ELLUCIDATION OF RPG4/RPG5-MEDIATED RESISTANCE AGAINST WHEAT STEM RUST 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

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ABSTRACT

Wheat stem rust, caused by *Puccinia graminis f. sp. tritici* (*Pgt*), is a major threat to barley and caused devastating reductions in yield and economic losses. The barley line Q21861 was identified as the best source of resistance to the potential threat posed by the highly virulent *Pgt* race TTKSK. Resistance to TTKSK was mapped to the telomeric region of chromosome 5HL, now designated as the *rpg4*-mediated resistance locus (RMRL). RMRL has been delimited into two tightly linked yet distinct loci, designated as RMRL1 and RMRL2. Three genes (*HvRga1*, *Rpg5*, and *HvAdf3*) were found at RMRL1 while gene(s) at RMRL2 remains unknown. BSMV-VIGS revealed that all three genes at RMRL1 are required for *rpg4*-mediated resistance, which follows the emerging theme of dual genetically linked NBS-LRR genes required for resistance. *HvAdf3* may play an important role in controlling host-specific resistance as suggested by AtADF4 in the *Arabidopsis-Pseudomonas syringae* plant-microbe interaction model.

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LITERATURE REVIEW

Stem Rust in Barley

Barley (*Hordeum vulgare* L.), one of the oldest and most important cultivated cereal crops, is the forth most widely grown crop after wheat, rice and corn with production of 47.5 million hectares globally in 2010. The United States is one of the major barley producing countries around world, and northern tier states from Minnesota to Washington constitutes the major barley producing region. North Dakota historically ranks number one for barley production, however in 2014 it fell behind Idaho for total production and Montana in acreage. As one of the 31 Hordeum species (Bothmer et. al., 1995), cultivated barley has a large genome of around 5000 Mb with 2n=14 chromosomes (Wenzl et. al., 2004). Although barley production for animal feed has dropped of considerably due to stiff competition from corn it is gaining importance as a specialty food and is essential for the malting industry and beer production.

However, several foliar, head, root and crown diseases can cause costly reductions to barley production. Although stem rust is not currently a major problem for production in the US, it was historically one of the most significant barley foliar diseases in the northern Great Plains of the United States and Prairie Provinces of Canada prior to the 1950s. Stem rust epidemics caused devastating reductions in yield and considerable economic damage. Barley losses due to stem rust is not as well recorded as it has been for wheat, however severe yield reduction of barley mirror wheat during epidemic years (Steffenson, 1991). Yield loss has been minimized in the northern Great Plains of the United States since deployment of a single dominant resistance gene, *Rpg1,* in barley cultivars in the mid-1940's (Steffenson, 1992). However, during the 1989 growing season, a new race of *P. graminis* f. sp. *tritici (Pgt),* designated QCCJ, was identified as virulent on barley cultivars containing *Rpg1* in North Dakota (Roelfs et al., 1991). After 1989,

this pathotype became one of the most common virulent types in the Great Plains, causing minor stem rust epidemics (Roelfs et al., 1993). In 1999, a new highly virulent race, *Pgt* race TTKSK (aka Ug99), was first reported in Uganda, Africa (Pretorius et al., 2000). This remarkable race of wheat stem rust has been shown to be virulent on over 97% of barley and approximately 70% of commercial wheat cultivars worldwide and is currently considered a major threat to barley and wheat production worldwide (Singh et al., 2008; Steffenson et al., 2012). It was reported that TTKSK has been confirmed in Kenya, Ethiopia, Sudan, Yemen (Singh et al., 2008) and Iran (Nazari et al., 2009). With consideration of wind dissemination and high virulence, TTKSK becomes a potential threat to barley and wheat production in US even though it has not reached North America yet.

The Pathogen-*Puccinia Graminis*

Puccinia graminis is the causal agent of stem rust also known as black rust. The rust fungus belongs to the Family Pucciniaceae, Order Pucciniales, Class Pucciniomycetes, subphylum Pucciniomycotina, and Phylum Basidiomycota. Within the species *P. graminis* there are subspecies given the designation "special forms" or *formae specialis*, which differ from each other by the most prominent primary gramineous hosts. There are three common *formae specialis* of *Puccinia graminis*: 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 infects oat and certain genotypes of barley. A standard set of differential wheat lines with different resistance genes were established in order to characterize the subdivisions within *formae specialis*, which are called races based on their different effectors/virulence genes and avirulence genes which allow the pathogen to have specific host specificities (Roelfs and Martens, 1988).

P. gaminirs is a heteroecious pathogen affecting two different hosts in order to complete its life cycle. The heterocious nature of *P. graminis* was first described by Anton deBarry in 1865. The alternate host (primarily common barberry: *Berberis vulgaris*) is required in the *P. gaminirs* life cycle in order to complete its sexual stage. Therefore *P. graminis* is macrocyclic, consisting of five different spore stages in its complete life cycle.

Teliospores, the overwintering structure residing in the residues of the primary grass host, germinate in the early spring. Following karyogamy and meiosis, four haploid thin-walled basidiospores are produced by a single telia. The colorless basidiospores specifically infect barberry, the alternative host. On the barberry leaves haploid mycelium are produced that colonize the barberry leaves when the basidiospores germinate. Pycnia form on the upper leaf surface from the haploid mycelium and receptive hyphae and pycniospores are produced by the pycnia. Fertilization occurs when pycniospores attach to receptive hyphae from different mating types. This dikaryotic status remains throughout the remaining stages of the life cycle. Pycnia that form in the alternate host barberry are vital for genetic recombination and emergence of new races as this sexual stage serves as the site for nuclei fusion from different mating types.

On the lower surface of the barberry leaves aecia form after the occurrence of fertilization, producing aeciospores that can only infect the primary gramineous hosts like wheat, barley or oat. Thus, susceptible barberry plants adjacent to cereal fields can serve as early season primary inoculum. Aeciospores germinate once they land on the leaves and form uredinium to elicit further infection. Usually, uredinium will erupt through the epidermis of the leaves or stems, causing large reddish brown pustules filling with urediniospores. This spore stage is also considered as polycyclic in the life cycle of stem rust since it can reinfect the cereal host and cycle over several generations during a single growing season. Epidemics will happen if multiple

generations of urediniospores affect primary host in the same region under favorable condition. Late in the growing season, uredinium will form telia that produce the black dikaryotic teliospores that allow the fungus to overwinter. Thus, the life cycle is completed.

Urediniospores are vital for eliciting epidemics since they are the only rust spores that can infect the host on which they are produced. The wind-disseminated spores are highly stable which can be carried by prevailing wind over thousands of miles. They attach on the leaf surface of the primary host. After the urediniospore germinate in a film of water on the leaf surface, the germ tubes form and elongate along the long axis of epidermal cells. The right-angled orientation will maximize the chances of encountering stomata as an entry point in order to facilitate further infections. Once the germ tube reaches a stomate, it swells to form an appresorium over the stomatal aperture instead of continuing growth. From the bottom of the appresorium a narrow penetration peg forms that enables the fungus to pass between guard cells and breach the hosts. The sub-stomatal vesicle forms under the stomatal pore and an infection hyphae emerges from the vesicles. The infection hyphae grow intracellularly in the apoplastic spaces and once it encounters a host mesophyll cell, the haustorial mother cell (hmc) forms from the tip. A narrow peg, produced by hmc will function as penetration structure that leads to further infection however the invagination doses not breach the host cells plasma membrane yet. The haustorium forms enveloped by the host. The haustorium, a specialized structure for extracting nutrients, is formed inside the host cell after penetration (Leonard and Szabo, 2005).

Genetic Resistance against Stem Rust in Barley

The most environmentally friendly and primary strategy to control stem rust in both barley and wheat is to incorporate resistance genes into commercial cultivars (Steffenson, 1992). There are eight resistance genes conferring resistance to *P. graminis* that have been identified in

barley, i.e. *Rpg1* (identified from Kindred), *Rpg2* (identified from Hietpas-5), *Rpg3* (identified from PI382313), *rpg4* (identified from Q21861), *Rpg5* (identified from Q21861), *rpg6* (identified from *H. bulbosum*), *rpgBH* (identified from Black Hulless CIho666), and *RpgU* (identified from Peatland) (Patterson *et al*., 1957; Jedel, 1990; Steffenson *et al*., 1984; Fox and Harder, 1995). Only the *Rpg1*, *rpg4* and *Rpg5* stem rust *R*-genes have been cloned and characterized (Brueggeman *et al*., 2002; Brueggeman *et al*., 2008, Wang *et al*. 2013, Arora *et al*., 2013). Further molecular investigations have been carried out in order to understand the pathway underlying the *Rpg1* resistance mechanisms (Nirmala *et al*., 2007; Nirmala *et al*., 2010; Nirmala *et al*., 2011).

The single dominant resistance gene, *Rpg1,* has provided durable resistance against *P. graminis* f. sp. *tritici* for over 70 years. Eradication of barberry (*Berberis vulgaris*) was a significant factor contributing to the remarkable durability of *Rpg1*. Due to the reduced genetic variability of the pathogen resulting from elimination of the sexual stage of *P. graminis* on barberry, the pathogen lost its ability to recombine virulence genes that gives rise to new virulent races (Kolmer, 2005). *Rpg1* has been incorporated into almost every commercial barley cultivar in the Upper Midwest region of the United States. Positional cloning techniques were utilized by Brueggemant et al to clone and characterize *Rpg1* in 2002, which identified RPG1 as a dual kinase protein with a unique combination of two tandem protein kinase domains (Brueggeman *et al.*, 2002).

During the 1989 growing season, a new race of *P. graminis* f. sp. *tritici (Pgt)*, designated as QCCJ, was identified to be virulent on barley cultivars containing *Rpg1* in North Dakota (Roelfs et al., 1991). Wind dissemination of a sexual population of *Pgt* in the Pacific Northwest was assumed as the source of *Pgt* QCCJ in the Great Plains (Kolmer, 2005). More than 18,000

barley accessions from USDA National Small Grains Collection were evaluated to identify resistance sources of QCCJ resistance, and Q21861, an unimproved barley line from the program of the International Maize and Wheat Improvement Center (CMMYT) in Mexico, was determined to be the best source of resistance (Jin et al., 1994a). The QCCJ resistance was mapped to the telomeric region of the long arm of barley chromosome 5H and designated as the *rpg4* locus (Jin et al., 1994b; Borovkova et al., 1995). The *rpg4* gene is an atypical resistance gene as it is recessive and temperature sensitive in nature (Sun et al., 1996; Jin et al., 1994a).

This line Q21861, also confers resistance to the rye stem rust (*Pgs*) isolate 92-MN-90, which can cause severe damage on barley even though it was first isolated from rye. This resistance was determined to be dominant and not temperature dependent and was designated as *Rpg5* (Sun et al., 1996). In a low-resolution mapping population, *Rpg5* was initially mapped to chromosome 5H, which co-segregates with *rpg4* (Sun et al., 1996). Hence, *Rpg5* was considered to be the resistance gene for the rye stem rust isolate *Pgs* 92-MN-90 (Sun et al., 1996). However, Sun and Steffenson showed *rpg4* and *Rpg5* are tightly linked yet distinct genes when analyzing their genetic data (Sun and Steffenson, 2005). Later in 2008, the analysis of a Steptoe (susceptible) x Q21861 high-resolution population also revealed that *rpg4* and *Rpg5* were distinct genes based on genotype and phenotype data (Brueggeman et al., 2008). The *Rpg5* gene was characterized with a high-resolution recombinant population consisting of 5,232 gametes (Sun and Steffenson, 2005). In the SQ recombinant population, *Pgt* QCCJ (*rpg4*) can elicit compatible reactions on recombinants that are resistant to *Pgs* 92-MN-90 (*Rpg5*). However, no reciprocal situation where a recombinant was resistant to *Pgt* QCCJ but susceptible to *Pgs* 92-MN-90 has been found in this population. Thus, it indicates that *Rpg5* together with the tightly linked *rpg4* gene could be required for resistance to *Pgt* pathotype QCCJ. As currently grown

cultivars do not contain the *rpg4/Rpg5* complex, *Pgt* QCCJ is still a potential threat to barley production in northern Great Plains of the United States (Sun and Steffenson, 2005). Another virulent race of wheat stem rust, *Pgt* race TTKSK, was identified in Uganda in 1999, and is virulent on almost all the barley lines in the primary germplasm pool including lines containing *Rpg1* (Steffenson and Jin, 2006). However, the *rpg4* resistance locus previously characterized and cloned provided a high level of resistance against TTKSK (Brueggeman et al., 2008; Steffenson et al., 2009).

