GENETICALLY DISSECTING THE RECESSIVE RPG4-MEDIATED WHEAT STEM RUST RESISTANCE LOCUS IN BARLEY

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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

MASTER OF SCIENCE

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ABSTRACT

The *rpg4*-mediated resistance locus (RMRL) in barley provides recessive resistance against wheat stem rust (*Puccinia graminis* f. sp. *tritici*) races including the virulent race TTKSK (a.k.a *Ug*99). Three genes (*HvAdfF3*, *Rpg5*, and *HvRga1*) at the RMRL are required together for resistance. Allele characterization of these three genes identified the dominant rye stem rust resistance gene *Rpg5* gene as the polymorphic *R*-gene conditioning the recessive *rpg4*-mediated wheat stem rust resistance. The *Rpg5* gene contains an atypical *R*-gene structure encoding a nucleotide-binding site, leucine rich repeat, and serine/threonine protein kinase (STPK) domains. Genetic analysis of crosses between the resistant cultivar Q21861 with a functional *Rpg5* allele and different susceptible varieties determined that most susceptible cultivars contain the susceptibility gene *HvPP2C.1* in place of *Rpg5* STPK domain. Genetic analyses determined that *HvPP2C.1* conditions the recessive nature of the reaction, possibly negatively regulating the signaling pathway initiated by the *Rpg5* kinase domain.

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DEDICATION

I dedicate this thesis to my family, my father Mr. Ravinder K. Arora, my mother Mrs. Raj R. Arora, and my loving sibling, Mr. Kapil Arora. Thank you.

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1. LITERATURE REVIEW

1.1. Historical significance of stem rust

Stem rust or black rust was historically the most devastating and widespread diseases of small grain crops including wheat, barley, oats and rye and was considered one of the biggest threats to world food security. It is caused by the obligate, biotrophic fungal pathogen *Puccinia graminis* (*P. graminis*). Stem rust is believed to have infected grasses for millions of years and it has probably infected grain crops since the beginning of their cultivation in the Fertile Crescent region. The earliest recorded documentation of plant diseases including stem rust is found in the Bible where the epidemics of cereal rusts and smuts were believed to be inflicted upon the Israelites as punishment to their sins (Deut, 28:22). The spores of *P. graminis* have been found in archeological sites containing wheat storage jars in Israel dating from 1300 BC (Kislev, 1982). Words about stem rust also appeared in the Mishnah, which described it as a kind of spreading plaque of wheat (Taanith, 3:5). Stem rust epidemics were considered a problem in ancient Greek and Rome empires and an ancient practice by the Romans as described by Numa Pompilius (715–672 BC) was to sacrifice red animals such as foxes, dogs, and cows to the rust God in the spring time festival known as the Robigalia hoping the god would be appeased and spare their wheat crops of the devastating red rust (Chester, 1946). Stem rust was observed and recognized as early as 384 B.C. making it one of the earliest studied plant diseases by ancient plant pathologists Aristotle and Theophrastus who associated cereal rust epidemics with warm, wet weather (Chester, 1946). As documented, a string of rainy seasons in the historical times resulted in high stem rust severity and reduced wheat harvests and is speculated to have played a major role in the fall of the Roman Empire. So, through prehistory until our current times stem rust has been threatening food security.

The parasitic nature of the stem rust fungus remained unknown until the 1700s, however, the European farmers were able to recognize the connection between barberry and stem rust epidemics in wheat in the 1600s. Laws banning the planting of barberry near wheat fields were first passed in France in 1660 (Chester, 1946). The cereal crops, barberry plant and possibly the stem rust pathogen were introduced to North America by European colonists. Barberry plants were brought to North America for several economic and practical uses such as, use of berries in jams and wine, yellow dye production, the construction of wooden hand tools, and for fast-growing, thorny hedges to retain domestic animals. Barberry banning laws to protect cereal crops were then enacted in several New England colonies of Massachusetts, Rhode Island, and Connecticut in the 18th century (Chester, 1946).

1.2. Major stem rust epidemics in North America

Records of the earliest epidemics and yield losses due to stem rust in colonial North America are scattered or have been lost. According to Hamilton (Hamilton, 1939), the north central region of the United States comprising southern Minnesota, northern Iowa and Wisconsin experienced a major wheat stem rust epidemic in 1878. Losses were estimated to be around 17-27% in the three states.

After the 1878 breakout, wheat and barley production of Midwestern United States and Prairie Provinces of Canada suffered a series of stem rust epidemics until the mid-twentieth century. Eight different regional epidemics occurred in 1904, 1916, 1923, 1925, 1935, 1937, 1953, and 1954 (Carleton, 1905; Stakman and Harrar, 1957) and resulted in catastrophic yield losses. Prior to the 1950s, wheat yield losses as high as 200 million bushels due to stem rust had been reported in a single growing season in the US alone. The epidemic of 1904 was more devastating and widespread than that of 1878 and initiated several breeding programs for

resistance to stem rust. The 1916 epidemic was the severest of all and caused about 70% wheat yield losses and abandonment of a considerable acreage of wheat in Minnesota (Roelfs, 1978). As a result of this an extensive barberry eradication program began in 1918 and about 500 million barberry bushes were removed before the program terminated in the late 1970s (Roelfs, 1982). Losses in the year 1923-25 ranged from 5 to 12% in wheat and about 0.8 to 3% in barley (Roelfs, 1978). Extensive breeding programs triggered by the 1904 and 1916 epidemics resulted in the development of rust-resistant wheat varieties. The first major wheat cultivar bred for resistance, Ceres, was released in 1926 (Dyck and Kerber, 1985), and marked the beginning of the 'Boom and Bust' cycles of plant breeding. The resistance was quickly overcome by race 56 of wheat stem rust and resulted in yet another major epidemic. This 1935 epidemic caused more than 50% yield losses in wheat and about 15% in barley. Considerable losses were observed again in 1937 due to the same reasons. After Ceres resistance was surpassed, cultivars with other resistance genes provided good protection from 1938 to 1950 and only trace amount of rust was found during that period in the country. A second boom and bust cycle happened in the early 1950s when another shift in the pathogen race negated the resistance genes deployed at that time (Stakman and Harrar, 1957), again causing losses nearing 50% in wheat. After the 1950s epidemics, wheat breeders began pyramiding several resistance genes into their varieties, which provided more durable resistance, ending the occurrence of the major stem rust epidemics (Peterson, 2001).

During all these epidemic years, the losses on barley due to stem rust were similar to that found on wheat, although not reported to be as severe (Steffenson, 1991). However, after the 1940s the barley stem rust epidemics were not as common as on wheat. This was due to the release of the stem rust-resistant barley variety Kindred in 1942, which provided durable

resistance against major stem rust races in barley for over 65 years (Steffenson, 1991). Stem rust became a problem in barley in the late 1980s due to the emergence of a new form of the pathogen originally race typed on wheat differentials as QCC (Martens *et al*., 1989) and later as QCCJ.

1.3. The pathogen- Puccinia graminis

The two Italian scientists, Felice Fontana and Giovanni Tozzetti, independently, were the first to describe the stem rust pathogen in the 1760s. It was named *Puccinia graminis* by Persoon in 1797. The observation that a number of autoecious (single host) rust fungi could have five different living or spore stages during their life cycle, was made by the Tulasne brothers, Charles and Louis during 1850s. They were the first to link the red (urediniospore) and black (teliospore) stages as different spores of the same organism. The heteroecious nature of the stem rust fungus remained a mystery until it was solved by Anton deBarry in 1865. He tried to infect wheat plants with the basidiospores of *P. graminis* in order to discover its subsequent life stages but was amazed by the lack of infection. With his awareness of the European farmers' belief and based on evidence from other scientists, that barberry was related to stem rust epidemics, he was successfully able to infect barberries with basidiospores and observe the remaining stages of *P. graminis*' lifecycle (Chester, 1946). Thus, barberry was shown to be the alternate host for stem rust with a life cycle involving two hosts and five spore stages. *P. graminis* was determined to be a heteroecious, macrocyclic fungus. This was an important and useful discovery in the field of plant pathology since the role of barberry in stem rust epidemics was confirmed and later many heteroecious rusts were discovered using the same knowledge.

P. graminis belongs to the phylum Basidiomycota, subphylum Pucciniomycotina, class Pucciniomycetes, order *Puccinia*les, and Family *Puccinia*ceae. In 1884, Eriksson discovered

host-specific subspecies or "special forms" or *formae specialis* of *P. graminis.* The broad host range of *P. graminis* includes more than 365 species of cereals and grasses and is commonly subdivided into *formae specialis*, that include: wheat stem rust, *P. graminis* f. sp. *tritici* (*Pgt*), which infects wheat and barley; rye stem rust, *P. graminis* f. sp. *secalis* (*Pgs*), which infects rye and barley; and oat stem rust, *P. graminis* f. sp. *avenae* (*Pga*), which only infects oat (Eriksson and Henning, 1894). Later in 1916, E.C. Stakman and others determined that within these *formae specialis* are further genetic subdivisions called races based on the specificity of interaction with host resistance genes. The evolution and generation of novel races can be produced as a result of genetic recombination during the life cycle of the fungus (Stakman and Levine, 1922).

The life cycle of *P. graminis* starts when its spores come in contact with the primary grass host. The first spore to initiate the infection is a urediniospore, which is highly stable and can be disseminated via winds for thousands of miles. As soon as a urediniospore lands on the aboveground plant tissue, it attaches itself to the surface and starts germinating. The presence of a film of water on the leaf surface is essential for urediniospore germination. The germinating urediniospore produces a germ tube that grows perpendicularly to the leaf surface or the long axis of epidermal cells. This right-angled orientation of germ tube is essential in order to maximize and facilitate encounter with and attachment to stomata. The germ tube stops growing and forms an appresorium when it reaches a stomatal opening. A narrow penetration peg appears from the lower surface of the appresorium, which passes through the stomata into the sub-stomatal cavity and produces a substomatal vesicle. An infectious hypha grows out from each end of the vesicle and continues to grow until it encounters a host mesophyll cell. The tip of the hypha develops a septum that differentiates into a haustorial mother cell (hmc) upon contact with the host. A narrow peg is then produced from the hmc to penetrate the host cell. Upon

penetration, a specialized hypha enlarges to form the feeding structure, called the haustorium (Leonard and Szabo, 2005). The double walled haustorium remains in the periplasmic space of the host cell and is responsible for extracting nutrients for urediniospore viability and multiplication. The urediniospores are dikaryotic and are formed on individual stalks within a structure called uredinium in such abundance that the leaf tissue erupts exposing spores on the outside in the form of a reddish brown pustule. Urediniospores are capable of clonal reproduction and re-infecting the primary hosts on which they are produced and therefore, serve as secondary inoculum during the summer. In conducive environments, the generation time from spore to secondary inoculum production is about two weeks. The rapid production of inoculum, coupled with the long distance that spores can move on the prevailing wind currents, allows quick spread of the pathogen over large areas resulting in an epidemic in a very short time period, especially when the environmental conditions are favorable and susceptible varieties are planted over wide acreage.

Late in the growing season, the stem rust fungus begins producing thick walled, two celled teliospores on the stems of maturing grass host. These are specialized survival structures for the fungus during the winter and are responsible for genetic recombination and evolution of new races of the fungus. In the spring, the dikaryotic teliospores germinate and produce four haploid basidiospores, the result of karyogamy and meiosis. Basidiospores cannot infect wheat plants but are rather forcibly ejected and carried away by air currents to a susceptible barberry leaf. On barberry, the resulting infections produce flask shaped structures called pycnia, which play an essential role in the sexual stage of the fungus. Pycnium is the site for the fusion of the haploid pycniospores and receptive hyphae of opposite mating types. This paired association between opposite mating restores the dikaryotic state and results in the formation of a dikaryotic

cup-shaped aecium. From the aecium, chains of dikaryotic aeciospores are produced, which are disseminated back to the gramineous host, thus completing the life cycle (Roelfs, 1985). Although new races can emerge by mutation events in the absence of alternate host barberry as exemplified in the epidemics that occurred after barberry eradication, however during the life cycle involving barberry, the infection of secondary host results in generation of new races due to genetic recombination during the pycnial stage.

1.4. Stem rust resistance in barley

Barley is an important crop in the Upper Midwestern region of the US and Prairie Provinces of Canada. It is the fourth largest cereal crop after corn, wheat and rice in the US. The US barley production has almost decreased by more than half since 1985 due to factors like increased corn production for feeding livestock and difficulties in achieving malting grade barley due to the insidious pathogen *Fusarium graminerarum* (Oliveira *et al*., 2012), but it still stands at about 200 million bushels annually as reported by USDA for year 2013-14. Out of the total barley production, most is used in the malting industry and a significant portion is still used for animal feed. A small percentage (~2%) is utilized for human food products. During the eight major stem rust epidemics that occurred between 1904 and 1954, barley was affected as well, but never as severely as wheat. Earnest breeding for resistance began in wheat after the stem rust epidemics of 1904 and 1916 and were intensified even more with the subsequent epidemics. Since barley has a much lower rank than wheat as a food crop for human consumption, it received noticeably less attention than wheat in the field of stem rust research.

The release of the first US wheat stem rust resistant barley cultivar was made possible due to the observations of an astute North Dakota Farmer. During the severe epidemic of 1935, a North Dakotan farmer named, Sam Lykken identified a single healthy plant in his heavily rusted

field of Wisconsin 37 barley (Lejeune, 1951). He saved that plant and increased its 18 seeds for six consecutive generations. Stem rust resistance in that genotype was later confirmed by the scientists at the North Dakota Agricultural Experiment Station and it was released as a commercial cultivar in 1942 as 'Kindred' (Lejeune, 1951). It remained the dominant cultivar in the Red River valley region for over a decade and the source of resistance was later identified as the *Rpg1* gene (Steffenson, 1991).

Another source of resistance was an unimproved barley line imported by the USDA from the Canton of Lucerne in Switzerland in 1914. Two resistant genotypes, Chevron and Peatland were derived from this seed lot and the resistance was shown to be governed by a single dominant gene called the *T* gene (Power and Hines, 1933), named for conferring resistance to the '*tritici*' *forma specialis* of *Puccinia graminis.* It was later changed to *Rpg1* for Reaction to *Puccinia graminis* (Sogaard and von Wettstein-Knowles, 1987). Kindred along with the two barley selections, Chevron and Peatland were the only source of stem rust resistance in barley in the Midwestern USA and Canada in the 1900s.

In total, eight genes conferring resistance to *P. graminis* have been identified and named in barley, *Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, *rpg6*, *rpgBH*, and *RpgU* . The *Rpg2 gene*, identified from Hietpas-5 (CIho 7124) (Patterson *et al*., 1957), *Rpg3*, from PI382313 (Jedel, 1990), *rpgBH* from Black Hulless (CIho 666) (Steffenson *et al*., 1984) and *RpgU* from Peatland (Fox and Harder, 1995) provide low levels of resistance and are difficult to phenotype, therefore none of these genes have been mapped (Sun and Steffenson, 2005). The *rpg6* gene was recently introgressed from *H. bulbosum* into *H. vulgare* (Fetch *et al*., 2009). It confers recessive resistance against *Pgt* races and maps to chromosome 6H. Only *Rpg1*, *rpg4* and *Rpg5* have been cloned (Brueggeman *et al*., 2002; Brueggeman *et al*., 2008, Wang *et al*. 2013, Arora *et al*., 2013)

and investigated at the molecular level (Nirmala *et al*., 2006; Nirmala *et al*., 2007; Nirmala *et al*., 2010; Nirmala *et al*., 2011).

The *R*-genes have been grouped into different classes on the basis of their protein domain structure (Martin *et al*., 2003). The majority of *R*-genes fall into the NBS-LRR family of genes characterized by a N-terminal nucleotide-binding site (NBS) and a C-terminal leucine rich repeat region (LRR). The proteins encoded by the second major family of *R*-genes contain at least one serine/threonine protein kinase (STPK) domain. The presence of the STPK domain in these proteins possibly suggests their function in plant signal transduction pathways. The *Rpg1* gene bears a novel *R*-gene structure containing two tandem STPK domains (Sun *et al*., 2004) and *Rpg5* which codes for an NBS-LRR-STPK has both these major protein domains (NBS-LRR and STPK) connected in a single transcript/protein (Brueggeman *et al*., 2008).

1.4.1. *Rpg1* resistance, cloning and characterization

Resistance factor in Kindred was found to be allelic to that in Peatland and Chevron confirming that a single gene, namely *Rpg1* confers resistance in both (Brueggeman *et al*., 2002). The *Rpg1* gene has been classified as a durable resistance gene (Johnson, 1984) due to its effectiveness in protecting barley from most pathotypes of *Puccinia graminis* f.sp. *tritici* (*Pgt*) for more than 65 years and the vast acreage (1.8 million hectares) over which cultivars with this resistance gene are grown (Steffenson, 1992). Identification and cloning of rust resistance genes is important to decipher the underlying mechanisms of resistance. The *Rpg1* gene was mapped to the short arm of barley chromosome 7H using morphological and molecular markers (Jin *et al*., 1993) (Kilian *et al*., 1997). Development of a Steptoe (universal susceptible parent) x Morex (*Rpg1* containing variety) mapping population and the subsequent genetic map development was the first step towards cloning of the *Rpg1* gene. Initial efforts to clone the *Rpg1* gene using a

map-based approach were focused on exploiting rice-barley synteny. Despite the excellent synteny between short arms of barley chromosome 7H and rice chromosome 6 and the perfect alignment of flanking markers, the *Rpg1* gene could not be found in the syntenous position in rice (Han *et al*., 1999). The subsequent development of a bacterial artificial chromosome (BAC) library from one of the mapping parents, Morex (Yu *et al*., 2000), combined with the invaluable markers obtained from the sequenced genomes of rice and the development of high quality genetic maps led to the cloning of the first barley stem rust resistance gene, *Rpg1* in 2002 (Brueggeman *et al*., 2002). The *Rpg1* gene identity was confirmed by high-resolution genetic and physical mapping and sequencing of multiple alleles from resistant and susceptible lines. Seventeen critical recombinants were selected from a total of 8,518 gametes to develop a high resolution genetic map by genotyping with co-dominant flanking markers and the *Rpg1* gene was delimited over a genetic distance of .21 cM. A physical map was constructed using a 330 kilobase BAC contig developed across the region by chromosome walking which positioned the gene over a physical distance of 110 kb (Brueggeman *et al*., 2002). Further allele sequencing from resistant and susceptible lines and a chance recombinant (AMS170) that had a crossover within the gene confirmed the identity of the *Rpg1* gene (Brueggeman *et al*., 2002).
Through genomic and cDNA sequence comparisons, *Rpg1* gene has been predicted to contain 14 exons in a genomic DNA sequence of 4,466 bp and code for an 837 aa (94.5 kDa) protein. The *Rpg1* gene product encodes a unique structure with two tandem kinase domains, a pseudokinase domain (pK1) and an active kinase domain (pK2), required together for resistance (Brueggeman *et al*., 2002; Nirmala *et al*. 2006) and shows no significant structural similarity with previously reported plant disease resistance genes.

