.46	0.01
HORVU0Hr1G024560	-12.62	0.00	-3.16	0.06
HORVU3Hr1G078940	-1.91	0.04	2.08	0.77
HORVU7Hr1G038770	-4.67	0.00	-1.20	1.00
HORVU3Hr1G088970	-1.03	1.00	3.79	0.01
HORVU5Hr1G016020	-3.29	0.04	1.19	1.00
HORVU3Hr1G007380	21.11	0.12	87.23	0.00
HORVU2Hr1G067250	1.17	1.00	4.54	0.70
HORVU3Hr1G060060	5.10	1.00	20.40	0.09
HORVU3Hr1G089910	-12.29	0.00	-3.42	0.00
HORVU7Hr1G098280	-2.65	0.01	1.36	1.00
HORVU7Hr1G081510	-22.69	0.00	-6.33	0.00
HORVU7Hr1G082850	5.16	1.00	18.77	0.09
HORVU4Hr1G009190	4.65	0.88	16.41	0.00
HORVU7Hr1G098410	-2.46	0.14	1.37	1.00
HORVU4Hr1G052450	27.38	0.00	91.13	0.00
HORVU2Hr1G056440	-1.46	1.00	2.24	0.48
HORVU4Hr1G010160	1.67	1.00	5.44	0.04
HORVU2Hr1G065060	2.69	1.00	8.62	0.00
HORVU5Hr1G053480	-4.92	0.00	-1.56	0.52
HORVU2Hr1G102190	1.51	1.00	4.75	0.01
HORVU3Hr1G074250	-11.48	0.00	-3.70	0.00

(continued)

Table A8. Differentially expressed gene in between virulent *rpg4/5* and avirulent *rpg4/5* inoculated sampled and its comparison with the non-inoculated controls (continued)

Feature ID	Non-inoculated vs Vir *rpg4/5* inoculated		Non-inoculated vs avr *rpg4/5* inoculated	
	Fold change[c]	Corrected P-value[d]	Fold change[c]	Corrected P-value[d]
HORVU7Hr1G038200	2.22	1.00	6.98	0.27
HORVU5Hr1G068360	2.87	1.00	8.98	0.16
HORVU2Hr1G079840	-1.11	1.00	2.77	0.07
HORVU1Hr1G030060	3.20	0.90	9.96	0.02
HORVU2Hr1G004720	5.36	0.41	16.54	0.00
HORVU7Hr1G116760	25.56	0.10	8.94	0.88
HORVU4Hr1G007420	-6.06	0.00	-18.69	0.00
HORVU5Hr1G038630	-5.71	0.00	-17.58	0.00
HORVU7Hr1G046050	1.59	1.00	-1.95	0.96
HORVU2Hr1G067250	1.17	1.00	4.54	0.70
HORVU4Hr1G060940	11.96	0.00	3.84	0.64
HORVU1Hr1G007800	1.70	1.00	-1.88	1.00
HORVU3Hr1G115420	27.69	0.02	9.09	1.00
HORVU5Hr1G071920	-8.88	0.00	-29.13	0.00
HORVU2Hr1G098110	3.81	0.01	1.15	1.00
HORVU7Hr1G116960	1.39	1.00	-2.39	0.96
HORVU5Hr1G081190	1.70	1.00	-1.97	1.00
HORVU2Hr1G060480	-10.00	0.00	-33.49	0.00
HORVU6Hr1G060370	68.80	0.00	21.98	0.27
HORVU5Hr1G084140	37.45	0.00	11.11	0.00
HORVU3Hr1G075870	-5.59	0.00	-18.92	0.00
HORVU7Hr1G040740	12.24	0.00	3.59	0.05
HORVU4Hr1G015260	-5.38	0.00	-18.34	0.00
HORVU5Hr1G011730	-3.89	0.10	-13.62	0.00
HORVU0Hr1G010880	62.44	0.00	19.40	0.32
HORVU1Hr1G089700	265.21	0.00	81.53	0.00

(continued)

Table A8. Differentially expressed gene in between virulent *rpg4/5* and avirulent *rpg4/5* inoculated sampled and its comparison with the non-inoculated controls (continued)

Feature ID	Non-inoculated vs Vir *rpg4/5* inoculated		Non-inoculated vs avr *rpg4/5* inoculated	
	Fold change[c]	Corrected P-value[d]	Fold change[c]	Corrected P-value[d]
HORVU0Hr1G002870	11.12	0.06	3.20	1.00
HORVU3Hr1G005500	-2.52	0.00	-9.16	0.00
HORVU6Hr1G039840	3.50	0.57	-1.04	1.00
HORVU6Hr1G033160	-3.80	0.00	-14.11	0.00
HORVU2Hr1G073370	-6.49	0.00	-24.82	0.00
HORVU2Hr1G040780	-5.27	0.00	-20.29	0.00
HORVU2Hr1G036960	-3.76	0.00	-14.53	0.00
HORVU2Hr1G065450	-2.75	0.00	-10.67	0.00
HORVU7Hr1G116750	20.65	0.17	5.63	1.00
HORVU6Hr1G090510	-1.53	1.00	-5.98	0.00
HORVU2Hr1G041280	-6.48	0.00	-25.57	0.00
HORVU5Hr1G095580	43.54	0.00	11.13	0.37
HORVU3Hr1G096910	-13.63	0.00	-54.67	0.00
HORVU2Hr1G082330	4.99	0.21	1.27	1.00
HORVU0Hr1G019640	23.78	0.07	6.19	1.00
HORVU7Hr1G046320	-4.61	0.00	-19.59	0.00
HORVU5Hr1G066280	-3.45	0.00	-14.76	0.00
HORVU3Hr1G055260	-3.66	0.06	-15.86	0.00
HORVU7Hr1G070870	1.32	1.00	-3.30	0.07
HORVU3Hr1G033870	26.19	0.06	6.38	1.00
HORVU3Hr1G027970	1.03	1.00	-4.34	0.11
HORVU1Hr1G071500	-1.79	1.00	-8.15	0.00
HORVU6Hr1G009360	-1.50	1.00	-6.96	0.00
HORVU6Hr1G026340	2.40	0.26	-1.93	0.76
HORVU2Hr1G098160	77.54	0.00	17.79	0.16
HORVU2Hr1G041080	-5.87	0.00	-27.97	0.00

(continued)

Table A8. Differentially expressed gene in between virulent *rpg4/5* and avirulent *rpg4/5* inoculated sampled and its comparison with the non-inoculated controls (continued)

Feature ID	Non-inoculated vs Vir *rpg4/5* inoculated		Non-inoculated vs avr *rpg4/5* inoculated	
	Fold change[c]	Corrected P-value[d]	Fold change[c]	Corrected P-value[d]
HORVU2Hr1G090730	1.89	1.00	-2.58	0.29
HORVU6Hr1G093520	118.93	0.00	24.07	0.38
HORVU5Hr1G082420	-7.66	0.00	-39.54	0.00
HORVU7Hr1G027560	10.19	0.11	1.99	1.00
HORVU5Hr1G124160	1.68	1.00	-3.16	0.48
HORVU1Hr1G070190	1.75	1.00	-3.11	0.11
HORVU2Hr1G065000	7.94	0.06	1.37	1.00
HORVU2Hr1G038940	10.08	0.00	1.74	1.00
HORVU7Hr1G096040	5.68	0.59	-1.13	1.00
HORVU3Hr1G055740	67.47	0.00	9.38	0.88
HORVU1Hr1G089540	230.90	0.00	26.43	0.11
HORVU1Hr1G089620	62.53	0.08	6.69	1.00

[a]Annotation of barley gene models (retrieved form http://webblast.ipk-gatersleben.de/barley_ibsc/downloads/)
[b]Top hits of given barley gene to to Arabidopsis *Arabidopsis* Col-o gene annotation (Araport11) (retrieved from https://www.araport.org/data/araport11)
[c]Fold change obtained using EdgeR bioconductor package (Robinson et al., 2010) embedded in CLC genomics
[d]False discovery rate corrected P-value calculated using EdgeR bioconductor package (A gene was considered significantly different between two compared groups if fold change >3 and corrected p-value <0.05)

Table A9. Gene enrichment analysis of differentially expressed gene in virulent vs avirulent *rpg4/5* inoculated Harrington in R bioconductor package TopGO

Sub - Ontology[a]	UP/ Down[b]	GO.ID[c]	GO Term[d]	Annotated[e]	Significant[f]	Expected	Rank[g]	Classic Fisher[h]
BP	Down	GO:0009408	response to heat	199	6	0.34	2	1.10E-06
BP	Down	GO:0009772	photosynthetic electron transport in photosystem II	10	2	0.02	9	0.00013
BP	Down	GO:0042542	response to hydrogen peroxide	62	3	0.11	11	0.00017
BP	Down	GO:0009409	response to cold	395	6	0.68	7	5.60E-05
BP	Down	GO:0009644	response to high light intensity	81	3	0.14	14	0.00037
BP	UP	GO:0009768	photosynthetic electron transport in photosystem I	23	10	0.05	1	7.80E-22
BP	UP	GO:0018298	protein-chromophore linkage	44	9	0.09	5	2.70E-16
BP	UP	GO:0009645	response to low light intensity stimulus	20	5	0.04	10	5.10E-10
BP	UP	GO:0009644	response to high light intensity	81	6	0.17	12	1.90E-08
BP	UP	GO:0010114	response to red light	71	6	0.15	11	8.30E-09
BP	UP	GO:0010218	response to far red light	61	5	0.13	13	1.80E-07
BP	UP	GO:0009637	response to blue light	82	5	0.17	15	8.20E-07
BP	UP	GO:0009769	photosynthesis, light harvesting in photosystem II	10	3	0.02	17	1.00E-06
BP	UP	GO:0009409	response to cold	395	7	0.83	22	1.80E-05
BP	UP	GO:0009723	response to ethylene	314	5	0.66	37	0.00051
CC	Down	GO:0009570	chloroplast stroma	740	10	1.22	5	0.0000003
CC	Down	GO:0009535	chloroplast thylakoid membrane	402	5	0.67	17	0.00052
CC	UP	GO:0009522	photosystem I	41	12	0.08	1	7.3E-24
CC	UP	GO:0010287	plastoglobule	80	9	0.16	13	4.7E-14
BP	UP	GO:0010228	vegetative to reproductive phase transition of meristem	192	4	0.4	38	0.00071

(continued)

Table A9. Gene enrichment analysis of differentially expressed gene in virulent vs avirulent *rpg4/5* inoculated Harrington in R bioconductor package TopGO (continued)

Sub - Ontology[a]	UP/ Down[b]	GO.ID[c]	GO Term[d]	Annotated[e]	Significant[f]	Expected	Rank[g]	Classic Fisher[h]
CC	UP	GO:0030076	light-harvesting complex	25	8	0.05	3	1.4E-16
CC	UP	GO:0009535	chloroplast thylakoid membrane	402	15	0.79	5	1.3E-15
CC	UP	GO:0009523	photosystem II	67	8	0.13	14	8E-13
CC	UP	GO:0009941	chloroplast envelope	682	11	1.34	19	7.8E-08
CC	UP	GO:0016021	integral component of membrane	4855	24	9.54	26	0.0000055
CC	UP	GO:0009517	PSII associated light-harvesting complex II	6	2	0.01	31	0.000057
MF	UP	GO:0016168	chlorophyll binding	40	11	0.08	1	1.5E-21
MF	UP	GO:0031409	pigment binding	22	9	0.04	2	1.2E-19
MF	UP	GO:0005515	protein binding	3606	20	7.21	8	0.0000099
MF	UP	GO:0046872	metal ion binding	3464	21	6.93	5	0.0000012

[a]Gene enrichment was done for 3 different sub-ontology: BP=Bilogical Process, CF=Cellular Component, MF=Molecular Function
[b]Represents if a gene is upregulated (UP) or downregulated (Down) in a differential gene expression analysis for host gene in in virulent vs avirulent *rpg4/5* inoculated Harrington
[c]Gene Ontology (GO) ID of a significantly enriched GO Term
[d]Significantly enriched GO term
[e]Total number of annotated gene in Arabiopsis for given GO term
[s]ignificant number of differentially expressed gene in this analysis that falls under a given GO term
[g]Ranking based on classic Fisher statistics
[h]A GO term is considered significantly enriched if the classic fisher value is <0.001

Table A10. Comparison between RNAseq mapping using *de novo* assembled transcripts vs barley RefSeq v1

De novo Transcript ID	Barley Gene[a]	Using Reference Genome[b]				Using *de novo* Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN29284_c0_g1_i1	HORVU5Hr1G115340	3932	23382	-2.53	0.00	861	4216	-3.65	0.01
TRINITY_DN42388_c0_g1_i7	HORVU6Hr1G031670	9181	23385	-1.17	1.00	87	403	-3.85	0.02
TRINITY_DN38785_c1_g1_i3	HORVU7Hr1G114650	35398	40508	1.91	0.00	506	257	3.13	0.02
TRINITY_DN28815_c0_g1_i1	HORVU2Hr1G041270	205	115	3.12	0.20	3151	1130	4.33	0.02
TRINITY_DN31634_c0_g1_i1	HORVU7Hr1G086520	6792	27915	-2.07	0.00	97	413	-3.34	0.05
TRINITY_DN11120_c0_g1_i1	HORVU4Hr1G000910	959	3710	-1.69	0.27	32	207	-5.00	0.01
TRINITY_DN10259_c0_g1_i1	HORVU7Hr1G112470	344	589	1.18	1.00	66	261	-3.12	0.05
TRINITY_DN24340_c0_g1_i1	HORVU7Hr1G006490	43207	161655	-1.64	0.01	696	3370	-3.73	0.02
TRINITY_DN37852_c0_g2_i2	HORVU7Hr1G098200	576	2029	-1.57	1.00	53	207	-3.23	0.04
TRINITY_DN14999_c0_g1_i2	HORVU2Hr1G020440	1375	4030	-1.26	1.00	259	1091	-3.15	0.03
TRINITY_DN12112_c0_g1_i1	HORVU5Hr1G022550	2645	1689	2.81	0.01	183	65	4.03	0.02
TRINITY_DN32808_c0_g1_i5	HORVU2Hr1G080430	1352	8108	-2.81	0.02	20	132	-5.31	0.05
TRINITY_DN40238_c0_g1_i2	HORVU3Hr1G018290	7	19	-1.05	1.00	21	910	-15.5	0.03
TRINITY_DN37451_c0_g1_i15	HORVU2Hr1G120170	143	931	-3.12	0.27	16	245	-7.91	0.04
TRINITY_DN16808_c1_g1_i1	HORVU2Hr1G080480	3602	18908	-2.25	0.00	923	4581	-3.99	0.00
TRINITY_DN37615_c1_g3_i1	HORVU6Hr1G041360	9	119	-1.81	1.00	187	858	-3.69	0.01
TRINITY_DN33504_c0_g1_i3	HORVU6Hr1G027650	43328	131818	-1.40	0.54	260	1658	-5.40	0.00
TRINITY_DN46106_c1_g1_i1	HORVU2Hr1G113350	3775	18183	-2.14	0.00	251	987	-3.18	0.03
TRINITY_DN36923_c4_g2_i1	HORVU7Hr1G036240	0	0	1.00	1.00	5	378	-24.7	0.00
TRINITY_DN25295_c0_g2_i1	HORVU3Hr1G092680	26815	78431	-1.31	0.74	15	129	-7.84	0.02
TRINITY_DN24165_c0_g1_i3	HORVU4Hr1G017030	130288	114596	2.43	0.02	3057	1353	3.74	0.00
TRINITY_DN44526_c0_g2_i9	HORVU0Hr1G018110	1483	4157	-1.27	1.00	27	174	-4.72	0.05
TRINITY_DN20448_c0_g1_i1	HORVU2Hr1G101150	20509	62228	-1.46	1.00	134	717	-4.82	0.01
TRINITY_DN32116_c0_g2_i3	HORVU3Hr1G111910	3683	7867	1.03	1.00	177	727	-3.54	0.04
TRINITY_DN26551_c0_g1_i2	HORVU2Hr1G089160	1212	3814	-1.39	0.86	121	517	-3.53	0.02