Plant Disease Resistance Genes

Within the past few decades, approximately 100 plant *R*-genes from different pathosystem have been cloned mainly via positional cloning techniques, however, more high throughput molecular techniques and tools are now being utilized. These *R*-genes could be grouped into different classes based on their protein domain structure, (Martin et al., 2003). There are two major classes in plant *R*-genes system so far, i.e. NBS-LRR R-genes having an N-terminal nucleotide-binding site (NBS) together with C-terminal leucine rich repeats (LRR), and another class consists of *R*-genes that have at least one serine/threonine protein kinase (S/TPK) domain. *Rpg1* encodes dual S/TPK domains (Brueggeman et al. 2002,) while *Rpg5* encodes an NBS-LRR-S/TPK domain containing protein (Brueggeman et al. 2008).

In many cases, compatible and incompatible reactions of host plants to different races of a pathogen is determined by dominant *R*-genes in the plant and corresponding dominant avirulence (Avr) genes in the pathogen, designated as gene-for gene theory (Flor 1971). The *Avr* genes and their products have been shown to be elicitor molecules that interact with *R* gene products, either directly or indirectly (Mansfield 2009; Stergiopoulos and de Wit 2009). On the other hand, the *R*-genes encode receptor molecules that can detect modification of effector

targeted molecules which are considered virulence targets in the host that are modified by virulence effector proteins secreted by pathogens. There are two models describing the interactions between host and pathogen in nature, designated as “guard” and “decoy” respectively (Van Der Hoom and Kamoun, 2008). Guardees which have functional kinase domain underlying basal resistance system in most cases will be protected by NBS-LRR proteins in the guard model while in the decoy model, decoy protein, which is a mimic of functional kinase domain underlying PAMP-triggered immunity pathway, will work solely to detect the pathogen protein without virulence function. However, a new picture with paired NBS-LRRs is emerging where both proteins are required for conferring resistance against pathogens. An “integrated decoy” model was established based on detailed studies on the *Arabidopsis thaliana* TIR-NLR pair RRS1 and RPS4 and CC-NLR pair RGA4 and RGA5 from rice, which could be applied to other resistance mechanisms where paired NBS-LRRs are required. In the integrated decoy model, direct interactions between effector and R-proteins have been observed and one of the paired NBS-LRR functions as a bait while the other one signals for further defense (Cesari et al. 2014).

Though several *R*-genes belonging to the nucleotide-binding site leucine-rich repeat (NBS-LRR) type have been cloned, little is known about how the interaction of pathogen elicitors with host receptors activates signal transduction cascades. However, the importance of protein kinases and phosphatases in the activation of early resistance defense responses have been shown by phosphatase and protein kinase inhibitor studies in vivo (Nirmala et al 2010).

In 2010, Nirmala et. al. showed that the rapid phosphorylation of the RPG1 protein, within 5 min of exposure to spores from avirulent but not virulent races of stem rust is required for the resistance response (Nirmala et al 2010). It was hypothesized that the effector was present

within the stem rust urediniospore in order to elicit this rapid phosphorylation and required for the urediniospores to germinate. The extensive effort put into characterizing *Rpg1* was warranted because of its remarkable durability. However, the virulence on *Rpg1* containing barley lines by the *Pgt* races QCCJ and more importantly TTKSK, which is considered a major threat to world food security, makes it more urgent to understand the molecular mechanisms underlying *rpg4*/*Rpg5* resistance which is effective against races QCCJ and TTKSK. The atypical recessive and temperature sensitive nature as well as the spatial and temporal interactions occurring between the host (barley) and parasite (wheat stem rust) during the *rpg4/Rpg5* stem rust resistance response and the elucidation of these phenomena will provide critical gaps in our basic understanding of resistance mechanisms.

Gene identification and cloning are required in order to characterize the functional mechanisms underlying the *Rpg* genes of barley. Map-based cloning is still the most effective strategy to identify barley *R*-genes, though the large (>5,000 Mb) repetitive barley genome makes it labor intensive and time consuming. The development of bacterial artificial chromosome (BAC) libraries constructed from the several barley cultivars with the first being the cv. Morex BAC library (Yu et al., 2000) has played a vital role in barley gene cloning, such as stem rust resistance genes *Rpg1* (Brueggeman et al., 2002) and *Rpg5* (Brueggeman et al., 2008). Physical contigs across the rust resistance regions carrying *Rpg1* (Brueggeman et al., 2002) and *rpg4*/*Rpg5* (Druka et al., 2000) have been established with the assistance of the cv. Morex BAC library.

The elucidation of the molecular mechanisms underlying stem rust resistance systems will accelerate the understanding of these stem rust resistance pathway which provide broad resistance to stem rust isolates suggesting that these early recognition mechanisms represent

holdovers of non race specific resistance mechanisms. A better understanding of these modes of resistance, that may include the *rpg4*-mediated resistance mechanism, may lead to better understanding of the interconnected between the early PAMP triggered Immunity responses and the later, higher amplitude effector triggered immunity responses, which result in a higher amplitude programmed cell death response.

Plant Disease Resistance Mechanisms

There are two primary defense mechanisms in plant immune system; one is pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) where the extracellular plasma membrane-localized pattern recognition receptors (PRRs) recognize the PAMPs such as chitin or flagellin and conduct the following signaling cascade, and the other one is effector-triggered immunity (ETI) in which host specific R-proteins recognize the pathogen effector directly or indirectly eliciting further robust hypersensitive response (HR) (Chisholm et al. 2006). In *Arabidopsis*, the best characterized PTI signaling is via FLS2, which is Flagellin Sensitive-2. In this system, the recognition of flagellin initiates FLS2 via LRR, causing phosphorylation of FLS2 and BAK1 (BRII-associated receptor kinase), which in turn elicits MAPK (mitogen-activated protein kinase) signaling cascade (Chinchilla et. al. 2007). During this process, both MAPK (MPK 3) and MAPK (MPK6) are phosphorylated, causing further expression of PTI-related genes (Rodriguez et al. 2010).

RPS5 (predicted nucleotide-binding site and leucine-rich repeats)-dependent resistance against *Pseudomonas syringae* is the best-characterized ETI response in *Arabidopsis thaliana*, in which involves recognition of the bacterial effector protein AvrPphB (Chisholm et al. 2006). PBS1 is a cytoplasmic serine/threonine protein kinase (S/TPK) and cleavage of PBS1 via AvrPphB triggers ETI (Shao et al. 2003). Recent studies suggested that RPS5/PBS1 fits in the

decoy model mentioned previously in which PBS1 is a mimic of another functional S/TPK, BIK1, underlying PTI signaling pathway due to the fact AvrPphB actually cleaves BIK1 without RPS5 presence (Zhang et al. 2010).

Recently, Porter et al. (2012) demonstrated that ADF4, a regulator of cytoskeletal dynamics required in PTI, actually plays a vital role in ETI signaling cascade. In this system, up-regulation of RPS5 was activated by phosphorylation of ADF4 via MPK3/6 involved in basal resistance mechanism. Thus, RPS5 was upregulated in the plant cell before the pathogen injection of the AvrPphB effector, which allows for the elicitation of a more robust host-specific defense reaction when effectors were secreted into cells via pathogen infection. Considering of the involvement of HvADF3 in *rpg4/Rpg5*-mediated resistance system, Rpg5 could be up-regulated in the cells by phosphorylation of ADF3 via PTI signaling cascade prior to pathogen infectio. Elucidation of connection between PTI and ETI could be vital in understanding the mechanism underlying the *rpg4/Rpg5* resistance pathway.

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CHAPTER ONE. 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[1][2]

Abstract

The *rpg4* gene confers recessive resistance to several races of wheat stem rust (*Puccinia graminis* f. sp. *tritici)* and *Rpg5* provides dominant resistance against isolates of the rye stem rust (*Puccinia graminis* f. sp. *secalis*) in barley. The *rpg4* and *Rpg5* genes are tightly linked on chromosome 5H and positional cloning using high-resolution populations clearly separated the genes, unambiguously identifying *Rpg5* but the identity of *rpg4* remained unclear. High-resolution genotyping of critical recombinants at the *rpg4*/*Rpg5* locus, designated here as RMRL (*rpg4*-mediated resistance locus) delimited two distinct yet tightly linked loci required for resistance, designated as RMRL1 and RMRL2. Utilizing virus-induced gene silencing, each gene at RMRL1, *Hv*Rga1 (a nucleotide binding site-leucine rich repeat (NBS-LRR) domain gene),

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[2] The material in this chapter was co-authored by X. Wang, J. Richards, T. Gross, A. Druka, A. Kleinhofs, B. Steffenson, M. Acevedo and R. Brueggeman X.W. performed SNP mapping, developed and performed the Rpg5 gene silencing experiments. J.R. developed the HvRga1 silencing construct and performed all experiments with the construct. T.G. developed the HvAdf3 silencing construct and performed all experiments with the construct. A.D helped in the conception of experimental design and manuscript preparation. A.K. helped in the conception of experimental design and manuscript preparation. B.S. developed the experimental design for stem rust evaluations and performed the analysis and interpretation of phenotyping data. M.A. developed the experimental design for stem rust evaluations and performed the analysis and interpretation of phenotyping data. R.B. developed the experimental design, analyzed and interpreted data. X.W. and R.B. wrote the manuscript.

Rpg5 (an NBS-LRR-protein kinase domain gene) and *Hv*Adf3 (an actin depolymerizing factor-like gene), were individually silenced followed by inoculation with *Pgt* race QCCJ. Silencing each gene changed the reaction type from incompatible to compatible, indicating that all three genes are required for *rpg4*-mediated resistance. This stem rust resistance mechanism in barley follows the emerging theme of unrelated pairs of genetically linked NBS-LRR genes required for specific pathogen recognition and resistance. It also appears that actin cytoskeleton dynamics may play an important role in determining resistance against several races of stem rust in barley.

Introduction

Stem rust epidemics in barley (*Hordeum vulgare*) can be caused by two biotrophic fungal pathogens, *Puccinia graminis* f. sp. *tritici* (*Pgt*), the wheat stem rust fungus, and *Puccinia graminis* f. sp. *secalis* (*Pgs*), the rye stem rust fungus. Prior to the 1950s, wheat stem rust was a devastating disease of barley and wheat in the upper Midwestern United States and Prairie Provinces of Canada until management was achieved by the deployment of genetic resistance. The single dominant *Rpg1* resistance gene has exclusively protected Midwestern barley cultivars against stem rust for nearly 70 years – a remarkable durability for otherwise easily breakable rust resistances (Steffenson, 1992). However, during the 1989 growing season, a new race of *Pgt*, named QCCJ was identified in North Dakota (Roelfs et al., 1991) as being virulent on barley cultivars containing *Rpg1*. The *Rpg1* virulence raised concern that Midwestern barley production would experience the return of stem rust epidemics prompting a search for resistance genes against this pathogen (Jin et al., 1994a).