The functional validation of the *Rpg1* gene (Brueggeman *et al*., 2002) was later done by *Agrobacterium* mediated stable transformation of the gene into a susceptible cv. Golden Promise (Horvath *et al*., 2003). Golden Promise lacks the *Rpg1* gene in its genome, presumed to be either deleted or highly diverged (Brueggeman *et al*., 2002). Higher levels of resistance to the pathogen were seen in several independent transformants as compared to the resistance source, Morex, thus confirming the role of *Rpg1* as an *R*-gene. Through expression analysis, *Rpg1* was shown to be constitutively and uniformly expressed in all plant organs with highest levels in the leaf epidermis than in the whole leaf (Rostoks *et al*., 2004), possibly indicating the detection at the leaf surface. The location of RPG5 protein is mostly cytosolic with small but significant amounts associated with membranes (Nirmala *et al*., 2006). The RPG1 protein has been shown to phosphorylate within 5 minutes of exposure to spores from avirulent but not virulent wheat stem rus races leading to disease resistance. RPG1 was rapidly phosphorylated when Morex plants were inoculated with the avirulent *Pgt* races MCCF and HKHJ but not with the virulent race QCCJ or a virulent *P. graminis* f. sp. *secalis* (*Pgs*) rye stem rust isolate 92-MN-90 (Nirmala *et al*., 2010). The *Rpg1* gene proved to be a durable *R*-gene for over six decades but emergence of *Rpg1* virulent races, such as QCCJ and TTKSK in the late 1989 and 1999, respectively, urged scientists to explore new sources of resistance effective against these exception stem rust races that overcame the broad *Rpg1* resistance mechanisms.

1.4.2. *rpg4*-mediated resistance locus or RMRL

A new race of *Pgt*, designated as QCCJ was identified in North Dakota during the 1989 growing season (Roelfs *et al*., 1991). The race was found to be virulent on barley containing *Rpg1*. QCCJ became one of the most predominant races on barley causing minor epidemics on Midwestern barley. The threat to barley production by this new stem rust race initiated the search

for effective resistance sources against QCCJ and over 18,000 barley accessions from the USDA National Small Grains collection were evaluated. The best source of resistance was discovered in the unimproved barley line Q21861 from the International Maize and Wheat Improvement Center CIMMYT (Jin *et al*., 1994a). Genetic studies revealed that resistance to race QCCJ in line Q21861 was conferred by a single recessive gene designated *rpg4*. The *rpg4* gene was genetically mapped to the long arm of barley chromosome 5H (Borovkova *et al*., 1995). Another source of resistance designated as the *Rpg5* gene was also identified in line Q21861. The *Rpg5* geneconfers dominant resistance to an isolate of rye stem rust, *Pgs* isolate 92-MN-90.. The single dominant *R*-gene, *Rpg5*, was also mapped to the long arm of chromosome 5H and initially cosegregated with the *rpg4* gene in a low resolution mapping population (Druka *et al*., 2000). A current threat to barley and wheat production is a highly virulent strain of stem rust, *Ug*99 (race TTKSK) detected in Uganda, Africa in 1999 (Pretorius *et al*., 2000) and since its emergence has spread across Eastern Africa and travelled over the red sea into areas of Yemen and Iran. This highly virulent race carries a unique combination of virulence genes and is capable of infecting more than 70% of the world's wheat cultivars (Singh *et al*., 2008) and more than 97% of barley cultivars, including those having *Rpg1* (Steffenson *et al*., 2012). The possible route of movement as predicted by Singh *et al* 2008 is through Eastern Africa to Middle East into West and South Asia. There have been no reports of this race in North America but once it reaches the breadbaskets, it will rapidly find its way to USA and Canada*Ug*99's high virulence potential and its ability to wipe away wheat and barley production across the World has raised concerns over world food security (Stoksad, 2007). Fortunately, the *rpg4*-mediated resistance locus or RMRL in the barley line Q21861 has been shown to confer effective resistance against race TTKSK (Steffenson *et al*., 2009).

The *Rpg5* gene alone confers resistance to *Pgs* isolate 92-MN90 but resistance against the *Pgt* races require both *Rpg5* and *rpg4*. The *rpg4*/*Rpg5* locus (currently RMRL1) was localized to the 70kb region on long arm of chromosome 5H. Similar to the *Rpg1* cloning, high-resolution genetic maps and the cv. Morex BAC library were used to develop physical contigs across the *rpg4/Rpg5* region on chromosome 5H (Druka *et al*., 2002; Brueggeman *et al*., 2008). Five candidate genes, two encoding NBS-LRR proteins (*Hv*Rga1 and *Hv*Rga2), two actin depolymerizing-like factors (*Hv*Adf2 and *Hv*Adf3) and a protein phosphatase 2C-like protein (*Hv*PP2C.1) were identified in the annotated sequence (Brueggeman *et al*., 2008). Since both *rpg4* and *Rpg5* genes were originally discovered in the unimproved barley line Q21861, equivalent regions of resistance were needed from the same line. Morex sequence along with a Q21861 lambda phage library were used to generate the Q21861 sequence. Interestingly, the *HvPP2C.1* gene was shown to be missing and replaced by a STPK coding gene in line Q21861. Sequence comparison of Morex and Q21861 suggested that *HvRga2* was the *Rpg5* gene since all other candidate gene showed no significant differences in primary DNA sequence or differences in expression at the transcript level from the susceptible and resistant cultivar. The only difference was that the Morex *Hv*Rga2 consisted of typical NBS-LRR domains and a PP2C gene a short distance downstream and the Q21861 *Hv*Rga2 allele consisted of NBS-LRR-S/TPK domains. Thus, the *HvRga2* allele from Q21861 was considered the best candidate *Rpg5* gene conferring resistance against the *Pgs* isolate, 92-MN-90.

The atypical *R*-gene structure of the *Rpg5* with NBS-LRR and STPK domains on a single mRNA transcript gene was determined using RT-PCR and northern blot analysis (Brueggeman *et al*., 2008). The *Rpg5* gene was predicted then shown by cDNA sequencing to contain seven exons in about 8504 bp of genomic sequence, encoding a predicted 1378 a.a. long protein. The

resistance function of the gene was validated using viral induced gene silencing (VIGS) where the post-transcriptional silencing of the candidate *Rpg5* gene in the resistant line Q21861 resulted in a shift from incompatibility to compatibility (Brueggeman *et al*.,2008).

The identity of the *rpg4* gene was still unknown due to the complexity underlying the *rpg4/Rpg5* resistance locus (Brueggeman *et al*. 2008; Brueggeman *et al*., 2009; Wang *et al*., 2013). For positional cloning and studying the recessive nature of the *rpg4*-mediated resistance, three high-resolution mapping populations, (Harrington × Q21861 [HQ], Multidominant 2 × Q21861 [MD2Q], and Steptoe × Q21861 [SQ]), representing a total of 5,223 recombinant gametes were developed (Brueggeman *et al*., 2008). Fourteen out of the 5233 recombinants were selected and using SNP analysis it was determined that the *Rpg5*-mediated *Pgs* and *rpg4*-mediated *Pgt* resistances co-segregate in mapping populations, HQ and MD2Q but segregate away from each other in the SQ population (Brueggeman *et al*.,2008; Brueggeman *et al*.,2009). Thus, a second gene was functionally polymorphic in the SQ population and was designated as *rpg4*-modifier element 1 (*Rme1*). VIGS analysis of the genes present at the RMRL1 determined that *Rpg5, HvRga1* and *HvAdf3* are all required for *rpg4*-mediated resistance, thus, the *rpg4*-mediated resistance locus (RMRL) is complex and requires the concerted action at least four genes (*Rpg5, HvRga1*, *HvAdf3* and *Rme1*) located at two tightly linked subloci (Wang *et al*., 2013). The two subloci have been given the designations RMRL1 and RMRL2. In case of wheat stem rust resistance, the current notion is to not to think of *rpg4* and *Rpg5* as distinct genes but as designations to define the *rpg4*-mediated resistance locus or RMRL.

1.5. Literature cited

Arora, D., Gross, T., and Brueggeman, R. 2013. Allele characterization of genes required for *rpg4*-mediated wheat stem rust resistance identifies *Rpg5* as the R-gene. Phytopathology 103:1153-1161.

Borovkova, I.G., Steffenson, B.J., and Jin, Y. 1995. Identification of molecular markers linked to the stem rust resistance gene *rpg4* in barley. Phytopathology 85:181-185.

Brueggeman, R., Rostoks, N., Kudrna, D., Kilian, A., Han, F., Chen, J., Druka, A., Steffenson, B., and Kleinhofs, A. 2002. The barley stem rust-resistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. Proc. Natl. Acad. Sci. USA 99:9328-9333.

Brueggeman, R., Druka, A., Nirmala, J., Cavileer, T., Drader, T., Rostoks, N., Mirlohi, A., Bennypaul, H., Gill, U., Kudrna, D., Whitelaw, C., Kilian, A., Han, F., Sun, Y., Gill, K., Steffenson, B., and Kleinhofs, A. 2008. The stem rust resistance gene *Rpg5* encodes a novel protein with nucleotide binding site, leucine-rich and protein kinase domains Proc. Natl. Acad. Sci. USA 105:14970-14975.

Carleton, M.A. 1905. Lessons from the grain-rust epidemic of 1904. Farmers bull. 219:1-24.

Chester, K.S. 1946. The Nature and Prevention of the Cereal Rusts as Exemplified in the Leaf Rust of Wheat. Waltham, MA:Chronica Botanica.

Deut. 28:22.1 Kings 8:37.

Druka, A., Kudrna, D., Han, F., Kilian, A., Steffenson, B., Frisch, D., Tomkins, J., Wing, R., and Kleinhofs, A. 2000. Physical mapping of barley stem rust resistance gene *rpg4*. Mol. Gen. Genet. 264:283-290.

Dyck, P.L. and Kerber, E.R. 1985. Resistance of the race-specific type. In: Roelfs AP, Bushnell WR (eds) The cereal rusts, vol II. Academic Press Inc., Orlando, FL, 469-500.

Eriksson, J. and Henning, E. 1894. Die Hauptresultate einer neuen Untersuchung uber die Getreideroste. *Z. Pflanzenkr.* 4: 66-73, 140-142, 197-203, 257-262.

Fetch, T.J., Johnston, P.A., and Pickering, R. 2009. Chromosomal location and inheritance of stem rust resistance transferred from *Hordeum bulbosum* into cultivated barley (*H. vulgare*). Phytopathology 99:339–343.

Fox, S.L. and Harder, D.E. 1995. Resistance to stem rust in selected barley lines and the inheritance of resistance to pathotype QCC. Canadian Journal of Plant Science 75:781–788.

Hamilton, L. M. 1939. Stem rust in the spring wheat area in 1878.Roehner, Bertrand M. 1996. Minn Hist. 20: 156–164.

Hammond-Kosack, K.E. and Jones, J.D.G. 1997. Plant disease resistance genes. Annual Review of Plant Physiology and Plant Molecular Biology 48:573-605.

Han F., Kilian A., Chen J.P., Kudrna D., Steffenson B., Yamamoto K., Matsumoto T., Sasaki T., and Kleinhofs A. 1999. Sequence analysis of a rice BAC covering the syntenous barley *Rpg1* region. Genome 42:1071-1076.

Horvath, H., Rostoks, N., Brueggeman, R., Steffenson, B., von Wettstein, D., and Kleinhofs, A. 2003. Genetically engineered stem rust resistance in barley using the *Rpg1* gene. Proc. Natl. Acad. Sci. USA 100:364-369.

Jedel, P.E. 1990. A gene for resistance to *Puccinia graminis* f. sp. *tritici* in PI 382313. Barley Genet. Newsl. 20:43-44.

Jin, Y., Steffenson, B.J., and Franckowiak, J.D. 1993. Linkage between the *Rpg1* gene for stem rust resistance and f5 locus on barley chromosome-1.Crop Sci. 33: 642–643.

Jin, Y., Steffenson, B.J., and Fetch, T.G.J. 1994a. Sources of resistance to pathotype QCC of *Puccinia graminis* f. sp. *tritici* in barley. Crop Sci. 34:285-8.

Jin Y., Steffenson B.J., and Miller J.D. 1994b. Inheritance of resistance to pathotypes QCC and MCC of *Puccinia graminis* f. sp. *tritici* in barley line Q21861 and temperature effects on the expression of resistance. Phytopathology 84:452-5.

Johnson, R. 1984. A critical analysis of durable resistance. Ann. Rev. Phytopathology 22:309-330.

Kilian, A., Chen, J., Han, F., Steffenson, B., and Kleinhofs, A. 1997. Towards map-based cloning of the barley stem rust resistance genes *Rpg1* and *rpg4* using rice as an intergenomic cloning vehicle. Plant Molecular Biology 35:187-195.

Kislev, M.E. 1982. Stem rust of wheat 3300 years old found in Isreal. Science 216:993-994.

Lejeune, A.J. 1951. The story of Kindred (L) barley. Barley Improv. Conf. Rept, Minneapolis, MN.

Leonard, K.J. and Szabo L.J. 2005. Stem rust of small grains and grasses caused by *Puccinia graminis*. Molecular Plant Pathology 6: 99-111.

Martens, J.W., Dunsmore, K.M., and Harder, D.E. 1989. Incidence and virulence of *Puccinia graminis* in Canada on wheat and barley in 1988. Can. J. Plant Pathol. 11: 424–430.

Martin, G.B, Bogdanove, A.J., and Sessa, G. 2003. Understanding the function of plant disease resistance proteins. Annu. Rev. Plant. Biol. 54:23-61.

Nirmala, J., Brueggeman, R., Maier, C., Clay, C., Rostoks, N., Kannangara, C.G., Wettstein D. von, Steffenson, B., and Kleinhofs, A. 2006. Subcellular localization and functions of the barley stem rust resistance receptor-like serine/threonine-specific protein kinase *Rpg1*. Proc. Natl. Acad. Sci. USA 103:7518-7523.

Nirmala, J., Dahl, S., Steffenson, B.J., Kannangara, C.G., Wettstein D. von, Chen, X., and

Kleinhofs, A. 2007. Proteolysis of the barley receptor-like protein kinase RPG1 by a proteasome pathway is correlated with *Rpg1*-mediated stem rust resistance. Proc. Natl. Acad. Sci. USA 104:10276-10281.

Nirmala, J., Drader, T., Chen, X., Steffenson, B., and Kleinhofs, A. 2010. Stem rust spores elicit rapid RPG1 phosphorylation. Mol. Plant Microbe Interact. 23:1635-1642.

Nirmala, J., Drader, T., Lawrence, P. K., Yin, C., Hulbert, S., Steber, C. M., Steffenson, B. J., Szabo, L. J., Wettstein, D. V., and Kleinhofs, A. 2011. Concerted action of two avirulent spore effectors activates reaction to *Puccinia graminis* 1 (*Rpg1*)-mediated cereal stem rust resistance. Proc. Natl. Acad. Sci. USA 108:14676-14681.

Oliveira, P.M., Mauch A., Jacobs F., Waters D.M., and Arendt E.K. 2012. Fundamental study on the influence of *Fusarium* infection on quality and ultrastructure of barley malt, International Journal of Food Microbiology 156: 32-43.

Patterson, F.L., Shands, R.G., and Dickson, J.G. 1957. Temperature and seasonal effects on seedling reactions of barley varieties to three races of *Puccinia graminis* f. sp. *tritici*. Phytopathology 47:395-402.

Peterson, P.D. 2001. Stem rust of wheat from enemy to modern foe. St Paul, MN:APS press.

Powers, L. and Hines, L. 1933. Inheritance of reaction to stem rust and barbing awns in barley crosses. J. Agric. Res. 46:1121-1129.

Pretorius, Z.A., Singh, R.P., Wagoire, W.W., and Payne, T. S. 2000. Detection of virulence to wheat stem rust resistance gene *Sr*31 in *Puccinia graminis*. f. sp. *tritici* in Uganda. Plant Dis. 84:203.

Roelfs, A.P. 1978. Estimated losses caused by rust in small grain cereals in the United States: 1918–1976. USDA Misc. Pub. 1363:1–85.

Roelfs, A.P. 1982. Effects of barberry eradication on stem rust in the United States. Plant Dis. 66:177-181.

Roelfs, A. P. 1985. Epidemiology in North America. The Cereal Rusts Vol. II: Diseases, distribution, epidemiology and control. 2: 403-434. Academic Press, Orlando, Fl.

Roelfs, A.P., Casper, D.H., Long, D.L., and Roberts, J.J. 1991. Races of *Puccinia graminis* in the United States in 1989. Plant Dis. 75:1127-30.

Rostoks, N., Steffenson, B.J., and Kleinhofs, A. 2004. Structure and expression of the barley stem rust resistance gene *Rpg1*. Physiology and Molecular Plant Pathology 64:91-101.

Singh, R.P., Hodson, D.P., Huerta-Espino, J., Jin, Y., Njau, P., and Wanyera, R. 2008. Will stem rust destroy the world's wheat crop? Advances in Agronomy 98:271-309.

Søgaard, B. and Wettstein-Knowles P. von. 1987. Barley: genes and chromosomes. Carlsberg Res. Commun. 52:123-196.

Stakman, E. C. and Harrar, J. G. 1957. Principles of Plant Pathology. Ronald Press, New York.

Stakman, E. C. and Levine, M. N. 1922. The determination of biologic forms of *Puccinia graminis* on Triticum spp. Minn. Agric. Exp. Stn. Tech. Bull. 8 pp.

Steffenson, B.J., Wilcoxson, R.D., and Roelfs, A.P. 1984. Inheritance of resistance to *Puccinia graminis* f. sp. *secalis* in barley. Plant Dis. 68:762-763.

Steffenson, B.J. 1991. Stem rust of barley: History, calamity, and control. Proc. 28th Barley Improvement Conference 3-8.

Steffenson, B.J. 1992. Analysis of durable resistance to stem rust in barley. Euphytica 63:153-167.

Steffenson, B. J., Jin, Y., Brueggeman, R. S., Kleinhofs, A., and Sun, Y. 2009. Resistance to stem rust race TTKSK maps to the *rpg4* /*Rpg5* complex of chromosome 5H of barley. Phytopathology 99:1135-1141.

Steffenson, B. J., Zhou, H., Chai, Y., and Grando, S. 2012. Vulnerability of cultivated and wild barley to African stem rust race TTKSK. Pages 149-155 in: Advances in Barley Science: Proc. of 11th International Barley Genetics Symposium. Li, C., Zhang, G., and Liu, X. eds. Zhejiang University Press, Hangzhou.

