INVESTIGATIONS ON STEM RUST RESISTANCE GENES IN BARLEY

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Dedication

This thesis is dedicated to my mother, Yufeng Gao, for her love and support.

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General Introduction

Barley and stem rust

Barley (*Hordeum vulgare* L.) is the fourth most widely grown cereal crop in the world after corn, rice and wheat. The United States ranks as one of the world's major barley producing countries with over 1 million cultivated hectares and annual production of about 5 million tonnes. Most of the barley cultivated in the United States is in the northern tier states from Minnesota in the east to Washington State in the west. Barley is used primarily for animal feed, but a substantial portion of the crop is also used in malting and specialty foods.

Barley production can be severely reduced by a number of different foliar, head, root and crown diseases. Stem rust, caused by *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn., is one of the most devastating diseases of barley. It causes shriveled grain and lodging, which greatly reduce yield. For barley, statewide yield losses as high as 15% were reported in North Dakota and Minnesota during epidemic years prior to 1940 (Steffenson, 1992). Ever since the release of barley cultivars with the resistance gene *Rpg1* in the mid-1940's, yield losses caused by stem rust have been minimal in the northern Great Plains even though pathotypes with virulence for *Rpg1* have been reported periodically (Steffenson, 1992). In 1990 and 1991, a pathotype of *P. graminis* f. sp. *tritici*, *Pgt*-QCCJ, with virulence for *Rpg1* caused minor stem rust epidemics in the northern Great Plains (Steffenson, 1992); however, since that time, no significant losses have been recorded in barley. Today, a new threat to both wheat and barley exists in the form of pathotype *Pgt*-TTKSK (original described race designation *Pgt*-TTKS with isolate synonym of Ug99), which was first characterized from Uganda in 1999 (Pretorius

et al., 2000). Pathotype *Pgt*-TTKSK can attack the vast majority of wheat and barley varieties cultivars worldwide and is spreading. After first being reported in Uganda, pathotype *Pgt*-TTKSK has been confirmed in Kenya, Ethiopia, Sudan, Yemen (Singh *et al.*, 2008) and Iran (Nazari *et al.*, 2009). It is predicted to move into North Africa, other parts of the Middle East, Asia and beyond, threatening much of the world's wheat and barley production.

Stem rust fungus *Puccinia graminis*

Stem rust or black rust is caused by the fungus *Puccinia graminis*. It is a heteroecious, macrocyclic rust fungus with the complete complement of five distinct spore stages. *P. graminis* needs an alternate host (primarily common barberry: *Berberis vulgaris*) to complete its sexual stage. The asexual uredinial stage can undergo repeated reproductive cycles on gramineous hosts every 14-20 days under favorable conditions. Because of the barberry eradication program in the United States during the early 20th century, aeciospores developing from infected barberry plants seldom initiate infection foci on cereal crops. Instead, the stem rust fungus survives during the winter as urediniospores on autumn-sown winter wheat in the southern Great Plains and is dispersed by wind northward to infect winter wheat in the central Great Plains and eventually spring-sown cereals in the northern Great Plains (Leonard and Szabo, 2005).

The diagnostic signs of stem rust are the brick-red uredinia containing masses of rust-colored urediniospores that form chiefly on the stems and leaf sheaths (Leonard and

Szabo, 2005). Based on the most prominent gramineous host attacked, *Puccinia graminis* was subdivided into different *formae speciales*, including *tritici* (wheat), *secalis* (rye), *avenae* (oat), *agrostidis* (*Agrostis* spp.), *poae* (bluegrass) and *airae* (Aira caespitosa) (Leonard and Szabo, 2005). In North America, barley can be attacked by two *formae speciales* of *Puccinia graminis*: *tritici* (the wheat stem rust pathogen) and *secalis* (the rye stem rust pathogen). To further characterize the pathotypes (races) of *P. graminis* f. sp. *tritici*, a standard set of differential wheat lines with different resistance genes were established (Jin *et al.*, 2008; Roelfs, 1993; Roelfs and Martens, 1988).

Rust resistance genes in barley

Incorporation of resistance genes into barley cultivars has been the primary and most economical strategy to control stem rust (Steffenson, 1992). Currently, seven genes for stem rust resistance have been identified in barley: *Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, *rpgBH*, and *rpg6*.