The barley line Q21861, originally from the International Maize and Wheat Improvement Center (CIMMYT), was identified as highly resistant to stem rust race QCCJ. The recessive and temperature sensitive *Pgt* race QCCJ resistance was mapped to the telomeric region of the long

arm of barley chromosome 5H and designated as the *rpg4* locus (Jin et al., 1994b; Borovkova et al., 1995). Q21861 also carries resistance against the rye stem rust (*Pgs*) isolate 92-MN-90, designated *Rpg5*, which also mapped to chromosome 5H co-segregating with *rpg4* in a low-resolution mapping population (Sun et al., 1996). However, analysis of a Steptoe (susceptible) x Q21861 high-resolution population identified five recombinant lines with *rpg4* and *Rpg5* segregating, indicating that the genes were distinct (Brueggeman et al., 2008).

A current threat to barley and wheat production worldwide and potential threat to production in North America is stem rust race TTKSK (aka Ug99) and its variants. Race TTKSK was first reported from Uganda, Africa in 1999 (Pretorius et al., 2000). It is considered as a major threat to world food security because it is virulent on over 97% of barley cultivars worldwide (Steffenson et al., 2012), including those having *Rpg1* (Steffenson and Jin, 2006) and approximately 70% of the world's wheat cultivars (Singh et al., 2008). Fortunately, the *rpg4* resistance locus previously characterized (Brueggeman et al., 2008) confers a high level of resistance against TTKSK (Steffenson et al., 2009).

Plant *R*-genes, representing innate receptors that identify and impart race-specific resistance against host-specific pathogens, have been cloned from many plant species. Prediction of the protein structures encoded by the >70 cloned R-genes, effective against a wide taxonomical range of pathogens, has shown that most fall into well-defined structural classes with the major class coding for nucleotide-binding site (NBS) and leucine-rich repeat (LRR) domain proteins (Martin et al., 2003). The second major class encodes proteins that contain at least one serine/threonine protein kinase (STPK) domain (Martin et al., 2003).

The NBS-LRR genes make up the largest family of plant disease resistance genes with 159 homologous sequences present in the Arabidopsis genome (Meyers et al., 2003) and 535

present in the rice genome (Zhou et al., 2004). NBS-LRR genes are frequently clustered as complex loci in the plant genomes, typically containing several copies of duplicated genes with high homology one to another as well as including NBS-LRR genes from different gene families that share little or no sequence homology (Leister, 2004; Hulbert et al., 2001). These complex loci commonly confer resistance to specific isolates or races of a pathogen presumably in a gene-for-gene relationship, implying a single dominant gene at the locus conferring resistance (Flor, 1971). However, it has been demonstrated for nine resistances mechanisms in both model plants and agronomically important crops, that two unrelated NBS-LRR genes are required to function together against specific races or isolates of the corresponding pathogen (Sinapidou et al., 2004; Peart et al., 2005; Ashikawa et al., 2008; Birker et al., 2009; Lee et al., 2009; Loutre et al., 2009; Narusuka et al., 2009; Okuyama et al., 2011; Yuan et al., 2011). Typically, the two NBS-LRR genes required for resistance in such systems are genetically linked. The one possible exception could be the tobacco N and NRG1 genes required for TMV resistance but this could simply be due to the lack of genetic linkage data and/or chromosome positions (Peart et al., 2005). The emerging theme of dual NBS-LRR resistance genes operating in concert for pathogen recognition to activate host-cell defense responses appears to be a common feature of plant innate immune systems.

R-proteins are presumed to detect pathogen challenge through direct protein-protein interaction with pathogen avirulence (AVR) proteins. However, direct interaction between R-proteins and their corresponding AVR-proteins have been demonstrated for relatively few interactions, where both the host R-gene and pathogen Avr-gene/s have been identified (Jia et al., 2000; Deslandes et al., 2003; Dodds et al., 2006; Ueda et al., 2006). Some NBS-LRR R-proteins are modular and protein fragments have been shown to be sufficient for initiation of defense

signaling (Eitas and Dangl, 2010). This modular activity in conjunction with the requirement of two or more R-proteins in a resistance complex suggests that pathogen recognition possibly require different combinations of domains from distinct proteins. The domain combinations of interacting R-proteins (i.e. NBS-LRRs and STPKs) in addition to the guard model (Van Der Biezen and Jones, 1998) and NBS-LRR proteins functioning in downstream signaling pathways (Caplan et. al., 2008) may explain why most yeast-two-hybrid experiments fail to detect direct R-protein-Avr protein interactions.

R-gene mediated detection of the pathogen activates signal transduction pathways involving phosphorylation cascades via the STPKs. The STPK domain containing *R*-proteins confer resistance to bacterial and fungal pathogens, and have been identified in a wide range of plant species (Martin et al., 1993; Song et al., 1995; Swiderski and Innes, 2001; Brueggeman et al., 2002; Sun et al., 2004; Brueggeman et al., 2008; Fu et al., 2009). The presence of STPK domains in this major class of R-genes suggests involvement in signaling cascades through phosphorylation activity (Hanks et al., 1988). However, STPK domains have also been shown to interact directly with AVR proteins, demonstrating the ability to recognize pathogen effectors through protein-protein interactions (Tang et. al., 1996). In *Arabidopsis,* resistance against *Pseudomonas syringae* strains that carry *AvrPphB* requires two genes, *RPS5*, a NBS-LRR gene, and *PBS1*, a STPK (Swiderski and Innes, 2001). The RPS5 immune receptor constitutively binds to the PBS1 effector target guarding the protein by detecting *AvrPphB* specific cleavage of PBS1 (Van der Hoon and Kamoun, 2008). The perception of PBS1 cleavage signals RPS5 to activate effector triggered immunity (ETI). Some cytosolic STPKs may serve as decoys and the interactions with the cognate effector triggers ETI but the true STPK effector target may be a protein involved in PAMP triggered immunity (PTI) (Block and Alfano, 2011). It has been

shown that the *AvrPphB* interaction with PBS1 may be a decoy-effector interaction guarded by RPS5 and the real effector target is the BIKI STPK that interacts with the PAMP-recognition receptor FLS2. (Block and Alfano, 2011). The *RPS5*/*PBS1* mediated resistance also has been shown to require an actin depolymerization factor, *AtADF4,* for resistance (Tian et al., 2009).

ADF proteins, in concert with other actin binding proteins, regulate actin filament dynamics leading to cytoskeleton rearrangement. Plant ADFs have been shown to play roles in biotic (Miklis et al., 2007; Tian et al., 2009) and abiotic stress responses (Ouellet et al., 2001), root formation (Thomas and Schiefelbein, 2002) and pollen tube growth (Chen et al., 2003). However, there is little information regarding the molecular mechanisms underlying these dynamic responses to environmental or developmental stimuli. Plant genomes have limited numbers of *Adf* genes as demonstrated by the presence of only 12 *Adf* genes in the sequenced genomes of *Arabidopsis* and rice (Feng et al., 2006). Minimal redundancy suggests that individual *Adfs*, although having concise function, are probably differentially modulated by diverse biotic, abiotic and developmental stimuli, involving phosphorylation and dephosphorylation-dependent signaling.

The *Arabidopsis thaliana* gene, *AtAdf4*, was recently shown to be required for *RPS5/PBS1*-mediated resistance against *Psuedomonas syringae* harboring the effector *AvrPphB* (Tian et al., 2009). Previous research had determined that cytoskeleton reorganization was a characteristic basal resistance or non-host resistance response (Kwon et al., 2008) with limited circumstantial evidence supported its role in specific or *R*-gene mediated resistance. Tian et al., 2009 demonstrated that *AtAdf4* was required for *RPS5*-initiated defense signaling, but was not involved in what is considered as the hallmark event of non-host resistance - cytoskeleton

polarization leading to callose deposition and resistance against pathogen entry at the cell periphery.

Previously we had reported that the *Hv*Adf2 gene at the *rpg4*/*Rpg5* locus was a candidate *rpg4* gene (Brueggeman et al., 2008; Brueggeman et al., 2009), however the research reported here eliminates *Hv*Adf2 as a candidate gene and shows that *rpg4*-mediated resistance is a complex system requiring the concerted action of several genes at the locus. The high-resolution mapping of resistance against *Pgt* races QCCJ identified two distinct, yet tightly linked loci required for wheat stem rust resistance: RMRL1 and RMRL2 (Fig. 1). The loci were genetically delimited to physical regions of ~70 and 220 kbp, respectively (Fig. 2). Post-transcriptional gene silencing using BSMV-VIGS determined that RMRL1 contains three genes required together for *Pgt* race QCCJ resistance, the NBS-LRR gene *Hv*Rga1, the NBS-LRR-STPK gene *Rpg5* and the actin depolymerizing factor-like gene, *Hv*Adf3. Silencing each gene present at RMRL1 resulted in a shift from incompatibility to compatibility in the resistant barley line Q21861. Our data demonstrates that *Pgt* race QCCJ stem rust resistance is conferred by a complex locus requiring the interaction of at least four tightly linked genes and that the system follows the emerging theme of two tightly linked yet unrelated NBS-LRR genes for resistance against a specific pathogen.

Results

Recombinant Analysis

Previous efforts to clone the *rpg4* and *Rpg5* genes resulted in the development of three high-resolution mapping populations (HQ, MD2Q and SQ), representing a total of 5,223 recombinant gametes (Brueggeman et al., 2008). The populations were developed by crossing the stem rust resistant line Q21861, known to carry Rpg1, *rpg4* and *Rpg5*, with three different

susceptible parents (Harrington, MD2 and Steptoe). Positional cloning based on these populations and a cv. Morex barley BAC library (Yu et al., 2000) resulted in the identification of the *Rpg5* gene encoding an NBS-LRR-STPK protein conferring resistance to the *Pgs* isolate 92-MN-90. This positional cloning analysis also identified a candidate *rpg4* gene, *Hv*Adf2, encoding a putative actin depolymerizing factor protein (Brueggeman et al., 2008; Brueggeman et al., 2009). This result was not conclusive because there was genetic data indicating that resistance against *Pgt* races QCCJ and TTKSK also required the *Rpg5* locus, and the SQ population contained a single recombinant (SQ55) that did not corroborate *Hv*Adf2 as the *rpg4* gene (Fig. 1).

Further analysis with new molecular markers of fourteen recombinants within or closely flanking the *rpg4*/*Rpg5* locus, now designated RMRL (*rpg4*-mediated resistance locus), showed that the SQ population segregated for an additional tightly linked locus required for wheat stem rust resistance, now designated RMRL2, that contained a gene that was not functionally polymorphic in the HQ and MD2Q crosses (Fig. 1). Lines defining RMRL (HQ1, HQ9, HQ18, SQ31, SQ41, SQ44, SQ46, SQ47, SQ48 and SQ55) were backcrossed to the parental lines that were not contributing to the genotype/phenotype at the loci. Homozygous plants for RMRL were selected after each round of backcrossing using marker assisted selection (MAS) with the *Rpg5*+ (Rsts1) and *rpg5*- (Psts1) markers (Table 3). The selected BC_2F_3 and BC_3F_3 recombinant lines were infected with *Pgt* races QCCJ and TTKSK and their rust reactions assayed (Table 1). This, in combination with the genotype analysis using SNP and STS markers, allowed identification of two distinct yet tightly linked loci, RMRL1 and RMRL2, required for resistance to *Pgt* races QCCJ and TTKSK (Figs. 1 and 2).