Stokstad, E. 2007. Deadly wheat fungus threatens world's breadbaskets. Science 315:1786-1787.

Sun, X., Cao, Y., Yang, Z., Xu, C., Li, X., and Wang, S. 2004. *Xa26*, a gene conferring resistance to *Xanthomonas oryzae* pv. *oryzae* in rice, encodes an LRR receptor kinase-like protein. The Plant Journal 37:517-527.

Sun, Y. and Steffenson, B.J. 2005. Reaction of barley seedlings with different stem rust resistance genes to *Puccinia graminis* f. sp. *tritici* and *P. g.* f. sp. *secalis*. Can. J. Plant. Pathol. 27:80-89.

Taanith, chapter 3:5.

Wang, X., Richards, J., Gross, T., Druka, A., Kleinhofs, A., Steffenson, B., Acevedo, M., and Brueggeman, R. 2013. The *rpg4*-mediated resistance to wheat stem rust (*Puccinia graminis*) in barley (*Hordeum vulgare*) requires *Rpg5*, a second NBS-LRR gene and an actin depolymerization factor. Mol. Plant-Microbe Interaction 26: 407-418.

Yu, Y., Tomkins, J., Waugh, R., Frisch, D., Kudrna, D., Kleinhofs, A., and Brueggeman, R. 2000. A bacterial artificial chromosome library for barley (*Hordeum vulgare* L.) and

identification of clones containing putative resistance genes. Theor. Appl. Genet. 101:1093-1099.

2. CHAPTER ONE: ALLELE CHARACTERIZATION OF GENES REQUIRED FOR *RPG4*-MEDIATED WHEAT STEM RUST RESISTANCE IDENTIFIES *RPG5* AS THE R-GENE

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Data deposition: The sequences reported in this paper have been deposited in the GenBank database under the following accession numbers. *Rpg5* genomic sequences deposited include the following: KC495438- KC495440, KC495442, KC495443 and KC529339 (barley lines WBDC040, WBDC214, WBDC225, Swiss *Hv*492, Swiss *Hv*611 and WBDC160): *Hv*Rga1 genomic sequences deposited include the following: KC433575- KC433575 and KC433589-KC433592 (WBDC 019, WBDC 040, WBDC 160, WBDC 214, WBDC 220, WBDC 225, Swiss *Hv*492, Swiss *Hv*584, Swiss *Hv*587, Swiss *Hv*611, Swiss *Hv*619, Swiss *Hv*645, Damon11-11, Harrington, Golden Promise, WBDC 269 and WBDC 323): *Hv*Adf3 genomic sequences deposited include the following: KC335499- KC335515 and KC335517- KC335518 (WBDC019, WBDC 040, WBDC 160, WBDC 214, WBDC 220, WBDC 225, WBDC 269, WBDC 323, Swiss *Hv*489, Swiss *Hv*489, Swiss *Hv*587, Swiss *Hv*611, Swiss *Hv*619, Swiss *Hv*645, Swiss *Hv*672, Damon11-11, MD2 and Golden Promise). The following sequences were previously deposited by Brueggeman *et al*. 2008. [Accession nos. EU812563 (genomic sequence from barley cultivar Morex): EU878778 (genomic sequence from barley line Q21861) EU883581-EU883583 (*Hv*Rga1genomic sequence from barley lines Harrington, MD2 and Steptoe, respectively): EU883788-EU883790 (*rpg5* allele (*Hv*Rga2) genomic sequence from barley lines Harrington, MD2, and Steptoe, respectively): and EU883791 (*Hv*Adf3 genomic sequence from barley line Steptoe)].

2.1. Abstract

A highly virulent form of the wheat stem rust pathogen, *Puccinia graminis* f. sp. *tritici* (*Pgt*) race TTKSK, is virulent on both wheat and barley presenting a major threat to world food security. The recessive and temperature sensitive *rpg4* gene is the only effective source of resistance identified in barley (*Hordeum vulgare* L.) against *Pgt* race TTKSK. Efforts to positional clone *rpg4* localized resistance to a small interval on barley chromosome 5HL, tightly linked to the rye stem rust (*Puccinia graminis* f. sp. *secalis*) resistance gene *Rpg5*. High-resolution genetic analysis and post transcriptional gene silencing of the genes at the *rpg4*/*Rpg5* locus determined that three tightly linked genes (*Rpg5*, *HvRga1* and *HvAdf3*) are required together for *rpg4*-mediated wheat stem rust resistance. Alleles of the three genes were analyzed from a diverse set of fourteen domesticated barley lines (*Hordeum vulgare*) and eight wild barley accessions (*Hordeum vulgare subsp. spontaneum*) to characterize diversity that may determine incompatibility (resistance). The analysis determined that *HvAdf3* and *HvRga1* code for predicted functional proteins that do not appear to contain polymorphisms determining the compatible (susceptible) interactions with the wheat stem rust pathogen and were expressed at the transcriptional level from both resistant and susceptible barley lines. The *HvAdf3* alleles shared 100% amino acid identity among all twenty-two genotypes examined. The *Pgt* race QCCJ susceptible barley lines with *HvRga1* alleles containing the limited amino acid substitutions unique to the susceptible varieties also contained predicted non-functional *rpg5* alleles. Thus, susceptibility in these lines is likely due to the non-functional RPG5 proteins. The *Rpg5* allele analysis determined that nine of the thirteen *Pgt* race QCCJ susceptible barley lines contain alleles that either code for predicted truncated proteins as the result of a single nucleotide substitution resulting in a stop codon at amino acid 161, a single cytosine indel causing a frame

shift and a stop codon at amino acid 217, or an indel that deleted the entire STPK domain. The three *Pgt* race QCCJ susceptible lines, Swiss landraces *Hv*489, *Hv*492 and *Hv*611 and the wild barley accession WBDC160, contain *rpg5* alleles predicted to encode full-length proteins containing a non-synonomous nucleotide substitution that results in the amino acid substitution E1287A. This amino acid substitution present in the uncharacterized C-terminal domain is not found in any resistant line and may be important to elicit the resistance reaction. These data suggest that *rpg4*-mediated resistance against many wheat stem rust pathogen races including *Pgt* race TTKSK rely on the *Rpg5* resistance gene, thus *rpg4*- and *Rpg5*-mediated resistance rely on a common *R*-gene and should not be considered completely distinct. The data also determined that *Rpg5* gene specific molecular markers could be used to detect *rpg4*-mediated wheat stem rust resistance for marker-assisted selection.

2.2. Introduction

Wheat stem rust caused by the biotrophic fungal pathogen *Puccinia graminis* f. sp. *tritici* was a devastating disease of wheat and barley grown in the northern Great Plains of the United States and Canada prior to successful management through resistance (*R*) gene deployment (Steffenson, 1992). The use of genetic resistance in both crops has been a major success, keeping stem rust in check for the past 70 years; however, the emergence of the highly virulent race of the wheat stem rust pathogen *P. graminis* f. sp. *tritici* race TTKSK in Africa and its spread has warranted concern for future food production security (Stoksad, 2007). Barley production is particularly vulnerable because *P. graminis* f. sp. *tritici* race TTKSK and its variant forms have been shown to be virulent on ≈97% of barley varieties from an extensive world barley collection of cultivated and wild barley tested at the seedling stage under controlled conditions (Steffenson *et al*., 2012). *P. graminis* f. sp. *tritici* race TTKSK is also virulent on U.S. barley varieties

carrying the *Rpg1* gene, the only major stem rust *R* gene deployed in commercial barley cultivars of the northern Great Plains (Steffenson, 1992). Barley is also vulnerable because the only effective *P. graminis* f. sp. *tritici* race TTKSK resistance known is the *rpg4/Rpg5* locus (Steffenson *et al*., 2009).

The recessive and temperature-sensitive *rpg4* stem rust *R* gene, presumed to control a gene-for-gene interaction between barley and several wheat stem rust races, including MCCF, QCCJ, and TTKSK, was mapped to a subtelomeric region of barley chromosome 5HL, cosegregating with the rye stem rust *R* gene *Rpg5* (Brueggeman *et al*., 2008). High-resolution mapping and positional cloning identified the *Rpg5* gene and determined that *rpg4* and *Rpg5* were distinct yet tightly linked (Brueggeman *et al*., 2008). The *Rpg5* gene was shown to encode a novel resistance protein containing three typical resistance domains: the nucleotide-binding site (NBS), leucine rich repeat (LRR), and a serine threonine protein kinase domain (S/TPK). Subsequent efforts at positional cloning of the *rpg4* gene have not yet identified the gene because of the complex genetic interactions underlying the locus and resistance mechanisms (Brueggeman *et al*., 2009; Wang *et al*., 2013). However, the recent genetic analysis of three high-resolution populations determined that the *rpg4*-mediated resistance locus (RMRL) is complex and requires at least four genes located at two tightly linked subloci (Wang *et al*., 2013). The two subloci have been given the designations RMRL1 and RMRL2 (Fig. 1A).

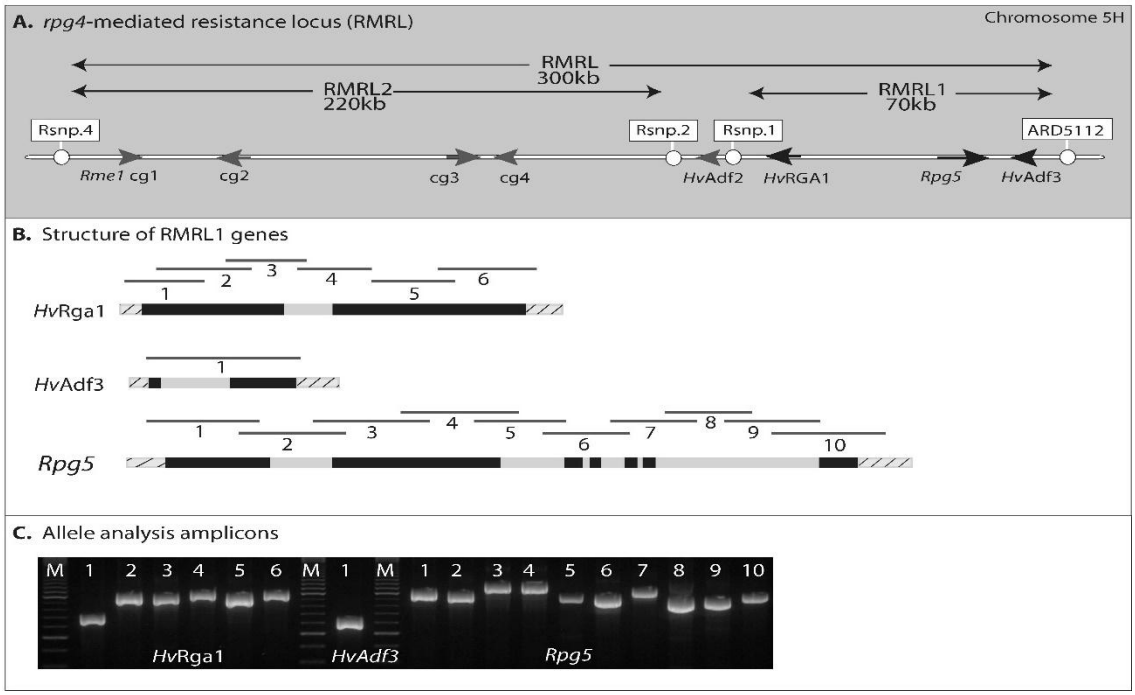

Figure 1. Physical map of the *rpg4/Rpg5*-mediated resistance locus and characterization of genes required for resistance. **A**, White horizontal bar represents the physical region containing the *rpg4*-mediated resistance locus (RMRL) and the two genetically defined subloci, RMRL1 and RMRL2, described by Wang *et al*. (Wang *et al*., 2013). White boxes indicate genetic markers delimiting the region and the black bars above indicate the RMRL, RMRL1, and RMRL2 delimited regions. Black arrows along the physical map indicate characterized genes in the RMRL1 region and *rpg4* modifier element 1 (*Rme1*) candidate genes (*Rme1* cg1–cg4) in the RMRL2 region. **B**, Gene structures of the genes at RMRL1 required for *rpg4*-mediated resistance. Horizontal bars represent the genomic DNA structures for *Rpg5*, *HvRga1*, and *HvAdf3*. Black indicates exons, and gray introns and dashed lines represent the 5′ and 3′ untranslated regions, respectively. Numbered black bars above indicate the positions of the amplicons used for allele sequencing. **C**, Agarose gel showing the amplicons used for direct sequencing of the *Rpg5*, *HvRga1*, and *HvAdf3* alleles amplified from the barley line Q21861. Genes are indicated below and the numbers above correspond to the fragments shown in **B**.

The *Rpg5*-mediated *P. graminis* f. sp. *secalis* and *rpg4*-mediated *P. graminis* f. sp. *tritici* resistances co-segregated at RMRL1 in two high-resolution mapping populations, Harrington × Q21861 and MD2 × Q21861, but segregate away from each other in the Steptoe × Q21861 population. The genetic data indicated that a second gene given the designation *rpg4*-modifier element 1 (*Rme1*) was functionally polymorphic in the Steptoe × Q21861 population and was

delimited to the tightly linked RMRL2 locus (Wang *et al*., 2013). The previously identified *Rpg5* gene that confers dominant resistance to isolates of the rye stem rust pathogen was shown by post-transcriptional gene silencing to be required in addition to two other genes at RMRL1 for resistance against *P. graminis* f. sp. *tritici* race QCCJ (Wang *et al*., 2013). The other genes at RMRL1 required for *P. graminis* f. sp. *tritici* race QCCJ resistance are a second unrelated NBS-LRR gene (*HvRga1*) and the actin depolymerization factor-like gene *HvAdf3* (Fig. 1). Mapping of *P. graminis* f. sp. *tritici* race TTKSK resistance in barley line Q21861 determined that resistance cosegregated with the *rpg4*/*Rpg5* locus in a small double-haploid population of 119 individuals from the cross Q21861 × Sm89010 (Steffenson *et al*., 2009). All the high-resolution recombinants examined also behave similarly to *P. graminis* f. sp. *tritici* race QCCJ and TTKSK, suggesting that resistance against the different wheat stem rust races is conferred by the same mechanisms at the *rpg4*/*Rpg5* locus (Wang *et al*., 2013).

The requirement of two closely linked yet unrelated NBS-LRR *R* genes is becoming a common theme to resistance mechanisms (Ashikawa *et al*., 2008; Birker *et al*., 2009; Lee *et al*., 2009; Loutre *et al*., 2009; Narusaka *et al*., 2009; Okuyama *et al*., 2011; Peart *et al*., 2005; Sinapidou *et al*., 2004; Wang *et al*., 2013; Yuan *et al*., 2011). Most of these mechanisms requiring dual NBS-LRR genes contain a genetically identifiable *R* gene with functional polymorphism and a second, closely linked nonpolymorphic NBS-LRR gene, which has been typically identified through mutagenesis or post-transcriptional gene silencing resulting in compatibility (Birker *et al*., 2009; Loutre *et al*., 2009; Narusaka *et al*., 2009; Okuyama *et al*., 2011; Peart *et al*., 2005; Sinapidou *et al*., 2004; Yuan *et al*., 2011). Alternatively, positional cloning of the rice blast *R* genes *Pikm* and *Pi-5* identified regions containing two NBS-LRR

genes: *Pikm1-TS/Pikm2- TS* and *Pi5-1/Pi5-2*, respectively. Complementation via transformation showed that both genes are required for resistance (Ashikawa *et al*., 2008; Lee *et al*., 2009).

Similar to the *rpg4*-mediated resistance in barley, it has been shown in *Arabidopsis* that an actin depolymerization factor, *AtAdf4*, is required for specific resistance against *Pseudomonas syringae* carrying the *AvrPphB* avirulence factor mediated by theRPS5/PBS1-resistance mechanism (Tian *et al*., 2009). Based on genome sequence analysis, it appears that there are limited *Adf* genes in plants; 12 in *Arabidopsis* and rice (Feng *et al*., 2006). This limited number of highly conserved genes appears to play a major role in many developmental (Chen *et al*., 2003; Thomas *et al*., 2002), abiotic (Ouellet *et al*., 2001), and biotic stress responses (Miklis *et al*., 2007, Tian *et al*., 2009), with the concise function determined by the developmental or environmental stimuli. The minimal redundancy within the plant genomes suggests that individual *Adf* genes are differentially modulated by these diverse stimuli involving phosphorylation- and dephosphorylation-dependent signaling. The identification of highly conserved ADF proteins via mutant analysis or by post-transcriptional gene silencing required for specific resistance responses suggests that, although they are required for a specific resistance pathway, they are certainly utilized for other resistance pathways and cellular functions as well. When pathogen challenge is recognized by a cognate resistance receptor, the ADFs must be precisely regulated to mount the defense response, probably via phosphorylation or dephosphorylation mechanisms. *R* gene dogma has historically stated that resistance loci contain single distinct *R* genes, yet the recent scrutiny of many *R* gene loci has led to the paradigm shift that many resistance loci are complexes containing several genes required for resistance; however, the only component identified by genetic analysis is the one that is functionally polymorphic.

Characterization of >8,000 recombinant gametes in three separate barley populations with the same resistance source (Q21861) and three different susceptible parents (Harrington, MD2, and Steptoe) could not separate the three candidate genes required for *rpg4*-mediated resistance present at the RMRL1 locus, indicating that recombination in the region was rare and the genes are typically transferred as a single genetic unit (Brueggeman *et al*., 2008; Wang *et al*., 2013). However, three recombination events in the Steptoe × Q21861 population determined that a second locus, designated RMRL2, contains a gene or genes required for *rpg4*-mediated resistance against *Puccinia graminis* f. sp. *tritici* races QCCJ and TTKSK. The RMRL2 segregates away from RMRL1 by 0.01 centimorgans in the Steptoe × Q21861 high-resolution mapping population and, thus, is tightly linked yet genetically distinct from RMRL1. The gene required for *rpg4*-mediated resistance at RMRL2, given the designation *Rme1*, has yet to be identified, although sequencing of the ≈200-kb genetically delimited RMRL2 region is completed and candidate *Rme1* genes have been identified (R. Brueggeman and X. Wang, *unpublished data*). These data suggest that *rpg4*- mediated resistance, previously considered the single *rpg4 R* gene, is actually a resistance complex requiring the concerted action of at least four genes at the RMRL (Wang *et al*., 2013). This research helped to elucidate the underlying molecular mechanisms determining compatibility and incompatibility and has helped lead to the understanding of important questions concerning rust resistance mechanisms in barley. However, we are still left to answer the fundamental questions of what genes are required for *rpg4*-mediated resistance against the wheat stem rust pathogens and what molecular markers can be used to select for *rpg4*-mediated resistance. Previous research analyzing *Rpg5* alleles from the single unimproved resistant barley line Q21861 and a limited number of susceptible genotypes suggested that a nonfunctional *rpg5* gene could be used to identify resistant or susceptible barley

genotypes containing what has been considered *rpg4*-mediated resistance against the wheat stem rust pathogen (Brueggeman *et al*., 2008; Wang *et al*., 2013). Here, we tested this hypothesis by analyzing alleles of the three genes recently identified as required for functional *rpg4*-mediated resistance from a total of 9 *P. graminis* f. sp. *tritici* race QCCJ-resistant (*rpg4*+) and 13 *P. graminis* f. sp. *tritici* race QCCJ-susceptible (*rpg4*–) barley genotypes representing a diverse group of cultivated and wild barley accessions from around the world (Table 1). The allele analysis confirmed that *Rpg5* is the polymorphic *R* gene at the *rpg4/Rpg5* locus and predicted that functional RPG5 proteins corresponded with *rpg4*-mediated wheat stem rust resistance. Thus, molecular markers developed based on polymorphisms in *rpg5* alleles predicted to encode nonfunctional proteins can be used to select for *rpg4/Rpg5*-mediated wheat stem rust resistance.