***Rpg1*.** The inheritance of resistance in barley to *P. graminis* f. sp. *tritici* was first investigated by Powers and Hines (1933). In cultivar Peatland, Powers and Hines (1933) identified a single dominant gene, which was originally designated "T" since it conferred resistance to the *tritici forma specialis* of *P. graminis*, but now designated *Rpg1* for Resistance to *Puccinia graminis* (Powers and Hines, 1933; Søgaard and von Wettstein-Knowles, 1987) . Several other studies on the genetics of resistance in Peatland, Chevron, or their derivatives corroborated the finding that a single dominant gene confers stem rust

resistance (Andrews, 1956; Brookins, 1940; Shands, 1939). *Rpg1* is present in almost every commercial barley cultivar in the Upper Midwest region of the United States and has provided durable resistance against *P. graminis* f. sp. *tritici* since the mid-1940's (Steffenson, 1992). *Rpg1* was cloned using a map-based method and encodes a receptor kinase-like protein with a unique combination of two tandem protein kinase domains (Brueggeman *et al.*, 2002).

***Rpg2*.** Patterson *et al.* (1957) reported adult stage resistance in cultivar Hietpas-5 (CIho 7124) and identified a resistance gene that was different from *Rpg1* in Chevron. This gene was designated as *Rpg2* (Patterson *et al.*, 1957; Søgaard and von Wettstein-Knowles, 1987).

***Rpg3*.** Jedel *et al.* (1989) identified a new gene for stem rust resistance in the Ethiopian landrace PI382313. This gene was different from *Rpg1* based on genetic studies using crosses between PI382313 and a number of barley accessions possessing *Rpg1* (Jedel *et al.*, 1989). The resistance gene in PI382313 was designated as *Rpg3* (Jedel, 1990).

***rpg4*.** In an effort to identify barley germplasm with resistance against wheat stem rust pathotype *Pgt*-QCCJ, Jin *et al.* (1994a) evaluated over 18,000 accessions and found barley line Q21861 (PI 584766) exhibited the highest level of resistance at both the seedling and adult plant stages. Genetic analyses with Q21861 indicated that resistance to

pathotype *Pgt*-QCCJ was conferred by a recessive gene designated as *rpg4* (Jin *et al.*, 1994b).

***Rpg5*.** In a subsequent study of the genetics of resistance in barley line Q21861 against the rye stem rust pathogen, Sun *et al.* (1996) reported a partially dominant gene that cosegregated with the *rpg4* locus. Based on high-resolution mapping, Brueggeman *et al.* (2008) resolved that the gene conferring rye stem rust resistance (previously *RpgQ*, now designated *Rpg5*) was different from *rpg4* and that both genes co-localized on a 70-kb region of chromosome 7(5H). The predicted *rpg4* gene encodes an actin depolymerizing factor-like protein, and *Rpg5* encodes an R protein with novel combinations of three domains: a nucleotide binding site (NBS) domain, a leucine-rich repeat (LRR) domain and a serine/threonine protein kinase (S/TPK) domain (Brueggeman *et al.*, 2008).

***rpgBH*.** Johnson and Buchannon (1954) reported that barley accession Black Hulless (CIho666) was resistant to *P. graminis* f. sp. *secalis*. By investigating progeny from crosses between Black Hulless and susceptible cultivars, Steffenson *et al.* (1984) found a single recessive gene (originally designated as the "S" gene, now as *rpgBH*) conferring resistance to pathotype HQ of *P. graminis* f. sp. *secalis* at the adult plant stage. Seedling tests by Sun and Steffenson (2005) showed that Black Hulless was only resistant to pathotype HQ of *P. graminis* f. sp. *secalis* at low temperature, but susceptible to all other *P. graminis* pathotypes/isolates tested.

***rpg6*.** By introgressing *Hordeum bulbosum* chromatin into cultivated barley chromosomes, Pickering *et al.* (2000) developed a number of lines with improved disease resistance. After testing these introgression lines against stem rust, Fetch *et al.* (2004) found two (119Y4 and 212Y1) resistant to pathotype *Pgt*-QCCJ. Subsequently, Fetch *et al.* (2009) investigated the inheritance of resistance in line 212Y1. The introgression was positioned on chromosome 6HS, a region where no other stem rust resistance genes have been reported. This new recessive gene from *H. bulbosum* was designated *rpg6* (Fetch *et al.*, 2009).