Analysis of the SQ population delimited *Pgt* race QCCJ resistance (*rpg4*-mediated resistance) to the distinct RMRL2, located up to 220 kbp distal of the genetically defined RMRL1 (Fig. 2). Five *Pgt* race QCCJ and TTKSK susceptible SQ recombinant lines SQ31, SQ41, SQ47, SQ48 and SQ55 all had Q21861 (resistant) genotype at the RMRL1 delimited by markers ARD5112 and Rsnp.1, with recombination occurring at, or near to the *Hv*Adf2 gene, and Steptoe (susceptible) genotype distal of the SNP markers Rsnp.1 or Rsnp.2 (Fig. 1). The QCCJ and TTKSK susceptible recombinant line SQ55 had Q21861 genotype extending the farthest distally with recombination occurring distal of the genetic marker Rsnp.2, positioning RMRL2 at least 1 kbp distal of the *Hv*Adf2 gene (Figs. 1 and 2). The *Pgt* race QCCJ resistant lines SQ44 and SQ46 have Q21861 genotype extending distal to Rsnp.3 with recombination and Steptoe genotype proximal of Rsnp.4; thus, Rsnp.4 flanks RMRL2 on the distal side (Figs. 1 and 2). The RMRL2 was defined by the markers Rsnp.4 and Rsnp.2, delimiting an ~ 220 kbp physical region (Fig 2) and contains yet to be identified gene/s required for *rpg4*-mediated resistance designated *Rme1* (*rpg4* modifier element 1). This physical region is present on the cv. Morex bacterial artificial chromosome (BAC) clones 64H24, 259B20, and 543P19 (Fig. 2B).

Phenotyping of the HQ and MD2Q populations with *Pgt* races QCCJ and TTKSK identified resistant recombinant lines (HQ1, HQ18, MD2Q28 and MD2Q29) with Q21861 genotype at RMRL1 and the susceptible parent genotypes at RMRL2, showing that the susceptible parents Harrington and MD2 must contain a *Rme1* allele that is functional for stem rust resistance (Fig. 1). These resistant HQ and MD2Q recombinant lines with susceptible parent genotypes distal of the markers Rsnp.1 and Rsnp.2 indicated that in addition to RMRL2 identified in the SQ population, other gene/s located at RMRL1 are also required for resistance against *Pgt* races QCCJ and TTKSK (Fig. 1). The genotypic and phenotypic analysis of the

recombinant lines from the HQ and MD2Q populations identified recombinants from each population delimiting resistance to *Pgt* races QCCJ and TTKSK (HQ18, HQ9, MD2Q27 and MD2Q28). The RMRL1 region required for wheat stem rust resistance in the HQ and MD2Q populations were identical to the ~70 kbp region delimiting *Rpg5*-mediated resistance against the rye stem rust isolate 92-MN-90 (Fig. 1). The marker R13-STS delimits RMRL1 proximally for both populations and for the MD2Q population the locus is delimited on the distal side by the marker Rsnp.2, but for the HQ population RMRL1 is delimited distally by Rsnp.1 (Figs. 1 and 2). Thus, the smallest region required for resistance to *Pgt* races QCCJ and TTKSK was delimited in the HQ population by the recombinants HQ18 and HQ9 corresponding to an ~70 kbp physical region. Three cosegregating genes (*Hv*Rga1, *Rpg5* and *Hv*Adf3) in the region were considered as candidate genes required for resistance against wheat stem rust races QCCJ and TTKSK (Figs. 1 and 2).

Table 1. Infection types of parental lines and recombinants in response to pathotypes of *Puccinia graminis* f. sp. *tritici* and an isolate of *Puccinia graminis* f. sp. *secalis* at the seedling stage.

	Pathotype				Isolate	
	QCCJ		TTKSK		92-MN-90	
Line	IT-M[a]	G R[b]	IT-M[a]	G R[b]	IT-M[a]	G R[b]
HQ1	0;1	R	0;1	R	0;	R
HQ9	3	S	3	S	3-	S
HQ18	0;1	R	0;1	R	0;	R
MDQ27	3	S	3+	S	3-	S
MDQ28	21	MR	0;	R	0;	R
MDQ29	12	R	0;1	R	0;	R
SQ31	3	S	3	S	0;	R
SQ36	3	S	3-	MS	23-	MR
SQ41	3	S	3	S	0;	R
SQ44	1	R	NA	NA	NA	NA
SQ46	21	MR	NA	NA	NA	NA
SQ47	0;1	R	3-	S	0;	R
SQ48	21	MR	3	S	0;	R
SQ55	3	S	3	S	0;1	R
Q21861	0;1	R	0;1	R	0;	R
Harrington	3	S	3	S	3	S
MD2	3	S	3	S	3	S
Steptoe	3	S	3	S	3	S
SM89010	3	S	3	S	3	S
Q asRpg5	32	S	NA	NA	NA	NA
Q asHvRga1	23	S	NA	NA	NA	NA
Q asHvAdf3	23	S	NA	NA	NA	NA
Q pBs	0;1	R	NA	NA	NA	NA

[a]IT-M is the mode infection type observed based on the 0 to 4 scale of Stakeman et al. (1962).

[b] GR is the general reaction with; R (resistant), MR (moderately resistant), MS (moderately susceptible), S (susceptible), and NA)data not available).

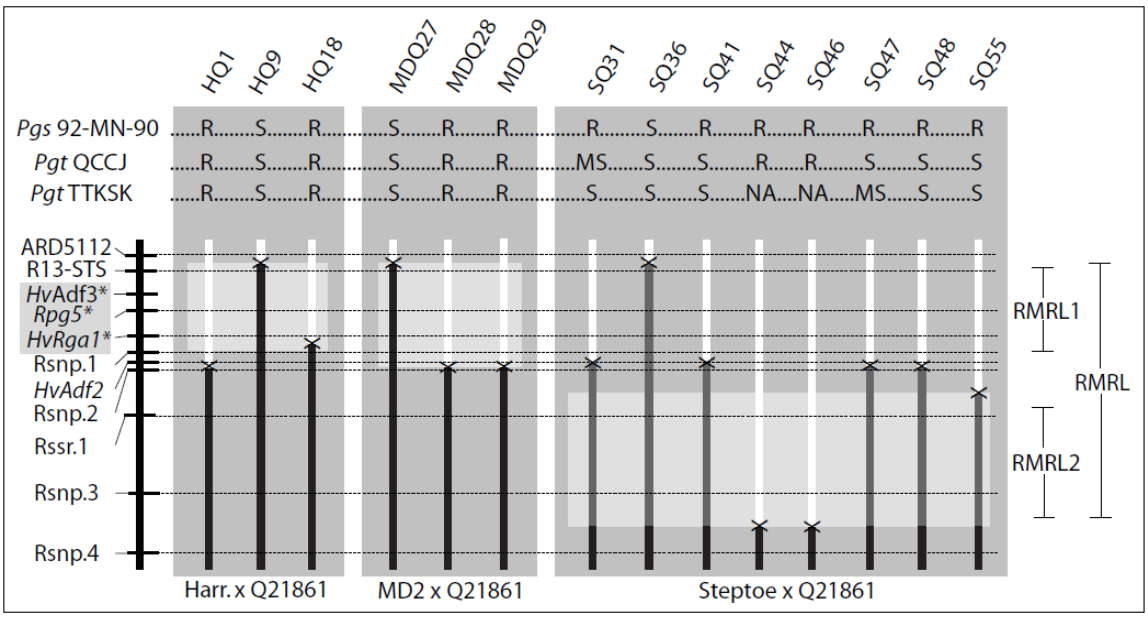

Figure 1. High-resolution analysis of recombinants genetically defining the *rpg4*/*Rpg5* locus. The general reactions of recombinant lines, designated at the top, to wheat stem rust races QCCJ and TTKSK or rye stem rust isolate 92-MN-90 are shown (R is resistant; MR is moderately resistant; MS is moderately susceptible, S is susceptible and NA is data not available). The vertical black bar on the left represents a physical map developed using the cv. Morex BAC sequence (GenBank accession number EU812563) and restriction mapping of unsequenced BACs 64h24 and 259b20. The order of genetic markers and candidate genes required for *rpg4*-mediated resistance are labeled to the left with candidate gene designated with an asterisk. Recombinant designations are given above the vertical bars representative of the genotypes of each recombinant defined by the genetic markers and genes to the left. White depicts Q21861 (resistant) genotype and black or dark grey represents the respective susceptible genotypes with black Xs showing the approximate region of recombination. The internal boxed regions represent the loci delimiting *rpg4*-mediated resistance in each population designated below. The bars to the right depict the regions delimiting the *rpg4*-mediated resistance locus (RMRL) and the RMRL1 and RMRL2 subloci.

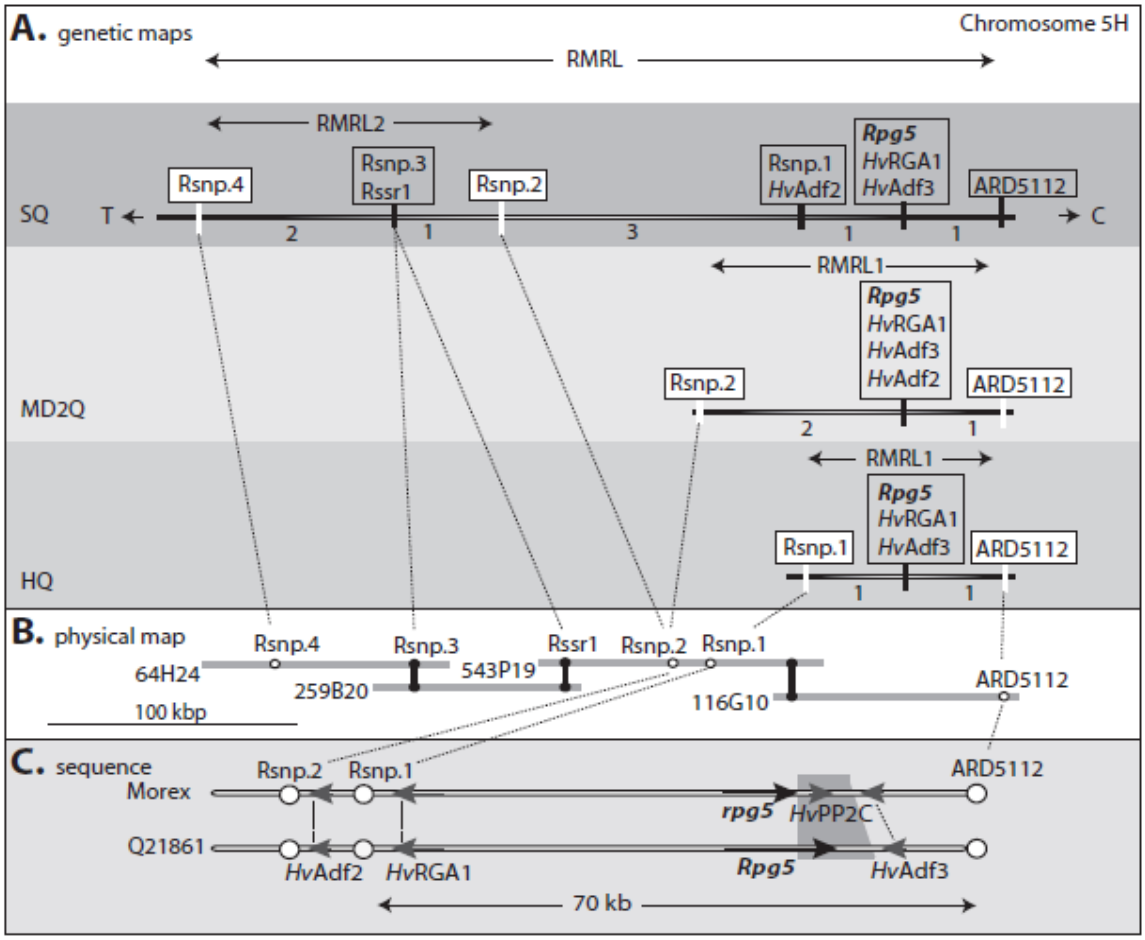

Figure 2. Genetic maps, physical map and sequence annotation of the *rpg4/Rpg5* region. A) The genetic maps from the three individual crosses (Steptoe/Q21861 (SQ), Multidominant 2/Q21861 (MD2Q) and Harrington/Q21861 (HQ)) used in the analysis are shown. The genetic markers and candidate genes are surrounded by boxes. White boxes indicate markers delimiting the regions required for *Pgt* QCCJ resistance (*rpg4*) also represented by the horizontal double-headed arrows. The number of recombinants identified between markers is shown below the horizontal bar representing the genetic region and the T and C indicate direction of the telomere and centromere, respectively. B) The cv. Morex BAC contig clones are represented by horizontal gray bars labeled to the left. Molecular markers delimiting the two regions required for *Pgt* QCCJ (*rpg4*)-mediated resistance are shown as white circles. The black circles indicate positions of annotated genes and molecular markers. C) The sequence and gene annotation of cv. Morex BAC clones (above) and Q21861 sequenced regions (below) are shown as horizontal bars. The region represents the ~ 70 kbp required for *Pgt* race QCCJ (*rpg4*-mediated) resistance delimited in the HQ population. Annotated genes are represented as arrows with gene designations. The scale is shown in kilobase pairs (kb).