Table 1. Reaction of barley lines to *Puccinia graminis* f. sp. *secalis* isolate 92-MN-90 and *P. graminis* f. sp. *tritici* pathotypes QCCJ, HKHJ and MCCF.

				Mode Infection Type (General reaction)			
				Pgs isolate	*Pgt* pathotype		
				92-MN-90	QCCJ	HKHJ	MCCF
Line	Origin	R5 Group	Genotype	*Rpg5+*	*rpg4+*	*Rpg1+*	*Rpg1 a/o rpg4+*
				IT-M (GR)	IT-M (GR)	IT-M (GR)	IT-M (GR)
Q21861	Mexico	1 R	*Rpg1+, rpg4+/Rpg5+*	0;1 (R)	0;1 (R)	0;1 (R)	0;1 (R)
G.P.	Chester, England	1 S	*rpg1-, Rpg4-/rpg5-*	3 (S)	3 (S)	3 (S)	3 (S)
MD2	Manitoba, Canada	1 S	*rpg1-, Rpg4-/rpg5-*	3 (S)	3 (S)	3 (S)	3 (S)
Steptoe	Washington, USA	2 S	*rpg1-, Rpg4-/rpg5-*	3 (S)	3 (S)	3 (S)	3 (S)
Morex	Minnesota, USA	2 S	*Rpg1+, Rpg4-/rpg5-*	3 (S)	3 (S)	21 (MR)	12 (R)
Harrington	Saskatchewan, Canada	3 S	*rpg1-, Rpg4-/rpg5-*	3-2 (MS)	3-2 (MS)	3 (S)	3-2 (MS)
*Hv*489	Trans, Switzerland	4 S	*rpg1-, Rpg4-/rpg5-*	3 (S)	3 (S)	3 (S)	3 (S)
*Hv*492	Scheid, Switzerland	4 S	*rpg1-, Rpg4-/rpg5-*	3 (S)	3 (S)	3 (S)	3 (S)
Hv584	Fuormis, Switzerland	3 R	*rpg1-, rpg4+/Rpg5+*	0;1 (R)	0;1 (R)	3 (S)	0;1 (R)
Hv587	Platta, Switzerland	3 R	*rpg1-, rpg4+/Rpg5+*	0;1 (R)	12 (R)	3 (S)	12 (R)
Hv611	Plan, Switzerland	4 S	*rpg1-, Rpg4-/rpg5-*	3 (S)	3 (S)	3 (S)	3 (S)
Hv619	Fetan, Switzerland	3 R	*rpg1-, rpg4+/Rpg5+*	10; (R)	12 (R)	3 (S)	10; (R)
Hv645	Scuol, Switzerland	1 S	*rpg1-, Rpg4-/rpg5-*	3 (S)	3 (S)	3 (S)	3 (S)
Hv672	Surava, Switzerland	3 S	*Rpg1+, Rpg4-/rpg5-*	3 (S)	3 (S)	12 (R)	12 (R)
Damon 11-11	Isreal	2 R	*rpg1-, rpg4+/Rpg5+*	0;1 (R)	12 (R)	3 (S)	0;1 (R)
WBDC 019	West Azerbaijan, Iran	3 S	*rpg1-, Rpg4-/rpg5-*	3 (S)	3 (S)	3 (S)	3 (S)
WBDC 040	Haifa, Isreal	4 R	*rpg1-, rpg4+/Rpg5+*	0;1 (R)	0;1 (R)	3 (S)	12 (R)
WBDC 160	Sweida, Syria	4 S	*rpg1-, Rpg4-/rpg5-*	3 (S)	3 (S)	3 (S)	3 (S)
WBDC 214	Samarkand, Uzbekistan	4 R	*rpg1-, rpg4+/Rpg5+*	0;1 (R)	10; (R)	3 (S)	21 (MR)
WBDC 220	Chimkent, Kazakhstan	4 R	*rpg1-, rpg4+/Rpg5+*	10; (R)	10; (R)	3 (S)	12 (R)
WBDC 225	Dushanbe, Tajikistan	4 R	*rpg1-, rpg4+/Rpg5+*	0;1 (R)	12 (R)	3 (S)	12 (R)
WBDC 269	Lebanon	3 S	*rpg1-, Rpg4-/rpg5-*	3 (S)	3 (S)	3 (S)	3 (S)

IT-M is the mode infection type observed based on a 0-4 scale of Stakman et al. (1962). GR is the general reaction: R (resistant), MR (moderatly resistant), MS (moderatly susceptible) and S (susceptible). R5 Groups were assigned based on the genotype of the *Rpg5* alleles.

2.3. Materials and methods

2.3.1. Barley germplasm

A collection of six cultivated barley lines (*Hordeum vulgare*), eight Swiss landraces (*H. vulagare*), and eight wild barley accessions (*H. vulgare* subsp. *spontaneum*) were phenotyped and alleles of *Rpg5*, *HvRga1*, and *HvAdf3* were analyzed. All of the barley cultivars utilized in this study, with the exception of 'Golden Promise', were previously selected for the positional

cloning of the *Rpg5* gene and in the effort to identify the *rpg4* gene (Brueggeman *et al*., 2008; Wang *et al*., 2013). The *P. graminis* f. sp. *tritici* race QCCJ susceptible Golden Promise was included because it is the most efficiently transformed barley genotype (Horvath *et al*., 2003). The eight Swiss landraces and seven of the wild barley accessions were previously utilized to determine allele diversity of the stem rust *R* gene *Rpg1* (Mirlohi *et al*., 2008). The eight wild barley accessions evaluated in this study have differential reactions to the rust isolates used and were collected from diverse regions of the Fertile Crescent, the center of barley origin (Table 1).

2.3.2. Stem rust isolates and phenotyping

The barley lines used in this study were phenotyped for their infection types (ITs) to single-pustule isolates (QCC-2, A5-MCCF, and R29M) of three wheat stem rust races (*P. graminis* f. sp. *tritici* races QCCJ, MCCF, and HKHJ, respectively) and the rye stem rust isolate 92- MN-90. The race designations given to the *P. graminis* f. sp. *tritici* isolates used were designated by wheat differentials, as described by Roelfs *et al*. (Roelfs *et al*., 1988). The *rpg4*/*Rpg5* resistance complex confers recessive and temperature-dependent resistance to *P. graminis* f. sp. *tritici* race QCCJ and TTKSK. The *Rpg1* gene is ineffective against both *P. graminis* f. sp. *tritici* races QCCJ and TTKSK. The *Rpg1* gene and the *rpg4*/*Rpg5* complex confer dominant and recessive or temperature-sensitive resistance to *P. graminis* f. sp. *tritici* race MCCF, respectively. The *Rpg1* gene confers specific resistance to the isolate *P. graminis* f. sp. *Tritici* race HKHJ; thus, HKHJ is virulent on lines containing the *rpg4*/*Rpg5* locus. The *Rpg5* gene alone confers dominant resistance to the rye stem rust isolate 92-MN-90. The stem rust evaluations were done at North Dakota State University. Fresh urediniospores, increased on the universal susceptible barley ‘Steptoe’, were applied to 7- to 9-day-old seedlings after primary leaves were fully expanded using a spore-oil suspension atomizer. The inoculum, 10 mg of

urediniospores per 1 ml of Soltrol oil, was applied to the seedlings at a rate of ≈0.1 mg/seedling. The seedlings were incubated as previously described by Steffenson *et al*. (Steffenson *et al*., 2009), except that, after incubation in a mist chamber and drying, the plants were returned to a growth chamber set to a 14-h photoperiod and day and night temperatures of 22.5 ± 1 and 19 ± 1°C, respectively. Fourteen days after inoculation, the ITs were assessed using the 0-to-4 scale modified for barley from the scale developed for wheat by Stakman *et al*. (Stakman *et al*., 1962), as previously described by Sun and Steffenson (Sun *et al*., 2005).

2.3.3. Allele sequencing and analysis

Genomic DNA was isolated from young barley leaves (7- to 10-day-old seedlings) from a single plant of each of the 22 selected barley lines. The plants were grown in 6-in. plastic pots containing Sunshine brand under cool fluorescent lights with a 14-h photoperiod and day and night temperatures of 22.5 ±1 and 19 ± 1°C, respectively. All DNA was isolated according to Edwards *et al*. (Edwards *et al*., 1991). Pairs of overlapping primers specific to each gene required for *rpg4*-mediated resistance (*Rpg5*, *HvRga1*, and *HvAdf3*) were designed to produce ≈1-kb overlapping amplicons. The primer sequences, sizes of amplicons produced, and relative positions of the *Rpg5*, *HvRga1*, and *HvAdf3* primers are given in Table 2 and Figure 1B and C. The polymerase chain reaction (PCR) reactions were carried out on all 22 barley genotypes and analyzed using all 17 primer combinations (Tables 1 and 2). The 25-µl PCR reactions consisted of ≈100 ng of gDNA, 20 pmol of each forward and reverse primers, 1× Go Taq Flexi Buffer (Promega Corp., Madison, WI), 1 U of Go Taq Flexi DNA polymerase, and 0.2 mM dNTPs. Amplification was performed in a Mastercycler pro (Eppendorf, Hauppauge, NY) thermocycler using the following parameters: 95°C for 4 min; 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. Amplification of each reaction was confirmed by

loading 10 µl of each PCR reaction onto 1% agarose gels. The remaining 20 µl of each reaction was purified using cycle pure spin columns (Omega Bio-Tek, Norcross, GA). Direct sequencing was performed by Genscript (Piscataway, NJ) on an ABI 3730xl (Applied Biosystems, Carlsbad, CA) using the respective forward and reverse primers. Contigs were generated for each allele and allele alignment was performed using Vecor NTI software (Life Technologies, Grand Island, NY). The deduced amino acid sequences for each group of alleles identified are given in Figure 2.

Table 2. Primers used for *Rpg5*, *HvRga1*, and *HvAdf3* allele analysis.

Fragment	Primers	Primer sequences	size	location*
Rpg5-1	*Rpg5*-F1/*Rpg5*-R1	CCGCCTACCACACCTCCGATTCCAC/TCAGGTTTGATGGCTGTCTCTGGAG	1087	-303/785
Rpg5-2	*Rpg5*-F2/*Rpg5*-R2	TGTGCCGGATGCAGATCTGGACCTC/GAAGTACATTGTTCGCTCTCCTCAGA	1048	492/1539
Rpg5-3	*Rpg5*-F3/*Rpg5*-R3	CATCAATGTTCTAACATGGCGTATC/GGGACATTCTGATGCTTTCCGGATC	1143	1396/2538
Rpg5-4	*Rpg5*-F4/*Rpg5*-R4	GGTGTTGTCATGTACAGTTGATCAC/CTATCTGAGATCTAACCGTGTATTG	1258	2351/3584
Rpg5-5	*Rpg5*-F5/*Rpg5*-R5	AGATGCACCTATCTGCATCGAGCAC/ATGTCGAGCCTGAGACTACTGACAC	1016	3422/4438
Rpg5-6	*Rpg5*-F6/*Rpg5*-R6	CCCCATACTTCAAATTACACTTCGCTGCC/GCAACCTTCATTCTGACAGACCATG	1019	4234/5252
Rpg5-7	*Rpg5*-F7/*Rpg5*-R7	CTATTCAGAAGTATGCACTGACCAG/GCACCGCAACTCCTGTGTGTTCGAC	1136	5023/6182
Rpg5-8	*Rpg5*-F8/*Rpg5*-R8	ACGCCTCCGAGGAGGAGGTTTACAG/GCTTTGGTTCAGCCGAACAGACGAG	985	5993/6977
Rpg5-9	*Rpg5*-F9/*Rpg5*-R9	GCTTGGGGATGCAAGCATAGAGCTG/GAGTGATGTGTATAGCTTTGGCGTG	1001	6775/7775
Rpg5-10	*Rpg5*-F10/*Rpg5*-R10	TGCATCTATCTGCTCATGCAAGGAG/AACAATATTCACCTGCGGCACCAAC	1082	7302/8383
*Hv*Rga1-1	*Hv*Rga1-F1/*Hv*Rga1-R1	TCAACCGCTTTCGCTCATATGCATG/GAAGTTGCTCTTCCGGATCCTGATC	740	-323/418
*Hv*Rga1-2	*Hv*Rga1-F2/*Hv*Rga1-R2	TTGGCCGCTCGAATGGCAGCAGATC/CCCGTGAATGAATGTGCGCAAATAG	1063	146/1208
*Hv*Rga1-3	*Hv*Rga1-F3/*Hv*Rga1-R3	GTAGTCATGATAAGATGGTGC/TTGCAATCCTACTAGTCCATACGA	939	949/1887
*Hv*Rga1-4	*Hv*Rga1-F4/*Hv*Rga1-R4	GATGGAATGTGGTCTAGGACTGTAG/TCTCTTTGCCTGGCAGATTCTTCTC	1086	1715/2800
*Hv*Rga1-5	*Hv*Rga1-F5/*Hv*Rga1-R5	GAATCCCCTGATAAGGCGATGGCTG/CTGCGTCAGCTTGCCTCTCAACTTG	1027	2526/3552
*Hv*Rga1-6	*Hv*Rga1-F6/*Hv*Rga1-R6	GCTACTTGAAGGTGACTGCACACAG/AACAGGTTTGCACTGCAGACAGTAG	1061	3189/4249
*Hv*Adf3-Q1	*Hv*Adf3-F1/*Hv*Adf3-QR1	ATCATACGAGCAGCTCCTGTC/CGATCCCATGCGCAGGTATGCCAAG	660	-80/581
*Hv*Adf3-s1	*Hv*Adf3-F1/*Hv*Adf3-MR1	ATCATACGAGCAGCTCCTGTC/AACCAAGCACTCAATTCTCGAAGAG	718	# -80/639

* location of amplicons is given as position of the 5' terminal nucleotide from the forward and reverse primers in relation to the ATG start codon with A being position 1 from the Q21861 sequence.

location of amplicons is given as position of the 5' terminal nucleotide from the forward and reverse primers in relation to the ATG start codon with A being position 1 from the Morex sequence.

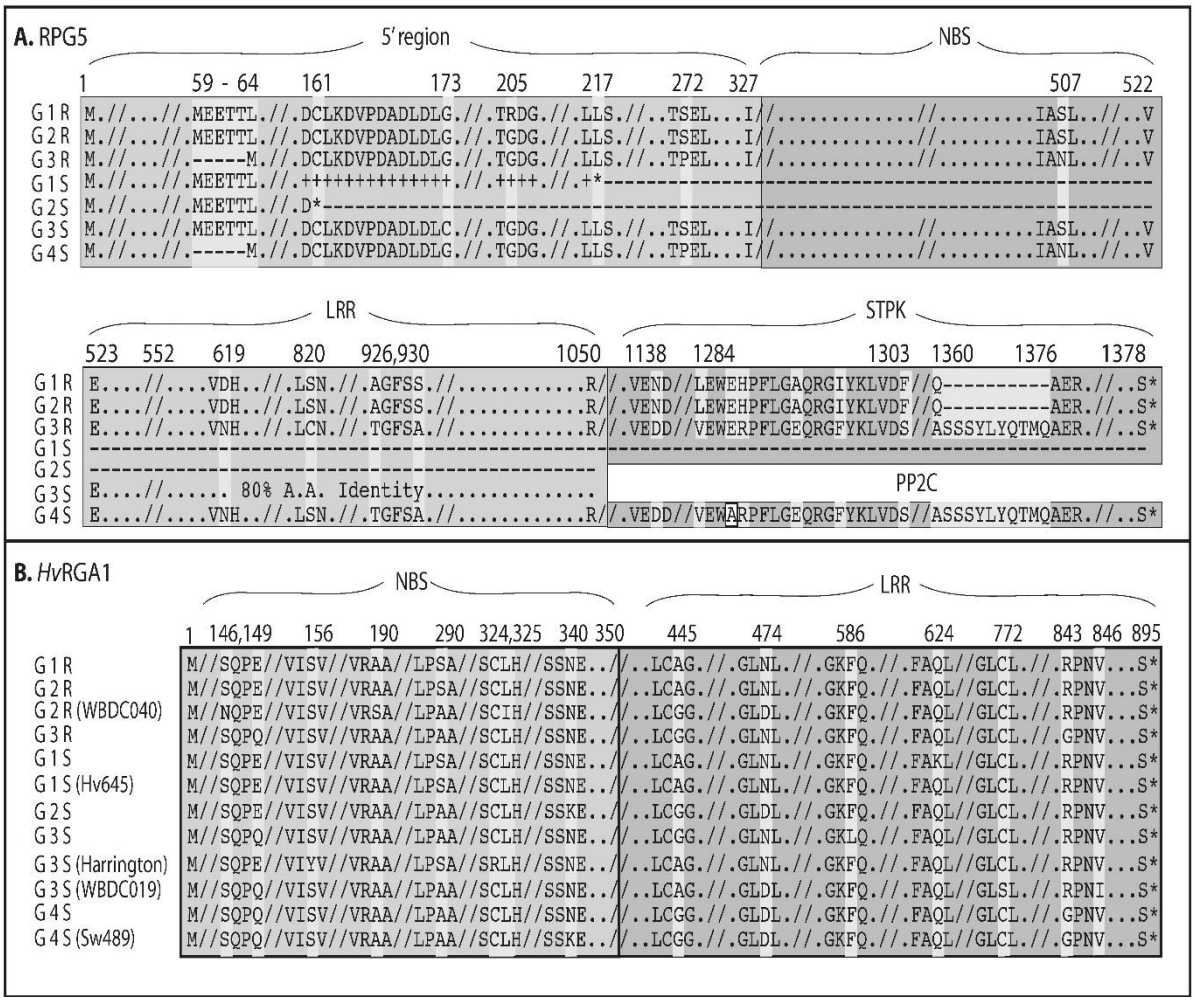

Figure 2. Protein domain structures and amino acid alignment for the *Rpg5*, *HvRga1*, and *HvAdf3* alleles. Boxes represent the protein domains with amino acid positions and domain labels shown above. Barley lines were grouped based on their RPG5 amino acid sequences and general reaction types with R = resistant and S = susceptible. Differences in amino acid sequences compared with group 1 resistant (G1R) are highlighted with light gray vertical bars. Barley lines contained in each group are as follows: GR1 (Q21861); GR2 (Damon 11-11, WBDC040, WBDC214, WBDC220, and WBDC225); GR3 (Hv584, Hv587, and Hv619); G1S (Golden Promise, MD2, and Hv645); G2S (Steptoe and Morex,); G3S (Harrington, Hv672, WBDC019, and WBDC269); and G4S (Hv489, Hv492, Hv611, and WBDC160).