Function of plant disease resistance proteins

By studying the inheritance of plant resistance and pathogen virulence in flax (*Linum usitatissimum*) and the flax rust fungus (*Melampsora lini*), respectively, H. H. Flor (1971) developed the gene-for-gene hypothesis. Disease resistance would be triggered upon specific interactions between a pathogen avirulence (*avr*) gene and corresponding plant disease resistance (*R*) gene. Resistance is commonly manifested through the induction of a hypersensitive response (HR), i.e. programmed cell death within infected tissues. Based on their structural motifs, R genes are now classified into five classes: (1) serine/threonine (S/T) protein kinase; (2) receptor-like protein with leucine-rich repeats (LRRs) domain, a putative nucleotide binding site (NBS), and an N-terminal leucine zipper (LZ) or other coiled-coil (CC) motif; (3) intracellular NBS-LRR proteins with an N-terminal domain similar to the Toll and Interleukin 1 receptor (TIR)

proteins; (4) proteins lacking an NBS but instead having a trans-membrane (TM) and an extracellular LRR (i.e., Cf proteins from tomato); and (5) rice *Xa21* protein that has a cytoplasmic serine/threonine kinase domain in addition to an extracellular LRR and a TM (Martin *et al.*, 2003). Also, there are a few R proteins that do not fit into these five classes, such as the toxin reductase gene *HM1* of maize (Johal and Briggs, 1992), powdery mildew resistance gene *RPW8* in *Arabidopsis* (Xiao *et al.*, 2001), and the stem rust resistance gene *Rpg1* in barley (Brueggeman *et al.*, 2002).

The barley stem rust resistance gene *Rpg1* is unique because it encodes a receptor kinase-like protein with two tandem protein kinase domains (Brueggeman *et al.*, 2002). Only a few other cloned R genes were reported to encode S/T protein kinases, including the *Pto* gene in tomato conferring resistance to bacterial speck caused by *Pseudomonas syringae* pv. *tomato* (Martin *et al.*, 1993), the *Pbs1* gene in *Arabidopsis* required for resistance to *Pseudomonas syringae* pv. *phaseolicola* (Swiderski and Innes, 2001), and the rice *Xa21* gene conferring resistance to *Xanthomonas oryzae* pv. *oryzae* (Song *et al.*, 1995). *Rpg1* showed homology to *Xa21* in the kinase domain, but *Xa21* contains an extracellular ligand-binding LRR domain, whereas *Rpg1* has no known receptor sequences (Brueggeman *et al.*, 2002). *Rpg1* shares more similarity with the tomato *Pto* gene, which also contains a S/T domain, but no obvious membrane-spanning or extracellular domain to serve as an external receptor (Brueggeman *et al.*, 2002; Martin *et al.*, 1993). In support of the receptor-ligand model, the direct physical interaction between the *Pto* gene product and corresponding avirulence gene (*AvrPto*) product has

been detected using the yeast two-hybrid system (Scofield *et al.*, 1996). Upon AvrPto-Pto recognition, Pto kinase is activated and induces phosphorylation of downstream components in the signal transduction pathways, ultimately leading to elicitation of HR, expression of defense-related genes, and an oxidative bust (Sessa and Martin, 2000). Pto autophosphorylation sites Thr-38 and Ser-198 appear to be required for AvrPto-Pto mediated HR (Sessa *et al.*, 2000). In significant parallels with the tomato *Pto* gene, the barley RPG1 protein has been shown to have autophosphorylation activity, and functional kinase activity is required for stem rust resistance (Nirmala *et al.*, 2006). Both protein kinase domains of RPG1 are required for disease resistance, although only one domain (pK2) is functional for autophosphorylation as determined by site-specific mutagenesis (Nirmala *et al.*, 2006). Further investigation revealed that, in response to avirulent pathotypes of *P. graminis* f. sp. *tritici,* RPG1 degrades to undetectable levels through a proteasome-mediated pathway (Nirmala *et al.*, 2007). In resistant barley lines, the degradation of RPG1 occurs rapidly (between 20-24 hours) after stem rust inoculation, and the degradation is correlated with disease resistance (Nirmala *et al.*, 2007). Another recent study reported that RPG1 protein is phosphorylated within five minutes after inoculation with avirulent stem rust urediniospores (Nirmala *et al.*, 2010). Nirmala *et al.* (2010) proposed that phorphorylation of RPG1 through interaction with an unknown stem rust urediniospore product is required for resistance to avirulent stem rust pathotypes. A similar R gene in *Arabidopsis* is the S/T kinase resistance gene *PBS1*, which is required for *RPS5*-mediated resistance to *Pseudomonas syringae* expressing avirulence gene *avrPphB* (Swiderski and Innes, 2001). Similar to RPG1 in barley, PBS1