Gene Annotation and Protein Domain Structures

The *Rpg5* gene codes for a 1,378-aa (151.6 kDa) protein that contains the NBS, LRR, and STPK predicted protein domains (Brueggeman et al., 2008). Allele analysis showed that all the susceptible parents contain a nonfunctional *Rpg5* allele with either an in frame stop codon leading to a truncated protein or the STPK domain was missing as in susceptible cvs. Harrington, Morex and Steptoe (Fig. 3). In these barley cultivars the STPK domain was replaced by a protein phosphatase 2C gene, designated *Hv*PP2C, presumably by an insertion/deletion event (Figs. 2C and 3)

The *Hv*Rga1 gene codes for a 895-aa (98.5 kDa) protein predicted to contain the typical NBS-LRR resistance-like protein domain structure. A BLAST n sequence homology comparison between the *Hv*Rga1 and *Rpg5* genes using low stringency determined that the two genes, although belonging to similar classes of NBS-LRR domain containing resistance proteins, have very little sequence homology with conservation only present among very short regions of the NBS conserved domains.

Sequence comparison between the Q21861 resistant and MD2 susceptible *Hv*Rga1 alleles showed 100% identity at the amino acid level (Fig. 3). Expression analysis using RT-PCR indicated that the alleles were expressed from both Q21861 and MD2 at similar levels (data not presented). The susceptible cv. Harrington contained an allele that differed from Q21861 by only a single aa (C324R). The susceptible cvs. Morex and Steptoe share identical aa sequence, different from the Q21861 allele by five amino acids S290A, K340N, A445G, M474D and F586L (Fig. 3).

*Hv*Adf3 is predicted to encode a small 144-aa (~15.8 kDa) protein that contains the domains indicative of a functional actin depolymerizing-like protein. The *Hv*Adf3 gene is highly

conserved with 100% aa similarity between the resistant and susceptible barley cultivars examined (Fig 3; Brueggeman et al., 2008). Expression Quantitative Trait Locus (eQTL) analysis of the *Hv*Adf3 transcript levels using the Affymetrix Barley1 Gene Chip suggested that the gene is differentially expressed between the susceptible line SM89010 and the resistant line Q21861 at 24 hours post-inoculation with *Pgt* race TTKSK (Moscou et al., 2011). However, we are currently conducting a thorough analysis of *Hv*Adf3 expression across several time points post-*Pgt* race QCCJ inoculation in resistant and susceptible barley lines using Q-PCR assays.

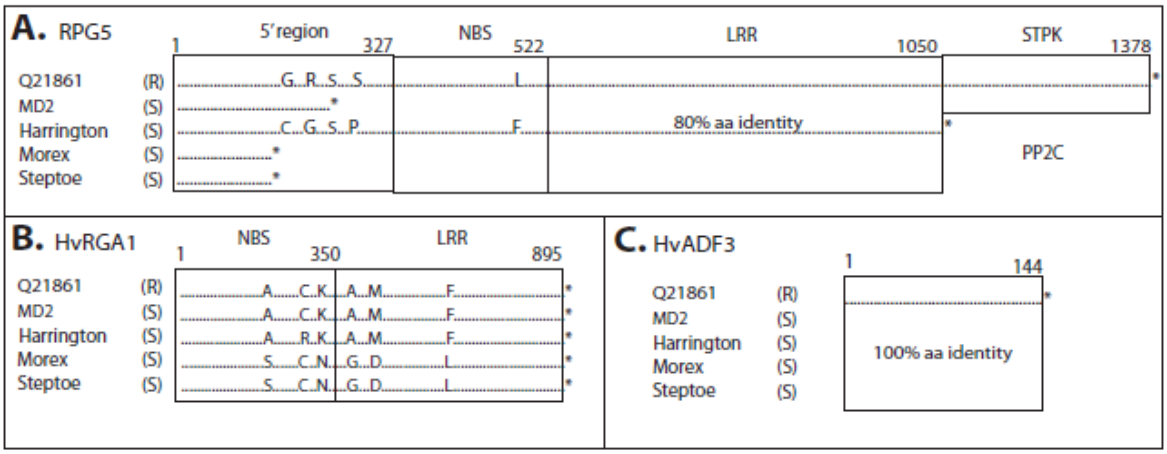

Figure 3. The protein domain structures and allele analyses for *Rpg5*, *Hv*Rga1 and *Hv*Adf3 genes. The boxes represent the different protein domains with the boundaries shown above as amino acid positions. The barley lines used in the allele analysis are labeled to the left with general reaction to stem rust isolates and races shown as R (resistant) or S (susceptible). The differences in aa sequence compared to the Q21861 resistance allele are indicated by single aa letter designations with the relative positions of the polymorphisms shown by amino acid numbering defining the boxes representing the protein domains. The LRR region of the *Rpg5* alleles of Harrington and Q21861 are diverged and shown as 80% amino acid identity. The *Hv*Adf3 gene is shown as 100% amino acid identity for the lines analyzed.

VIGS of Genes at the Rpg5 Locus

To determine which candidate gene/s at RMRL1 are required for resistance against races QCCJ and TTKSK, the *Barley Stripe Mosaic Virus* (BSMV) mediated virus-induced gene silencing (VIGS) system was used to post-transcriptionally silence each gene in barley line Q21861. Following VIGS, the seedlings were inoculated with *Pgt* race QCCJ. The BSMV-VIGS

constructs were developed containing 253, 275 and 291 bp of antisense cDNA from the *Hv*Rga1, *Hv*Adf3 and *Rpg5* genes, respectively (Fig. 4A and 4B; Table 3). The BSMV-VIGS infected plants were inoculated with *Pgt* race QCCJ and after a 14-17 day incubation period, rust infection types were classed either as compatible or incompatible. As controls we included virus inoculated controls (BSMV-MCS) and mock inoculated Q21861. The infection types varied on the plants inoculated with gene specific BSMV constructs, ranging from resistant to susceptible, but we did not observe plants with susceptible infection type on the BSMV-MCS virus inoculated controls or mock inoculated controls. The resistant infection types observed on the as*Rpg5*, as*Hv*Rga1 and as*Hv*Adf3 silenced plants was most probably the result of silencing variability, commonly observed between plants in VIGS experiments. Individual BSMV-as*Rpg5* plants tested by q-PCR ranged from 0 to 79% silencing of *Rpg*5 compared to the virus mock inoculated controls. This result indicated that the silencing among plants in our experiments also had high variability even when virus symptoms and severity were similar. Infection of Q21861 with the BSMV-as*Hv*Rga1, BSMV-as*Rpg5*, BSMV-as*Hv*Adf3 or BSMV-MCS constructs produced similar BSMV symptoms including stunted plants with distorted, mottled and striped leaves indicating systemic BSMV infection. No such symptoms were observed in the mock-inoculated plants. After inoculation with *Pgt* race QCCJ, there was no indication of the rust-specific compatible interaction in Q21861 plants pre-inoculated with BSMV-MCS; however, silencing of any of the three candidate genes using BSMV-as*Hv*Rga1, BSMV-as*Rpg5* and BSMV-as*Hv*Adf3 constructs followed by inoculation with *Pgt* race QCCJ resulted in development of clearly compatible rust specific symptoms in Q21861 and as expected in cv. Steptoe, the QCCJ susceptible control. The compatible symptoms were not observed in the BSMV-MCS inoculated controls and mock inoculated controls (Fig. 4C). It must be noted that

the BSMV-as*Hv*Rga1, -as*Rpg5* and -as*Hv*Adf3 plants exhibited mesothetic reactions typical in barley ranging from 1 to 3+, based on a modified 0-4 scale of Stakman et al., 1962 (Sun and Steffenson, 2005). However, the susceptible infection types (or compatible reactions) ranging from 3- to 3+, characterized by the development of large 2-4 mm pustules surrounded by chlorosis were not observed on BSMV-MCS virus inoculated controls or the virus mock inoculated Q21861 plants. The BSMV-MCS virus inoculated and mock inoculated controls consistently exhibited small (<1mm) pustules surrounded by small chlorotic or necrotic regions defining an incompatible stem rust interaction (Fig. 4C; Table 1). Many of the stem rust pustules that developed within the chlorotic stripes of the BSMV-as*Rpg5*, -as*Hv*Rga1 and –as*Hv*Adf3 infected plants were elongated following the stripe patterns of the virus infection (Fig. 4C). These elongated pustules were scored as compatible interactions (infection type 3+). The BSMV-MCS inoculated Q21861 seedling controls only exhibited incompatible stem rust infection types even in the regions of the leaf with severe virus symptoms.