(continued)

Table A10. Comparison between RNAseq mapping using *de novo* assembled transcripts vs barley RefSeq v1 (continued)

De novo Transcript ID	Barley Gene[a]	Using Reference Genome[b]				Using *de novo* Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN38405_c0_g2_i8	HORVU1Hr1G064490	40025	78574	1.11	1.00	196	887	-3.44	0.03
TRINITY_DN27766_c0_g1_i2	HORVU7Hr1G094240	53816	36805	2.88	0.00	807	249	4.89	0.00
TRINITY_DN42132_c0_g2_i4	HORVU3Hr1G065720	12291	33689	-1.23	1.00	25	244	-6.19	0.02
TRINITY_DN39931_c0_g2_i9	HORVU1Hr1G029270	1012	4993	-2.14	0.02	1342	5070	-3.09	0.03
TRINITY_DN14954_c0_g1_i3	HORVU0Hr1G003890	43443	35695	2.45	0.16	632	277	3.20	0.03
TRINITY_DN20493_c0_g1_i1	HORVU5Hr1G050510	7879	17934	-1.05	1.00	385	2398	-5.33	0.01
TRINITY_DN36791_c1_g1_i1	HORVU4Hr1G044210	0	0	1.00	1.00	14	556	-20.9	0.00
TRINITY_DN36791_c1_g1_i8	HORVU4Hr1G044210	0	0	1.00	1.00	11	522	-22.9	0.00
TRINITY_DN36791_c1_g1_i2	HORVU2Hr1G127580	0	0	1.00	1.00	43	1429	-23.7	0.00
TRINITY_DN36791_c1_g1_i9	HORVU4Hr1G044210	0	0	1.00	1.00	8	1029	-57.2	0.00
TRINITY_DN36791_c0_g1_i1	HORVU5Hr1G021540	26	284	-2.42	1.00	62	1096	-11.7	0.00
TRINITY_DN43589_c1_g1_i20	HORVU6Hr1G041720	19791	74193	-1.70	0.00	10	80	-6.53	0.04
TRINITY_DN77386_c0_g1_i1	HORVU5Hr1G064200	367	1695	-1.87	1.00	13	103	-4.91	0.03
TRINITY_DN39675_c1_g1_i1	HORVU4Hr1G066430	1106	3016	-1.28	1.00	116	595	-4.05	0.01
TRINITY_DN29507_c1_g1_i1	HORVU5Hr1G109710	43506	32658	2.83	0.01	2737	1355	3.00	0.04
TRINITY_DN43662_c0_g1_i8	HORVU5Hr1G008050	14504	12533	2.19	0.15	1014	311	4.12	0.03
TRINITY_DN38828_c0_g3_i1	HORVU7Hr1G002460	424	1585	-1.64	1.00	322	1499	-3.70	0.03
TRINITY_DN65107_c0_g1_i1	HORVU4Hr1G081810	208	342	1.10	1.00	158	54	4.04	0.01
TRINITY_DN40333_c0_g1_i7	HORVU3Hr1G068110	84457	309794	-1.68	0.07	132	688	-4.19	0.05
TRINITY_DN55241_c0_g1_i1	HORVU4Hr1G059260	2121	24326	-6.01	0.00	12	132	-6.95	0.03
TRINITY_DN26991_c0_g1_i2	HORVU2Hr1G079840	14029	94237	-3.12	0.00	162	1158	-5.68	0.01
TRINITY_DN13681_c0_g1_i1	HORVU5Hr1G053480	2710	20956	-3.19	0.00	120	677	-3.88	0.02
TRINITY_DN24410_c0_g1_i1	HORVU5Hr1G084140	15444	9282	3.34	0.00	424	190	3.51	0.04
TRINITY_DN37501_c0_g1_i1	HORVU7Hr1G020110	32	439	-5.71	0.04	80	832	-7.04	0.00
TRINITY_DN43692_c3_g1_i1	HORVU4Hr1G002020	1710	2729	1.50	1.00	63	415	-5.02	0.01

(continued)

Table A10. Comparison between RNAseq mapping using *de novo* assembled transcripts vs barley RefSeq v1 (continued)

De novo Transcript ID	Barley Gene[a]	Using Reference Genome[b]				Using *de novo* Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN39748_c0_g1_i3	HORVU7Hr1G046320	119798	56472	4.21	0.02	1307	401	5.36	0.04
TRINITY_DN24283_c0_g1_i1	HORVU5Hr1G066280	111926	52984	4.24	0.02	3427	1170	4.67	0.03
TRINITY_DN28815_c0_g2_i1	HORVU2Hr1G041280	117003	59766	3.91	0.00	1201	432	4.32	0.03
TRINITY_DN37451_c0_g1_i2	HORVU3Hr1G007500	1469	48691	-14.4	0.00	23	191	-5.64	0.03
TRINITY_DN37451_c0_g1_i6	HORVU3Hr1G007500	1469	48691	-14.4	0.00	19	291	-7.47	0.01
TRINITY_DN37451_c0_g1_i14	HORVU3Hr1G007500	1469	48691	-14.4	0.00	19	342	-9.94	0.02
TRINITY_DN47241_c0_g1_i1	HORVU5Hr1G095580	7796	4063	3.92	0.02	185	70	4.56	0.02
TRINITY_DN30941_c0_g1_i2	HORVU4Hr1G063350	477	24489	-24.8	0.00	40	485	-7.55	0.02
TRINITY_DN30941_c0_g1_i1	HORVU4Hr1G063350	477	24489	-24.8	0.00	8	442	-17.8	0.01
TRINITY_DN18883_c0_g1_i1	HORVU2Hr1G036960	123290	66403	3.83	0.00	6424	2412	3.96	0.00
TRINITY_DN37227_c1_g1_i1	HORVU6Hr1G016860	3311	1112	5.54	0.01	34	9	6.05	0.02
TRINITY_DN63469_c0_g1_i1	HORVU4Hr1G051010	1095	17448	-8.43	0.00	30	477	-8.99	0.02
TRINITY_DN29706_c0_g2_i1	HORVU1Hr1G070190	5150	1946	5.34	0.00	121	25	5.44	0.01
TRINITY_DN20138_c0_g1_i1	HORVU3Hr1G055740	2932	891	7.65	0.00	96	12	8.89	0.00
TRINITY_DN37451_c0_g1_i19	HORVU3Hr1G007500	1469	48691	-14.4	0.00	22	211	-5.99	0.02
TRINITY_DN69791_c0_g1_i1	HORVU6Hr1G026340	4452	1829	4.59	0.00	250	74	4.48	0.01
TRINITY_DN38644_c0_g1_i7	HORVU2Hr1G041410	539	4505	-3.73	0.00	18	160	-5.83	0.02
TRINITY_DN21359_c0_g2_i1	HORVU7Hr1G042790	164	12845	-37.0	0.00	3	167	-15.1	0.01
TRINITY_DN47359_c0_g1_i1	HORVU7Hr1G042790	164	12845	-37.0	0.00	2	99	-19.1	0.00
TRINITY_DN15045_c0_g1_i1	HORVU2Hr1G059920	326	9136	-12.2	0.00	16	394	-13.2	0.00
TRINITY_DN33431_c0_g1_i1	HORVU3Hr1G084210	1355	21862	-7.80	0.00	266	4050	-12.4	0.00
TRINITY_DN35523_c1_g2_i3	HORVU4Hr1G007420	9555	6514	3.04	0.00	669	317	3.40	0.02
TRINITY_DN551_c0_g1_i1	HORVU5Hr1G081190	2956	1856	3.31	0.01	196	66	4.81	0.01
TRINITY_DN11356_c0_g1_i1	HORVU5Hr1G095580	7796	4063	3.92	0.02	241	97	4.37	0.02

(continued)

Table A10. Comparison between RNAseq mapping using *de novo* assembled transcripts vs barley RefSeq v1 (continued)

De novo Transcript ID	Barley Gene[a]	Using Reference Genome[b]				Using *de novo* Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN43333_c0_g1_i1	HORVU6Hr1G016480	53	1982	-20.3	0.00	12	187	-14.1	0.01
TRINITY_DN11250_c0_g1_i1	HORVU6Hr1G093520	5535	1985	5.05	0.00	264	79	5.34	0.01
TRINITY_DN57452_c0_g1_i1	HORVU3Hr1G088970	1659	15989	-3.97	0.00	127	955	-5.20	0.01
TRINITY_DN45663_c1_g1_i7	HORVU3Hr1G027970	9322	3408	4.39	0.02	286	82	5.30	0.04
TRINITY_DN34315_c0_g1_i2	HORVU2Hr1G073370	50736	25510	3.79	0.02	2596	888	4.40	0.03
TRINITY_DN34631_c0_g1_i2	HORVU7Hr1G082850	643	6015	-3.53	0.03	30	200	-4.61	0.05

[a]Barley gene corresponding to the top hit of given *de novo* transcript

[b]Data represent the output obtained from mapping of orignial data to barley RefSeq v1

[c]Data represent the output obtained from mapping of unmapped read to *de novo* assembled transcripts. Unmapped reads are the reads that did not map to either of barley RefSeq v1 and *Pgt* SCCL genome

[db]The sum of reads of ll the sample representing virulent *rpg4/5* samples that mapped to the barley gene

[eb]The sum of reads of all the sample representing avirulent *rpg4/5* samples that mapped to the barley gene

[fb]The fold change between sample inoculated with virulent *rpg4/5* isolates vs avirulent *rpg4/5* inoculated isolates for the barley gene

[gb]FDR corrected P-value for fold change of the barley gene

[dc]The sum of reads for all the sample representing virulent *rpg4/5* samples that mapped to the *de novo* assemble transcript

[ec]The sum of reads for all the sample representing avirulent *rpg4/5* samples that mapped to the *de novo* assemble transcript

[fc]The fold change between sample inoculated with virulent *rpg4/5* isolates vs avirulent *rpg4/5* inoculated isolates for the *de novo* assemble transcript

[gc]FDR corrected P-value for fold change of the *de novo* assemble transcript

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN42508_c1_g1_i2	PGTG_20734	1430	574	1.01	1.00	1430	574	3.31	0.04
TRINITY_DN41619_c1_g1_i1	PGTG_11395	221	95	1.05	1.00	221	95	3.84	0.01
TRINITY_DN36302_c0_g1_i2	PGTG_09766	1284	264	-1.18	0.63	1284	264	6.71	0.01
TRINITY_DN44358_c2_g2_i1	PGTG_13207	155	1364	-1.28	0.92	155	1364	-5.33	0.03
TRINITY_DN43054_c0_g1_i3	PGTG_22023	437	154	1.23	0.52	437	154	4.21	0.01
TRINITY_DN43728_c0_g1_i4	PGTG_14391	939	359	1.49	0.06	939	359	3.70	0.02
TRINITY_DN39596_c0_g2_i9	PGTG_20631	23	896	1.13	0.90	23	896	-23.82	0.01
TRINITY_DN44600_c1_g1_i1	PGTG_04896	722	281	1.07	1.00	722	281	3.73	0.01
TRINITY_DN45340_c0_g2_i6	PGTG_15995	2750	1332	1.27	0.68	2750	1332	3.30	0.02
TRINITY_DN44755_c0_g1_i5	PGTG_00085	75	15	1.01	1.00	75	15	7.72	0.00
TRINITY_DN39336_c0_g1_i6	PGTG_00164	176	74	2.04	0.11	176	74	3.58	0.04
TRINITY_DN39376_c0_g1_i11	PGTG_00366	498	46	1.34	0.89	498	46	15.51	0.00
TRINITY_DN41759_c0_g1_i2	PGTG_00742	430	157	1.18	0.82	430	157	4.37	0.03
TRINITY_DN41951_c1_g2_i2	PGTG_01101	331	138	1.17	0.86	331	138	3.24	0.03
TRINITY_DN46232_c5_g1_i9	PGTG_01402	376	126	-1.10	1.00	376	126	4.65	0.01
TRINITY_DN40973_c0_g1_i2	PGTG_01863	266	78	-1.12	0.98	266	78	5.29	0.01
TRINITY_DN44500_c0_g1_i8	PGTG_02378	423	88	1.07	1.00	423	88	5.95	0.00
TRINITY_DN41214_c0_g3_i2	PGTG_02514	418	127	-1.04	1.00	418	127	4.32	0.00
TRINITY_DN40597_c0_g1_i2	PGTG_02561	1396	666	-1.01	1.00	1396	666	3.02	0.02
TRINITY_DN43933_c1_g1_i2	PGTG_02601	486	233	1.33	0.39	486	233	3.11	0.03
TRINITY_DN41900_c1_g1_i5	PGTG_02602	1	185	-1.37	1.00	1	185	-25.50	0.00
TRINITY_DN42283_c1_g1_i8	PGTG_02654	346	136	-1.12	1.00	346	136	3.95	0.02
TRINITY_DN42283_c1_g1_i5	PGTG_02654	366	147	-1.12	1.00	366	147	3.74	0.02
TRINITY_DN35813_c0_g1_i6	PGTG_02682	254	80	1.11	1.00	254	80	3.90	0.02
TRINITY_DN40601_c0_g1_i2	PGTG_02680	115	26	2.32	0.04	115	26	6.69	0.00

(continued)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN44206_c0_g1_i10	PGTG_02767	37	4	-1.06	1.00	37	4	6.92	0.00
TRINITY_DN37554_c0_g1_i2	PGTG_02815	167	61	1.11	1.00	167	61	3.64	0.04
TRINITY_DN44618_c1_g1_i7	PGTG_02894	369	90	-1.11	1.00	369	90	5.70	0.00
TRINITY_DN44618_c1_g2_i3	PGTG_02894	8	309	-1.11	1.00	8	309	-17.54	0.02
TRINITY_DN45421_c0_g1_i11	PGTG_02911	0	100	1.04	1.00	0	100	-21.68	0.03
TRINITY_DN45421_c0_g1_i10	PGTG_02911	0	106	1.04	1.00	0	106	-22.06	0.02
TRINITY_DN45421_c0_g1_i7	PGTG_02911	0	138	1.04	1.00	0	138	-25.67	0.02
TRINITY_DN45421_c0_g1_i8	PGTG_02911	0	186	1.04	1.00	0	186	-30.69	0.02
TRINITY_DN44444_c0_g1_i5	PGTG_02944	523	211	-1.12	1.00	523	211	3.53	0.04
TRINITY_DN46378_c2_g1_i3	PGTG_03545	191	55	-1.72	0.20	191	55	5.13	0.00
TRINITY_DN40976_c0_g1_i12	PGTG_03552	409	178	-1.29	0.33	409	178	3.10	0.04
TRINITY_DN35700_c0_g1_i1	PGTG_03645	129	681	-2.56	0.06	129	681	-3.94	0.02
TRINITY_DN41512_c0_g1_i2	PGTG_04311	219	95	-1.06	1.00	219	95	3.48	0.03
TRINITY_DN44989_c1_g2_i3	PGTG_04572	1565	417	1.07	1.00	1565	417	4.93	0.01
TRINITY_DN44321_c1_g1_i1	PGTG_04690	948	218	1.02	1.00	948	218	5.22	0.01
TRINITY_DN38990_c1_g1_i2	PGTG_04707	134	50	1.06	1.00	134	50	3.72	0.03
TRINITY_DN42299_c0_g1_i6	PGTG_04883	407	91	-2.18	0.53	407	91	5.58	0.01
TRINITY_DN44713_c0_g1_i20	PGTG_04940	131	51	1.06	1.00	131	51	4.21	0.03
TRINITY_DN46181_c3_g2_i6	PGTG_05067	989	363	1.01	1.00	989	363	3.92	0.01
TRINITY_DN43427_c0_g1_i7	PGTG_05095	102	26	1.32	0.75	102	26	5.32	0.00
TRINITY_DN43706_c0_g1_i2	PGTG_05247	113	43	1.14	0.99	113	43	3.95	0.02
TRINITY_DN41107_c4_g1_i1	PGTG_05284	89	25	1.15	0.87	89	25	5.18	0.01
TRINITY_DN35738_c0_g1_i1	PGTG_05713	2	629	-6.74	0.27	2	629	-22.01	0.01
TRINITY_DN45072_c1_g1_i4	PGTG_06314	447	136	-1.06	1.00	447	136	4.87	0.01
TRINITY_DN43686_c0_g1_i4	PGTG_06316	218	75	-1.12	1.00	218	75	4.02	0.02
TRINITY_DN46321_c5_g1_i2	PGTG_06667	1721	352	1.12	0.94	1721	352	6.45	0.00