2.3.4. RNA extraction

RNA was extracted from leaf tissue collected at the second-leaf stage from three plants for each of the 22 selected barley lines. The plants were grown in 6-in. plastic pots containing Sunshine brand potting mix 1 supplemented with Osmocote 14-14-14 at 14.3 g/pot in a

temperature-controlled green-house with a 14-h photoperiod and day and night temperatures of 22 and 18°C, respectively. The RNA was extracted using a Trizol mini method modified from Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Secondary seedling leaf tissue (≈4 cm) was ground in 1.5-ml microcentrifuge tubes with 1 ml of Trizol reagent (prewarmed at 65°C) using disposable microfuge pestles. After complete maceration of leaf tissue, the sample was placed in a 65°C water bath for 10 min. The homogenized mixture was centrifuged at 9,000 rpm for 10 min to pellet the cellular debris and ≈1 ml of the supernatant was transferred to a new 1.5-ml microcentrifuge tube. Chloroform (400 µl) was added, the solution was vortexed for 5 s, and the samples were left at room temperature for 5 min prior to 15 min of centrifugation at 9,000 rpm in a 4°C bench-top centrifuge. Supernatant (≈400 µl) was transferred to a new 1.5-ml microcentrifuge tube and 100 µl of isopropanol and 100 µl of a 0.8 M sodium citrate + 1.2 M sodium chloride solution was added. The mixture was homogenized by gentle inversion and centrifuged at 13,000 rpms for 15 min at 4°C. The pelleted RNA was gently washed with ice-cold 75% ethanol and left to dry for 10 min after pipetting out the excess ethanol. The RNA was then resuspended in 30 µl of ultrapure water containing 0.003% RNAsin. The integrity of the RNA was determined by the visualization of four ribosomal RNA bands after separation on a 0.5× Tris-borate- EDTA, 1% agarose gel.

Table 3. Primers used for expression analysis of the *Rpg5*, *HvRga1*, and *HvAdf3* alleles.

Gene	Primers	Primer sequences	Amplicon size	location*
Rpg5	QST Kin F1/*Rpg5 R7.3*	AAAATGCGCCACACGGAACTTCA/GCAACCTTCATTCTGACAGACCATG	354/748	4505/5252
*Hv*Rga1	R4-F2/R4-R4	TTGGCCGCTCGAATGGCAGCAGATC/TCTCTTTGCCTGGCAGATTCTTCTC	1227/2655	146/2800
*Hv*Adf3	ADF3 F2/ADF3 R3	CCAACCTCCGGATCCATCAACAATG/GTCGTCCATCTTGTACACCACGAAC	117/356	-22/334

Amplicon sizes are given for cDNA/gDNA from the Q21861 sequence. The location of amplicons is given as position of the 5′ terminal nucleotide from the forward and reverse primers in relation to the ATG start codon, with A being position 1 from the Q21861 gDNA sequence.

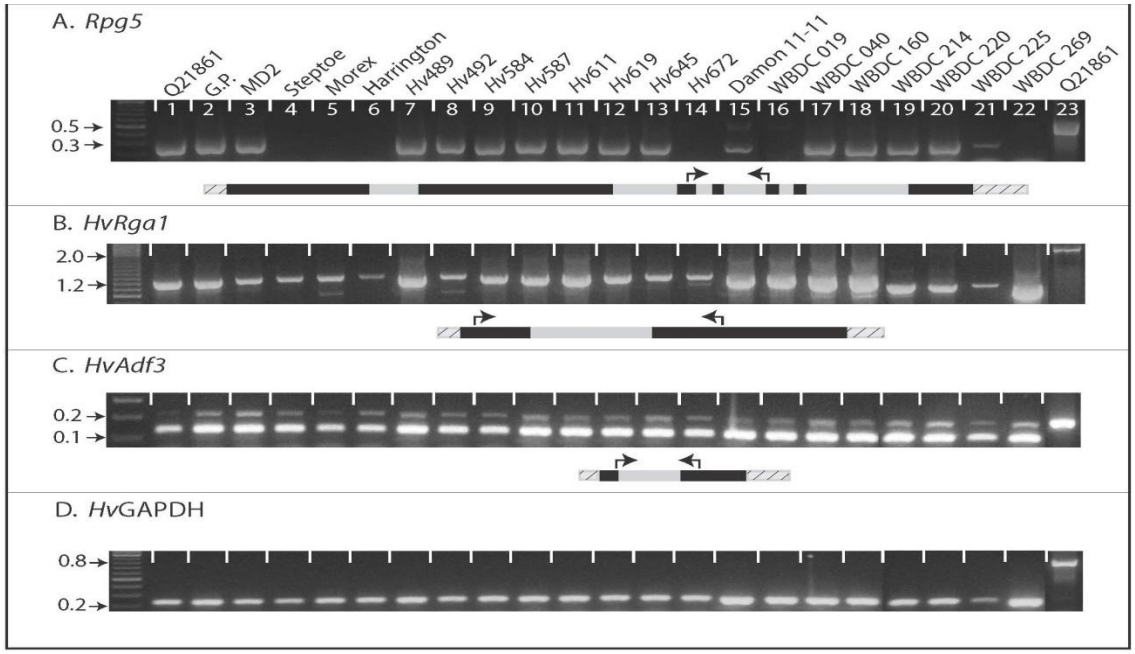

Figure 3. Expression analysis of the *Rpg5*, *HvRga1,* and *HvAdf3* alleles. Lines analyzed are labeled at the top. All lanes used cDNA template, except the last lane is Q21861 genomic DNA and the first lane is a 100-bp molecular marker. Horizontal bars below the gel images represent the gene structure for each gene analyzed; black represents exons, and gray introns and dashed lines are the 5′ and 3′ prime untranslated regions, respectively. Numbered arrows to the left indicate the approximate size of cDNA and genomic DNA amplicons. **A**, *Rpg5* analysis; **B**, *HvRga1* analysis; **C**, *HvAdf3* analysis; and D, *Hv*GAPDH housekeeping gene.

2.3.5. Reverse transcription and reverse-transcription PCR

RNA obtained from seedling tissue of each line was converted into cDNA using the Promega GoScript reverse-transcription (RT) system following the manufacturer's standard procedure. RNA (≈0.5 µg) was added to RT mix for each genotype, normalized based on the intensity of the bands visualized on the agarose gel, and checked using a Qubit Flourometer. The 20-µl RT reactions were diluted 1:1 with ultrapure H2O and RT-PCR was set up using exon-specific primer pairs that amplified across at least 1 intron from each of the genes required for *rpg4*-mediated resistance (*Rpg5*, *HvRga1*, and *HvAdf3*). The 25-µl RT-PCR reaction mix was the same as described for genomic DNA, except that 2 µl of the diluted cDNA was used as the template. Amplification was performed in a Mastercycler pro (Eppendorf) thermocycler using

the following parameters: 95°C for 1 min; 35 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 30 s; followed by 72°C for 5 min for GAPDH, *Rpg5*, and *HvAdf3* amplification. To amplify the *HvRga1*-specific cDNA fragment, 33 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min were used, keeping the other conditions the same. The resistant barley line Q21861 was used as the genomic control for each amplicon. The RT reactions were visually normalized using primers specific to the GAPDH housekeeping gene. The *Hv*GAPDH primers used were previously reported by Horvath *et al*. (Horvath *et al*., 2003). The primer sequences, amplicon sizes, and positions of the genes on the Q21861 genomic template are given in Table 3 and Figure 3.

2.4. Results

2.4.1. Stem rust phenotyping

The *P. graminis* f. sp. *tritici* race QCCJ susceptible and resistant control genotypes utilized in these experiments reacted as expected, with the resistant line Q21861 (*rpg4+*, *Rpg5+*) exhibiting resistant ITs and the susceptible controls Steptoe, 'Morex', 'Harrington', Golden Promise, and line MD2 (*rpg4–*, *rpg5–*) exhibiting susceptible ITs (Table 1). The Swiss landraces *Hv*584, *Hv*587, and *Hv*619 showed *P. graminis* f. sp. *tritici* race QCCJ-resistant ITs, with medians ranging from 0;1 to 12. The Swiss landraces *Hv*489, *Hv*492, *Hv*611, *Hv*645, and *Hv*672 exhibited susceptible *P. graminis* f. sp. *tritici* race QCCJ phenotypes, with the median IT for all being 3. The wild barley accessions Damon 11-11, WBDC040, WBDC214, WBDC220, and WBDC225 showed *P. graminis* f. sp. *tritici* race QCCJ resistance, with the median ITs ranging from 0;1 to 12. The wild barley accessions WBDC019, WBDC160, and WBDC269 showed *P. graminis* f. sp. *tritici* race QCCJ susceptibility, with median ITs of 3. The *P. graminis* f. sp. *tritici* race QCCJ-resistant Swiss landraces and wild barley accessions were previously identified as

resistant to *P. graminis* f. sp. *tritici* race MCCF, with the exception of Damon 11-11, which was not included in the previous study. However, only one genotype contained an intact *Rpg1* gene and was resistant to the *Rpg1*-avirulent *P. graminis* f. sp. *tritici* race HKHJ, suggesting that the race MCCF resistance in the majority of the resistant accessions was conferred by a second source of wheat stem rust resistance, presumed to be the *rpg4* gene (Mirlohi *et al*., 2008). The wheat stem rust *P. graminis* f. sp. *tritici* race MCCF phenotyping data (Table 1) were consistent with the general reaction designations of lines utilized in previous studies (Brueggeman *et al*., 2002; Mirlohi *et al*., 2008).

The *P. graminis* f. sp. *secalis* isolate 92-MN-90 stem rust phenotyping data on all the susceptible and resistant control lines reacted as expected, with the resistant line Q21861 (*Rpg5*+), exhibiting the resistant IT 0;1 and the susceptible Steptoe, Morex, Harrington, Golden Promise, and experimental line MD2 (all *rpg5*–) exhibiting susceptible ITs, with the median IT ranging from 3-2 to 3 (Table 1). These data were similar to the phenotyping data previously reported for these lines (Brueggeman *et al*., 2008). The general reactions for *P. graminis* f. sp. *secalis* isolate 92-MN-90 were very similar to those of *P. graminis* f. sp. *tritici* race QCCJ, suggesting that resistance to both wheat and rye stem rust pathogens is conferred by a functional *rpg4*/*Rpg5* resistance locus. The *H. spontaneum* line Damon 11-11 exhibited a resistant IT, suggesting that Damon 11-11 contains a functional *rpg4*/*Rpg5* resistance complex.

2.4.2. Allele sequencing and analysis

Allele analysis of the *HvAdf3* gene showed a very high level of gene conservation, with only two synonymous nucleotide substitutions identified in the 22 barley genotypes examined. The barley landrace *Hv*672 had a single-nucleotide substitution, G233C, that resulted in no change to the amino acid sequence. The *H. spontaneum* line WBDC019 also contained a single

synonymous nucleotide substitution, C245T, that resulted in no change in the amino acid sequence. The 100% conservation of the *Hv*ADF3 protein suggests that no functional polymorphism exists between the genotypes examined; thus, differences at *HvAdf3* did not contribute to the compatible or incompatible interaction with the wheat stem rust pathogen in the genotypes tested.

The allele analysis of the *HvRga1* gene determined that this NBS-LRR domain gene was predicted to encode a full-length 895-amino-acid resistance-like protein from all the alleles examined. There were eight amino acid sequence differences among the nine *P. graminis* f. sp. *tritici* race QCCJ-resistant alleles examined. Compared with the Q21861-resistant allele, considered a group 1 resistant genotype, the S146N, E149Q, A190S, S290A, L325I, A445G, N474D, and R843G amino acid substitutions (Fig. 2) were present in *P. graminis* f. sp. *tritici* race QCCJ-resistant genotypes and, therefore, considered not to effect the incompatible (resistant) interaction with the pathogen. The group 1 susceptible lines MD2 and Golden Promise contained one amino acid substitution, Q624K, that was unique to these susceptible genotypes. The group 3 susceptible Harrington contained the substitutions S156Y and C324R that were unique to this susceptible cultivar. The group 3 susceptible accession WBDC019 contained the two substitutions C772S and V846I, unique to this wild barley accession. Although these differences could result in nonfunctional *Hv*RGA1 proteins, these alleles were present with predicted nonfunctional *rpg5* alleles containing mutations that resulted in truncated proteins. Thus, the compatible interaction with *P. graminis* f. sp. *tritici* race QCCJ in these susceptible barley genotypes was probably due to the nonfunctional *rpg5* alleles.

The *Rpg5* allele analysis determined that all of the resistant genotypes contained predicted functional RPG5 proteins; however, there was significant allele diversity that provides

some hint to regions important for stem rust resistance function. The barley line Q21861, the original source of *rpg4* and the variety utilized in the cloning of the *Rpg5* gene, was given the designation group 1 resistant (Fig. 2; Table 1). The *Rpg5* allele analysis determined that the four *H. spontaneum* wild barley accessions WBDC040, WBDC214, WBDC225, and Damon 11-11 contained *Rpg5* alleles that only differed from the Q21861 allele by the single amino acid substitution R205G (Fig. 2) and were given the designation group 2 resistant (Table 1). The genotypes containing the group 3 resistant alleles, including *Hv*584, *Hv*587, and *Hv*619, had the greatest *Rpg5* diversity for an allele encoding a functional *P. graminis* f. sp. *tritici* race QCCJ and *P. graminis* f. sp. *Secalis* isolate 92-MN-90 stem rust *R* gene. The diversity of the group 3 allele compared with the Q21861 allele includes a 5-amino-acid deletion at the N terminus (amino acids 59 to 63) and a 10-aminoacid insertion at the C terminus (amino acids 1,367 to 1,376). The group 3 resistant alleles also contained single-nucleotide substitutions that introduced 15 amino acid substitutions (L64M, R205G, S272P, S507N, D619N, S820C, A926T, S930A, N1138D, L1284V, H1288R, A1293E, I1297F, F1303S, and Q1366A) (Fig. 2).
The group 1 susceptible genotypes (MD2, Golden Promise, and *Hv*645) contain a single cytosine insertion within the first exon, resulting in a frame shift and a predicted truncated RPG5 protein that results in a stop codon at amino acid position 217 (Fig. 2).

The group 2 and group 3 susceptible genotypes (Harrington, Morex, Steptoe, *Hv*672, WBDC019, and WBDC269) code for a predicted nonfunctional RPG5 protein due to the insertion or deletion event leading to the deletion of the protein kinase domain and insertion of the *HvPP2C.1* gene (Fig. 2) (Brueggeman *et al*., 2008; Wang *et al*., 2013).

The group 4 susceptible genotypes (*Hv*489, *Hv*492, *Hv*611, and WBDC160) code for a predicted full-length RPG5 protein that was identical to the group 3 resistance allele, except for

the two amino acid differences S820C and E1287A. The Q21861 (group 1 resistant) allele contained the serine residue at position 820; thus, this amino acid substitution probably does not contribute to a nonfunctional RPG5 protein. However, the alanine at position 1,287 is unique to this susceptible allele and is considered the polymorphism that is responsible for the compatible interaction with the wheat stem rust pathogen. It must be further explained that the *HvRga1* allele in the group 4 susceptible genotypes are identical to the group 3 resistant *HvRga1* alleles, except for the aspartic acid residue at position 474. However, the WBDC040 group 2 resistant accession contains aspartic acid at position 474 (Fig. 2); thus, this difference in the *HvRga1* allele probably does not contribute to susceptibility in the group 4 susceptible genotypes.

2.4.3. Expression analysis

RT-PCR was run for all the genotypeswith primer pairs specific to each gene. The housekeeping gene GAPDH was amplified from all 22 cDNAs and Q21861 genomic DNA to determine the integrity of the cDNA samples (Fig. 3). The *Hv*GAPDH primers amplified the ≈250-bp cDNA amplicon, showing that all 22 samples contained GAPDH expressed at the mRNA level. The RT-PCR reactions using the *HvAdf3*-specific and *HvRga1*-specific primers amplified a 117-bp band for the *HvAdf3* gene and a 1,227-bp band for the *HvRga1* gene. The sizes correspond to the expected amplicon sizes, showing that these are amplified from cDNA with introns spliced out. The *HvAdf3* cDNA produced a weak band the size of the genomic amplicon (356 bp) that is due to either nonspliced mRNA or genomic contamination. However, the mRNA was treated with DNAase prior to cDNA synthesis and a genomic band did not amplify from the other primer combinations, suggesting that it may be the product of an unspliced or alternate splice form. The amplification, although a bit variable, determined that both *HvAdf3* and *HvRga1* genes are expressed at the transcript level from all 22 genotypes

analyzed. The *Rpg5*-specific amplification showed expression in all the resistant genotypes. Some of the susceptible genotypes (i.e., those with an intact STPK domain and a stop codon at amino acid position 217) and the group 4 susceptible lines with a full-length *rpg5* allele were expressed at the transcript level. Thus, all genotypes except the group 2 susceptible genotypes (Steptoe, Morex, Harrington, *Hv*672, and wild barley accessions WBDC019 and WBDC269) produced the expected 354-bp cDNA band corresponding to the LRR to STPK junction, with introns three and four properly spliced. The results show that the *rpg5*-susceptible alleles containing the STPK domain are expressed at the transcript level but are predicted to code for truncated proteins and the susceptible alleles missing the STPK domain do not have *Rpg5* STPK region transcripts as expected. All the RT-PCR reactions were run with a Q21861 genomic control to show the size of the amplicons without the introns spliced out. Using the Q21861 genomic DNA as the template in each reaction produced amplicon of 748, 2,655, and 356 bp for *Rpg5*, *HvRga1*, and *HvAdf3*, respectively (Fig. 3).

2.5. Discussion

The Upper Midwest region of the United States and the central Prairie Provinces of Canada comprise the largest barley production region in North America. Currently, the only known stem rust *R* gene deployed in barley cultivars grown in the region is the single *Rpg1* gene. This gene has conferred remarkably durable resistance, considering the vast acreage on which varieties containing *Rpg1* have been grown and the disease pressure exerted; however it is ineffective against *P. graminis* f. sp. *Tritici* race QCCJ and, more importantly, race TTKSK and its lineage. This leaves barley production in North America vulnerable to stem rust epidemics should *P. graminis* f. sp. *tritici* race TTKSK be introduced.