can autophosphorylate, and the cleavage of PBS1 by AvrPphB is required for RPS5-mediated resistance (Shao *et al.*, 2003). It is proposed that the cleavage product of PBS1 possibly binds to RPS5 to activate resistance (Shao *et al.*, 2003). However, with RPG1, no degradation product has been detected, and the corresponding *AvrRpg1* gene has not been identified. Thus, the mechanism of *Rpg1*-mediated resistance is still unclear.

Two closely linked barley stem rust resistance genes, *rpg4* and *Rpg5*, were recently isolated by map-based cloning (Brueggeman *et al.*, 2008). *Rpg5* encodes a protein containing three typical R protein domains: NBS, LRR, and S/T protein kinase. The combination of all three domains in a single transcript is unusual (Brueggeman *et al.*, 2008). The NBS-LRR gene family is the largest group of R genes in plants, with two functionally distinct groups, TIR-NBS-LRR and CC-NBS-LRR. Genome-wide analysis of disease resistance gene homologues revealed 149 NBS-LRR genes in *Arabidopsis* and approximately 500 NBS-LRR genes in rice (Meyers *et al.*, 2003; Monosi *et al.*, 2004). However, no TIR-NBS-LRR genes were found in rice, and the absence of TIR-NBS-LRR in cereal species suggests the loss of these genes in the early angiosperm ancestors of the cereal lineage (McHale *et al.*, 2006; Monosi *et al.*, 2004). For NBS-LRR proteins, the amino-termini (TIR or CC) are thought to act as an interaction platform for downstream signaling partners; the NBS domain contains characteristic motifs for ATP binding and hydrolysis for releasing signals, whereas the LRR domain may be involved in the modulation of activation (Belkhadir *et al.*, 2004). The NBS-LRR domains of *Rpg5* share high similarity with the rice resistance gene *Pi-ta*, while the S/T kinase domain of *Rpg5*

shows significant similarity to the tomato R gene *Pto* (Brueggeman *et al.*, 2008). Interestingly, *Pto* requires the function of an NBS-LRR gene *Prf* for resistance (Salmeron *et al.*, 1996). Also, the *Arabidopsis* S/T kinase resistance gene *PBS1* requires an NBS-LRR gene *RPS5* for resistance (Swiderski and Innes, 2001). As mentioned above, *Rpg5* is a novel R gene with the presence of all three domains, and allele analysis of the susceptible barley cultivar Harrington proves that the S/T kinase domain is required for resistance (Brueggeman *et al.*, 2008).

The predicted barley *rpg4* gene encodes an actin-depolymerizing factor-like (Adf) protein (Brueggeman *et al.*, 2008). Actin microfilaments are known to play important roles in all aspects of plant growth and have also been reported to be involved in non-host resistance in higher plants (Kobayashi *et al.*, 1997). The polymerized, filamentous state of actin is required for plants to block fungal penetration (Kobayashi *et al.*, 1997). In *Arabidopsis*, cytochalasin E, an inhibitor of actin microfilament polymerization, severely compromised non-host resistance against wheat powdery mildew in *eds1 Arabidopsis* mutants, indicating the important function of actin cytoskeleton organization during non-host resistance (Yun *et al.*, 2003). However, the report of actin-depolymerizing factor-like protein in gene-for-gene interactions is novel and needs further investigation.