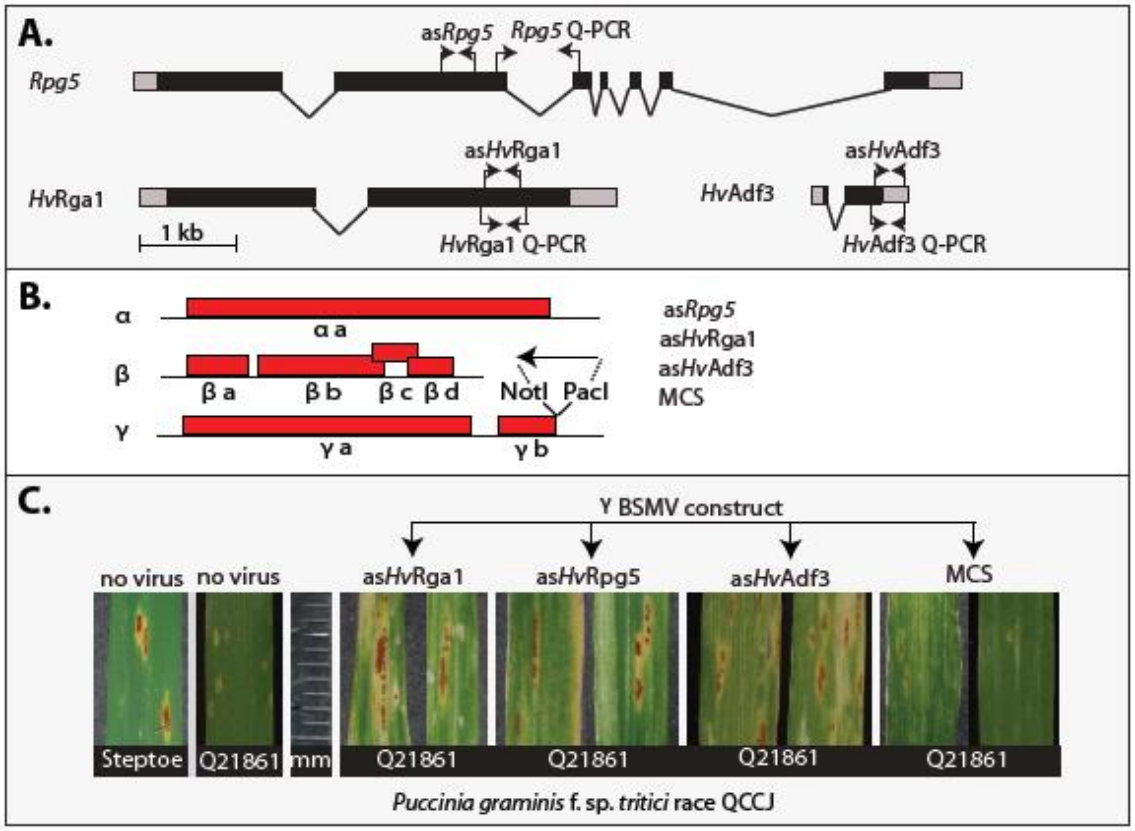

Figure 4. BSMV-VIGS of candidate genes required for race QCCJ resistance. A) Gene structures of *Rpg5*, *Hv*Rga1 and *Hv*Adf3. The black bars represent the exons and intervening thin lines represent the introns. Arrows represent the position of primer pairs used to design BSMV-VIGS antisense constructs (as*Rpg5*, as*Hv*Rga1 and as*Hv*Adf3) and primers used for Q-PCR analysis (*Rpg5*-QPCR, *Hv*Rga1 QPCR and *Hv*Adf3-QPCR). **B)** BSMV tripartite genome showing α, β and γ genomes with the arrow representing the antisence insertions for the as*Rpg5*, as*Hv*Rga1, as*Hv*Adf3 and MCS antisense constructs. **C)** VIGS of the *Rpg5*, *Hv*Rga1 and *Hv*Adf3 genes in *Pgt* race QCCJ resistant barley line Q21861 results in a shift from incompatibility to compatibility with *Pgt* race QCCJ. Barley seedlings were inoculated with *Rpg5*, *Hv*Rga1, HvAdf3 antisense (as) or pBluescript MCS control BSMV-VIGS constructs. Steptoe and Q21861 are susceptible and resistant virus mock-inoculated controls, respectively. All BSMV-VIGS experiments were done in the Q21861 resistant line. The scale in millimeters is shown in the lane labeled mm.

Transcript Analysis

The q-PCR analysis determined that the BSMV-*asRpg5*, BSMV-as*Hv*Rga1 and BSMV-as*Hv*Adf3 inoculated plants had significantly reduced levels of the corresponding mRNA transcript averaging 60%, 39% and 54% decrease in relative expression as compared to the BSMV-MCS virus inoculated controls, respectively (Table 2). The BSMV-MCS controls were

subjected to the same regimen of treatments as the gene specific constructs with the only differences being the absence of the gene specific antisense sequence. The gene specific BSMV constructs showed no significant effect on the relative expression levels of the genes not targeted for silencing demonstrating that the virus infection with the constructs used in these experiments had no effect on the expression of the other genes at RMRL1 (Table 2). Analysis using available barley sequencing data, NCBI non redundant nucleotide database; NCBI EST (Hordeum) and HarvEST; Barley 1.83 Assembly 36 (http://www.harvest-web.org) determined that the LRR regions which were targeted by the VIGS silencing constructs for the *Rpg5* and *Hv*Rga1 genes identified one gene family member for *Rpg5* with 73% sequence homology (Gen Bank accession nos. AK370199.1) and no closely related genes were identified in barley with the *Hv*Rga1 sequence. These data were supported by BAC hybridization experiments and both Southern and Northern blot analysis. An *Hv*Rga1 NBS-LRR specific probe was hybridized to Southern blots producing a single band (Fig. 5) suggesting it does not belong to a gene family. A Rpg5 NBS-LRR specific probe was also hybridized to Northern and Southern blots also producing single bands (Fig. 5). Additionally, BAC hybridization analysis revealed that *Rpg5* belongs to a gene family containing two genes, *Rpg5* and a closely related gene encoding a S/TPK. The S/TPK gene with high homology to the RPG5 protein kinase (89% nucleic acid identity) does not appear to be connected to an NBS-LRR region with homology to the *Rpg5* LRR domain that was targeted for silencing (Fig. 4)., These data suggest that the BSMV-VIGS constructs should be specifically silencing *Rpg5* and *Hv*Rga1.

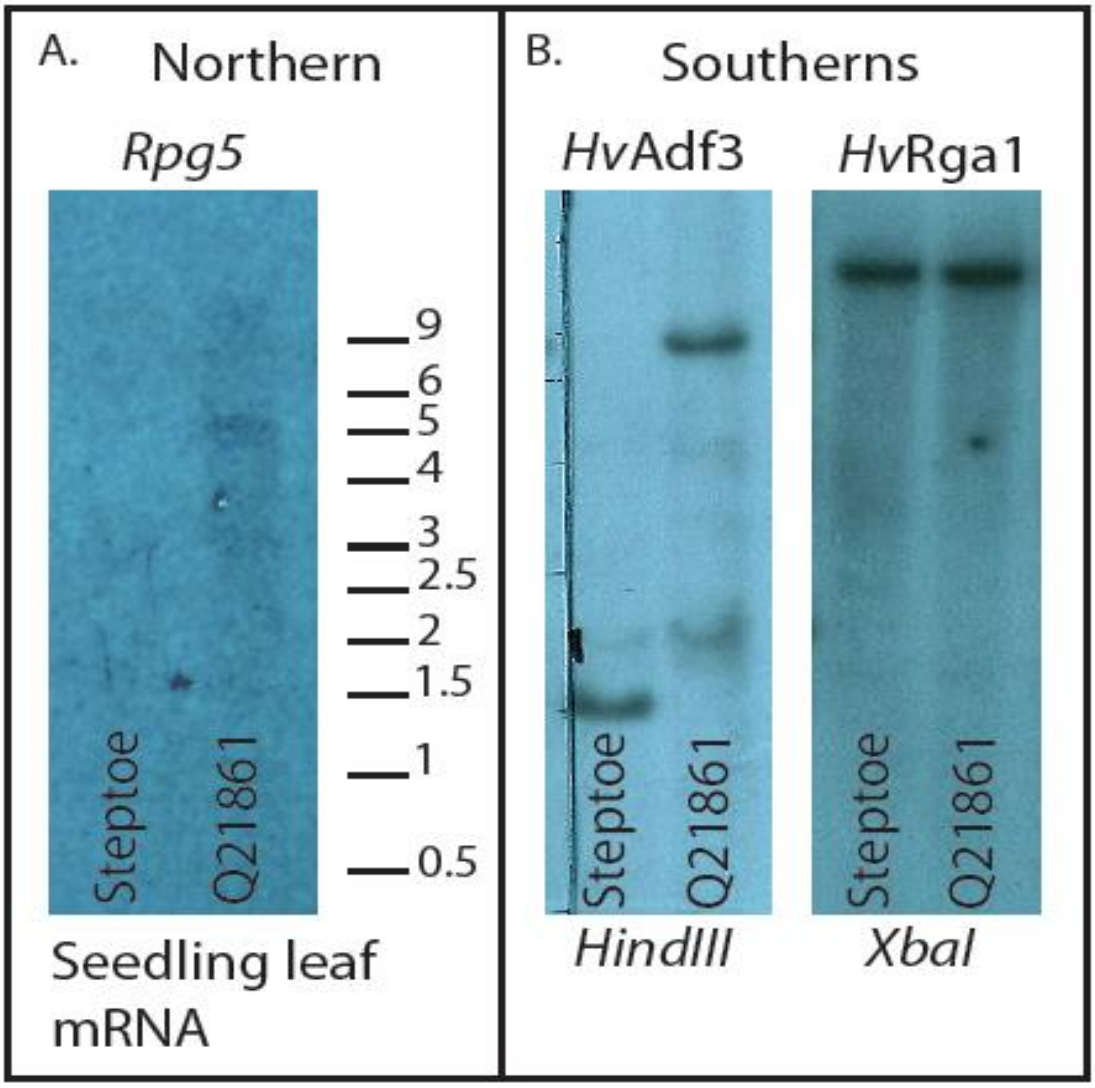

Figure 5. Northern and Southern blots for *Rpg5*, *Hv*Adf3 and *Hv*Rga1. **A)** Northern blot showing a single band specifically hybridizing to an approximately 5 kb mRNA in barley line Q21861. The approximate positions of RNA molecular markers are shown on the right. **B)** Southern blots showing specific hybridization of *Hv*Adf3 and *Hv*Rga1 probes to total genomic DNA from the barley lines Steptoe and Q21861.

Analysis using available barley sequencing data and Southern analysis determined that *Hv*Adf3 is a member of a family of Adf genes, which includes the three Adf genes (*Hv*Adf1, *Hv*Adf2 and *Hv*Adf3), present at RMRL. The *Hv*Adf2 is the most closely related to *Hv*Adf3 (86 % nucleic acid homology) and *Hv*Adf1 has 80% homology to *Hv*Adf2, but is diverged from *Hv*Adf3 with BLASTn analysis showing no significant similarity between *Hv*Adf1 and *Hv*Adf3.

We designed the BSMV-as*Hv*Adf3 construct to target the 3' UTR, a region of *Hv*Adf3 that is completely diverged from *Hv*Adf2 and *Hv*Adf1, thus we are confident that the BSMV-VIGS construct is specifically silencing only the targeted gene.

Table 2. QPCR analysis of *Rpg5*, *HvRga1* and *HvAdf3* gene expression after silencing by BSMV-VIGS shown as relative expression levels compared to the BSMV-MCS virus inoculated control.

	BSMV Construct								
	as*Rpg5*			**as*Hv*Rga1**			**as*Hv*Adf3**		
Gene	**Re[a]**	**SD[b]**	**MED[c]**	**Re[a]**	**SD[b]**	**MED[c]**	**Re[a]**	**SD[b]**	**MED[c]**
Rpg5	40*	7	-60%	100	18	0%	98	37	-2%
*Hv*Rga1	104	25	4%	61*	43	-39%	116	3	16%
*Hv*Adf3	122	19	22%	105	29	5%	46*	17	-54%

[a] Relative expression (RE) levels are a percent of the BSMV-MCS virus control expression levels normalized against the barley ubiquitin gene.
[b] Standard deviation (SD) was calculated from values obtained from three biological replicates.
[c] The mean expression difference (MED) is in comparison to the expression levels in the BSMV-MCS controls.
* Values are significantly different at □=.01 using chi-square analysis.

Discussion

Research of stem rust resistance genes in barley has resulted in the cloning and partial characterization of *Rpg1* from the cv. Morex (Brueggeman et al., 2002; Kleinhofs et. a., 2009) and *Rpg5* from barley line Q21861 (Brueggeman et al., 2008). We previously reported a genetically defined interval delimiting a small physical region of ~1 kbp containing only a single gene (*Hv*Adf2) believed to be the candidate *rpg4* gene (Brueggeman et al., 2008; Brueggeman et al., 2009). The *Hv*Adf2 gene was identified by virtue of somewhat ambiguous recombinant analysis that either included or eliminated resistance to *Pgt* race QCCJ (Brueggeman et al.,

2008). However, using backcrossing experiments described in this manuscript, we eliminated *Hv*Adf2 as a *rpg4* candidate gene, and more importantly identified two distinct yet tightly linked loci that are required for *rpg4*-mediated resistance against *Pgt* races QCCJ and TTKSK. The first region (RMRL2) is up to 220 kbp distal to the *Hv*Adf2 gene, and was detected only in the SQ population. The second region (RMRL1) detected in the HQ and MD2Q populations lies up to 70 kbp proximal to *Hv*Adf2 gene (Figs. 1 and 2). These genetic data eliminating *Hv*Adf2 as the candidate *rpg4* gene also were confirmed by BSMV-VIGS analysis showing that silencing of the *Hv*Adf2 gene had no effect on the *Pgt* race QCCJ incompatible infection type on line Q21861 (Chai, 2011).