The only known source of resistance effective against *P. graminis* f. sp. *tritici* race TTKSK in barley is the *rpg4* gene, warranting further research to understand this complex resistance mechanism. Recent research identified three genes at the RMRL that are required for wheat stem rust race QCCJ resistance and, presumably, TTKSK resistance. This conclusion is supported by the observation that all recombinants used to genetically dissect the *rpg4/Rpg5* locus behave similarly to both races (Brueggeman *et al*., 2008; Wang *et al*., 2013). Because resistance to *P. graminis* f. sp. *tritici* races QCCJ and TTKSK conferred at the *rpg4*/*Rpg5* locus has been shown to be genetically similar (Wang *et al*., 2013), QCCJ was used as a TTKSK surrogate in this study due to the restrictions on the use of TTKSK in North America.

The recent identification of the three genes at RMRL required for resistance (Wang *et al*., 2013) prompted our research to determine whether *P. graminis* f. sp. *secalis* and *P. graminis* f. sp. *tritici* resistance or susceptibility correlated with functional or nonfunctional *Rpg5, HvRga1*, or *HvAdf3* genes. To test the hypothesis that polymorphisms in one of these three genes should determine compatibility or incompatibility with the pathogen, we sequenced alleles from a diverse collection of cultivated and wild barley accessions. A comprehensive evaluation of a germplasm collection of 74 barley landraces from Switzerland on the reaction type to diverse pathotypes of *P. graminis* f. sp. *tritici* and isolates of *P. graminis* f. sp. *secalis* revealed that three lines designated *Hv*584, *Hv*587, and *Hv*619 were resistant to *P. graminis* f. sp. *secalis* isolate 92-MN-90 and *P. graminis* f. sp. *tritici* races QCCJ and TTKSK, presumed to be conferred by functional *rpg4*/*Rpg5* resistance alleles (Mirlohi *et al*., 2008; Steffenson *et al*., 2007; Steffenson *et al* 2012). It was also determined that the lines were resistant to the wheat stem rust race MCCF but did not have a functional *Rpg1* gene. Thus, it was proposed that a functional *rpg4* gene conditioned the *P. graminis* f. sp. *tritici* race MCCF resistance as well (Mirlohi *et al*., 2008).

Recent allelism tests between Q21861 and *Hv*584 determined that the two lines contain alleles of *rpg4/Rpg5* (R. Brueggeman, *unpublished data*). Therefore, we included the set of Swiss landraces and wild barley accessions that were previously characterized for *Rpg1* allele diversity (Mirlohi *et al*., 2008) in our set of genotypes used for *Rpg5*, *HvRga1*, and *HvAdf3* allele analysis. The wild barley accession Damon 11-11 was also included because it contains resistance to *P. graminis* f. sp. *secalis* isolate 92-MN-90 and *P. graminis* f. sp. *tritici* races MCCF and QCCJ that was previously mapped to the *rpg4*/*Rpg5* locus (Alsop *et al*., 2008).

All the barley genotypes containing resistance to *P. graminis* f. sp. *tritici* race QCCJ contain a predicted functional *Rpg5* allele and most of the susceptible genotypes examined contain a predicted truncated nonfunctional *rpg5* allele (Table 1; Fig. 2). The exceptions are the susceptible *H. vulgare* landraces *Hv*489, *Hv*492, and *Hv*611 and the *H. spontaneum* line WBDC160, which are predicted to encode full-length RPG5 proteins. Interestingly, these alleles contain a non-synonymous nucleotide substitution that results in the amino acid substitution E1287A that is unique to these four susceptible genotypes compared with all the resistant alleles. All resistant alleles contain glutamate at position 1,287, which is frequently an amino acid involved in protein active or binding sites. It is quite possible that the substitution with the nonreactive alanine residue, rarely involved in protein function, results in the loss of stem rust function by causing a loss of protein–protein interaction or activity. The group 4 susceptible genotypes also contain *HvRga1* and *HvAdf3* alleles with amino acid differences compared with the Q21861 allele but that are shared with other resistant genotypes, suggesting that susceptibility is not due to nonfunctional *HvRga1* or *HvAdf3* alleles. Thus, we have identified a new susceptible *Rpg5* allele that can be attributed to this single amino acid difference. It appears

that this amino acid in the C-terminal region is important for stem rust resistance function and will be a target for future functional analysis of the RPG5 protein.

The data presented here suggest that the rye stem rust *R* gene *Rpg5*, previously considered distinct from *rpg4*, is the polymorphic *R* gene required for *rpg4*- and *Rpg5*-mediated resistance, and the polymorphisms in the *Rpg5* gene could provide perfectly linked markers to determine whether barley lines or cultivars contain *rpg4*-mediated resistance to the wheat stem rust pathogens. These markers could also be utilized to screen barley lines to determine whether newly identified sources of TTKSK resistance are novel. Although the rye and wheat stem rust resistance mechanisms rely on the same *R* gene component, the requirement of the *Rme1* gene and the recessive nature of the wheat stem rust resistance versus the dominant nature of the rye stem rust resistance suggests that the two stem rust pathogens interact differently with the *Rpg5* gene and function via different resistance mechanisms.

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2.7. Literature cited

Alsop, B. P., Kilian, A., Carling, J., Pickering, R. A., and Steffenson, B. J. 2008. Comparison of DArT marker-based linkage analysis and multiple disease resistance QTL in two wild × cultivated barley populations. Plant & Animal Genomes XVI Conference P322. http://www.intl-pag.org/ 16/abstracts/PAG16_P05c_322.html

Ashikawa, I., Hayashi, N., Yamane, H., Kanamori, H., Wu, J., Matsumoto, T., Ono, K., and Yano, M. 2008. Two adjacent nucleotide-binding site– leucine-rich repeat class genes are required to confer Pikm-specific rice blast resistance. Genetics 180:2267-2276.

Birker, D., Heidrich, K., Takahara, H., Narusaka, M., Deslandes, L., Narusaka, Y., Reymond, M., Parker, J. E., and O'Connell, R. 2009. A locus conferring resistance to *Colletotrichum*

higginsianum is shared by four geographically distinct *Arabidopsis* accessions. Plant J. 60:602-613.

Brueggeman, R., Druka, A., Nirmala, J., Cavileer, T., Drader, T., Rostoks, N., Mirlohi, A., Bennypaul, H., Gill, U., Kudrna, D., Whitelaw, C., Kilian, A., Han, F., Sun, Y., Gill, K., Steffenson, B., and Kleinhofs, A. 2008. The stem rust resistance gene *Rpg5* encodes a novel protein with nucleotide binding site, leucine-rich and protein kinase domains. Proc. Natl. Acad. Sci. USA 105:14970-14975.

Brueggeman, R., Rostoks, N., Kudrna, D., Kilian, A., Han, F., Chen, J., Druka, A., Steffenson, B., and Kleinhofs, A. 2002. The barley stem rustresistance gene *Rpg1* is a novel disease-resistance gene with homology to receptor kinases. Proc. Natl. Acad. Sci. USA 99:9328-9333.

Brueggeman, R., Steffenson, B. J., and Kleinhofs, A. 2009. The *rpg4/ Rpg5* stem rust resistance locus in barley; resistance genes and cytoskeleton dynamics. Cell Cycle 8:977-981.

Chen, Y., Cheung, A. Y., and Wu, H. M. 2003. Actin-depolymerizing factor mediates Rac/Rop GTPase-regulated pollen tube growth. Plant Cell 15:237-249.

Chomczynski, P. and Sacchi, N. 1987. Single-step method of RNA isolation by acid Guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156.

Edwards, K., Johnstone, C., and Thompson, C. 1991. A simple and rapid method for preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. 19:1351.

Feng, Y., Liu, Q., and Xue, Q. 2006. Comparative study of rice and *Arabidopsis* actin-depolymerizing factors gene families. J. Plant Physiol. 163:69-79.

Horvath, H., Rostoks, N., Brueggeman, R., Steffenson, B., von Wettstein, D., and Kleinhofs, A. 2003. Genetically engineered stem rust resistance in barley using the *Rpg1* gene. Proc. Natl. Acad. Sci. USA 100:364-369.

Lee, S.-K., Song, M.-Y., Seo, Y.-S., Kim, H.-K., Ko, S., Cao, P.-J., Suh, J.- P., Yi, G., Roh, J.-H., Lee, S., An, G., Hahn, T.-R., Wang, G.-L., Ronald, P., and Jeon, J.-S. 2009. Rice Pi5-mediated resistance to *Magnaporthe oryzae* requires the presence of two coiled-coil–nucleotide-binding– leucine-rich repeat genes. Genetics 181:1627-1638.

Loutre, C., Wicker, T., Travella, S., Galli, P., Scofield, S., Fahima, T., Feuillet, C., and Keller, B. 2009. Two different CC-NBS-LRR genes are required for Lr10-mediated leaf rust resistance in tetraploid and hexaploid wheat. Plant J. 60:1043-1054.

Miklis, M., Consonni, C., Bhat, R. A., Lipka, V., Schulze-Lefert, P., and Panstruga, R. 2007. Barley *MLO* modulates actin-dependent and actinindependent antifungal defense pathways at the cell periphery. Plant Physiol. 144:1132-1143.

Mirlohi, A., Brueggeman, R., Drader, T., Nirmala, J., Stephenson, B. J., and Kleinhofs, A. 2008. Allele sequencing of the barley stem rust resistance gene *Rpg1* identifies regions relevant to disease resistance. Phytopathology 98:910-918.

Narusaka, M., Shirasu, K., Noutoshi, Y., Kubo, Y., Shiraishi, T., Iwabuchi, M., and Narusaka, Y. 2009. RRS1 and RPS4 provide a dual resistance gene system against fungal and bacterial pathogens. Plant J. 60:218-226.

Okuyama, Y., Kanzaki, H., Abe, A., Yoshida, K., Tamiru, M., Saitoh, H., Fujibe, T., Matsumura, H., Shenton, M., Galam, D. C., Undan, J., Ito, A., Sone, T., and Terauchi, R. 2011. A multi-faceted genomics approach allows the isolation of rice *Pia*-blast resistance gene consisting of two adjacent NBS-LRR protein genes. Plant J. 66:467-479.

Ouellet, F., Carpentier, E., Cope, J., Monroy, A. F., and Sarhan, F. 2001. Regulation of a wheat actin-depolymerizing factor during cold acclimation. Plant Physiol. 125:360-368.

Peart J. R., Mestre, P., Lu, R., Malcuit, I., and Baulcombe, D. C. 2005. NRG1, a CC-NB-LRR protein, together with N, a TIR-NB-LRR protein, mediates resistance against *Tobacco mosaic virus*. Curr. Biol. 15:968-973.

Roelfs, A. P. and Martens, J. W. 1988. An international system of nomenclature for *Puccinia graminis* f. sp. *tritici*. Phytopathology 78:526- 533.

Sinapidou, E., Williams, K., Nott, L., Bahky, S., Tor, M., Bittner-Eddy, P., and Beynon, J. 2004. Two TIR:NB:LRR genes are required to specify resistance to *Peronospora parasitica* isolate Cala2 in *Arabidopsis*. Plant J. 38:898-909.

Stakman, E. C., Stewart, D. M., and Loegering, W. Q. 1962. Identification of physiological races of *Puccinia graminis* f. sp. *tritici*. U.S. Dep. Agric. Agric. Res. Serv. Publ. No. E617.
Steffenson, B. J. 1992. Analysis of durable resistance to stem rust in barley. Euphytica 63:153-167.
Steffenson, B. J., Jin, Y., Brueggeman, R. S., Kleinhofs, A., and Sun, Y. 2009. Resistance to stem rust race TTKSK maps to the *rpg4*/*Rpg5* complex of chromosome 5H of barley. Phytopathology 99:1135-1141.

Steffenson, B. J., Olivera, P., Roy, J. A., Jin, Y., Smith, K. P., and Muehlbauer, G. J. 2007. A walk on the wild side: Mining wild wheat and barley collections for rust resistance genes. Aust. J. Agric. Res. 58:532- 544.

Steffenson, B. J., Zhou, H., Chai, Y., and Grando, S. 2012. Vulnerability of cultivated and wild barley to African stem rust race TTKSK. Pages 149-155 in: Advances in Barley Science. Proc. 11th Int. Barley Genet. Symp. C. Li, G. Zhang, and X. Liu, eds. Zhejiang University Press, Hangzhou, China.

Stokstad, E. 2007. Deadly wheat fungus threatens world's breadbaskets.Science 315:1786-1787.

Sun, Y., and Steffenson, B. J. 2005. Reaction of barley seedlings with different stem rust resistance genes to *Puccinia graminis* f. sp. *tritici* and *Puccinia graminis* f. sp. *secalis*. Can. J. Plant Pathol. 27:80-89.

Thomas, P. and Schiefelbein, J. 2002. Cloning and characterization of an actin depolymerizing factor gene from grape (*Vitis vinifera* L.) expressed during rooting in stem cuttings. Plant Sci. 162:283-288.

Tian, M., Chaudhry, F., Ruzicka, D. R., Meagher, R. B., Staiger, C. J., and Day, B. 2009. *Arabidopsis* actin-depolymerizing factor *AtADF4* mediates defense signal transduction triggered by the *Pseudomonas syringae* effector *AvrPphB*. Plant Physiol. 150:815-824.

Wang, X., Richards, J., Gross, T., Druka, A., Kleinhofs, A., Steffenson, B., Acevedo, M., and Brueggeman, R. 2013. The *rpg4*-mediated resistance to wheat stem rust (*Puccinia graminis*) in barley (*Hordeum vulgare*) requires *Rpg5*, a second NBS-LRR gene and an actin depolymerization factor. Mol. Plant-Microbe Interaction 26: 407-418.

Yuan, B., Zhai, C., Wang, W., Zeng, X., Xu, X., Hu, H., Lin, F., Wang, L., and Pan, Q. 2011. The Pik-p resistance to *Magnaporthe oryzae* in rice is mediated by a pair of closely linked CC-NBS-LRR genes. Theor. Appl. Genet. 122:1017-1028.

3. CHAPTER TWO: A PROTEIN PHOSPHATASE 2C GENE AT THE *RPG4*-MEDIATED RESISTANCE LOCUS DETERMINES THE RECESSIVE NATURE OF THE ONLY *UG*99 RESISTANCE IN BARLEY

3.1. Abstract

The *rpg4*-mediated resistance locus (RMRL) in barley (*Hordeum vulgare*) provides recessive and temperature sensitive resistance against several wheat stem rust races (*Puccinia graminis* f. sp. *tritici*) including races QCCJ and the highly virulent race TTKSK (a.k.a Ug99). Previous research identified three genes required for wheat stem rust resistance (*HvAdf3*, *Rpg5*, and *HvRga1*) at the ~ 70kbp *rpg4*-mediated resistance locus 1 (RMRL1) using high-resolution mapping and viral induced gene silencing. The dominant rye stem rust resistance gene *Rpg5* is predicted to have the typical *R*-gene domains including the nucleotide-binding-site (NBS), leucine-rich-repeat (LRR) and serine threonine protein kinase (STPK) domains. *Rpg5* appears to be the gene conditioning compatible or incompatible interactions with the wheat stem rust races because it was recently discovered as the only polymorphic gene correlating with resistance and susceptibility at the delimited RMRL1 region. Sequencing *rpg5* susceptible alleles showed that the alleles can be divided into four groups. The group 1 susceptible lines have an intact STPK domain but also contain a single cytosine insertion causing a frame shift mutation resulting in a premature stop codon at amino acid position 217 (Golden Promise and MD2). The group 2 and 3 susceptible lines contain an insertion/deletion region having a predicted functional protein phosphatase 2C gene (*HvPP2C.1*) in place of the *Rpg5* STPK domain (Steptoe and Sm89019). The group 4 susceptible genotypes code for a predicted full-length RPG5 protein that was

identical to the group 3 resistant allele, except for the two amino acid differences S820C and E1287A. Analysis of F_2 progeny from crosses between Q21861 and group 1 and 2 susceptible lines segregated in 1: 3 ratio (resistant:susceptible) with the group 1 susceptible crosses where the *HvPP2C.1* gene is present but in a 3:1 ratio (resistant:susceptible) with the group 2 susceptible crosses where the *HvPP2C.1* gene is absent. Thus, it appears that the *Rpg5* gene previously identified as the dominant rye stem rust resistance gene also imparts *rpg4*-mediated wheat stem rust resistance. *Rpg5* behaves as a dominant gene in the absence of the *HvPP2C.1* gene but its resistance function is suppressed by the presence of *HvPP2C.1* resulting in a recessive segregation at the locus. The data suggests that the *rpg4* and *Rpg5 R*-gene component may not be distinct genes and the difference in the dominant or recessive nature of resistance is due to the *HvPP2C.1* gene acting as a dominant susceptibility factor that suppresses *Rpg5*-mediated resistance against the wheat stem rust races including *Ug*99.

3.2. Introduction

Stem rust is a widespread and devastating disease of small grain crops caused by the obligate biotrophic fungal pathogen *Puccinia graminis*. Two *P. graminis* forma speciales, *P. graminis* f. s. *tritici* (*Pgt*) the wheat stem rust and *P. graminis* f. s. *secalis* (*Pgs*) the rye stem rust, can both infect and cause considerable damage in barley. The dominant *R*-gene, *Rpg1* in barley has provided durable resistance against most of the races of *Pgt* for over 65 years (Steffenson, 1992). The Midwestern emergence of a new *Pgt* race, QCCJ in 1989, and the emergence of a highly virulent race TTKSK (Ug99) in Uganda Africa in 1999, which are virulent on *Rpg1* containing barley raised major alarm for Midwestern barley production. A thorough search of the primary barley germplasm pool of over 14,000 accessions, identified the unimproved barley line Q21861 as the best source of resistance (Jin *et al*., 1994a; Steffenson *et al*., 2009). The stem rust

resistance in line Q21861 is conferred by the *rpg4*-mediated resistance locus (RMRL) at the subtelomeric region of barley chromosome 5H. The RMRL is complex and contains two tightly linked subloci, designated as RMRL1 and RMRL2 (Wang *et al*., 2013; Arora *et al*., 2013). RMRL1 harbors the NBS-LRR-STPK rye stem rust *R*- gene *Rpg5*, the NBS-LRR gene *HvRga1* and the actin depolymerizing factor like gene *HvAdf3*. The RMRL1 alone confers dominant resistance to isolates of rye stem and is conferred by *Rpg5*, yet tests still need to be done to determine in *HvAdf3* and *HvRga1* are also required for *Rpg5*-mediated rye stem rust resistance. However, recessive resistance against the *Pgt* races QCCJ and presumably TTKSK is known to require the interaction of the other two genes present at RMRL2, *HvAdf3* and *HvRga1*, in addition to *Rpg5* and at least one gene at the RMRL1 (Wang *et al*., 2013). The *Rpg5* gene encodes a nucleotide binding site (NBS), a leucine rich repeat (LRR) and a serine threonine protein kinase (STPK) domain. STPK domains are predicted to be involved in signal transduction via phosphorylation cascades, which in turn activates resistance responses (Hanks and Hunter, 1995). The activation of resistance responses via the STPK may be the function of the *Rpg5* domain in resistance, however, several STPK domains have been shown to be effector targets that are guarded by the typical NBS-LRR resistance gene following the guard-guardee model (van der Hoorn and Kamoun, 2008). Thus, the *Rpg5* STPK domain may function in either way.