(continued)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN40754_c0_g1_i4	PGTG_06668	204	81	-1.05	1.00	204	81	3.54	0.04
TRINITY_DN45823_c3_g1_i1	PGTG_07033	259	87	1.01	1.00	259	87	4.17	0.04
TRINITY_DN42828_c0_g3_i4	PGTG_07148	258	59	-1.08	1.00	258	59	5.92	0.00
TRINITY_DN46145_c4_g1_i9	PGTG_07239	97	20	1.09	1.00	97	20	5.49	0.01
TRINITY_DN46349_c5_g1_i6	PGTG_07250	1830	914	1.18	0.88	1830	914	3.07	0.02
TRINITY_DN45931_c2_g2_i11	PGTG_07313	119	45	1.09	1.00	119	45	3.87	0.00
TRINITY_DN44166_c0_g1_i7	PGTG_07339	309	100	-1.08	1.00	309	100	4.00	0.01
TRINITY_DN43934_c1_g1_i1	PGTG_07371	2	99	1.46	0.66	2	99	-10.73	0.03
TRINITY_DN43746_c0_g2_i5	PGTG_07396	216	88	-1.21	0.78	216	88	3.60	0.04
TRINITY_DN44696_c1_g2_i4	PGTG_07409	289	114	1.08	1.00	289	114	3.76	0.02
TRINITY_DN41943_c1_g1_i15	PGTG_08305	48	757	1.02	1.00	48	757	-7.71	0.02
TRINITY_DN41943_c1_g1_i4	PGTG_08305	28	544	1.02	1.00	28	544	-8.84	0.02
TRINITY_DN46257_c0_g2_i5	PGTG_08661	941	349	1.33	0.20	941	349	3.77	0.00
TRINITY_DN43738_c0_g1_i6	PGTG_08763	365	127	1.09	1.00	365	127	3.99	0.02
TRINITY_DN40403_c0_g1_i4	PGTG_08790	417	133	1.09	1.00	417	133	4.30	0.00
TRINITY_DN39384_c1_g1_i8	PGTG_08831	89	16	2.88	0.27	89	16	6.59	0.01
TRINITY_DN40618_c0_g1_i6	PGTG_09037	593	189	1.19	0.75	593	189	4.39	0.00
TRINITY_DN42953_c1_g1_i11	PGTG_09082	97	20	-1.10	1.00	97	20	4.83	0.02
TRINITY_DN43296_c0_g3_i10	PGTG_09137	281	52	-1.02	1.00	281	52	5.99	0.00
TRINITY_DN43093_c3_g1_i17	PGTG_09749	295	120	-1.40	0.32	295	120	3.79	0.03
TRINITY_DN44233_c1_g2_i3	PGTG_10130	112	36	-1.00	1.00	112	36	4.18	0.01
TRINITY_DN45859_c0_g1_i7	PGTG_10162	492	115	1.10	1.00	492	115	6.08	0.03
TRINITY_DN40908_c0_g1_i4	PGTG_10246	489	77	-1.08	1.00	489	77	8.50	0.00
TRINITY_DN45636_c6_g1_i5	PGTG_10301	489	175	1.10	1.00	489	175	3.79	0.05
TRINITY_DN33607_c0_g1_i4	PGTG_10301	19	427	1.10	1.00	19	427	-11.42	0.02
TRINITY_DN39312_c0_g1_i4	PGTG_10503	249	1950	-1.17	0.91	249	1950	-4.74	0.04

(continued)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN45396_c3_g1_i3	PGTG_10521	127	36	1.39	0.55	127	36	4.85	0.02
TRINITY_DN41130_c0_g1_i1	PGTG_11089	147	42	1.13	1.00	147	42	4.62	0.00
TRINITY_DN43302_c1_g1_i2	PGTG_11239	1121	296	1.18	1.00	1121	296	5.00	0.01
TRINITY_DN46254_c0_g1_i5	PGTG_11460	623	256	-1.37	0.36	623	256	3.69	0.01
TRINITY_DN45500_c2_g1_i1	PGTG_11621	1117	227	1.21	0.58	1117	227	6.64	0.00
TRINITY_DN28324_c0_g1_i5	PGTG_11665	164	70	1.26	0.67	164	70	3.63	0.03
TRINITY_DN41606_c0_g1_i9	PGTG_22757	358	140	1.23	1.00	358	140	3.15	0.03
TRINITY_DN39909_c0_g1_i2	PGTG_11906	876	325	1.22	0.88	876	325	3.90	0.03
TRINITY_DN40937_c0_g1_i4	PGTG_11925	230	47	-1.00	1.00	230	47	7.01	0.01
TRINITY_DN41478_c1_g1_i3	PGTG_12260	291	94	-1.15	0.95	291	94	4.10	0.03
TRINITY_DN44474_c0_g1_i13	PGTG_12324	304	95	1.48	0.14	304	95	4.15	0.02
TRINITY_DN41391_c0_g1_i7	PGTG_12330	2232	810	-1.00	1.00	2232	810	3.77	0.00
TRINITY_DN44226_c2_g1_i9	PGTG_12691	360	110	-1.01	1.00	360	110	4.91	0.03
TRINITY_DN40651_c0_g1_i3	PGTG_13265	1013	469	1.28	0.78	1013	469	3.38	0.05
TRINITY_DN40386_c0_g1_i1	PGTG_13270	94	36	1.06	1.00	94	36	4.08	0.05
TRINITY_DN44000_c2_g2_i9	PGTG_13528	234	26	-1.01	1.00	234	26	11.52	0.00
TRINITY_DN39762_c2_g1_i4	PGTG_13691	28	554	-3.43	0.00	28	554	-7.95	0.02
TRINITY_DN43233_c0_g1_i7	PGTG_13933	481	134	1.41	0.64	481	134	5.76	0.00
TRINITY_DN43233_c0_g1_i5	PGTG_13933	400	120	1.41	0.64	400	120	5.37	0.00
TRINITY_DN43003_c0_g1_i1	PGTG_13944	3501	1265	1.12	0.98	3501	1265	4.22	0.00
TRINITY_DN32294_c0_g1_i4	PGTG_13979	4	135	-1.22	1.00	4	135	-11.92	0.02
TRINITY_DN43573_c0_g1_i6	PGTG_14036	180	55	1.02	1.00	180	55	4.66	0.01
TRINITY_DN40641_c0_g1_i6	PGTG_14118	13	272	-1.60	0.05	13	272	-8.88	0.02
TRINITY_DN44838_c3_g2_i1	PGTG_14602	199	43	1.01	1.00	199	43	6.57	0.00
TRINITY_DN45168_c1_g1_i1	PGTG_14617	56	493	1.79	0.29	56	493	-5.77	0.02
TRINITY_DN42561_c0_g1_i7	PGTG_14940	1115	356	-1.06	1.00	1115	356	4.51	0.00

(continued)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN40617_c0_g1_i9	PGTG_15095	715	160	-1.08	1.00	715	160	6.29	0.01
TRINITY_DN36299_c10_g2_i1	PGTG_15174	16301	8057	1.55	0.19	16301	8057	3.10	0.05
TRINITY_DN41932_c0_g1_i16	PGTG_15191	212	30	-1.02	1.00	212	30	7.82	0.00
TRINITY_DN38135_c0_g2_i4	PGTG_15326	32	575	-3.71	0.29	32	575	-10.60	0.02
TRINITY_DN41430_c0_g1_i3	PGTG_15465	205	95	1.75	0.22	205	95	3.53	0.03
TRINITY_DN40927_c0_g1_i4	PGTG_15486	535	170	2.17	0.03	535	170	4.79	0.00
TRINITY_DN43393_c0_g1_i4	PGTG_15554	460	75	-1.08	1.00	460	75	7.62	0.00
TRINITY_DN39717_c0_g1_i4	PGTG_15755	606	204	-1.09	1.00	606	204	4.04	0.01
TRINITY_DN39717_c0_g1_i1	PGTG_15755	509	233	-1.09	1.00	509	233	3.41	0.03
TRINITY_DN45763_c2_g2_i1	PGTG_15815	378	107	1.39	1.00	378	107	5.16	0.00
TRINITY_DN33809_c0_g1_i2	PGTG_15926	278	39	1.94	0.27	278	39	7.95	0.00
TRINITY_DN42496_c0_g2_i1	PGTG_16216	119	49	1.35	0.57	119	49	4.06	0.03
TRINITY_DN43158_c0_g1_i7	PGTG_16474	1737	789	1.12	1.00	1737	789	3.31	0.00
TRINITY_DN41862_c0_g2_i4	PGTG_16718	886	371	-1.18	1.00	886	371	3.49	0.00
TRINITY_DN45823_c4_g1_i3	PGTG_16969	408	18	1.07	1.00	408	18	29.05	0.00
TRINITY_DN45823_c4_g1_i1	PGTG_16969	278	22	1.07	1.00	278	22	23.30	0.00
TRINITY_DN46220_c2_g2_i5	PGTG_16991	191	47	1.02	1.00	191	47	4.46	0.03
TRINITY_DN45751_c2_g2_i3	PGTG_17178	718	128	-1.04	1.00	718	128	7.31	0.00
TRINITY_DN42378_c2_g1_i5	PGTG_17365	21	260	1.03	1.00	21	260	-9.34	0.03
TRINITY_DN41469_c1_g2_i2	PGTG_17672	282	128	1.03	1.00	282	128	3.48	0.03
TRINITY_DN19988_c0_g1_i1	PGTG_17756	134	41	2.49	0.40	134	41	4.42	0.02
TRINITY_DN44364_c1_g1_i16	PGTG_18142	0	157	-5.44	0.24	0	157	-32.71	0.01
TRINITY_DN40300_c0_g1_i1	PGTG_18356	269	118	1.16	0.87	269	118	3.45	0.03
TRINITY_DN43618_c2_g1_i7	PGTG_18443	88	16	1.17	0.80	88	16	5.57	0.01
TRINITY_DN44727_c0_g2_i5	PGTG_18685	209	84	1.09	1.00	209	84	3.46	0.01
TRINITY_DN42443_c0_g1_i1	PGTG_18718	323	153	1.09	1.00	323	153	3.09	0.04

(continued)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN42443_c0_g1_i4	PGTG_18718	325	156	1.09	1.00	325	156	3.00	0.04
TRINITY_DN40281_c1_g1_i1	PGTG_18976	250	67	1.01	1.00	250	67	5.39	0.00
TRINITY_DN44337_c6_g2_i1	PGTG_19039	133	60	-1.05	1.00	133	60	3.33	0.04
TRINITY_DN38943_c0_g3_i1	PGTG_19211	2290	576	1.33	1.00	2290	576	5.93	0.00
TRINITY_DN42479_c0_g1_i1	PGTG_19325	252	33	1.11	1.00	252	33	8.92	0.00
TRINITY_DN42479_c0_g1_i5	PGTG_19325	675	176	1.11	1.00	675	176	4.73	0.02
TRINITY_DN46018_c3_g1_i3	PGTG_19453	109	9202	-1.53	1.00	109	9202	-47.30	0.00
TRINITY_DN43714_c0_g1_i4	PGTG_19496	100	12	-1.10	1.00	100	12	9.22	0.00
TRINITY_DN39003_c0_g2_i2	PGTG_19500	444	104	-1.01	1.00	444	104	6.03	0.00
TRINITY_DN39003_c0_g2_i6	PGTG_19500	432	95	-1.01	1.00	432	95	5.89	0.00
TRINITY_DN39190_c0_g1_i3	PGTG_19794	333	141	1.08	1.00	333	141	3.41	0.04
TRINITY_DN42845_c2_g5_i1	PGTG_19912	237	54	2.35	0.37	237	54	4.86	0.04
TRINITY_DN42371_c1_g1_i1	PGTG_19991	489	114	-1.12	1.00	489	114	5.34	0.00
TRINITY_DN44358_c1_g2_i1	PGTG_20020	1247	337	1.31	0.45	1247	337	6.27	0.04
TRINITY_DN42098_c0_g1_i5	PGTG_20023	474	235	1.11	1.00	474	235	3.05	0.05
TRINITY_DN45203_c2_g3_i2	PGTG_20056	222	107	1.22	0.63	222	107	3.17	0.03
TRINITY_DN46004_c3_g2_i8	PGTG_20106	424	70	1.68	0.02	424	70	8.32	0.00
TRINITY_DN43377_c0_g2_i1	PGTG_20106	347	161	1.68	0.02	347	161	3.26	0.05
TRINITY_DN43428_c1_g1_i1	PGTG_22572	432	148	-1.11	1.00	432	148	4.68	0.02
TRINITY_DN41705_c0_g1_i8	PGTG_20331	221	97	1.47	0.20	221	97	3.34	0.03
TRINITY_DN40844_c0_g1_i1	PGTG_20660	1287	285	1.06	1.00	1287	285	6.72	0.00
TRINITY_DN41745_c0_g1_i1	PGTG_20711	595	115	1.40	1.00	595	115	7.30	0.01
TRINITY_DN45060_c0_g2_i2	PGTG_20746	272	126	-1.13	0.91	272	126	3.53	0.03
TRINITY_DN44090_c0_g1_i14	PGTG_20901	102	27	1.68	0.09	102	27	5.74	0.02
TRINITY_DN45131_c0_g2_i1	PGTG_21086	175	63	1.02	1.00	175	63	3.84	0.02
TRINITY_DN45670_c6_g3_i3	PGTG_21144	113	36	1.31	0.37	113	36	3.94	0.02