Genetic engineering of crops to enhance resistance

Incorporation of durable disease resistance into crop cultivars is a great challenge for breeders in reducing losses to biotic agents and securing crop production.

Conventional breeding methods have vastly improved disease resistance in many crop cultivars. However, these breeding methods have some disadvantages: e.g. crosses/backcrosses are usually time-consuming and sexual incompatibility between species prevents transferring novel genes across species barriers. Moreover, it is difficult for conventional breeding programs to react to the evolution of new virulent variants in pathogen populations. With our growing knowledge of plant-pathogen interactions and isolation of plant disease resistance genes, attempts have been made to incorporate disease resistance genes into plants using genetic engineering. Transgenic technology offers breeders a powerful tool for developing traits that are otherwise difficult to achieve through conventional breeding and can even facilitate the introduction of genes from species outside the cultivated gene pool. The potential for transgene technology has been well demonstrated by the commercialization of *Bt* maize and *Bt* cotton for insect control, where the incorporation of the insecticidal genes from *Bacillus thuringiensis* has led to increased yields and reduction in insecticide applications (Shelton *et al.*, 2002). For engineered resistance to fungal diseases, a broad range of genes has been utilized by researchers, including the expression of various antifungal proteins (i.e. pathogenesis-related proteins, chitinases, glucanases, ribosome-inactivating proteins, etc.) and expression of R genes on the basis of specific interactions between R and Avr genes (Melchers and Stuiver, 2000). With respect to antifungal proteins, chitinase, a hydrolytic enzyme capable of degrading cell walls of most filamentous fungi, has been widely used for genetic engineering to enhance fungal resistance in plants (Punja, 2001). Brogue *et al.* (1991) reported that transgenic tobacco expressing a bean chitinase gene showed

enhanced resistance to fungal pathogen *Rhizoctonia solani* (Brogue *et al.*, 1991). Transformation of rice with a chitinase gene under the CaMV 35S promoter generated transgenic plants with resistance to *Rhizoctonia solani*, the sheath blight pathogen (Lin *et al.*, 1995). Many other transgenic plant species with chitanase also exhibit reduced fungal disease symptoms (Punja, 2001). Different from antifungal proteins, R-gene mediated resistance often results in a hypersensitive response triggered by specific recognition between a pathogen Avr gene product and host R gene product. Genetic engineering enables breeders to efficiently transfer R genes from resistant landraces, wild relatives or sexually incompatible plant species into adapted cultivars. For example, expression of the tomato *Cf-9* gene, which confers resistance to races of *Cladosporium fulvum*, has been shown to induce a rapid hypersensitive cell death in transgenic tobacco and potato in response to Avr9 peptide injection (Hammond-Kosack *et al.*, 1998). Also, the barley stem rust resistance *Rpg1* was successfully transferred into the susceptible cultivar Golden Promise, providing an even higher level of resistance to stem rust than that expressed in the original source of the gene, cultivar Morex (Horvath *et al.*, 2003). For stem rust control in wheat, researchers are aiming to package three or more resistance genes into a single gene construct and introduce them together into adapted cultivars. This process will eliminate undesirable genes with quality and yield defects that are carried along in linkage drag and at the same time hasten the breeding process (Dennis *et al.*, 2008). Due to the complexity and diversity of host-pathogen interactions for fungal diseases, the production of transgenic resistant plants has lagged behind compared to the successful commercialization of transgenic crop plants with herbicide, insect, and virus disease

resistance. However, the tremendous progress made in research holds great promise for using genetic engineering techniques to provide commercially useful broad-spectrum disease resistance for many crops (Punja, 2001).