We have identified an *HvPP2C.1* gene at RMRL1 by genetic analysis that is present at the locus in many susceptible barley varieties and appears to act as a dominant susceptibility factor determining the recessive nature of the *rpg4/Rpg5*-mediated wheat stem rust resistance.

Signal transduction triggered due to various biotic and abiotic stimuli is an essential phenomenon occurring in plants. An important regulatory aspect of signal transduction is the

ability of plants to reverse or turn off the signaling process. *R*- gene mediated signal transduction in plants is achieved through protein phosphorylation cascades mediated by protein kinase activity. Another class of proteins known as phosphatases plays a crucial role in turning these signaling cascades off through dephosphorylation. Thus, phosphorylation mediated signaling mechanisms in plants are regulated by opposing protein kinase and protein phosphatase activity (Cohen,2000). Genome sequence information has determined that PP2Cs are a major class of serine/threonine phosphatases in plants and the recurrent theme is that the superfamily of plant PP2Cs negatively-regulate signaling pathways by their kinase opposing dephosphorylation activity.

Extensive studies on protein kinases in different eukaryotic systems have shown that most of them share a highly conserved functional domain (Manning *et al*., 2002 ; Caenepeel *et al*., 2004; Champion *et al*., 2004). On the other hand, there is little information on PP2C characterization since they are highly diverse in terms of structure and have different catalytically active domains and motifs (Luan, 2003; Moorhead *et al*., 2007). Most of the studies dealing with PP2C characterization in plants have been conducted in model organisms, Rice and Arabidopsis. About 80 candidate PP2C-type phosphatase candidates in Arabidopsis and 78 in Rice have been discovered and could be classified into 10-13 groups (Singh *et al*., 2010; Xue *et al*., 2008). There is a limited knowledge available on the substrate specificity of PP2Cs, however, the large number of PP2Cs in plant genomes suggests that individual phosphatases may have tight specificity in substrate binding and specific control of signaling mechanisms (Schweighofer *et al*.,2004).

Plant PP2Cs are emerging as a key player in stress signaling (Moorhead *et al*., 2007). However, there are only three examples of PP2C proteins negatively regulating disease

resistance pathways in plants. These PP2C proteins negatively regulate responses elicited by PAMP receptors and result in susceptibility. In Arabidopsis, the Mitogen activated protein Kinase Phosphatase (MKP) AP2C1 physically interacts with the FLS2 receptor and overexpression of AP2C1 results in insensitivity to the PAMP flg22 of *Botrytis cinerea* and reduced FLS2/flg22 binding (Gomez-Gomez *et al*., 2001; Schweighofer *et al*., 2007), thus compromising innate immunity. In rice the PAMP receptor kinase Xa21 physically interacts with the PP2C protein XB15 that was shown to negatively regulate the innate immune response elicited by the receptor kinase Xa21 against *Xanthomonas oryzae* (Park *et al*., 2008). In another study, a phytochrome-associated protein phosphatase type 2C (PAPP2C) in Arabidopsis was shown to interact with the atypical disease resistance protein RPW8.2 and negatively regulates salicylic acid-dependent basal defense against powdery mildew in Arabidopsis (Wang *et al*., 2012).

In this study, we demonstrate the presence of an *HvPP2C.1* gene at the RMRL1 stem rust resistance locus in barley through extensive genetic analysis and validate its function through post transcriptional gene silencing using viral induced gene silencing (VIGS). We hypothesize that *HvPP2C.1* negatively regulates stem rust resistance signaling activated by the kinase domain of *Rpg5* gene against the wheat stem rust races QCCJ and TTKSK but not the rye stem rust isolates including 92-MN-90.

3.3. Materials and methods

3.3.1. *HvPP2C.1* genetic analysis

To genetically analyze the *HvPP2C.1* gene, F_1 and F_2 generations were obtained by crossing four different wheat stem rust susceptible barley cultivars in the two major susceptible groups 1 and 2 and the resistant genotype Q21861 (Fig 4.). Susceptible genotypes included

Steptoe, Sm89019, Golden Promise (GP) and Multidominant 2 (MD2). The RMRL region has already been sequenced for all the cultivars used for this experiment (Brueggeman *et al*., 2008, Arora *et al*., 2013). The F_1s and F_2s obtained from four different crosses were phenotyped for their infection types (ITs) to single-pustule derived isolates of wheat stem rust race QCCJ. Race QCCJ was used as a TTKSK surrogate in this study due to the restrictions on the use of TTKSK in North America. Stem rust inoculations and evaluation were performed as described in Arora *et al*., 2013. ITs were assessed using the 0-to-4 scale modified for barley as previously described by Sun and Steffenson (Sun *et al*., 2005) and ITs of 0, 10, 12; 2, 21; 2-3, 3-2 and 3, 3+ were considered resistant; moderately resistant; moderately susceptible and susceptible reactions respectively (Table 4). The number of F_2 seed used, observed resistant: susceptible ratio, chi square analysis and the fitness of the observed ratio to the expected ratio for each cross are all provided in Table 4.

3.3.2. *HvPP2C.1* cloning

To transiently express the *HvPP2C.1* gene in barley for performing complementation assays and functional analysis, a BSMV gamma (γ) RNA infectious cDNA clone (pSL38.1) was modified by deleting the gene that encodes the gamma ORF a (γa) protein and inserting an adaptor cassette that contained restriction sites for directional cloning of any gene of interest. Site directed mutagenesis was utilized to change two nucleotides which substituted the start methionine codon of the BSMV γa protein with isoleucine (M1I) and the same mutation event also introduced a unique *Eag*I restriction site. The γa gene was deleted by digestion with *Eag*I and *Cla*I and replaced with a DNA cassette containing NotI and PacI restriction sites. This BSMV expression construct/infectious clone was designated RSB1393. The full length *HvPP2C.1* gene was then amplified by RT-PCR from stem rust susceptible genotype Sm89010

cDNA with gene specific NotI and PacI adaptor primers and cloned into the NotI and PacI restriction sites of RSB1393. The integrity of the full-length gene was tested through sequencing and the positive construct was named DDS32 (Fig 6). The predicted gene structure of the *HvPP2C.1* gene was determined by comparison of the Sm89010 cDNA with the genomic sequence using Vector NTI software and NCBI nucleotide BLAST. The *HvPP2C.1* gene was predicted to be 1149 bases and predicted to encode a 383 amino acid protein (42kDa).

3.3.3. *HvPP2C.1* functional analysis: VIGS

The BSMV vector was used to post-transcriptionally silence the *HvPP2C.1* gene with its antisense RNA as previously described (Holzberg *et al*., 2002). A 121-bp *HvPP2C.1* cDNA fragment was generated by PCR from DDS32 and ligated in antisense (as) orientation into the BSMV-VIGS infectious clone Psl38.1 and designated DDS34 (Fig 6). The γ BSMV-as*HvPP2C.1* or DDS34, α and β genomes of the tripartite BSMV virus were in vitro transcribed with the mMessage mMachine T7 kit (Ambion), and the T7 promoter following the manufacturers standard protocol. The infectious BSMV RNA was then inoculated onto the Q21861 X Steptoe F_1 plants (having one copy of the functional *Rpg5* gene and one copy of the functional *HvPP2C.1* gene) at the two-leaf stage. The remainder of the experiment was performed as described for *Rpg5* VIGS in Brueggeman *et al*., 2008; Wang *et al*., 2013. After VIGS inoculation the BSMV infected plants were challenged with *Pgt* race QCCJ. The plants were scored for compatibility or incompatibility at 12 days and 18 days post-fungal infection (Table 5). The parents, Q21861 and Steptoe as well as F1 progeny were used as the resistant and susceptible virus-inoculated and -uninoculated controls. The BSMV-MCS construct, which containing BSMV's full length γ genome with an antisense insert of pBlue script *E. coli* vector

sequence known not to be present within the barley genome was used as the virus inoculated control. Tissue samples for qRT-PCR were collected 14 days after fungal infection.

3.4. Results

3.4.1. *HvPP2C.1* genetic analysis

The requirement of two NBS-LRR containing *R*-gene like domain proteins that typically provide dominant resistance against biotrophic pathogens prompted the further evaluation of crosses made between the resistant parent Q21861 and different susceptible parents. Of primary interest were the susceptible parental lines containing non-functional *Rpg5* alleles with and without the insertion/deletion event leading to the presence or absence of the functional *HvPP2C.1* gene. We phenotyped Steptoe, Sm89010, Golden Promise (GP), and Multidominant 2 (MD2) x Q21861 F_1 plants to determine if the *Rpg5/HvRga1* mediated resistance behaved as a dominant or recessive resistance gene in the presence or absence of the functional *HvPP2C.1* gene. The F_1 progeny from two crosses containing a single copy of the *HvPP2C.1* gene from the susceptible parent (Sm89010 and Steptoe) were inoculated with *Pg*t race QCCJ resulting in susceptible IT scores similar to the susceptible parent for each cross. The F_2 segregation data resulted in a ratio of 3 susceptible for every 1 resistant, indicating that the resistance was recessive in the presence of a single copy of *HvPP2C.1 gene* (Table 4; Fig 4 C, 4D). The F_1 progeny from the two crosses between the susceptible cultivar Golden Promise and MD2 which contained non-functional *Rpg5* alleles due to a single cytosine insertion which caused a frame shift mutation resulting in a predicted truncated protein (Arora et al., 2013; Fig 2) were also assayed with *Pgt* race QCCJ. These two susceptible parents contain a predicted non-functional *Rpg5* allele without the insertion/deletion that replaces the STPK domain with the functional *HvPP2C.1*. The F_1 progeny from these crosses assayed with *Pgt* race QCCJ resulted in resistant

ITs similar to those of the resistant parent Q21861 and the F_2 progenies segregated for 3 resistant for every 1 susceptible, indicating that the *Rpg5* behaves as a dominant gene in the *rpg4*-mediated wheat stem rust resistance mechanism in the absence of a functional *HvPP2C.1* gene (Fig 4B, 4E). The data suggests that the *Rpg5*/*HvRga1* genes function as dominant resistance genes against *Pgt* race QCCJ in the absence of *HvPP2C.1* suggesting that that the PP2C protein may function as a dominant susceptibility factor in the *rpg4*-mediated resistance mechanism.

3.4.2. *HvPP2C.1* functional analysis

To validate the *HvPP2C.1* gene as the dominant susceptibility factor the *HvPP2C.1* gene was silenced in the presence of a functional *Rpg5* allele. However, since the *PP2C.1* gene is an insertion deletion event that eliminates the STPK domain which is required for resistance, the functional *Rpg5* allele is perfectly linked to the functional *HvPP2C.1* gene. Thus, the only possible way to test if silencing *HvPP2C.1* in the presence of *Rpg5* resulted in a shift from compatibility to incompatibility was post transcriptional gene silencing utilizing BSMV-VIGS in F_1 progeny plants.

As expected, the Q21861 X Steptoe F_1 progeny plants with BSMV-as *HvPP2C.1* infection showed lower ITs after inoculating with QCCJ as compared to the F_1s with no BSMV-VIGS. The virus uninoculated control F_1 progeny plants showed intermediate ITs ranging from of 21; to or 213 (moderately susceptible), which shifted to a range from 0;1 to 21 (resistant to moderately resistance) on the BSMV-as*HvPP2C.1* infected seedlings.

The virus-uninoculated susceptible parent Steptoe with no functional copies of *Rpg5* and two functional copies of *HvPP2C.1* showed ITs ranging from 231 to 3+ (moderately susceptible to susceptible). The Steptoe seedlings infected with BSMV-as*HvPP2C.1* remained susceptible with ITs ranging from 3 to 3+ (susceptible). However, the F_1 plants inoculated with the virus

control BSMV-MCS, which does not contain the gene specific antisense fragment specific to *HvPP2C.1* also showed a shift from susceptibility to resistance. The BSMV-MCS inoculated F_1s showed ITs ranging from 0;1 to 21; (resistant to moderately resistance) which where indistinguishable from the F_1s inoculated with DDS34 construct (BSMV-as*HvPP2C.1*). The data obtained from these experiments are summarized in table 5.

Table 4. F_1 reactions and F_2 analyses to determine the fitness of segregation ratios obtained for crosses made to validate the *HvPP2C.1* as a dominant susceptibility gene.

Genetic Cross	**F1 reaction/phenotype (S-susceptible, R-Resistant)**	**No. of F_2 plants with resistant IT**	**No. of F_2 plants with susceptible IT**	**Expected R:S ratio**	**Chi square value; degrees of freedom**	**Fitness with the expected ratio**
Q x Steptoe	S	28	79	1:3	0.0495; 1	Good fit
Q x Sm89010	S	15	57	1:3	0.67; 1	Good fit
Q x GP	R	80	32	3:1	0.76; 1	Good fit
Q x MD2	R	60	28	3:1	2.18; 1	Good fit

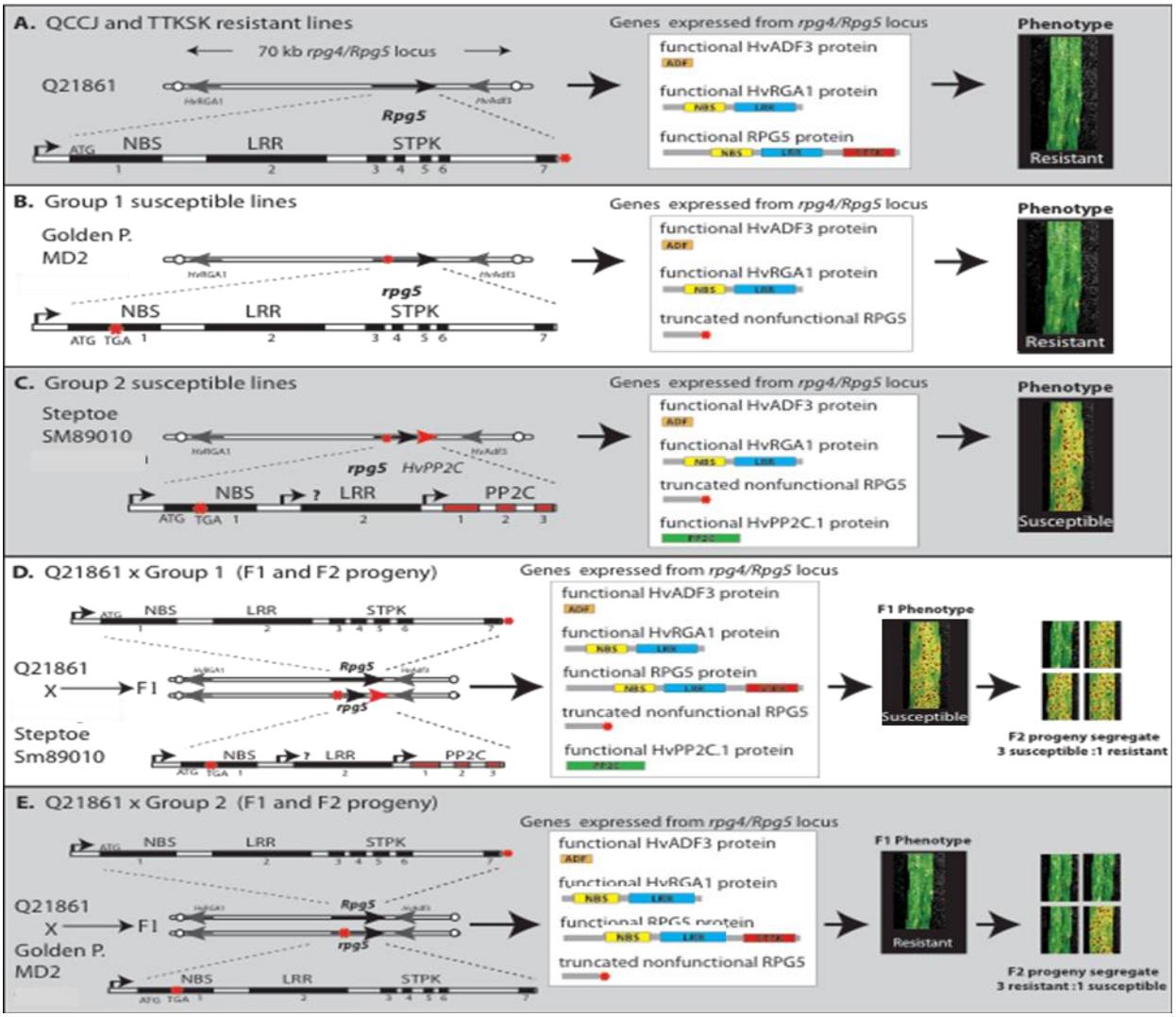

Figure 4. Predicted gene and protein architecture at RMRL for parental haplotypes and F1 progeny as well as the predicted functional proteins translated from each loci from the parental and F1 progeny and the observed typical reactions of the F1 and F2s progeny after QCCJ inoculation.

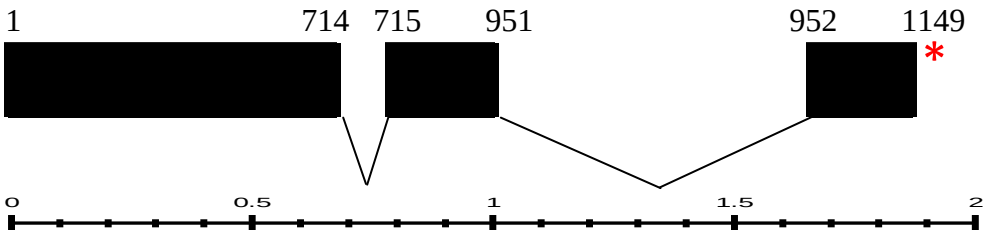

Figure 5. Predicted full length gene architecture of *HvPP2C.1* gene shown to scale. Black solid bars represent exons and lines joining black bars represent introns. Asterisk represents a stop codon. Numbers above the solid bars represent the starting and ending and thus, size of each exon.