(continued)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN41112_c0_g1_i2	PGTG_21210	559	146	1.85	0.58	559	146	5.17	0.00
TRINITY_DN42440_c1_g3_i3	PGTG_21347	1968	709	-1.14	1.00	1968	709	4.13	0.01
TRINITY_DN45044_c0_g1_i2	PGTG_21581	284	119	-1.08	1.00	284	119	3.32	0.04
TRINITY_DN43475_c2_g1_i2	PGTG_21720	216	23	1.41	0.94	216	23	10.13	0.00
TRINITY_DN41553_c0_g1_i4	PGTG_22084	154	48	2.78	0.01	154	48	4.97	0.02
TRINITY_DN40975_c0_g1_i2	PGTG_22097	518	149	2.26	0.03	518	149	4.76	0.01
TRINITY_DN38837_c0_g1_i5	PGTG_22196	523	228	1.06	1.00	523	228	3.25	0.04
TRINITY_DN44835_c0_g1_i3	PGTG_22267	662	76	1.38	0.15	662	76	11.93	0.00
TRINITY_DN5955_c0_g1_i2	PGTG_22339	1	85	-3.69	0.86	1	85	-11.77	0.05
TRINITY_DN34862_c0_g1_i1	PGTG_22446	589	30	3.89	0.04	589	30	23.57	0.00
TRINITY_DN44281_c2_g1_i10	PGTG_01889	710	264	1.42	0.52	710	264	4.04	0.01
TRINITY_DN40231_c1_g2_i2	PGTG_09457	691	239	1.19	0.90	691	239	4.39	0.03
TRINITY_DN39438_c0_g1_i3	PGTG_16470	717	183	1.14	0.96	717	183	5.99	0.00
TRINITY_DN43768_c1_g2_i2	PGTG_15693	2733	650	2.47	0.00	2733	650	6.83	0.00
TRINITY_DN41404_c0_g1_i2	PGTG_01916	581	204	1.04	1.00	581	204	4.09	0.03
TRINITY_DN43795_c0_g1_i3	PGTG_12713	4120	1937	1.15	0.85	4120	1937	3.26	0.04
TRINITY_DN43795_c0_g1_i1	PGTG_12713	32	1157	1.15	0.85	32	1157	-18.87	0.01
TRINITY_DN43004_c0_g2_i2	PGTG_05022	238	102	1.16	0.87	238	102	3.44	0.03
TRINITY_DN43004_c0_g2_i3	PGTG_05022	260	118	1.16	0.87	260	118	3.16	0.05
TRINITY_DN43681_c1_g1_i1	PGTG_11729	486	229	1.36	0.52	486	229	3.02	0.04
TRINITY_DN44010_c0_g1_i6	PGTG_12809	111	17	-1.31	1.00	111	17	6.96	0.00
TRINITY_DN44010_c0_g1_i3	PGTG_12809	101	15	-1.31	1.00	101	15	6.60	0.01
TRINITY_DN44010_c0_g1_i4	PGTG_12809	232	51	-1.31	1.00	232	51	5.04	0.04
TRINITY_DN39953_c0_g1_i2	PGTG_14526	466	203	1.11	1.00	466	203	3.35	0.02
TRINITY_DN42107_c1_g1_i1	PGTG_19501	744	215	1.05	1.00	744	215	4.12	0.01
TRINITY_DN44544_c4_g2_i9	PGTG_00386	3182	859	1.11	1.00	3182	859	5.07	0.03

(continue)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN44372_c0_g1_i2	PGTG_03886	528	246	1.17	0.77	528	246	3.19	0.03
TRINITY_DN44437_c0_g2_i2	PGTG_04154	353	148	1.69	0.01	353	148	3.78	0.00
TRINITY_DN45012_c0_g1_i2	PGTG_02391	452	145	-1.12	0.96	452	145	3.89	0.02
TRINITY_DN41454_c0_g2_i2	PGTG_21262	484	157	-1.89	0.09	484	157	4.06	0.03
TRINITY_DN42225_c4_g1_i5	PGTG_15040	171	54	1.19	0.66	171	54	4.93	0.00
TRINITY_DN42225_c4_g1_i7	PGTG_15040	447	176	1.19	0.66	447	176	3.57	0.01
TRINITY_DN43016_c1_g2_i7	PGTG_06180	229	89	-1.01	1.00	229	89	3.78	0.01
TRINITY_DN46183_c1_g2_i2	PGTG_17815	801	328	1.13	1.00	801	328	3.72	0.01
TRINITY_DN44239_c0_g2_i2	PGTG_09284	323	120	1.31	0.37	323	120	4.12	0.01
TRINITY_DN45950_c7_g2_i5	PGTG_09333	249	126	1.07	1.00	249	126	3.01	0.04
TRINITY_DN44251_c0_g3_i1	PGTG_08873	227	81	-1.28	1.00	227	81	4.27	0.04
TRINITY_DN45170_c0_g1_i8	PGTG_16654	531	97	1.01	1.00	531	97	8.36	0.04
TRINITY_DN44976_c4_g1_i3	PGTG_15442	12737	5822	1.16	1.00	12737	5822	3.41	0.02
TRINITY_DN46194_c0_g1_i1	PGTG_00451	343	2364	-1.23	0.55	343	2364	-4.69	0.05
TRINITY_DN43418_c0_g1_i2	PGTG_00556	227	73	-1.19	0.82	227	73	4.07	0.02
TRINITY_DN42636_c1_g1_i1	PGTG_00643	138	52	-1.09	1.00	138	52	3.42	0.05
TRINITY_DN42763_c1_g1_i1	PGTG_00679	108	33	-1.26	0.43	108	33	4.49	0.04
TRINITY_DN44667_c0_g2_i1	PGTG_01210	289	111	-1.23	1.00	289	111	3.90	0.00
TRINITY_DN37645_c0_g1_i1	PGTG_01415	232	33	1.08	1.00	232	33	9.17	0.00
TRINITY_DN39086_c0_g1_i9	PGTG_01415	82	17	1.08	1.00	82	17	6.31	0.02
TRINITY_DN38299_c0_g1_i3	PGTG_01594	395	146	-1.15	0.83	395	146	3.37	0.02
TRINITY_DN43999_c0_g1_i2	PGTG_02069	1520	424	-1.72	0.68	1520	424	4.90	0.00
TRINITY_DN42174_c0_g1_i1	PGTG_02151	133	1174	-1.85	0.29	133	1174	-6.19	0.02
TRINITY_DN43366_c0_g2_i10	PGTG_02224	515	145	1.05	1.00	515	145	4.54	0.01
TRINITY_DN42797_c1_g1_i2	PGTG_02394	1085	441	1.29	0.81	1085	441	3.45	0.01
TRINITY_DN45824_c1_g1_i2	PGTG_02459	544	176	1.23	0.95	544	176	4.55	0.01

(continue)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN42284_c1_g1_i8	PGTG_02717	512	57	1.30	0.30	512	57	10.68	0.00
TRINITY_DN46347_c13_g4_i2	PGTG_02834	11995	3297	1.23	1.00	11995	3297	5.05	0.00
TRINITY_DN41138_c1_g1_i3	PGTG_03129	163	49	1.15	0.81	163	49	4.87	0.00
TRINITY_DN42825_c0_g2_i4	PGTG_03134	457	190	1.10	1.00	457	190	3.96	0.04
TRINITY_DN45445_c2_g1_i18	PGTG_03393	73	24	1.01	1.00	73	24	4.39	0.01
TRINITY_DN45202_c3_g1_i12	PGTG_03458	197	80	-1.10	1.00	197	80	3.84	0.04
TRINITY_DN43536_c0_g1_i2	PGTG_03556	401	160	1.35	0.54	401	160	3.60	0.02
TRINITY_DN41790_c0_g1_i1	PGTG_03894	269	78	1.01	1.00	269	78	5.17	0.01
TRINITY_DN44872_c0_g2_i2	PGTG_04280	70	12	1.39	1.00	70	12	6.61	0.00
TRINITY_DN44872_c0_g2_i1	PGTG_04280	80	13	1.39	1.00	80	13	6.25	0.01
TRINITY_DN42339_c0_g1_i5	PGTG_04377	494	176	-1.25	1.00	494	176	3.60	0.03
TRINITY_DN39531_c0_g1_i1	PGTG_04583	1209	587	1.51	0.41	1209	587	3.19	0.04
TRINITY_DN44946_c0_g1_i5	PGTG_04684	360	126	1.04	1.00	360	126	3.92	0.01
TRINITY_DN46310_c4_g1_i7	PGTG_04825	206	53	1.04	1.00	206	53	4.70	0.01
TRINITY_DN45706_c0_g2_i2	PGTG_04845	8	143	1.41	0.52	8	143	-9.10	0.05
TRINITY_DN45596_c3_g2_i4	PGTG_04847	1048	416	1.13	0.97	1048	416	3.89	0.04
TRINITY_DN45388_c2_g2_i2	PGTG_04913	125	41	-1.04	1.00	125	41	4.17	0.02
TRINITY_DN45905_c2_g1_i4	PGTG_04939	399	84	1.06	1.00	399	84	7.60	0.00
TRINITY_DN45475_c1_g1_i7	PGTG_05143	61	731	1.06	1.00	61	731	-8.43	0.03
TRINITY_DN45475_c1_g1_i10	PGTG_05143	44	2304	1.06	1.00	44	2304	-29.59	0.00
TRINITY_DN38049_c0_g2_i2	PGTG_05144	10	1110	1.83	0.33	10	1110	-36.64	0.00
TRINITY_DN38049_c0_g2_i5	PGTG_05144	18	2012	1.83	0.33	18	2012	-51.96	0.00
TRINITY_DN38049_c0_g2_i1	PGTG_05144	1	2560	1.83	0.33	1	2560	-155.80	0.00
TRINITY_DN44781_c0_g2_i3	PGTG_05352	203	43	1.18	0.78	203	43	5.34	0.01
TRINITY_DN45536_c3_g2_i3	PGTG_05701	814	333	1.17	0.77	814	333	3.66	0.02
TRINITY_DN45209_c1_g1_i12	PGTG_05744	55	18	1.08	1.00	55	18	4.54	0.04

(continue)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN45067_c1_g1_i9	PGTG_06440	1713	632	-1.78	0.24	1713	632	3.89	0.00
TRINITY_DN44879_c0_g1_i11	PGTG_06508	36	558	-1.12	1.00	36	558	-7.15	0.04
TRINITY_DN45287_c0_g1_i7	PGTG_06518	215	91	1.10	1.00	215	91	3.86	0.03
TRINITY_DN46243_c0_g2_i6	PGTG_06736	576	203	-1.05	1.00	576	203	3.66	0.03
TRINITY_DN46042_c5_g3_i2	PGTG_07001	2407	722	-1.43	0.75	2407	722	4.49	0.00
TRINITY_DN45723_c5_g1_i1	PGTG_07198	410	209	1.09	1.00	410	209	3.08	0.02
TRINITY_DN44684_c0_g1_i3	PGTG_07655	199	52	1.77	0.61	199	52	5.99	0.02
TRINITY_DN44310_c2_g2_i1	PGTG_07656	92	40	2.19	0.04	92	40	3.60	0.03
TRINITY_DN45197_c4_g1_i1	PGTG_07707	866	344	1.10	1.00	866	344	3.66	0.01
TRINITY_DN39933_c2_g1_i3	PGTG_07930	305	101	-1.05	1.00	305	101	4.58	0.01
TRINITY_DN41386_c0_g1_i9	PGTG_07938	776	230	1.51	0.29	776	230	4.95	0.00
TRINITY_DN41386_c0_g1_i8	PGTG_07938	548	265	1.51	0.29	548	265	3.38	0.04
TRINITY_DN44832_c1_g1_i2	PGTG_08416	62	12	-1.03	1.00	62	12	5.72	0.01
TRINITY_DN44260_c4_g1_i7	PGTG_08591	378	112	1.27	0.44	378	112	5.16	0.01
TRINITY_DN46305_c1_g3_i8	PGTG_08794	72	18	-1.06	1.00	72	18	5.02	0.02
TRINITY_DN39331_c0_g2_i4	PGTG_08868	213	25	-1.11	1.00	213	25	10.23	0.00
TRINITY_DN20260_c0_g1_i2	PGTG_09125	4643	437	1.01	1.00	4643	437	21.34	0.03
TRINITY_DN20260_c0_g1_i1	PGTG_09125	6288	596	1.01	1.00	6288	596	20.24	0.02
TRINITY_DN41198_c0_g1_i1	PGTG_09258	201	52	2.78	0.16	201	52	5.89	0.00
TRINITY_DN44031_c0_g1_i2	PGTG_09510	344	149	-1.06	1.00	344	149	3.71	0.02
TRINITY_DN44031_c0_g1_i5	PGTG_09511	606	117	-1.20	1.00	606	117	7.58	0.00
TRINITY_DN45039_c1_g1_i3	PGTG_09640	38	496	-1.10	1.00	38	496	-6.78	0.02
TRINITY_DN37306_c0_g1_i8	PGTG_09843	451	195	1.14	0.94	451	195	3.79	0.02
TRINITY_DN40349_c0_g2_i6	PGTG_09868	501	193	1.39	0.48	501	193	3.89	0.02
TRINITY_DN46083_c0_g2_i2	PGTG_09876	552	156	1.02	1.00	552	156	5.20	0.04
TRINITY_DN40847_c0_g1_i6	PGTG_09991	567	261	1.02	1.00	567	261	3.05	0.03

(continue)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN40587_c1_g1_i2	PGTG_10000	237	27	2.66	0.09	237	27	11.30	0.00
TRINITY_DN40587_c1_g1_i7	PGTG_10000	237	39	2.66	0.09	237	39	7.80	0.00
TRINITY_DN40587_c1_g1_i5	PGTG_10000	1143	371	2.66	0.09	1143	371	5.26	0.02
TRINITY_DN40587_c1_g1_i12	PGTG_10000	242	93	2.66	0.09	242	93	4.18	0.02
TRINITY_DN45716_c0_g1_i5	PGTG_10150	87	25	-1.08	1.00	87	25	5.13	0.01
TRINITY_DN34091_c0_g1_i2	PGTG_10327	125	15	3.05	0.04	125	15	10.44	0.00
TRINITY_DN41602_c1_g1_i5	PGTG_10554	65	1049	1.10	1.00	65	1049	-8.00	0.02
TRINITY_DN45998_c0_g1_i10	PGTG_10559	108	23	1.02	1.00	108	23	5.50	0.01
TRINITY_DN41926_c0_g1_i4	PGTG_10618	952	272	4.15	0.04	952	272	5.69	0.01
TRINITY_DN45043_c1_g1_i16	PGTG_11133	0	180	-1.22	0.64	0	180	-14.22	0.05
TRINITY_DN46105_c3_g1_i20	PGTG_11384	38	6	1.12	1.00	38	6	7.91	0.00
TRINITY_DN45272_c2_g1_i2	PGTG_11486	105	44	-1.02	1.00	105	44	3.45	0.03
TRINITY_DN35294_c0_g1_i1	PGTG_11625	351	166	3.28	0.01	351	166	3.60	0.03
TRINITY_DN39863_c0_g1_i2	PGTG_22585	167	60	-2.32	0.54	167	60	3.89	0.02
TRINITY_DN35888_c0_g2_i1	PGTG_12079	2288	213	10.75	0.03	2288	213	18.76	0.00
TRINITY_DN40729_c0_g1_i2	PGTG_12128	166	55	1.09	1.00	166	55	4.32	0.03
TRINITY_DN37143_c5_g1_i1	PGTG_12354	1380	518	1.08	1.00	1380	518	3.81	0.03
TRINITY_DN44396_c0_g1_i4	PGTG_12695	304	84	-1.46	0.87	304	84	4.98	0.00
TRINITY_DN45150_c1_g2_i2	PGTG_12736	67	18	-1.07	1.00	67	18	4.73	0.01
TRINITY_DN22036_c0_g1_i2	PGTG_12759	68	13	-1.29	1.00	68	13	6.78	0.00
TRINITY_DN44728_c0_g1_i1	PGTG_12889	626	139	1.20	0.69	626	139	6.23	0.00
TRINITY_DN44243_c1_g1_i2	PGTG_12898	473	139	-1.13	1.00	473	139	4.56	0.02
TRINITY_DN39247_c0_g2_i3	PGTG_13026	2710	523	-1.27	0.74	2710	523	7.37	0.00
TRINITY_DN38814_c0_g1_i2	PGTG_13160	8	323	-2.66	0.15	8	323	-11.22	0.02
TRINITY_DN44397_c1_g1_i1	PGTG_13352	2343	1116	-1.06	1.00	2343	1116	3.12	0.04
TRINITY_DN39539_c0_g1_i1	PGTG_13402	486	236	1.17	0.84	486	236	3.07	0.00