Transgene silencing in plants

In the development of transgenic plants, it is critical to have stable expression of transgenes in order to obtain the desired novel traits. However, transgene expression levels may vary among independent transgenic lines, a phenomenon that can be caused by the sites of integration, transgene copy number, genetic background of the host, and environmental conditions (Zhong, 2001). In 1990, a transgene co-suppression phenomenon was reported by van der Krol *et al.* (1990) in an attempt to increase flower pigmentation in petunia by transferring more copies of flavonoid genes, but instead yielded white flowers. Co-suppression is now recognized as post-transcriptional gene silencing (PTGS), a sequence-specific gene silencing phenomenon that has also been found in fungi and animals (review, Vance and Vaucheret, 2001). In addition to PTGS, transgene silencing also can occur at the transcriptional level (TGS), which is often associated with heavily methylated and inactive promoter sequences (review, Meyer and Saedler, 1996). Although early reports of transgene silencing are mostly in dicots, it is apparent that transgene silencing is also very common in monocots (review, Iyer *et al.*, 2000). For example, in rice, Kumpatla *et al.* (1997) introduced a *Bt* gene (*Btt yIIIA*) with a selectable herbicide resistance gene (*bar*) and found silencing of *bar* in R_1 plants due to methylation of the *Ubi1* promoter driving *bar*. In barley, by particle bombardment with a

plasmid containing the *bar* and *uidA* genes, Wan and Lemaux (1994) generated large numbers of independently transformed fertile barley plants and found that several T_1 lines had an intact *bar* gene insert, but were sensitive to herbicide, indicating the occurrence of transgene silencing.

Three natural pathways of RNA silencing have been revealed in plants: (1) cytoplasmic small interfering RNAs (siRNAs) silencing that is important for virus infection defense; (2) silencing of endogenous messenger RNAs by microRNAs (miRNAs) to regulate gene expression; and (3) RNA silencing associated with DNA methylation and suppression of transcription to protect the genome from transposons (review, Baulcombe, 2004). Both miRNAs and siRNAs are 21-25 nucleotides long, but they consist of two different classes of small RNAs: miRNAs are encoded by miRNA genes, while siRNAs are processed from long double-stranded RNA precursors (Bartel and Bartel, 2003). All of these three pathways involve double-stranded RNA (dsRNA) as an intermediate, which is cleaved into short 21-25 nucleotide RNAs by an enzyme, Dicer, with RNase III domains. Mature siRNAs are incorporated into a complex called RISC (RNA-induced silencing complex), while mature miRNAs are incorporated into a similar ribonucleoprotein complex known as the miRNP (miRNA ribonucleoprotein particle). The RISC endonuclease cleaves mRNA based on the complementarity between the siRNA and target mRNA region, whereas miRNP attenuates translation by binding to the 3'-untranslated regions (UTR) of the target mRNA. Some heterochromatic siRNAs are

thought to be involved in histone methylation of chromatin, triggering TGS (review, Bartel and Bartel, 2003).

In developing transgenic plants, it is not possible to introduce a defined number of transgenes into specific positions in the recipient genome, regardless of whether the *Agrobacterium*-mediated transformation or particle bombardment method is used. Independent transgenic lines usually differ in transgene copy numbers, integration sites, and transgene arrangements in the genome, resulting in variability of transgene expression levels. Transgene expression levels are thought to relate to PTGS according to the RNA threshold model, which hypothesizes that transgenes produce a level of RNA that exceeds a critical threshold, thereby triggering a silencing mechanism that removes all homologous RNAs (review, Stam *et al.*, 1997). The RNA threshold hypothesis was supported by a study on transgenic tobacco plants expressing *Tobacco Etch Virus* (*TEV*) coat protein, where a 12- to 22-fold reduction of transgene transcript occurred in resistant plant tissues after *TEV* infection (Lindbo *et al.*, 1993). Also, based on a systematic study of transgene expression in *Arabidopsis thaliana*, Schubert *et al.* (2004) demonstrated that RNA silencing was triggered if the transcript level of a transgene exceeded a gene-specific threshold (Schubert *et al.*, 2004). However, PTGS is not always associated with high transgene expression levels. For example, co-suppression of the pigmentation gene *chs* in *Petunia hybrida* was neither related to a high level nor a low level of transgene expression (Van Blokland *et al.*, 1994). Other factors, such as the integration sites of

transgenes, methylation of transgenes, and presence of homologous endogenous genes may also be involved in PTGS (review, Stam *et al.*, 1997).