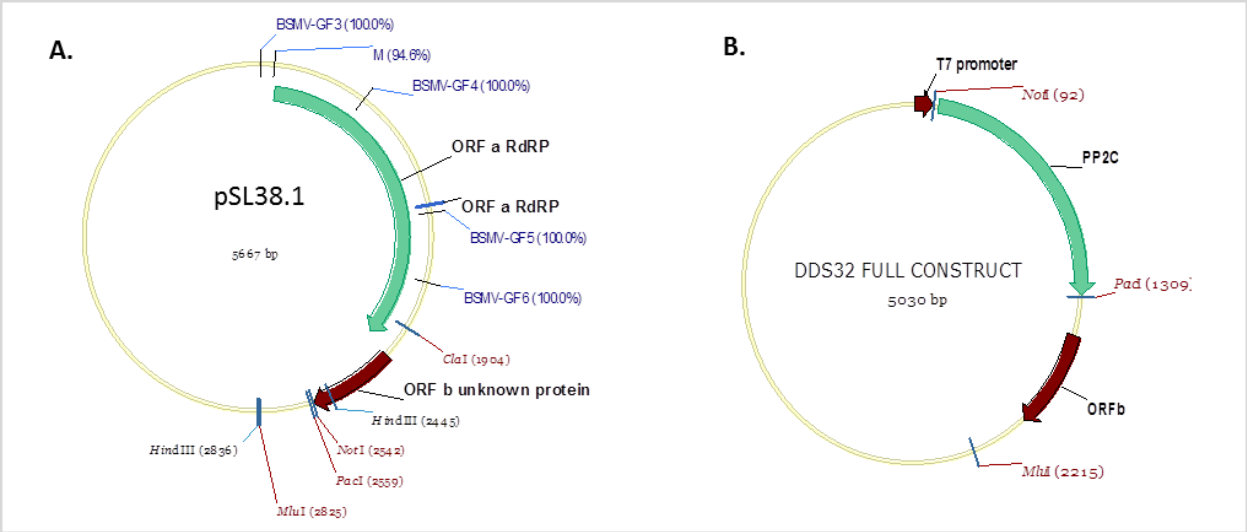

Figure 6. pSL38.1 and DDS32 plasmid DNA. **A**, pSL38.1 plasmid DNA with full length BSMV γ genome cloned into an *E.coli* based cloning vector, with green and red solid arrow representing γ ORFa and γ ORFb respectively. **B,** DDS32, with γ ORFa replaced with full length *HvPP2C.1* gene, shown by solid green arrow.

Table 5. Infection types (IT) of parental lines (Q21861 and Steptoe) and F1s (Q X S) in response to pathotypes of *Puccinia graminis* f. sp. *tritici* QCCJ obtained for *HvPP2C.1* functional analysis using VIGS.

F1 (34 + QCCJ)		S(34 + QCCJ)		F1(MCS +QCCJ)		S(MCS +QCCJ)		F1 (QCCJ)		S(QCCJ)		Q(QCCJ)	
12th day	18th day	12th day	18th day	12th day	18th day	12th day	18th day	12th day	18th day	12th day	18th day	12th day	18th day
1;2	23-	32	3	21;	21	32	32	21; (ONE LESION 3)	21	33+	3+	01;	0;1
1,	2	32	3+	0;1	-	3	3	21	213	33+	3+	01;	0;1
12	1	23	3	1;	1	33+	3+	21;3	2	33+	3+	01;	0;1
1;	12	32	33+	1;2	2	32	3	213	21	231	3	01;	0;1
21;	21	32	33+	12;	2	33+	32	21;	2			0;1	0;1
1;	12	3	3	1;2	32	32	3						
10;	21	32	33+	1;	21	3	3						
12	21	23	3	0;1	2	33+	3						
01;	21			1;	-								
0;1	1			1;	1								
0;1	2			21;	12								
1,	2			10;	33-								
1;0	21			1;	3								

[(34+QCCJ) QCCJ inoculated and virus infected with *HvPP2C.1* silencing construct; (MCS+ QCCJ), QCCJ inoculated positive virus control; (QCCJ), QCCJ inoculated with no virus infection].

3.5. Discussion

The two *R*-genes, *Rpg5* and *Hv*Rga1, required for *rpg4*-mediated resistance raised the intriguing question of why the NBS-LRR-STPK domain resistance gene *Rpg5* behave as a dominant *R*-gene against the *Pgs* isolate 92-MN-90 but behaves as a recessive resistance gene against *Pgt* races QCCJ and TTKSK. Traditionally, recessive resistance is considered the result of a non-functional allele or a dominant functional susceptibility factor. Transcript analysis of *Rpg5* and *HvRga1* and the fact that *Rpg5* behaves as a dominant resistance gene against the Rye stem rust pathogen suggested that the genes would function as dominant *R*-genes in the traditional gene-for-gene fashion as was proposed by H.H. Flor in dominant rust resistance mechanisms in Flax. Interestingly, the allele analysis of the genes in the rpg4/Rpg5 region required for rpg4-mediated resistance, determined that most of the susceptible barley varieties contain the insertion/deletion event, which removes the STPK domain of *Rpg5* and replaces it with a predicted functional *HvPP2C.1* gene. However, non-functional *rpg5* alleles were identified and designated the group 1 susceptible genotypes that had non-functional *rpg5* alleles as a result of a single cytosine insertion resulting in a frame shift mutation and a stop codon at amino acid position 217. Analysis of all the crosses used to characterize *rpg4* as a recessive resistance gene were made with susceptible varieties containing the *HvPP2C.1* insertion. Thus, we hypothesized that the functional *HvPP2C.1* gene that is not present in the resistant cultivar could potentially function as a dominant susceptibility factor, conferring susceptibility when present as a single copy in the F_1 generation and segregate 1:3 for resistance and susceptibility in the F_2 generations. We tested this hypothesis by making crosses with the group 1 susceptible lines without the *HvPP2C.1* gene and the group 2 susceptibles containing the *HvPP2C.1* gene and assaying the F1 and F2 individuals with *Pgt* race QCCJ. Interestingly, three crosses with

susceptible parents containing the *HvPP2C.1* gene behaved as predicted. Previous F_2 phenotype analysis of one of the populations we tested and two different populations made with susceptible parents containing an intact *HvPP2C.1* allele (Morex, Steptoe, and Sm89010 x Q21861) revealed 1:3 segregation for resistance:susceptibility, further demonstrating the recessive nature of *rpg4* mediated resistance in the presence of the *HvPP2C.1* gene . The other three crosses examined were made with the susceptible barley lines, Golden Promise, MD2 and OSU6, which contain the non-functional *Rpg5* allele due to the nucleotide insertion but still have the PK domain present without the functional *HvPP2C.1* gene. Golden Promise, MD2 and OSU6 were crossed with Q21861 and the F_1 progeny from these crosses were assayed for reaction type to *Pgt* race QCCJ. The F_1 progeny of the two crosses were resistant to *Pgt* race QCCJ suggesting that the *HvPP2C.1* dominant susceptibility factor hypothesis may be correct. We also tested the F_2 progeny from the Golden Promise x Q21861 population and determined that the resistance:susceptibility segregated 3:1 as a single dominant resistance gene determined to be the *rpg4*/*Rpg5* locus. This data suggests that *HvPP2C.1* may be a dominant susceptibility factor that represses the resistance reaction triggered by the *Rpg5* and *HvRga1 R*-genes. Although the genetic data strongly suggests that *HvPP2C.1* could be a dominant susceptibility factor, further functional evaluation is required to validate this hypothesis. The plant tissues obtained as part of the preliminary VIGS experiment have been collected and are being stored at -80^{o}C. In the future, the stored tissue will be utilized for q-RTPCR analyses, which should allow us to determine if the construct is specifically silencing *HvPP2C.1*. This information may provide a starting point from which we can begin forming testable hypotheses for understanding the mechanisms underlying the HvPP2C action on the resistance mechanisms as well as to

understand why the virus itself is changing the infection types from compatibility to incompatibility*HvPP2C.1*.

3.6. Literature cited

Arora, D., Gross, T., and Brueggeman, R. 2013. Allele characterization of genes required for *rpg4*-mediated wheat stem rust resistance identifies *Rpg5* as the R-gene. Phytopathology 103: 1153-1161.

Brueggeman, R., Druka, A., Nirmala, J., Cavileer, T., Drader, T., Rostoks, N., Mirlohi, A., Bennypaul, H., Gill, U., Kudrna, D., Whitelaw, C., Kilian, A., Han, F., Sun, Y., Gill, K., Steffenson, B., and Kleinhofs, A. 2008. The stem rust resistance gene *Rpg5* encodes a novel protein with nucleotide binding site, leucine-rich and protein kinase domains. Proc. Natl. Acad. Sci. USA 105:14970-14975.

Caenepeel, G. Charydczak, S. Sudarsanam, T. Hunter, and G. Manning. 2004. The mouse kinome: discovery and comparative genomics of all mouse protein kinases. Proc. Natl. Acad. Sci. USA 101:1707-11712.

Champion, A., Kreis, M., Mockaitis, K., Picaud, A., and Henry, Y. 2004. Arabidopsis kinome: after the casting. Funct. Integr. Genomics 4: 163-187.

Cohen, P. 2000. The origins of protein phosphorylation. Nat. Cell Biol. 4: 127-130.

Hanks, S. K. and Hunter, T. 1995. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J. 9:576-596

Holzberg, S., Brosio, P., Gross, C., and Pogue, G.P. 2002. Barley stripe mosaic virus-induced gene silencing in a monocot plant. Plant J. 30: 315-327.

Gomez-Gomez, L., Bauer, Z., and Boller, T. 2001. Both the extracellular leucine rich repeat and the kinase activity of FLS2 are required for flagellin binding and signaling in Arabidopsis. Plant Cell 13:1155-1163.

Jin, Y., Steffenson, B.J., and Fetch, T.G.J. 1994a. Sources of resistance to pathotype QCC of *Puccinia graminis* f. sp. *tritici* in barley. Crop Sci. 34:285-8.

Lammers, T. and Lavi, S. 2007. Role of Type 2C protein phosphatases in growth regulation and in cellular stress signaling. Crit. Rev. Biochem. Mol. Biol. 42:437-461.

Manning, G., Whyte, D. B., Martinez, R., Hunter, T., and Sudarsanam, S. 2002. The protein kinase complement of the human genome. Science 298:1912-1934.

Luan, S. 2003. Protein phosphatases in plants. Annu. Rev. Plant. Biol. 54:63-92.

Moorhead, G.B.G., Trinkle-Mulcahy, L., and Ulke-Leme´e, A. 2007. Emerging roles of nuclear protein phosphatases. Nat Rev Mol Cell Biol. 8:234-244.

Park, C.J., Peng, Y., Chen, X., Dardick, C., Ruan, D., Bart, R., Canlas, P.E., and Ronald, P.C. 2008. Rice *XB15*, a protein phosphatase 2C, negatively regulates cell death and *XA21*-mediated innate immunity. PLoS Biol. 6, e231.

Schweighofer, A., Hirt, H., and Meskiene, I. 2004. Plant PP2C phosphatases: emerging functions in stress signaling. Trends Plant Sci. 9:236-243.

Singh, A., Giri, J., Kapoor, S., Tyagi, AK., and Pandey, G.K. 2010. Protein phosphatase complement in rice: genome-wide identification and transcriptional analysis under abiotic stress conditions and reproductive development. BMC Genomics 11:435.

Steffenson, B.J. 1992. Analysis of durable resistance to stem rust in barley. Euphytica 63:153-167.

Steffenson, B. J., Jin, Y., Brueggeman, R. S., Kleinhofs, A., and Sun, Y. 2009. Resistance to stem rust race TTKSK maps to the *rpg4* /*Rpg5* complex of chromosome 5H of barley. Phytopathology 99:1135-1141.

Sun, Y. and Steffenson, B.J. 2005. Reaction of barley seedlings with different stem rust resistance genes to *Puccinia graminis* f. sp. *tritici* and *P. g.* f. sp. *secalis*. Can. J. Plant Pathol. 27:80-89.

van der Hoorn R.A. and Kamoun, S. 2008. From guard to decoy: A new model for perception of plant pathogen effectors. Plant Cell 20:2009-2017.

Wang, W.M., Ma, X.F., and Zhang, Y. 2012. PAPP2C interacts with the atypical disease resistance protein RPW8.2 and negatively regulates salicylic acid-dependent defense responses in *Arabidopsis*. Molecular Plant 5:1125-37.

Wang, X., Richards, J., Gross, T., Druka, A., Kleinhofs, A., Steffenson, B., Acevedo, M., and Brueggeman, R. 2013. The *rpg4*-mediated resistance to wheat stem rust (*Puccinia graminis*) in barley (*Hordeum vulgare*) requires *Rpg5*, a second NBS-LRR gene and an actin depolymerization factor. Mol. Plant-Microbe Interaction 26:407-418.

Xue, T., Wang, D., Zhang, S., Ehlting, J., and Ni, F. 2008. Genome-wide and expression analysis of protein phosphatase 2C in rice and Arabidopsis. BMC Genomics 9:550

4. PROLOGUE

More than 60 stem rust (*Sr*) resistance genes have been identified in wheat and hundreds of *Pgt* races have been identified utilizing single *R*-gene wheat differentials. However, in barley only 6 stem rust resistance genes (Resistance to Puccinia graminis, *Rpg*) have been identified and of these, only 2, the *Rpg1* gene and the *rpg4*/*Rpg5* resistance complex, have been shown to be effective. The presence of only two effective resistance sources in barley, despite the screening of thousands of lines from the primary barley germplasm pool, suggests that an ancient molecular arms race between the host and pathogen giving rise to multiple specific *R*-genes and virulence/avirulence genes did not occur and that barley is a recently emerged host. Also, the fact that both effective resistance sources in barley, *Rpg1* and *rpg4/Rpg5*, provide broad resistance to most *Pgt* races, indicates that the mode of resistance is broad and doesn't follow the typical race specific resistance mechanisms as seen in the wheat-*Pgt* pathosystem and was described by Flor in the Flax-Flax rust pathosystem (Flor, 1956). This hypothesis is also supported by the recent functional analyses of the *Rpg1* resistance mechanism, where it was shown that the RPG1 protein undergoes phosphorylation/activation within five minutes of avirulent stem rust spore landing on the leaf surface (Nirmala *et al*., 2010). Also, the two spore localized proteins eliciting this rapid resistance response are large atypical effectors (Nirmala *et al*., 2011) more in line with a rapid Pathogen Associated Molecular Pattern (PAMP) triggered immunity (PTI) or nonhost resistance response.

The *Rpg1*-mediated stem rust mechanism has been studied in great detail both genetically and functionally and thus, is being used as a model to further understand the *rpg4/Rpg5* stem rust resistance mechanisms in barley. We have preliminary data suggesting that the *Rpg5* gene is induced within twelve hours of pathogen interaction suggesting that it may represent an early

resistance response, possibly elicited by a PAMP similar to the *Rpg1* gene. The early pre-haustorial activation of these resistance mechanisms suggest that barley had or still has an early defense mechanisms for the stem rust pathogen, which primes the resistance response or blocks pathogen colonization early in the infection process.

Rpg1 had remained remarkably durable and effective, yet the pathogen has evolved in regions where sexual populations still persist, the Pacific Northweastern USA and Africa. Races QCCJ and TTKSK have evolved in the PNW and Africa, respectively, that are virulent on *Rpg1*, warranting the characterization of the *rpg4/Rpg5* resistance mechanisms, the only effective resistance known to either of these *Pgt* races (Steffenson *et al*., 2009). Since *Rpg1* is the only gene deployed in barley till date, the barley production is quite vulnerable as these new races emerge. The goal now is to deploy RMRL into Midwestern barley varieties with *Rpg1* resistance to provide effective resistances to all *Pgt* races including the highly virulent race TTKSK and its lineage races. Unlike *Rpg1* resistance, RMRL resistance is complex and involves the interaction of more than one gene, which makes it harder for the scientists to work with and understand the underlying mechanisms (Wang *et al*., 2013; Arora *et al*., 2013). To comprehend the individual function of each gene in providing complete resistance, it requires an understanding of how these genes interact or come together in space and time when challenged by the pathogen. Some of the other important aspects of the resistance response which will allow for the understanding of the biological significance of each include transcriptional regulation of the genes involved, functional characterization of the interactions between the proteins to determine if they are direct or indirect, and if indirect, which model such as guard-guardee or decoy model does this interaction fit. Another very important aspect to consider is that this only effective *Ug*99 resistance source provides a kind of resistance, which is dominant against *Pgs* isolate but

recessive as well as temperature sensitive against these *Pgt* races. We have found a dominant susceptibility factor (*HvPP2C.1*) acting at the resistance locus which codes for a functional phosphatase and possibly plays a role in shutting off the resistance mechanism initiated by the kinase domain of the *Rpg5* gene in case of *Pgt* isolates. Genetic analysis has generated strong evidences for *HvPP2C.1* presence being the cause for the recessive nature of the resistance; however, due to the complex nature of this resistance locus and involvement of more than one gene in providing resistance or susceptibility, extensive functional analysis is required to validate any hypotheses. Once the mechanism of the recessive resistance is elucidated, it may provide a model for other recessive resistances found in other important crop species, such as wheat. Understanding the recessive and temperature sensitive nature of this resistance mechanism may provide clues to how to stabilize the resistance when deploying it into commercial varieties.

4.1. Literature cited

Arora, D., Gross, T., and Brueggeman, R. 2013. Allele characterization of genes required for *rpg4*-mediated wheat stem rust resistance identifies *Rpg5* as the R-gene. Phytopathology 103: 1153-1161.

Flor H.H. 1956. The complementary system in flax and flax rust. Adv. Genet. 8:29-54.

Nirmala, J., Drader, T., Chen, X., Steffenson, B., and Kleinhofs, A. 2010. Stem rust spores elicit rapid RPG1 phosphorylation. Mol. Plant Microbe Interact. 23:1635-1642.

Nirmala, J., Drader, T., Lawrence, P. K., Yin, C., Hulbert, S., Steber, C. M., Steffenson, B. J., Szabo, L. J., Wettstein, D. V., and Kleinhofs, A. 2011. Concerted action of two avirulent spore effectors activates reaction to *Puccinia graminis* 1 (*Rpg1*)-mediated cereal stem rust resistance. Proc. Natl. Acad. Sci. USA 108:14676-14681.

Steffenson, B. J., Jin, Y., Brueggeman, R. S., Kleinhofs, A., and Sun, Y. 2009. Resistance to stem rust race TTKSK maps to the *rpg4*/*Rpg5* complex of chromosome 5H of barley. Phytopathology 99:1135-1141.

Wang, X., Richards, J., Gross, T., Druka, A., Kleinhofs, A., Steffenson, B., Acevedo, M., and Brueggeman, R. 2013. The *rpg4*-mediated resistance to wheat stem rust (*Puccinia graminis*) in barley (*Hordeum vulgare*) requires *Rpg5*, a second NBS-LRR gene and an actin depolymerization factor. Mol. Plant-Microbe Interaction 26: 407-418.