(continue)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN45869_c3_g1_i2	PGTG_13407	216	92	-1.21	0.72	216	92	3.48	0.01
TRINITY_DN43636_c0_g1_i5	PGTG_13491	1350	395	1.02	1.00	1350	395	4.93	0.00
TRINITY_DN35932_c1_g1_i1	PGTG_13514	4	1801	-1.36	1.00	4	1801	-34.59	0.00
TRINITY_DN40713_c0_g1_i9	PGTG_13696	118	35	1.35	0.49	118	35	4.53	0.04
TRINITY_DN43330_c0_g1_i3	PGTG_13846	307	125	1.41	0.54	307	125	3.90	0.02
TRINITY_DN44735_c0_g1_i4	PGTG_13906	576	194	-1.07	1.00	576	194	3.96	0.02
TRINITY_DN43905_c1_g1_i6	PGTG_13919	82	16	-1.15	0.93	82	16	7.86	0.00
TRINITY_DN45067_c1_g1_i12	PGTG_13964	73	24	1.52	0.14	73	24	4.85	0.03
TRINITY_DN43066_c1_g2_i3	PGTG_14115	235	46	1.63	0.06	235	46	8.22	0.00
TRINITY_DN43066_c1_g2_i8	PGTG_14115	24	554	1.63	0.06	24	554	-14.71	0.02
TRINITY_DN45319_c0_g2_i3	PGTG_14197	187	80	1.31	0.35	187	80	3.30	0.03
TRINITY_DN44712_c0_g5_i4	PGTG_14259	440	168	1.48	0.32	440	168	4.24	0.02
TRINITY_DN44410_c1_g1_i2	PGTG_14311	223	61	-1.04	1.00	223	61	4.55	0.05
TRINITY_DN39523_c0_g1_i2	PGTG_14328	3	94	-1.01	1.00	3	94	-8.77	0.04
TRINITY_DN45302_c2_g1_i2	PGTG_14350	306	99	1.52	0.29	306	99	4.24	0.01
TRINITY_DN39530_c0_g1_i1	PGTG_14351	652	184	1.51	0.10	652	184	4.99	0.00
TRINITY_DN39031_c0_g2_i1	PGTG_14366	163	20	2.48	0.02	163	20	9.69	0.00
TRINITY_DN34461_c0_g1_i1	PGTG_14373	75	26	1.48	0.79	75	26	4.18	0.02
TRINITY_DN44965_c1_g1_i7	PGTG_14435	539	269	1.10	1.00	539	269	3.01	0.04
TRINITY_DN46279_c0_g1_i5	PGTG_14588	89	40	2.70	0.03	89	40	4.65	0.03
TRINITY_DN46132_c3_g2_i4	PGTG_15001	572	228	-1.07	1.00	572	228	3.81	0.02
TRINITY_DN44865_c0_g1_i12	PGTG_15427	82	18	1.01	1.00	82	18	6.25	0.01
TRINITY_DN37752_c0_g1_i2	PGTG_15471	450	192	1.05	1.00	450	192	3.43	0.02
TRINITY_DN78831_c0_g1_i1	PGTG_15484	1	71	1.05	1.00	1	71	-13.88	0.02
TRINITY_DN38736_c0_g1_i4	PGTG_15540	464	202	1.39	0.12	464	202	3.46	0.03
TRINITY_DN41055_c0_g1_i1	PGTG_15606	410	181	1.60	0.64	410	181	3.42	0.03

(continue)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN45328_c0_g2_i6	PGTG_15927	827	186	1.28	0.85	827	186	5.36	0.03
TRINITY_DN46018_c4_g2_i1	PGTG_16114	32	4852	-5.52	0.36	32	4852	-55.58	0.00
TRINITY_DN45659_c3_g2_i2	PGTG_16251	61	15	-1.09	1.00	61	15	7.02	0.00
TRINITY_DN40779_c0_g1_i1	PGTG_16392	157	70	-1.10	1.00	157	70	3.49	0.03
TRINITY_DN45065_c0_g1_i1	PGTG_13853	103	1336	-2.42	1.00	103	1336	-6.62	0.04
TRINITY_DN41111_c0_g1_i6	PGTG_16505	399	103	1.03	1.00	399	103	5.49	0.00
TRINITY_DN41111_c0_g1_i3	PGTG_16505	424	126	1.03	1.00	424	126	4.77	0.00
TRINITY_DN43942_c0_g1_i5	PGTG_16741	1690	599	-1.02	1.00	1690	599	3.75	0.00
TRINITY_DN43220_c0_g1_i2	PGTG_16800	1168	459	-1.16	0.88	1168	459	3.50	0.03
TRINITY_DN44149_c10_g1_i5	PGTG_16836	1843	432	1.70	0.36	1843	432	6.15	0.01
TRINITY_DN41966_c1_g1_i11	PGTG_16871	918	212	1.33	1.00	918	212	6.71	0.00
TRINITY_DN41339_c0_g1_i2	PGTG_16948	192	71	-1.19	0.86	192	71	4.57	0.03
TRINITY_DN42695_c2_g1_i4	PGTG_17151	185	36	-1.14	0.92	185	36	6.69	0.01
TRINITY_DN44215_c0_g2_i6	PGTG_17236	227	103	-1.03	1.00	227	103	3.32	0.03
TRINITY_DN41777_c0_g1_i3	PGTG_17305	2306	1012	1.04	1.00	2306	1012	3.32	0.05
TRINITY_DN42592_c1_g1_i5	PGTG_17493	262	122	1.04	1.00	262	122	3.37	0.03
TRINITY_DN40917_c0_g1_i6	PGTG_17533	289	130	1.17	0.87	289	130	3.44	0.04
TRINITY_DN38925_c1_g1_i2	PGTG_17717	359	51	-1.04	1.00	359	51	9.73	0.00
TRINITY_DN41823_c0_g1_i1	PGTG_18357	708	332	1.05	1.00	708	332	3.08	0.04
TRINITY_DN42206_c0_g1_i2	PGTG_18760	186	30	2.10	0.71	186	30	5.65	0.02
TRINITY_DN42625_c0_g1_i5	PGTG_19126	115	16	1.14	1.00	115	16	10.37	0.00
TRINITY_DN45491_c1_g2_i1	PGTG_19528	503	169	2.68	0.32	503	169	4.90	0.03
TRINITY_DN39941_c2_g1_i2	PGTG_19830	205	39	-1.15	0.98	205	39	8.08	0.00
TRINITY_DN43799_c2_g3_i5	PGTG_20074	346	70	1.62	0.69	346	70	7.86	0.00
TRINITY_DN43799_c2_g3_i15	PGTG_20074	690	123	1.62	0.69	690	123	7.62	0.00
TRINITY_DN43799_c2_g3_i12	PGTG_20074	766	162	1.62	0.69	766	162	6.66	0.00

(continue)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN43799_c2_g3_i9	PGTG_20074	499	120	1.62	0.69	499	120	5.98	0.00
TRINITY_DN43799_c2_g3_i19	PGTG_20074	553	131	1.62	0.69	553	131	5.80	0.00
TRINITY_DN43799_c2_g3_i16	PGTG_20074	818	215	1.62	0.69	818	215	5.74	0.00
TRINITY_DN43799_c2_g3_i13	PGTG_20074	837	235	1.62	0.69	837	235	5.52	0.01
TRINITY_DN43799_c2_g3_i14	PGTG_20074	514	131	1.62	0.69	514	131	5.48	0.00
TRINITY_DN43799_c2_g3_i18	PGTG_20074	625	193	1.62	0.69	625	193	4.78	0.00
TRINITY_DN43799_c2_g3_i4	PGTG_20074	346	115	1.62	0.69	346	115	4.17	0.01
TRINITY_DN43014_c0_g1_i7	PGTG_17029	2051	419	1.12	1.00	2051	419	7.30	0.00
TRINITY_DN42793_c0_g1_i4	PGTG_00016	1195	590	1.13	1.00	1195	590	3.06	0.03
TRINITY_DN45391_c1_g1_i4	PGTG_19195	810	351	1.16	1.00	810	351	3.67	0.02
TRINITY_DN39056_c0_g1_i2	PGTG_17247	2284	962	1.54	0.61	2284	962	3.69	0.01
TRINITY_DN38585_c0_g1_i3	PGTG_16913	695	276	-1.13	1.00	695	276	3.60	0.01
TRINITY_DN23412_c0_g1_i2	PGTG_13519	1	323	-41.79	0.00	1	323	-12.61	0.03
TRINITY_DN23412_c0_g1_i1	PGTG_13519	4	3584	-41.79	0.00	4	3584	-111.31	0.00
TRINITY_DN40779_c0_g1_i7	PGTG_16392	160	58	-1.10	1.00	160	58	4.40	0.03
TRINITY_DN38902_c0_g1_i2	PGTG_04980	1383	702	1.51	0.69	1383	702	3.01	0.04
TRINITY_DN45252_c1_g3_i3	PGTG_03181	85	829	1.04	1.00	85	829	-5.99	0.02
TRINITY_DN45516_c0_g1_i8	PGTG_20914	2	169	1.08	1.00	2	169	-11.92	0.02
TRINITY_DN45565_c2_g1_i2	PGTG_10868	33	379	1.07	1.00	33	379	-5.10	0.03
TRINITY_DN45043_c1_g1_i22	PGTG_11133	2	277	-1.22	0.64	2	277	-17.05	0.01
TRINITY_DN45043_c1_g1_i5	PGTG_11133	0	258	-1.22	0.64	0	258	-21.05	0.02
TRINITY_DN39133_c0_g1_i5	PGTG_21950	146	23	-1.15	1.00	146	23	8.95	0.01
TRINITY_DN45184_c0_g5_i1	PGTG_09600	10	201	-5.47	0.10	10	201	-10.30	0.02
TRINITY_DN41350_c0_g2_i1	PGTG_05795	2987	1400	1.53	0.06	2987	1400	3.23	0.02
TRINITY_DN39564_c1_g2_i3	PGTG_01413	583	43	-1.15	0.86	583	43	19.90	0.00
TRINITY_DN40424_c0_g1_i2	PGTG_14974	340	110	1.15	1.00	340	110	3.77	0.03

(continue)

Table A11. Comparison between RNAseq mapping using de novo assembled transcripts vs *Pgt* race SCCL genome (continued)

De novo Transcript ID	*PGT* Gene[a]	Using Reference Genome[b]				Using de novo Transcript[c]			
		Vir-sum[db]	Avr sum[eb]	FC[fb]	FDR p-value[gb]	Vir sum[dc]	Avr sum[ec]	FC[fb]	FDR p-value[gb]
TRINITY_DN45043_c1_g1_i14	PGTG_11133	1	821	-1.22	0.64	1	821	-109.91	0.00
TRINITY_DN42457_c0_g2_i3	PGTG_01320	844	357	1.12	1.00	844	357	3.22	0.02
TRINITY_DN44976_c4_g2_i1	PGTG_15441	5135	1901	1.03	1.00	5135	1901	4.27	0.02
TRINITY_DN42955_c3_g1_i2	PGTG_04648	175	71	2.25	0.01	175	71	3.89	0.02
TRINITY_DN45448_c3_g1_i6	PGTG_03181	403	98	1.04	1.00	403	98	5.71	0.00
TRINITY_DN45448_c3_g1_i3	PGTG_03181	371	151	1.04	1.00	371	151	3.44	0.04
TRINITY_DN38482_c0_g1_i1	PGTG_22152	479	158	-2.72	0.20	479	158	4.91	0.01
TRINITY_DN43795_c0_g3_i8	PGTG_17411	20	650	1.49	1.00	20	650	-14.55	0.04
TRINITY_DN43017_c0_g1_i3	PGTG_13989	516	47	1.52	1.00	516	47	12.29	0.00
TRINITY_DN43017_c0_g1_i5	PGTG_12051	283	71	-1.67	1.00	283	71	4.99	0.01
TRINITY_DN43017_c0_g1_i6	PGTG_13989	416	111	1.52	1.00	416	111	5.24	0.02
TRINITY_DN6253_c0_g1_i1	PGTG_21023	673	17	-1.56	1.00	673	17	30.52	4.63E-05

[a]*Pgt* gene corresponding to the top hit of given *de novo* transcript

[b]Data represent the output obtained from mapping of original data to *Pgt* race SCCL genome

[c]Data represent the output obtained from mapping of unmapped read to de novo assembled transcripts. Unmapped reads are the reads that did not map to either of barley RefSeq v1 and *Pgt* SCCL genome

[db]The sum of reads of all the virulent *rpg4/5* samples that mapped to the *Pgt* gene

[eb]The sum of reads of all the avirulent *rpg4/5* samples that mapped to the *Pgt* gene

[fb]The fold change between virulent *rpg4/5* isolates vs avirulent *rpg4/5* isolates for the *Pgt* gene

[gb]FDR corrected P-value for fold change of the *Pgt* gene

[dc]The sum of reads for all the sample representing virulent *rpg4/5* samples that mapped to the *de novo* assembled transcript

[ec]The sum of reads for all the sample representing avirulent *rpg4/5* samples that mapped to the *de novo* assembled transcript

[fb]The fold change between sample inoculated with virulent *rpg4/5* isolates vs avirulent *rpg4/5* inoculated isolates for the *de novo* assembled transcript

[gb]FDR corrected P-value for fold change of the *de novo* assembled transcript

Table A12. Variants associated with *Pgt* virulence on *rpg4/5*

SC[a]	Position [b]	Gene ID[c]	CSEP [d]	Annotation[e]	AA	Consequence	AA change[f]	Codon change
2.131	67552	*PGT*G_19496	NO	FRA10AC1 [Rhodotorula toruloides NP11]	243	missense_variant	K/R	aAg/aGg
2.1	1842294	*PGT*G_00438	NO	hypothetical protein *PGT*G_00438	126	5_prime_UTR_variant	-	-
2.75	111760	*PGT*G_16718	YES	phosphatidylethanolamine-binding PEBP [Rhodotorula toruloides NP11]	295	missense_variant	A/V	gCc/gTc
2.17	404952	*PGT*G_06872	NO	related to phospho phosphatase 2C	425	5_prime_UTR_variant	-	-
2.17	404865	*PGT*G_06872	NO	related to phospho phosphatase 2C	425	missense_variant	E/D	gaG/gaC
2.17	404949	*PGT*G_06872	NO	related to phospho phosphatase 2C	425	5_prime_UTR_variant	-	-
2.3	165457	*PGT*G_10718	NO	NADH-ubiquinone oxidoreductase	879	missense_variant	S/N	aGc/aAc
2.3	156704	*PGT*G_10716	NO	hypothetical protein *PGT*G_10716	993	missense_variant	N/H	Aac/Cac
2.44	567104	*PGT*G_12898	NO	hypothetical protein *PGT*G_12898	1197	inframe_deletion	E/-	GAA/-
2.25	1067860	*PGT*G_08749	NO	enhancer of mRNA-decapping 3 [Rhodotorula toruloides NP11]	618	missense_variant	T/N	aCc/aAc
2.41	122536	*PGT*G_13122	NO	hypothetical protein *PGT*G_13122	498	missense_variant	M/I	atG/atC
2.53	221088	*PGT*G_14032	NO	hypothetical protein *PGT*G_14032	379	missense_variant	I/L	Atc/Ctc
2.106	116208	*PGT*G_18718	NO	likely mitochondrial ribosomal	374	missense_variant	A/T	Gcc/Acc
2.53	358549	*PGT*G_14065	NO	COP9 signalosome complex subunit 7b-like isoform X1	356	missense_variant	T/A	Acc/Gcc