Virus-induced gene silencing (VIGS)

Based on an RNA silencing mechanism, virus-induced gene silencing (VIGS) was developed as a gene function analysis tool to specifically silence or knock down target genes in plants. When virus vectors carrying inserts from a host gene are introduced into plants, antiviral defenses generate siRNAs that target and silence the corresponding host mRNA. In *Nicotiana benthamiana*, Kumagai *et al.* (1995) first demonstrated that the expression of the endogenous phytoene desaturease (PDS) gene was knocked down after infection with a recombinant *Tobacco Mosaic Virus* (*TMV*) strain carrying a partial PDS gene sequence, resulting in leaves with a bleached white phenotype (Kumagai *et al.*, 1995). The same bleaching phenotype was observed in *Nicotiana benthamiana* after infection with *Potato Virus X* (*PVX*) vectors carrying PDS gene inserts (Ruiz *et al.*, 1998). Using a recombinant *PVX* vector, Thomas *et al.* (2001) investigated the size constraints for triggering PTGS and found that a minimum of 23 nucleotides with complete identity was sufficient to direct silencing of target mRNA (Thomas *et al.*, 2001). Initially, VIGS was mostly performed on *Nicotiana benthamiana*, due to the limitation of suitable viral vectors. Fortunately, the development of new viral vectors has expanded the range of dicot plant species where VIGS can be employed, including tomato (Liu *et al.*, 2002a), potato (Brigneti *et al.*, 2004), and *Arabidopsis* (Burch-Smith *et al.*, 2006; Pflieger *et al.*, 2008). A viral vector using modified *Barley Stripe Mosaic Virus*

(*BSMV*) has now successfully been used for VIGS in monocot plants such as barley and wheat (Holzberg *et al.*, 2002; Scofield *et al.*, 2005). A comprehensive list of viruses used for VIGS on different hosts was reviewed by Unver and Budak (2009).

Conventional loss-of-function approaches for plants generally involve chemical or physical mutagenesis, T-DNA insertion, or transposon insertion techniques, which are time-consuming and sometimes result in embryo lethal mutations. VIGS can overcome these limitations and generate a rapid phenotype without plant transformation or causing embryonic lethality. VIGS provides a powerful tool to study the functions of disease defense genes in plants. The basis of this technique is to induce susceptibility in resistant host plants by silencing genes required for disease resistance. For example, using *N* gene-transformed *Nicotiana benthamiana* plants, Liu *et al.* (2002b) silenced *Rar1*-, *EDS1*- and *NPR1/NIM1*-like genes by *Tobacco Rattle Virus* (*TRV*)-mediated VIGS and demonstrated that these genes are required for *N*-mediated resistance. Using VIGS, other disease defense related genes also have been identified, such as *NbSGT1* and *NbSKP1* for *N*-gene mediated *TMV* resistance (Liu *et al.*, 2002c), tobacco chloroplast carbonic anhydrase (CA) gene for *Pto:avrPto*-mediated HR (Slaymaker *et al.*, 2002), and *Nicotiana Protein Kinase 1* (*NPK1*) gene for viral and bacterial resistance (Jin *et al.*, 2002). Recently, several VIGS systems have been successfully developed for monocotyledonous hosts, including *BSMV*-mediated VIGS for barley and wheat (Holzberg *et al.*, 2002; Scofield *et al.*, 2005), *Brome Mosaic Virus* (*BMV*) mediated VIGS for barley, rice and maize (Ding *et al.*, 2006), and several other potential virus vectors (review, Scofield and Nelson,

2009). In wheat, Scofield *et al.* (2005) demonstrated that silencing the NBS-LRR gene *Lr21* using *BSMV*-VIGS could convert incompatible interactions into compatible ones. Additionally, the *RAR1*, *SGT1*, and *HSP90* genes were implicated in this *Lr21*-mediated leaf rust resistance. In a recent study of the barley stem rust resistance gene *Rpg5*, Brueggeman *et al.* (2008) reported that silencing *Rpg5* in resistant barley seedling plants could result in susceptible reactions. With further refinements of VIGS vectors and associated methodology, significant advances will be made in large-scale functional genomics analyses for plant disease resistance research.