The genetic analysis indicated that RMRL2, located distal of the Rsnp.2 marker, only segregated for resistance in the SQ population. This suggests that the gene required for *rpg4*-mediated resistance at RMRL2, designated *Rme1*, is a functional allele from Q21861 and non-functional from Steptoe. The resistant HQ and MD2Q recombinant lines HQ1, HQ18, MD2Q28 and MD2Q29 contain Q21861 genotype at RMRL1 and susceptible genotype at RMRL2, suggesting that Harrington and MD2 are susceptible due to a non-functional gene at RMRL1, but contain functional *Rme1* alleles that cooperate with the genes at RMRL1 to provide resistance against *Pgt* races QCCJ and TTKSK. The *Pgt* susceptible parent Harrington does not contain a functional *Rpg5* allele (Fig. 3), and the resistant HQ18 recombinant combines susceptible cv. Harrington genotype from Rsnp.2, including *Hv*Adf2, and distal, with Q21861 genotype at RMRL1, indicating that Harrington is susceptible due to the lack of a functional gene at RMRL1 (Fig. 1).

The smallest RMRL1 interval required for race QCCJ resistance, defined by the HQ recombinant lines HQ18 and HQ9, contains three candidate genes, *Hv*Rga1, *Rpg5* and *Hv*Adf3

(Figs. 1 and 2). The *Rpg5* gene was considered the most likely candidate gene required for resistance to *Pgt* races QCCJ and TTKSK at RMRL1 because it confers resistance to rye stem rust isolate 92-MN-90 (Brueggeman et al., 2008). Analysis of *Rpg5* alleles from multiple *Pgt* races QCCJ and TTKSK resistant and susceptible barley lines showed that a predicted functional allele corresponded perfectly with resistance (data not presented). The other two genes in the region, *Hv*Rga1 and *Hv*Adf3, were not considered as good candidates required for *rpg4*-mediated resistance because allele sequences were highly conserved among cultivars resistant and susceptible to *Pgt* races QCCJ and TTKSK with respect to amino acid identity. Resistant and susceptible *Hv*Rga1 alleles were 100% identical or had a few minor amino acid substitutions and all *Hv*Adf3 resistant and susceptible alleles had 100% amino acid conservation (Fig. 3; Brueggeman et al., 2008). However, all three genes present at the locus contain protein domains implicated in defense responses and could have differential transcription regulation. Since no recombinant lines were identified separating these genes in a population representing 5,232 recombination events, the function of each gene was examined independently using post-transcriptional gene silencing.

The BSMV-VIGS system is an important tool that can be utilized to test the function of genes by post-transcriptional gene silencing in barley and wheat (Hein et al., 2005; Holzberg et al., 2002; Scofield et al., 2005). We utilized VIGS to silence each gene at RMRL1 to determine which gene/s are required for *rpg4*-mediated resistance against *Pgt* race QCCJ. Based on infection type assays with *Pgt* race QCCJ and the corresponding Q-PCR data, it was unexpectedly determined that *Rpg5, Hv*Rga1 and *Hv*Adf3 are all required for *rpg4*-mediated resistance. These data determined that *rpg4*-mediated resistance is not determined by a single

gene but requires concerted interaction of three genes at RMRL1 and at least one additional gene at RMRL2 (Fig. 2).

The *Rpg5* gene is a unique disease resistance gene containing the R-protein domains, NBS-LRR and STPK, typically present in two separate proteins representing two of the major classes of R-genes (Brueggeman et al., 2008). However, the recent cloning of the wheat tan spot susceptibility gene *Tsn1* has revealed a dominant STPK-NBS-LRR susceptibility factor which may act in a reverse gene-for-gene interaction with the necrotrophic fungal pathogen *Stagonospora nodorum* (Faris et al., 2010). The *Hv*Rga1 gene located ~20 kbp distal of *Rpg5* with inverted gene orientation is predicted to encode a typical NBS-LRR domain R-protein (Figs. 2C and 3B). Silencing of either *Rpg5* or *Hv*Rga1 resulted in similar compatible (susceptible) phenotypes (Fig. 4C and Table 1) indicating that neither the *Rpg5* or *Hv*Rga1 NBS-LRR domain containing R-genes alone are capable of eliciting the resistance reaction. Thus, it appears that both genes are required together for *rpg4*-mediated resistance against *Pgt* race QCCJ. This research demonstrates that resistance to stem rust race QCCJ, presumed to harbor a specific *rpg4-AVR* gene, requires two unrelated NBS-LRR domain genes for pathogen recognition or to elicit the race specific resistance response. Recent data has suggested that some NBS-LRR genes are modular and the discrete modules or protein fragments are sufficient to initiate defense signaling (Eitas and Dangl, 2010). This suggests that NBS-LRR proteins that act coincident in defense complexes may interact in different combinations of domain structure to recognize unique effector molecules or to trigger different resistance pathways. The RPG5 and *Hv*RGA1 proteins may interact forming heterodimers with both proteins required for the recognition of the wheat stem rust avirulence genes encoded by *Pgt* races QCCJ and TTKSK. However, the possibility exists that one of the two NBS-LRR domain containing genes functions

downstream of the other possibly guarding a virulence target or is involved in down stream signaling.

The predicted RPG5 protein contains an STPK domain implicating its involvement in a signaling pathway that may be activated upon *Rpg5*/*Hv*Rga1 pathogen recognition. The recognition may trigger phosphorylation of the STPK domain and provide the initial step in a phosphorylation-signaling cascade resulting in the resistance response. Recent research has shown that the RPG1 stem rust resistance protein, containing a functional STPK domain similar to but not closely related to the *Rpg5* STPK, undergoes a rapid phosphorylation in response to incompatible *Pgt* races. The phosphorylation is specific to the resistance response suggesting that it may be the initial signaling event in the stem rust resistance mechanism (Nirmala et al., 2010). Alternatively, the STPK domain may act as an effector target that is guarded by the NBS-LRR protein domains. Studies with RIN4, PBS1 and Pto show that NBS-LRR guard proteins may constitutively bind to the effector targets, which are STPK proteins in the case of PBS1 and Pto. In the *Rpg5* system it is possible that the NBS-LRR guard and STPK guardee are encoded by a single gene and present in a single protein.

The *HvAdf3* requirement for resistance was surprising because it was originally presumed that *Rpg5* would be the gene at the locus required for resistance. Four genes, including the gene present at RMRL2, required for resistance may raise concerns about the specificity of our silencing experiments. However, after three replications of the VIGS experiments with the three gene specific constructs and the control virus the results were consistently reproduced determining that the specific silencing of each gene had an effect in converting incompatible to compatible reactions between the pathogen, *Pgt* race QCCJ, and the normally resistant barley line Q21861. Recent research has determined that BSMV infection affects susceptibility to

fungal pathogens in wheat and perhaps barley as well (Tufan et al., 2011). Our experiments were performed with a proper BSMV-VIGS control vector (BSMV-MCS), which did not show any affect on the incompatible interactions between *Pgt* race QCCJ and the resistant line Q21861. All of our silencing comparisons were also made as a percent of gene expression compared to the BSMV-MCS control and it was shown that the gene specific constructs were specifically silencing the targeted gene. Thus, we are confident that the shift from resistance to susceptibility was a specific response to the silencing of the targeted genes and not due to the BSMV infection.

The requirement of the actin depolymerizing factor for resistance is intriguing and we would like to determine if the *Hv*Adf3 gene is required for this specific resistance reaction or if it is required for many different resistance pathways and is a component of non-race specific resistance. Experiments are underway to test the specificity of the *Hv*Adf3 silencing response to other stem rust races and pathogen species. Tian et al. (2009) have shown that the *Arabidopsis AtAdf4* gene is required for the *RPS5*/*PBS1* resistance reaction against *P. syringae* harboring *AvrPphB*, setting precedence for the requirement of NBS-LRR, STPK and ADF protein domains together for race specific resistance against a pathogen harboring a specific AVR protein. Interestingly, the Arabidopsis RPS5 NBS-LRR domain (Genbank accession nos. AEE28851.1) shares 23% amino acid identity and 40% similarity with the RPG5 NBS-LRR domain (Genbank accession nos. ACG68417.1) and no significant similarity with the NBS-LRR domain of *Hv*Rga1 (Genbank accession nos. EU878778.1). The PBS1 STPK (accession nos. AED91858.1) also shows 53% amino acid identity and 66% similarity to the RPG5 STPK (accession nos. ACG68417.1). The actin depolymerizing factor-like gene, *Hv*Adf3, shown to be required for resistance in this study also has 52% amino acid identity and 76% similarity with *At*Adf4 (AT5G59890). We suspect that the *Rpg5*/*Hv*Rga1/*Hv*Adf3 mediated stem rust resistance in

barley may be functionally similar to the RPS5/PBS1/*At*ADF4 mediated *P. Syringae* resistance in Arabidopsis and are utilizing the Arabidopsis model for future functional analysis research on stem rust resistance in barley.

A recent study using eQTL analysis of a SM89010/Q21861 double haploid population indicated that the SM89010 susceptible allele of the *Hv*Adf3 gene is up regulated and the differential regulation is in response to infection by race TTKSK (Moscou et al., 2011). The data presented from the eQTL study suggested that up regulation of *Hv*Adf3 may determine susceptibility and *Hv*Adf3 may function as a dominant susceptibility factor that potentially determines the recessive nature of *rpg4*-mediated resistance. The results presented here are contradictory to the hypothesis put forth by Moscou et al., 2011 because we determined that silencing of *Hv*Adf3 resulted in a shift from resistance to susceptibility in the line Q21861. This result suggests that the ADF protein is required for resistance similar to that reported for the RPS5/*At*ADF4 resistance mechanism in *Arabidopsis* against *P. syringae* (Tian et al., 2009).

We have demonstrated that a complex genetic system consisting of at least four genes at two tightly linked loci interact to confer *rpg4*-mediated *Pgt* race QCCJ resistance. The high-resolution mapping determined that resistance to *Pgt* race TTKSK, previously mapped to the region in a low resolution population (Steffenson et al., 2009), maps to the same tightly linked genetic intervals, RMRL1 and RMRL2, identified with *Pgt* race QCCJ, indicating that the same host resistance genes in the three populations analyzed provide resistance to both pathogen races. This research characterizing RMRL1 and RMRL2 was performed using *Pgt* race QCCJ because of the limitations on the use of race TTKSK. However, it is still important to individualy test the requirment of the *Rpg5, Hv*Rga1 and *Hv*Adf3 genes in race TTKSK resistance and this research is underway.

The identification and validation of the *rpg4* gene has eluded our efforts, but now we are beginning to unravel its complexities. The new information presented here begins to define the *rpg4*-mediated resistance locus (RMRL) and will be utilized to identify and validate the remaining *Rme1* gene that is still unknown. We delimited the RMRL2 locus and generated low pass sequence of the ~220 kb region. Three candidate *Rme1* genes have been identified including a heat shock protein 70 (*Hv*Hsp70), a zinc finger SEC14 protein (*Hv*ZF-SEC14) and a third actin depolymerization like protein (*Hv*Adf1). We are currently generating complete sequence of the region by ion torrent sequencing technology and validation of the candidate genes is underway.