(continued)

Table A12. Variants associated with *Pgt* virulence on *rpg4/5* (continued)

SC[a]	Position [b]	Gene ID[c]	CSEP [d]	Annotation[e]	AA	Consequence	AA change[f]	Codon change
2.3	718087	*PGT*G_10874	NO	hypothetical protein *PGT*G_10874	572	missense_variant	D/N	Gac/Aac
2.19	213473	*PGT*G_08059	YES	hypothetical protein *PGT*G_08059	435	5_prime_UTR_variant	-	-
2.1	628179	*PGT*G_00156	YES	glucan endo-1,3-alpha-glucosidase Agn1	508	missense_variant	D/A	gAt/gCt
2.2	176607	*PGT*G_01415	NO	f-box pof6	1085	missense_variant	S/C	tCc/tGc
2.17	486322	*PGT*G_06894	NO	NADH-ubiquinone oxidoreductase	748	missense_variant	I/V	Atc/Gtc
2.16	800171	*PGT*G_07313	NO	S-adenosyl-L-methionine-dependent methyltransferase	210	missense_variant	A/T	Gct/Act
2.33	322974	*PGT*G_11449	NO	DNA RNA polymerase	673	missense_variant	H/Q	caT/caG
2.45	156708	*PGT*G_13608	NO	hypothetical protein *PGT*G_13608	281	5_prime_UTR_variant	-	-

(continued)

Table A12. Variants associated with *Pgt* virulence on *rpg4/5* (continued)

SC[a]	Gene ID[c]	CSEP[d]	AA change[f]	Virulent *rpg4/5* isolates								
				370C[g]	640C	A14	A-15	A-21	A48	HKHJ	R29J	R29M
2.131	*PGT*G_19496	NO	K/R	1	1	1	1	1	1	1	1	1
2.1	*PGT*G_00438	NO	-	1	1	1	1	2	1	1	1	1
2.75	*PGT*G_16718	YES	A/V	1	1	1	1	0	1	1	1	1
2.17	*PGT*G_06872	NO	-	1	1	1	1	0	1	1	1	1
2.17	*PGT*G_06872	NO	E/D	1	1	1	1	0	1	-	1	1
2.17	*PGT*G_06872	NO	-	1	1	1	1	0	1	-	1	1
2.3	*PGT*G_10718	NO	S/N	1	1	1	1	1	1	1	1	1
2.3	*PGT*G_10716	NO	N/H	1	1	1	1	1	1	1	1	1
2.44	*PGT*G_12898	NO	E/-	1	1	1	1	0	1	1	1	1
2.25	*PGT*G_08749	NO	T/N	1	1	1	1	0	1	1	1	1
2.41	*PGT*G_13122	NO	M/I	1	1	1	1	0	1	1	1	1
2.53	*PGT*G_14032	NO	I/L	1	1	1	1	0	1	1	1	1
2.53	*PGT*G_14065	NO	T/A	1	1	1	1	0	1	1	1	1
2.106	*PGT*G_18718	NO	A/T	1	1	1	1	0	1	1	1	1
2.3	*PGT*G_10874	NO	D/N	1	1	1	1	1	1	0	1	1
2.19	*PGT*G_08059	YES	-	1	-	1	-	0	1	-	1	1
2.1	*PGT*G_00156	YES	D/A	1	2	2	1	0	1	-	2	-
2.2	*PGT*G_01415	NO	S/C	1	1	1	1	0	1	0	1	1
2.17	*PGT*G_06894	NO	I/V	1	1	1	1	0	1	0	1	1
2.16	*PGT*G_07313	NO	A/T	1	1	1	1	0	1	0	1	1
2.33	*PGT*G_11449	NO	H/Q	1	1	1	1	0	1	0	1	1
2.45	*PGT*G_13608	NO	-	2	1	1	2	0	2	1	1	1

(continued)

Table A12. Variants associated with *Pgt* virulence on *rpg4/5* (continued)

				Avirulent *rpg4/5* isolates							
SC[a]	Gene ID[c]	CSEP[d]	AA change[f]	79_2	7241SP2	72_00	79_1	79_20	P8416	R11C	QCCJ
2.131	*PGT*G_19496	NO	K/R	0	0	0	0	0	0	0	0
2.1	*PGT*G_00438	NO	-	2	2	2	2	2	2	2	2
2.75	*PGT*G_16718	YES	A/V	0	0	0	0	0	0	0	0
2.17	*PGT*G_06872	NO	-	0	0	-	0	0	0	0	0
2.17	*PGT*G_06872	NO	E/D	0	0	0	0	0	0	0	0
2.17	*PGT*G_06872	NO	-	0	0	0	0	0	0	0	0
2.3	*PGT*G_10718	NO	S/N	0	0	0	0	0	0	2	0
2.3	*PGT*G_10716	NO	N/H	0	0	0	0	0	0	0	0
2.44	*PGT*G_12898	NO	E/-	0	0	-	-	-	0	0	0
2.25	*PGT*G_08749	NO	T/N	0	0	0	0	0	0	0	0
2.41	*PGT*G_13122	NO	M/I	0	0	0	0	0	0	0	0
2.53	*PGT*G_14032	NO	I/L	0	0	0	0	0	0	0	0
2.53	*PGT*G_14065	NO	T/A	0	0	0	0	0	0	0	0
2.106	*PGT*G_18718	NO	A/T	0	0	0	0	0	0	0	0
2.3	*PGT*G_10874	NO	D/N	0	0	0	0	0	0	0	0
2.19	*PGT*G_08059	YES	-	0	0	0	0	-	-	0	0
2.1	*PGT*G_00156	YES	D/A	0	0	0	0	0	0	0	0
2.2	*PGT*G_01415	NO	S/C	0	0	0	0	0	0	0	0
2.17	*PGT*G_06894	NO	I/V	0	0	0	0	0	0	0	0
2.16	*PGT*G_07313	NO	A/T	0	0	0	0	0	0	0	0
2.33	*PGT*G_11449	NO	H/Q	0	0	0	0	0	0	0	0
2.45	*PGT*G_13608	NO	-	2	2	2	2	2	2	2	2

(continued)

Table A12. Variants associated with *Pgt* virulence on *rpg4/5* (continued)

SC[a]	Gene ID[c]	CSEP[d]	AA change[g]	Avirulent *rpg4/5* isolates						
				81AC28	A5	AC12	81AC34	81AC46	A12	WM1
2.131	*PGTG*_19496	NO	K/R	0	0	0	1	0	0	0
2.1	*PGTG*_00438	NO	-	2	2	2	1	2	2	2
2.75	*PGTG*_16718	YES	A/V	0	0	0	1	0	0	0
2.17	*PGTG*_06872	NO	-	0	0	0	1	0	0	0
2.17	*PGTG*_06872	NO	E/D	0	0	0	1	0	0	0
2.17	*PGTG*_06872	NO	-	0	0	0	1	0	0	0
2.3	*PGTG*_10718	NO	S/N	0	0	0	0	0	0	2
2.3	*PGTG*_10716	NO	N/H	0	0	0	0	0	0	2
2.44	*PGTG*_12898	NO	E/-	0	0	0	1	0	0	0
2.25	*PGTG*_08749	NO	T/N	0	0	0	0	0	0	0
2.41	*PGTG*_13122	NO	M/I	0	0	0	0	0	0	0
2.53	*PGTG*_14032	NO	I/L	0	0	0	0	0	0	0
2.53	*PGTG*_14065	NO	T/A	0	0	0	0	0	0	0
2.106	*PGTG*_18718	NO	A/T	0	0	0	0	0	0	0
2.3	*PGTG*_10874	NO	D/N	0	0	0	0	0	0	0
2.19	*PGTG*_08059	YES	-	0	0	0	-	0	0	0
2.1	*PGTG*_00156	YES	D/A	0	0	0	1	0	-	0
2.2	*PGTG*_01415	NO	S/C	0	0	0	0	0	0	0
2.17	*PGTG*_06894	NO	I/V	0	0	0	0	0	0	0
2.16	*PGTG*_07313	NO	A/T	0	0	0	0	0	0	0
2.33	*PGTG*_11449	NO	H/Q	0	0	0	0	0	0	0
2.45	*PGTG*_13608	NO	-	2	2	2	2	2	0	2

[a]Supercontig of *Pgt* race SCCL reference genome
[b]Position in the SC where the variants were identified
[c]*Pgt* gene containing the variants
[d]If the given gene is a Candidate Secreted Effector Protein or not?
[e]Annotaion of a given gene that was obtained from conducting a BlastP search in Blast2GO with automated settings

[f]Amino acid change associated with given variant (AA before change/AA after mutation)
[g]0-Homozygous reference, 1-Heterozygoys,2-Homozygous alternate allele

Table A13. Field assay of line using *Pgt* race TTKSK

Year[a]	Field #[b]	Genotype	Rep	Stage-1[c]			Stage-2[c]		
				Rating[d]	Conversion value[e]	CI[f]	Rating[d]	Conversion value[e]	CI[f]
2015		Conlon	1	TMS	0.4	0.4	5MS	0.8	4
			1	TMS	0.4	0.4	5MS	0.8	4
			2	10MSS	0.9	9	15MSS	0.9	13.5
			2	10MSS	0.9	9	15MSS	0.9	13.5
			1	15MS	0.8	12	20MS	0.8	16
			1	15MS	0.8	12	20MS	0.8	16
			2	15MS	0.8	12	20MSS	0.9	18
			2	15MS	0.8	12	20MSS	0.9	18
			2	20MS	0.8	16	25MSS	0.9	22.5
			2	20MS	0.8	16	25MSS	0.9	22.5
	FAR14-94A-1	HQ1BC4/ConlonBC4F3	1	TMR	0.4	0.4	5MSMR	0.6	3
			1	TMR	0.4	0.4	5MSMR	0.6	3
			2	1MRMS	0.6	0.6	5MRMS	0.6	3
			2	1MRMS	0.6	0.6	5MRMS	0.6	3
	FAR14-94A-2	HQ1BC4/ConlonBC4F3	2	TMR	0.4	0.4	5MR	0.4	2
			2	TMR	0.4	0.4	5MR	0.4	2
			1	TMR	0.4	0.4	10MRMS	0.6	6
			1	TMR	0.4	0.4	10MRMS	0.6	6
			2	TMS	0.4	0.4	5MS	0.8	4
	FAR14-94A-2	HQ1BC4/ConlonBC4F3	2	TMS	0.4	0.4	5MS	0.8	4
	FAR14-94A-4	HQ1BC4/ConlonBC4F3	1	5MS	0.8	4	10MS	0.8	8
			1	5MS	0.8	4	10MS	0.8	8

(continued)

Table A13. Field assay of line using *Pgt* race TTKSK (continued)

Year[a]	Field #[b]	Genotype	Rep	Stage-1[c]			Stage-2[c]		
				Rating[d]	Conversion value[e]	CI[f]	Rating[d]	Conversion value[e]	CI[f]
	FAR14-94B-1	HQ1BC4/ConlonBC4F3	1	5MS	0.8	4	10MS	0.8	8
			1	5MS	0.8	4	10MS	0.8	8
			2	TMS	0.4	0.4	5MS	0.8	4
			2	TMS	0.4	0.4	5MS	0.8	4
	FAR14-94B-2	HQ1BC4/ConlonBC4F3	1	5MSMR	0.6	3	10MS	0.8	8
			1	5MSMR	0.6	3	10MS	0.8	8
	FAR14-94B-3	HQ1BC4/ConlonBC4F3	1	5MS	0.8	4	10MS	0.8	8
			1	5MS	0.8	4	10MS	0.8	8
			2	TMS	0.4	0.4	5MS	0.8	4
			2	TMS	0.4	0.4	5MS	0.8	4
	FAR14-94B-4	HQ1BC4/ConlonBC4F3	1	5MS	0.8	4	10MS	0.8	8
			1	5MS	0.8	4	10MS	0.8	8
			2	TMS	0.4	0.4	5MS	0.8	4
			2	TMS	0.4	0.4	5MS	0.8	4
	FAR14-95A-1	HQ1BC4/ConlonBC4F3	1	5MSMR	0.6	3	10MS	0.8	8
			1	5MSMR	0.6	3	10MS	0.8	8
			2	TMS	0.4	0.4	5MS	0.8	4
			2	TMS	0.4	0.4	5MS	0.8	4
	FAR14-95A-2	HQ1BC4/ConlonBC4F3	1	5MS	0.8	4	5MS	0.8	4
			1	5MS	0.8	4	5MS	0.8	4
			2	TMS	0.4	0.4	5MSMR	0.6	3
			2	TMS	0.4	0.4	5MSMR	0.6	3
	FAR14-95A-3	HQ1BC4/ConlonBC4F3	1	1MS	0.8	0.8	5MS	0.8	4
			1	1MS	0.8	0.8	5MS	0.8	4
			2	TMS	0.4	0.4	5MS	0.8	4
			2	TMS	0.4	0.4	5MS	0.8	4

(continued)

Table A13. Field assay of line using *Pgt* race TTKSK (continued)

Year[a]	Field #[b]	Genotype	Rep	Stage-1[c]			Stage-2[c]		
				Rating[d]	Conversion value[e]	CI[f]	Rating[d]	Conversion value[e]	CI[f]
	FAR14-95A-4	HQ1BC4/ConlonBC4F3	1	1MS	0.8	0.8	1MS	0.8	0.8
			1	1MS	0.8	0.8	1MS	0.8	0.8
			2	TMS	0.4	0.4	10MSS	0.9	9
			2	TMS	0.4	0.4	10MSS	0.9	9
		Pinnacle	1	20mss	0.9	18	25mss	0.9	22.5
		Pinnacle	2	10MSS	0.9	9	15MSS	0.9	13.5
		Pinnacle	2	15MS	0.8	12	30MSS	0.9	27
		Q21861	1	TMR	0.4	0.4	5MRMS	0.6	3
		Q21861	1	TMR	0.4	0.4	TMR	0.4	0.4
		Q21861	1	TMR	0.4	0.4	TMR	0.4	0.4
		Q21861	1	TMR	0.4	0.4	TMR	0.4	0.4
		Q21861	1	TMR	0.4	0.4	TMR	0.4	0.4
		Q21861	1	TMR	0.4	0.4	TMR	0.4	0.4
		Q21861	1	TMR/5MR	0	2	TMR/5MR	0	0
		Q21861	1	TMR	0.4	0.4	TMS	0.4	0.4
		Q21861	2	5MR	0.4	2	10MRMS	0.6	6
		Q21861	2	15MSS	0.9	13.5	20MSS	0.9	18
		Q21861	2	TMR	0.4	0.4	TMR	0.4	0.4
		Q21861	2	TMR	0.4	0.4	TMR	0.4	0.4
		Q21861	2	TMR	0.4	0.4	TMR	0.4	0.4
		Q21861	2	TMR	0.4	0.4	TMR	0.4	0.4
		Q21861	2	TMR	0.4	0.4	TMS	0.4	0.4
	FAR14-1-1	Q21861/Pinnacle BC3F3	1	15MS	0.8	12	25MSS	0.9	22.5
			2	15MSS	0.9	13.5	15MSS	0.9	13.5

(continued)

Table A13. Field assay of line using *Pgt* race TTKSK (continued)

Year[a]	Field #[b]	Genotype	Rep	Stage-1[c]			Stage-2[c]		
				Rating[d]	Conversion value[e]	CI[f]	Rating[d]	Conversion value[e]	CI[f]
	FAR14-1-2	Q21861/Pinnacle BC3F3	1	20MSS	0.9	18	30MSS	0.9	27
			2	10MSS	0.9	9	15MSS	0.9	13.5
	FAR14-1-3	Q21861/Pinnacle BC3F3	1	25MSS	0.9	22.5	25MSS	0.9	22.5
			2	15MS	0.8	12	15MSS	0.9	13.5
	FAR14-1-4	Q21861/Pinnacle BC3F3	1	20MSS	0.9	18	25MSS	0.9	22.5
			2	5MSS	0.9	4.5	10MSS	0.9	9
		Steptoe	1	15MSMR	0.6	9	20MSS	0.9	18
		Steptoe	1	15MSS	0.9	13.5	20MSS	0.9	18
		Steptoe	1	20MSS	0.9	18	25MSS	0.9	22.5
		Steptoe	1	20MSS	0.9	18	30MSS	0.9	27
		Steptoe	1	25MSS	0.9	22.5	30MSS	0.9	27
		Steptoe	1	25MSS	0.9	22.5	30MSS	0.9	27
		Steptoe	1	25MSS	0.9	22.5	30S	1	30
		Steptoe	1	30MSS	0.9	27	40MSS	0.9	36
		Steptoe	2	5MS	0.8	4	5MS	0.8	4
		Steptoe	2	10MS	0.8	8	20MSS	0.9	18
		Steptoe	2	15MSS	0.9	13.5	20MSS	0.9	18
		Steptoe	2	15MS	0.8	12	25MSS	0.9	22.5
		Steptoe	2	20MSS	0.9	18	25MSS	0.9	22.5
		Steptoe	2	20MSS	0.9	18	30MSS	0.9	27
		Steptoe	2	30MSS	0.9	27	40MSS	0.9	36
		Steptoe	2	25MSS	0.9	22.5	30MSS	0.9	27
2011		Harrington	1				1MSS	0.9	0.9
		Harrington	2				TS	1	1
		HQ1	1				1MRMS	0.6	0.6
		HQ1	1				TMRMS	0.6	0.6

(continued)

Table A13. Field assay of line using *Pgt* race TTKSK (continued)

		Rating[d]	Conversion value[e]	CI[f]	Rating[d]	Conversion value[e]	CI[f]
HQ1	2				5RMR	0.3	1.5
HQ1	2				5RMR	0.3	1.5
Pinnacle	1				10S	1	10
Pinnacle	2				TS	1	1

[a]Year on which the screening was done. The data from the resistance parent of Conlon NIL, HQ1 was not available in year 2015, so the field scoring data from 2011 was used in this study for the comparison between parents and the NILs

[b]Field number given by barley pathology program at North Dakota State University

[c]The scoring was done at two stages. At heading stage (stage-1) and hard-dough stage (stage-2) (Zadoks et al., 1974; Case et al., 2018). The comparison was done using the stage 2 score

[d]The modified cobb scale was used to record the disease severity of stem rust (Peterson et al., 1948, McIntosh et al., 1995). Base on the size and type of the uredinia, the infection response (IR) were categorized into resistant (R), moderately resistant (MR), intermediate (M), moderately susceptible (MS), and susceptible (S), or intermediate of any two categories (Roelfs et al., 1992, McIntosh et al., 1995).

[e]The conversion used for calculating the coefficient of infection as recommended by Yu et al. (2011). Immune = 0.0, R = 0.2, MR = 0.4, MRMS or MSMR =0.6, MS = 0.8, MSS or SMS=0. 9 and S = 1.0

[f]The coefficient of infection obtained by multiplying severity with the conversion value as recommended by Yu et al. (2011). (For example: 25MSS = 25 X0.9 = 22.5)

Table A14. Seedling assay of NILs and their parental types using *Pgt* race QCCJB and HKHJC

Line	HKHJC		QCCJB	
	Categorial IT[a]	Quantitiave score[b]	Categorial Score[a]	Quantitiave score[b]
Q21861	0;1	0.875	1;	1.625
Q21861	0;1	0.875	0;1	0.875
Q21861	0;1	0.875	;1,2	1.2
Q21861	0;1	0.875	2,1;	2.45
Q21861	0;1	0.875	0;1	0.875
Q21861	dead	-	0;1	0.875
HQ1	3-	3.5	2,1	2.75
HQ1	3-2	3.375	2,3-1	3.05
HQ1	3-2	3.375	;1,2	1.2
HQ1	3-3	3.625	2,3-	3.125
HQ1	3-2	3.375	-	-
Harrington	3-2	3.375	3,3+	4.125
Harrington	3-2	3.375	3-2	3.375
Harrington	3,3-	3.875	dead	-
Harrington	3-3	3.625	3-2	3.375
Harrington	3-	3.5	2,3-	3.125
Harrington	3-3	3.625	2,3-	3.125
Harrington	3,3-	3.875	2,3-	3.125
Harrington	3,3-	3.875	-	-
Harrington	3-	3.5	-	-
Steptoe	3-	3.5	3-2	3.375
Steptoe	3-	3.5	3-	3.5
Steptoe	3-2	3.375	3-3	3.625
Steptoe	3-2	3.375	3,3-	3.875
Steptoe	3-2	3.375	3-2	3.375
Steptoe	3-2	3.375	3-3	3.625
Steptoe	2,3-	3.125	3-3	3.625
Steptoe	-	-	3,3-	3.875
Morex	2,1	2.75	3-3	3.625
Morex	2,1	2.75	3-2	3.375
Morex	1,2	2.25	3-2	3.375
Morex	1;2	1.65	3-	3.5
Morex	1;2	1.65	3-	3.5
Morex	-	-	2,3-	3.125
Morex	-	-	2,3-	3.125
Conlon Wild	1;	1.625	3-3	3.625

(continued)

Table A14. Seedling assay of NILs and their parental types using *Pgt* race QCCJB and HKHJC (continued)

Line	HKHJC		QCCJB	
	Categorial IT[a]	Quantitiave score[b]	Categorial Score[a]	Quantitiave score[b]
Conlon Wild	1,2;	2.15	3-2	3.375
Conlon Wild	1;2	1.65	3-2	3.375
Conlon Wild	2,1	2.75	3-	3.5
Conlon Wild	-	-	3,3-	3.875
Conlon Wild	-	-	-	-
Conlon Wild	-	-	-	-
Pinnacle Wild	2,1	2.75	3,3-	3.875
Pinnacle Wild	2,1	2.75	3-3	3.625
Pinnacle Wild	escape	-	3-2	3.375
Pinnacle Wild	2,1	2.75	3,3-	3.875
Pinnacle Wild	2	3	3-3	3.625
Pinnacle Wild	2,1	2.75	3,3-	3.875
Pinnacle Wild	-	-	-	-
Conlon NIL	1;	1.625	1;	1.625
Conlon NIL	1;	1.625	1;	1.625
Conlon NIL	1;	1.625	1;	1.625
Conlon NIL	1;	1.625	1,2	2.25
Conlon NIL	;1	0.875	1;2	1.65
Conlon NIL	;1	0.875	1;	1.625
Conlon NIL	1;	1.625	-	-
Pinnacle NIL	3-	3.5	3,3-	3.875
Pinnacle NIL	3-3	3.625	3-3	3.625
Pinnacle NIL	3-3	3.625	3-3	3.625
Pinnacle NIL	3-3	3.625	3,3-	3.875
Pinnacle NIL	3-3	3.625	3	4
Pinnacle NIL	3-3	3.625	3,3+	4.125
Pinnacle NIL	3-3	3.625	Dead	-

[a]The infection type was assessed using 0-4 scale modified for barley (Stakman et al., 1962; Steffenson et al., 2009)

[b]The quantitative score obtained using the transformation scale provide by Zhou et al. (2014). The categoricalIT "0" was coded as 0.0; IT "0;" or ";" as 0.5, IT "1" as 2.0, IT "2" as 3, IT "3-"as 3.5, IT "3" as 4.0, and IT "3+" as 4.5. A single score was give 100 % of coded score (example: Catergorical IT of 3 = Quantitative score of 4). A categorical IT with two IT were converted using formula: (75 % of 1st IT + 25% of 2nd IT; example: IT of 3-3 = (0.75 * 3.5+0.25*4) =3.625. A categorical IT with three IT were converted using formula: (60 % of 1st IT + 30% of 2nd IT+10% of 3rd IT; example: IT of 2,3-1 = (0.6*3+0.3 * 3.5+0.1*2) =3.05).

Table A15. Primer sequences of iSelect markers designed to saturate the region harboring *Rrr1*

iSelect Marker	Forward Primer[a]	Reverse Primer[b]
11_10217	ACAACCACAAAATTTGGTCTCC	TGCTATCGTTTTGCATGATAGG
SCRI_RS_182353	CGGTGTATAGGATCTTGTTTGC	AGCTTCAGATCATCACAATCG
11_20104	TTTGATGGTTCTCAAATGAAG	TGTAAATTTGAAATCTGGGTTGG
SCRI_RS_44795	AGCGAAGGACTCTGGGTTTAC	AAGGCTGCCAAGGCCAAGAAG
SCRI_RS_205647	CTTCGTGCTACAATTCTTGGTC	TAGTTACGCCATGGATGGAATG
11_11490	CACATCATGGAATCGCTGTC	TTAGTAATAGTAGGCCATTTTGG
12_20186	CAAAGCATCATCACGTATTCAG	GGTGCTCCTCTTCGTTGTATC
SCRI_RS_3429	CGAGCCTCGTCGTCAACCTC	CTCTCTTCTCTCTGTGGGATGG
11_11497	ATGATATCCCATAGGCATGAGC	AAGGCCAGGGAGGTCAAG
SCRI_RS_32778	TCCTGAGGCTAAGTTGCTGTT	TTCTTGGTTTTTGGGTGCAT
12_30759	CCGGTGTTGAACTGGAAGTG	ACCTTCTACTCCAAGGTGGTG
12_30238	TTGCTTCTCATCTTATCGGTAG	TGCAGTAGTCAACTTGTTCAGC
11_11185	CATAAAATTGGAGTTGGCTCTC	CTCAACGCTTTACCTTTTTACC
12_20317	CCACTGTTTTTGCTCCTCGT	GGAACAGTGTCATGATCTGCAT
11_10385	GGCGAGCTTGAGGAGGTTG	CCAGGTCGAGGAGTTTTACG
SCRI_RS_155688	GACAATTAGACGAAAGGATCATGG	CAGCTAATTTAGTGCCCGTTC
11_21452	GAAAGGCATGAGGGGAATGG	GGGTACTTGAACTCGATGACG
11_10902	AGTAGCCATCGTCATCCATATC	TTAGGAGTCCATCAATCAATGTC
11_10741	CAAGTGGATAATCACCCAGAAG	GATCCCACCAACAACTATCTTG
SCRI_RS_224438	TGGAAATGGTGATATCGATGC	GCGTGTTTCATCATTCAGTAAG
11_20324	GAGATGGTCAACAACAAGAAGG	CCTCACAGTTTGTGAAGAGATG
SCRI_RS_131479	GCTCTCATGTCAGTCTTTTCAAC	GTCAGGAGGTTAGCTGTTGATG
11_11464	AGGCATAGTGCAACCAGAGG	TGTGCACGATAAACACCACA
12_30162	AGATGTGAAGACGGAGCTGTAG	CAAAAACAACACCCAAGGTC

[a]A 22 nucleotide CS1 adaptor sequence (5'-ACACTGACGACATGGTTCTACA-3') was attached to forward primer

[b]A 22 nucleotide CS2 adaptor sequence (5'-TACGGTAGCAGAGACTTGGTCT-3') was attached to reverse primer

Table A16. Seedling assay of Q21861/Pinnalce NIL progenies using *Pgt* race QCCJB and HKHJC

Pgt race	QCCJB								HKHJC	
	Experiment 1				Experiment 2				Experiment 1	
	Rep 1		Rep 2		Rep 1		Rep 2		Rep 1	
RIL #	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]
GH593	escape	-	1,2;	2.15	0;1	0.88	0;1	0.88	2,3-	3.13
GH595	3-2	3.38	2,3-	3.13	3,3-	3.88	3,3-	3.88	0;	0.50
GH596	2,1	2.75	2,3-	3.13	3,3-	3.88	3,3-	3.88	0;	0.50
GH597	1;2	1.65	escape	-	0;1	0.88	0;1	0.88	-	-
GH598	;1	0.88	1;	1.63	3,3-	3.88	0;	0.50	3.00	4.00
GH599	0;	0.50	0;	0.50	0;1	0.88	0;	0.50	0;	0.50
GH601	;1	0.88	1;	1.63	0;	0.50	0;	0.50	0;	0.50
GH602	3-2	3.38	3-2	3.38	3.00	4.00	3+3	4.38	0;	0.50
GH603	0; or escape	0.5 or escape	2,3-	3.13	3-2	3.38	3-	3.50	3.00	4.00
GH604	2,3-	3.13	2,3-	3.13	3-3	3.63	3,3-	3.88	0;	0.50
GH605	2,1,3-	2.75	1,2;	2.15	3,3-	3.88	3-3	3.63	2,3-	3.13
GH606	3,3-	3.88	escape	-	3-3	3.63	0;	0.50	;1	0.88
GH608	3,3-	3.88	3-3	3.63	3,3-	3.88	3,3+ & 0;	-	3	4.00
GH609	2.00	3.00	2,3-1	3.05	3-3	3.63	3,3+	4.13	3	4.00
GH610	0; or escape	0.5 or escape	;1,2	1.20	3-3	3.63	3,3+	4.13	;1	0.88
GH611	1;	1.63	1;2	1.65	0;1	0.88	0;1	0.88	0;	0.50
GH612	1;	1.63	;1	0.88	0;	0.50	0;1	0.88	0;1	0.88
GH613	0; or escape	0.5 or escape	0; or escape	0.5 or escape	0;	0.50	0;	0.50	0;	0.50
GH614	3,3-2	3.75	3-3	3.63	3-2	3.38	3,3+	4.13	-	-
GH615	-	-	2,1	2.75	0;1	0.88	-	-	-	-

(continued)

Table A16. Seedling assay of Q21861/Pinnalce NIL progenies using *Pgt* race QCCJB and HKHJC (continued)