Recent research on stem rust resistance in barley has raised many questions, but the ones we are most interested in answering are why is *rpg4*-mediated resistance recessive and temperature sensitive and is the resistance an early response or pre-haustorial form of resistance, similar to what is expected in a non-host resistance mechanism? We have strong genetic evidence showing that the recessive nature of rpg4-mediated resistance against the wheat stem rust pathogens is determined by the presence of a *Hv*PP2C gene in place of the STPK domain of the majority of the *Pgt* races QCCJ and TTKSK susceptible barley lines. Crosses between Q21861 and these susceptible genotypes resulted in susceptible F_1 progeny whereas crosses between Q21861 and susceptible genotypes containing a nonfunctional *rpg5* allele with the *Hv*PP2C gene absent results in resistant F_1 progeny (Data not shown). Thus, the *Rpg5* gene in the absence of the HvPP2C protein and presence of a functional *Rme1* allele appears to behave as a dominant R-gene mediating wheat stem rust resistance otherwise considered *rpg4*-mediated resistance. The genetic data suggests that the *Hv*PP2C may act as a dominant susceptibility factor, a possible hypothesis is the PP2C activity suppresses the resistance response mediated by the RPG5 protein kinase similar to the Rice Xa21 protein kinase mediated resistance being

suppressed by the XB15 protein Phosphatase 2C gene (Park et al., 2008). Further research and validation of the *Hv*PP2C gene is required to elucidate this complex genetic interaction, but this research may answer the question of *rpg4*-mediated recessive resistance. Perhaps the other questions will also be answered once the remaining *Rme1* gene required for wheat stem rust resistance is characterized and the temporal and spatial occurrence of the host-pathogen interactions are elucidated.

Materials and Methods

Genetic and Physical Mapping

The high-resolution genetic map of RMRL was constructed from the progeny of the crosses Steptoe/Q21861, Harrington/Q21861 and MD2/Q21861 as described in Brueggeman et al. (2008). Fourteen lines were identified with recombinations occurring between the SNP marker Rsnp.4 and RFLP marker ARD5112. In this study, these fourteen recombinants were genotyped at RMRL using SNP and SSR markers (Figs. 1 and 2) developed utilizing sequence generated from the cv. Morex BAC contig spanning the *rpg4*/*Rpg5* region (Brueggeman et. al., 2008; GenBank accession number EU812563).

Molecular Markers

The SNP and STS markers were developed using predicted genes or low copy regions identified from the cv. Morex BAC sequences as described in Druka et al., (2000). The alleles were sequenced from the parental lines using the primers described in Table 3. Amplifications were performed in a Mastercycler pro programmable thermocycler (Eppendorf, Hauppauge, NY, USA) at 95°C for 4 min, followed by 35 cycles of 95°C for 30 sec, 62°C for 1 min, and 72°C for 1 min; followed by 72°C for 5 min. The PCR amplification reactions contained ~100ng of genomic DNA, 0.2mM dNTP mix, 30 pmol of each forward and reverse gene specific primers

(Table 3), 1.5 µl of RedTaq DNA polymerase (Sigma, St. Louis, MO, USA), and 3 µl of 10X RedTaq reaction buffer in 30 µl volume. The physical locations of the primers used for each marker are shown in Table 3 relative to the cv. Morex BAC sequence or Q21861 genomic sequence from the RMRL (GenBank accession number EU812563 or EU878778, respectively) or the low copy HindIII subclone sequences from BAC 64H24.

Table 3. Primer sequences used for marker development, VIGS constructs and Q-PCR

Designation	primer	sequence	location	amplicon
asRpg5	Rpg5-NF2	TATGCGGCCGCAGGATTGCCTGCCCTCACTGCTCTC	27,925 *	291 [c]
	Rpg5-PR2	TATTTAATTAAGACTCTGTGCCAGCAGCACCTGCAC	28,215 *	
Rpg5 Q-PCR	RpgQ-F6	AGATGCACCTATCTGCATCGAGGAC	28,129 *	193 [c]
	RpgQ-R6	ATGTCGAGCCTGAGACTACTGACAC	29,144 *	
asHvRga1	Rga1-PF1	TATTTAATTAACAAGATGAAGCTATGCCCTACCTTG	9,121 *	253 [c]
	Rga1-NR1	TATGCGGCCGCTGCAATTTCCACTGTTGCTTGGCAC	8,868 *	
HvRga1 Q-PCR	R4-JR-F8	TTATGCCTTGTGGGCAGCAAAGGA	9,163 *	372 [c]
	R4-JR-R8	TCTTGGCCGTGCACAAGCAAAGATG	8,793 *	
asHvAdf3	Adf3-PF2	ATATTAATTAACTACGACCTGGACTTCGTGTCGGAG	44,282 *	275 [c]
	Adf3-NR2	ATAGCGGCCGCCGATCCCATGCGCAGGTATGCCAAG	44,007 *	
HvAdf3 Q-PCR	Adf3-F2	CCAACCTCCGGATCCATCAACAATG	44,609 *	355 [c]
	Adf3-R3	AGTCCTCCGACACGAAGTCCAGGTC	44,254 *	
Rsts1	LRK-F1	GGTGGATCGAAGAGAATGGAACTGC	28,210 *	1,046 [g]
	LRK-R1	GCAACCTTCATTCTGACAGACCATG	29,245 *	
Psts1	RpgQ-F6	AGATGCACCTATCTGCATCGAGGAC	155,877 [#]	841 [g]
	PP2C-R2	CCCGAGGTTTGCCGATGAAGAGAGTC	156,717 [#]	
Rsnp.1	R4-F22	CGGATATAGGGAGAAGGGTTTGATG	106,177 [#]	922 [g]
	R4-R22	GTCGTCCATCTTGTACACCACGAAC	105,256 [#]	
Rsnp.2	R4-F25	GCTAGCTTTGTGCCGTGCATGTATC	104,731 [#]	1,053 [g]
	R4-R25	AGATTCCGAGGGACATACATGCAAG	103,679 [#]	
Rsnp.3	Rsnp3-F1	CTACGAGCATATGCACCCTATAGTC	615	867 [g]
	Rsnp3-R1	ATCCTGTTGCAAATGCACCCTGGAG	1,481	
Rsnp.4	Rsnp4-F1	ACCCGGACATCGGCCATGTTTCATC	24	536 [g]
	Rsnp4-R1	TAAGATCCCATACAAGTCTGCCCG	559	
R13 STS	A12-F13	CGCCCGACGAAAGAGAACGACAATG	55,016 *	1336 [g]
	A12-R13	GGGCCACCGACACTGTAGCACTC	56,350 *	
Rssr1	RB_SSR1-F1	CACATCCACCCATGGTTGTTGAGAG	59,853 *	304 [g]
	RB_SSR1-R1	CTTCACTGGTACCAGTTCGACCGAG	60,155 *	

[*] the location is based on the Q21861 sequence (Gen Bank Accession number EU878778).
[#] the location is based on the Morex sequence (Gen Bank Accession number EU812563).

[c] The amplicon size is given for cDNA.
[g] The amplicon size is given for genomic DNA.

Disease Phenotyping

The recombinant lines and the parents were inoculated with *Pgt* race QCCJ according to previously established methods (Steffenson et al., 2009). Infection types were assessed 12 to 14 days post-inoculation using a 0-4 scale modified from the one developed for wheat by Stakman

et al. 1962 (Sun and Steffenson, 2005). The IT data was simplified in Table 1 to show the most common ITs (i.e. the IT mode) and the general reaction. Classification of recombinant lines into resistant and susceptible categories was based on the reaction of the parents to *Pgt* races QCCJ and TTKSK and *Pgs* isolate 92-MN-90. The recombinant lines were analyzed several times with *Pgt* race QCCJ at the University of Minnesota and North Dakota State University (Table 1). Plants were grown and assayed for their disease reaction as previously described (Brueggeman et al., 2008). Infection type analysis using *Pgt* race TTKSK at the seedling stage was performed in the Biosecurity level-3 (BSL-3) facility in St Paul, MN.

BSMV VIGS

BSMV-VIGS constructs were developed using the BSMV γRNA-based vector previously described by Hein et al. (2005). Construction of the *Hv*Rga1, *Rpg5* and *Hv*Adf3 gene specific constructs was performed by amplifying 253-291 bp cDNA fragments from the *Hv*Rga1, *Hv*Adf3 and *Rpg5* cDNAs (GenBank accession number EU878778) using gene specific primers with 5 prime terminal *Not*I or *Pac*I adaptor sequences in the combinations shown in Table 3. Constructs, plant growth and infection protocols were performed as described in Brueggeman et al. (2008).

Quantitative Real-Time PCR

Tissue samples were taken from the different virus infected plants and controls at the time of fungal inoculation 14-17 days post-virus inoculation. The three biological replicates were bulks of three random gene specific BSMV infected plants, virus inoculated BSMV-pBs control plants or mock inoculated control plants. Total RNA was extracted using a modified Trizol extraction method as described in Brueggeman et al. (2008). To generate first strand cDNA, 1 μg of total RNA was annealed with 2 μm oligo-dT primer in a 20 μl reaction and converted to single

stranded cDNA using Reverse Transcription System (Promega, Madison, WI, USA) following the manufacturer's recommended procedure. The 20 µl RT-PCR reactions were diluted with 40 µl of H_2O to 60 µl and 5 µl aliquots of the diluted cDNA template were used as template for amplification with gene specific primers using iQ SYBR Green Supermix (BIO-RAD, Hercules, CA, USA) in 25 µl reactions following the manufactures standard procedure. Primer sequences for *Rpg5*, *Hv*Adf3 and *Hv*Rga1 were designed to specifically amplify 193, 355 and 372 bp fragments of cDNA, respectively. The QPCR amplicons were outside the regions targeted by the BSMV constructs to avoid amplification from the virus encoded antisense cDNA fragments. The sequences of gene specific primers used for Q-PCR analysis are given in Table 3. The barley *Ubi1* gene primer sequences are described in Rostoks et al. (2003). Q-PCR was performed in a CFX96 Real-Time System thermocycler (BIO-RAD, Hercules, CA, USA) using the following parameters; 2 min at 50°C and hot start for 15 min at 95°C followed by 40 cycles, each consisting of 15 s denaturing at 95°C, 20 s annealing at 60°C, 30 s primer extension at 72°C and 15 s data acquisition at a temperature specific for each PCR fragment. Quantification of the targeted gene mRNA transcript (*Rpg5*, *Hv*Rga1 and *Hv*Adf3) and the reference gene (*Ubi1*) was determined using standard curves generated using external cDNA standards covering 8 magnitudes (10pg down to 1ag). Three experimental replicates of Q-PCR experiments were performed and expression levels were normalized to the mean expression level of ubiquitin for each biological replicate. Normalized percent values of the three biological replicates for each construct were used to calculate the average expression level of the VIGS targeted genes and standard deviations. The values for Table 2 are given as expression levels as a percent of the BSMV-MCS virus inoculated control. A chi squared test was used to compare expression levels with significance assessed @ α =.01.

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Author Contributions

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X.W. performed SNP mapping, developed and performed the *Rpg5* gene silencing experiments. J.R. developed the *Hv*Rga1 silencing construct and performed all experiments with the construct. T.G. developed the *Hv*Adf3 silencing construct and performed all experiments with the construct. A.D helped in the conception of experimental design and manuscript preparation. A.K. helped in the conception of experimental design and manuscript preparation. B.S. developed the experimental design for stem rust evaluations and performed the analysis and interpretation of phenotyping data. M.A. developed the experimental design for stem rust evaluations and performed the analysis and interpretation of phenotyping data. R.B. developed the experimental design, analyzed and interpreted data. X.W. and R.B. wrote the manuscript.

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