Pgt race	QCCJB								HKHJC	
	Experiment 1				Experiment 2				Experiment 1	
	Rep 1		Rep 2		Rep 1		Rep 2		Rep 1	
RIL #	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]
GH616	1;2	1.65	0; or escape	0.5 or escape	0;	0.50	0;	0.50	2;1	2.15
GH617	escape	-	0; or escape	0.5 or escape	0;	0.50	0;	0.50	3-2	3.38
GH618	;1	0.88	;1	0.88	0;	0.50	0;	0.50	3	4.00
GH619	3-	3.50	2,3-	3.13	3,3-	3.88	3,3-	3.88	0;	0.50
GH620	3,3-	3.88	3.00	4.00	3-3	3.63	3,3+	4.13	3	4.00
GH621	2.00	3.00	2;3-	2.30	3,3-	3.88	3	4.00	-	-
GH622	0; or escape	0.5 or escape	0; or escape	0.5 or escape	0; & 3-	-	0;1	0.88	;1	0.88
GH623	1.00	2.00	2,1	2.75	3-2	3.38	-	-	;1	0.88
GH624	1;	1.63	;1	0.88	0;	0.50	0;	0.50	0;	0.50
GH625	3-2	3.38	2,3-	3.13	3-3	3.63	3,3-	3.88	0;	0.50
GH626	;1,2	1.20	1;	1.63	-	-	-	-	-	-
GH627	escape	-	3-2	3.38	3,3-	3.88	0;1	0.88	-	-
GH628	3-2	3.38	3-2	3.38	3	4.00	3,3+	4.13	;1	0.88
GH629	2,1,3-	2.75	2,1	2.75	3-3	3.63	3,3+	4.13	0;	0.50
GH630	3-2	3.38	escape	-	3,3-	3.88	3,3+	4.13	0;	0.50
GH631	0; or escape	0.5 or escape	1;	1.63	0;1	0.88	0;1	0.88	2	3.00
GH632	;1,2	1.20	-	-	0;	0.50	0;	0.50	0;	0.50
GH633	;1,2	1.20	0; or escape	0.5 or escape	;1,2 & 3,3-	-	;1,2	1.20	;1	0.88
GH647	3	4.00	3+3	4.38	3-	3.50	3,3-	3.88	3	4.00
GH648	1,2;	2.15	;1,2	1.20	;1,2	1.20	0;1	0.88	;1	0.88
GH649	2,3-1	3.05	3,3-	3.88	3-2	3.38	3,3+	4.13	0;	0.50
GH650	2,1;	2.45	1;2	1.65	0;	0.50	0;	0.50	0;	0.50
GH634	2.00	3.00	3-2	3.38	3,3+	4.13	3,3+	4.13	0;	0.50

(continued)

Table A16. Seedling assay of Q21861/Pinnalce NIL progenies using *Pgt* race QCCJB and HKHJC (continued)

Pgt race	QCCJB								HKHJC	
	Experiment 1				Experiment 2				Experiment 1	
	Rep 1		Rep 2		Rep 1		Rep 2		Rep 1	
RIL #	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]
GH652	0; or escape	0.5 or escape	0; or escape	0.5 or escape	0;	0.50	0;	0.50	;1	0.88
GH653	0; or escape	0.5 or escape	1;	1.63	0;1	0.88	0;1	0.88	;1	0.88
GH655	3-3	3.63	3,3-	3.88	3,3+	4.13	3,3+	4.13	0;	0.50
GH657	1;	1.63	;1	0.88	0;1 & 3-3	-	;1,2	1.20	3-2	3.38
GH659	-	-	3-2	3.38	3+3	4.38	0;1	0.88	;1	0.88
GH660	0; or escape	0.5 or escape	1;	1.63	0;	0.50	0;	0.50	0;	0.50
GH661	2	3.00	1,2;	2.15	0;1	0.88	3+3	4.38	3-3	3.63
GH662	;1,2	1.20	1,2	2.25	0;	0.50	0;	0.50	2	3.00
GH663	3,3-	3.88	3,3+	4.13	3-3	3.63	3,3-	3.88	2,3-	3.13
GH664	1.00	2.00	1;	1.63	3,3-	3.88	3,3-	3.88	3,3+	4.13
GH665	1.00	2.00	0; or escape	0.5 or escape	0;	0.50	0;	0.50	-	-
GH666	3-	3.50	3,3-	3.88	3,3+	4.13	3-3	3.63	0;	0.50
GH667	0; or escape	0.5 or escape	1,2;	2.15	0;1 & 3,3-	-	1;2 & 3	-	-	-
GH668	;1	0.88	1;2	1.65	0;	0.50	0;	0.50	0;	0.50
GH669	;1	0.88	;1	0.88	0;	0.50	0;1	0.88	;1,2	1.20
GH670	1;	1.63	;1	0.88	0;1	0.88	0;	0.50	;1	0.88
GH671	;1	0.88	0;	0.50	0;	0.50	0;1	0.88	2,1	2.75
GH673	3,3-	3.88	3	4.00	3,3+	4.13	3,3+	4.13	3	4.00
GH674	1,2	2.25	2,1;	2.45	3-2	3.38	3,3- & 2,1;	-	3,3+	4.13
GH675	1;	1.63	1;2	1.65	0;1	0.88	0;	0.50	;1	0.88
GH676	3-2	3.38	2,3-	3.13	3-2,1	3.20	3,3+	4.13	3	4.00
GH677	3-	3.50	3-3	3.63	3,3+	4.13	3,3-	3.88	3+	4.50
GH678	0; or escape	0.5 or escape	2,3-	3.13	3	4.00	3,3+	4.13	3,3+	4.13

(continued)

Table A16. Seedling assay of Q21861/Pinnalce NIL progenies using *Pgt* race QCCJB and HKHJC (continued)

Pgt race	QCCJB								HKHJC	
	Experiment 1				Experiment 2				Experiment 1	
	Rep 1		Rep 2		Rep 1		Rep 2		Rep 1	
RIL #	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]
GH658	3-2	3.38	2,1,3-	2.75	3-3 & 2,1;	-	3+ & ;1	-	0;	0.50
GH679	1;	1.63	;1,2	1.20	0;	0.50	0;	0.50	;1,2	1.20
GH680	3,3-	3.88	3-3	3.63	3,3-	3.88	3,3+	4.13	0;1	0.88
GH681	1;	1.63	0; or escape	0.5 or escape	0;	0.50	0;	0.50	1;	1.63
GH682	2,3-	3.13	2,3-	3.13	3-2	3.38	3-2	3.38	3	4.00
GH684	2,3-1	3.05	2,1,3-	2.75	3-2	3.38	3-	3.50	0;1	0.88
GH685	3-2	3.38	2,1	2.75	0;1	0.88	;1,2	1.20	3-2	3.38
GH686	3-2	3.38	3-3	3.63	3,3-	3.88	3-2	3.38	-	-
GH687	1,2;	2.15	0; or escape	0.5 or escape	0;1	0.88	0;	0.50	1;	1.63
GH688	2,1	2.75	3-3	3.63	3,3-	3.88	3,3-	3.88	3	4.00
GH689	0; or escape	0.5 or escape	-	-	-	-	-	-	0;	0.50
GH690	3-2	3.38	3-3	3.63	3-2	3.38	3,3-	3.88	0;	0.50
GH691	0; or escape	0.5 or escape	0; or escape	0.5 or escape	0;	0.50	0;1	0.88	0;	0.50
GH692	2,1;	2.45	2,1	2.75	3,3+	4.13	3-3	3.63	3	4.00
GH693	1;2	1.65	1;	1.63	0;	0.50	0;	0.50	1,2	2.25
GH694	;1	0.88	1;	1.63	0;	0.50	0;	0.50	1;	1.63
GH695	1;	1.63	0; or escape	0.5 or escape	3,3+	4.13	3,3-	3.88	3-3	3.63
GH696	2	3.00	2,3-	3.13	3,3+	4.13	3,3-	3.88	3,3-	3.88
GH697	1;	1.63	0;1	0.88	0;	0.50	0;	0.50	0;	0.50

(continued)

Table A16. Seedling assay of Q21861/Pinnalce NIL progenies using *Pgt* race QCCJB and HKHJC (continued)

Pgt race	QCCJB								HKHJC	
	Experiment 1				Experiment 2				Experiment 1	
	Rep 1		Rep 2		Rep 1		Rep 2		Rep 1	
RIL #	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]
GH698	0;	0.50	0; or escape	0.5 or escape	0;1	0.88	-	-	3-	3.50
GH699	1;	1.63	1;	1.63	3	4.00	-	-	-	-
GH700	3+3	4.38	2,3-	3.13	0;	0.50	0;	0.50	;1	0.88
GH701	1;	1.63	;1	0.88	3,3-	3.88	3-2	3.38	3,3+	4.13
GH702	3,3-2	3.75	3,2	3.75	;1,2	1.20	3-2 & 0;1	-	3,3-	3.88
GH703	-	-	-	-	3,3-	3.88	3,3-	3.88	3+	4.50
GH705	3-2	3.38	2,3-	3.13	3,3+	4.13	3-3	3.63	;1	0.88
GH706	1;	1.63	0; or escape	0.5 or escape	3,3+	4.13	0; & 3,3+	-	0;	0.50
GH707	1;2	1.65	1;	1.63	0;	0.50	0;1 & 3	-	0;	0.50
GH708	1;	1.63	1;2	1.65	0;	0.50	0;1	0.88	0;	0.50
GH709	1;	1.63	0; or escape	0.5 or escape	0;	0.50	0;	0.50	0;1	0.88
GH710	1;	1.63	1;2	1.65	0;	0.50	-	-	;1	0.88
GH712	2,3-1	3.05	3-2	3.38	3-3	3.63	3-3	3.63	3,3-	3.88
GH714	0; or escape	0.5 or escape	1;	1.63	-	-	-	-	0;	0.50
GH716	;1	0.88	;1	0.88	0;	0.50	0;	0.50	-	-
GH717	;1	0.88	1,2;	2.15	3-2	3.38	3,3-	3.88	0;	0.50
GH718	1,2;	2.15	3+3	4.38	3,3+	4.13	3-3	3.63	3,3-	3.88
GH721	2,1,3-	2.75	2,1	2.75	3,3-	3.88	3,3-	3.88	3,3-	3.88
GH723	1,2;	2.15	0; or escape	0.5 or escape	0;	0.50	0;	0.50	;1	0.88
GH724	3,2,3+	3.75	2,3-3	3.25	3-2 & 1,2;	-	-	-	0;	0.50
GH722	3	4.00	3,3-	3.88	-	-	-	-	-	-

(continued)

Table A16. Seedling assay of Q21861/Pinnalce NIL progenies using *Pgt* race QCCJB and HKHJC (continued)

Pgt race	QCCJB								HKHJC	
	Experiment 1				Experiment 2				Experiment 1	
	Rep 1		Rep 2		Rep 1		Rep 2		Rep 1	
RIL #	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]	IT[a]	Score[b]
GH725	1;2	1.65	1;2	1.65	-	-	0;1	0.88	;1	0.88
GH726	0; or escape	0.5 or escape	1;	1.63	0;	0.50	0;	0.50	;1	0.88
GH729	;1	0.88	0; or escape	0.5 or escape	0;	0.50	0;	0.50	2,1;	2.45
GH730	3	4.00	2,3	3.25	3,3-	3.88	3,3+	4.13	3,3-	3.88
GH731	;1	0.88	1,2	2.25	1;2 & 0;	-	0;1	0.88	0;1	0.88
GH732	3-3	3.63	3,3-	3.88	3-3	3.63	3-2	3.38	3,3-	3.88
GH734	0; or escape	0.5 or escape	0; or escape	0.5 or escape	0;	0.50	0;1	0.88	1,2	2.25
GH735	3-2	3.38	3-3,2	3.60	3,3+	4.13	3	4.00	3	4.00
GH736	-	-	-	-	3,3+	4.13	3	4.00	3-2	3.38
GH737	0;1	0.88	1;	1.63	0;	0.50	0;	0.50	1;2	1.65
GH738	2,3-	3.13	2,3-	3.13	3,3-	3.88	3-2	3.38	3,3-	3.88
Q21861	0;	0.50			0;	0.50	0;	0.50	0;	0.50
Pinnacle	3,3-	3.88			3-2	3.38	3,3+	4.13	;1	0.88
Morex	3,3-	3.88			3	4.00	3	4.00	0;	0.50
Pinnacle NIL	-	-			3,3-	3.88	3.00	4.00	3.00	4.00
Harrington					3-	3.50	3-2	3.38	2,3-	3.13
Steptoe	3,3-	3.88			3	4.00	3,3-	3.88	3+	4.50

[a]The infection type was assessed using 0-4 scale modified for barley (Stakman et al., 1962; Steffenson et al., 2009)

[b]The quantitative score obtained using the transformation scale provide by Zhou et al. (2014). The conversion of categorical IT to quantitative score is explained in Appendix Table A14.

APPENDIX B. SUPPLEMENTARY FIGURE

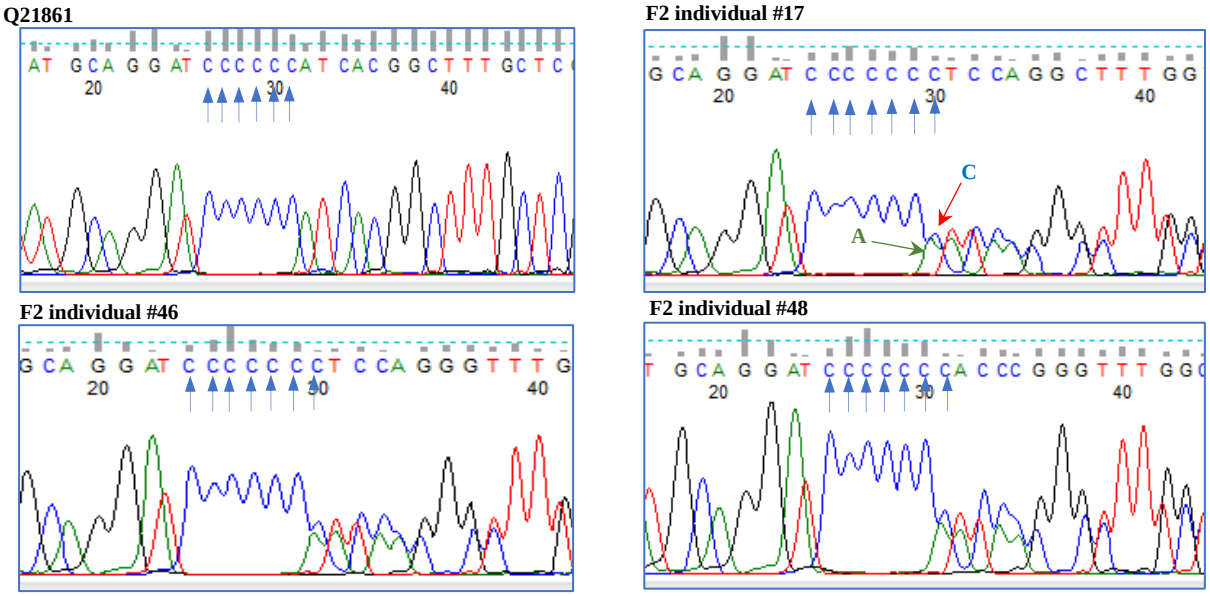

Figure B1. Chromatograms of the sequencing results obtained from sequencing amplicons of Swiss-Hv645/Conlon RMRL-NIL derived F_2 individuals RMRL-NIL. The amplicon was produced with the PCRGBS-C-insert F/ PCRGBS-C-insert R primer pair that amplifies 172bp from a region in 1st exon of *Rpg5* allele. A functional *Rpg5* contains a series of six cytosine bases as shown in (A) for barley line Q21861 (represented by six blue arrows). A non-functional *Rpg5* contains a c-insertion, making a series of 7 C bases, as shown in F_2 individuals #17 (B), #46 (C) and #48 (D). A heterozygous individual will show a double peak, as shown in (B). The red arrow represents a c-insertion and green arrow represents base 'A'.