Chapter 1

Highly expressed RPG1 protein in a five-copy *Rpg1*-transgenic barley line results in susceptibility to stem rust

Introduction

Barley (*Hordeum vulgare* L.) is the fourth most widely grown cereal crop in the world after corn, rice and wheat. In the United States, barley is grown on over 1 million hectares and is primarily used for cattle feed and also malt in the brewing industries. Historically, stem rust, caused by *Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn., has been a major biotic constraint to barley production in the region and caused a number of major epidemics prior to 1940 (Roelfs, 1978). Since 1942, the losses caused by stem rust have been minimal due to the release of barley cultivars with stem rust resistance (Roelfs, 1978). Genetic studies revealed that the resistance in these cultivars is due to a single dominant gene, initially called "T" since it conferred resistance to the *tritici forma specialis* of *P. graminis*, but now designated *Rpg1* for Resistance to *Puccinia graminis* (Powers and Hines, 1933; Søgaard and von Wettstein-Knowles, 1987). *Rpg1* was the first stem rust resistance gene identified in barley and remains the principal source of stem rust resistance in nearly every malting barley cultivar in the northern Great Plains region of North America. The gene was positioned on the extreme subteleomeric region of the short arm of barley chromosome 1 (7H) using molecular markers (Kilian *et al.*, 1994). Later, Brueggeman *et al.* (2002) developed a high-resolution genetic and physical map of the *Rpg1* region and identified three candidate genes. Only one could be the likely *Rpg1* gene based on sequence comparisons between susceptible and resistance alleles (Brueggeman *et al.*, 2002). Successful map-based cloning and sequence analysis of *Rpg1* revealed that it encodes a receptor kinase-like protein with two tandem protein kinase domains (Brueggeman *et al.*, 2002). Structure of the predicted *Rpg1* gene product

is similar to the tomato *Pto* gene product, a serine/threonine kinase that confers resistance against *Pseudomonas syringae* by direct interaction with AvrPto protein and the development of a hypersensitive response (HR) (Sessa and Martin, 2000).

To explore the molecular mechanism of *Rpg1*, Rostoks *et al.* (2004) characterized the structure of *Rpg1* mRNA and measured the expression of the *Rpg1* gene using quantitative real-time PCR. *Rpg1* transcript intrinsically has alternative splicing variants and is transcribed at relatively uniform and low levels in almost all organs of barley and at all developmental stages. However, Rostoks *et al.* (2004) also found that in leaf epidermis, the *Rpg1* transcript level was up to 30 times higher than in whole leaves. Nirmala *et al.* (2006) investigated the subcellular localization and phosphorylation properties of the RPG1 protein. The protein was present mainly in the cytosol, but also on the plasma membrane and intracellular membranes. Analysis of barley *Rpg1* loss-of-function mutants revealed that both of the kinase domains are required for resistance, but only the second domain has kinase function activity (Nirmala *et al.*, 2006). Further study on the phosphorylation and proteolysis of RPG1 found that, after infection with avirulent stem rust pathotypes, the protein disappeared to an undetectable level in barley seedlings and that RPG1 degradation is correlated with, but is not alone sufficient to confer disease resistance (Nirmala *et al.*, 2007). Another recent study revealed that the phosphorylation of RPG1 protein occurred within five minutes after inoculation with urediniospores of avirulent stem rust pathotypes, suggesting that it may have a role in the very early response against stem rust infection (Nirmala *et al.*, 2010).

After the map-based cloning of *Rpg1*, Horvath *et al.* (2003) developed transgenic stem rust resistant barley lines by transferring *Rpg1* from barley cultivar (cv.) Morex into the susceptible cv. Golden Promise by *Agrobacterium*-mediated transformation. Initial transformation yielded 42 primary transgenic (T_0) plants, eight of which were characterized using Southern blot analysis to estimate the number of transgene copies. T_1 progeny of 23 primary T_0 plants were tested for stem rust resistance at the seedling stage and 21 had plants with highly resistant or resistant reactions (Horvath *et al.*, 2003). Transgenic line H228.2c, containing a single copy *Rpg1* insertion, showed a 3:1 segregation ratio for resistance : susceptibility among the T_1 progeny when selfed, indicating the sufficiency of one *Rpg1* copy for resistance. Interestingly, transgenic line H228.19, with five copies of *Rpg1*, showed a wide range of different infection types among the T_1 progeny. One susceptible T_1 seedling from H228.19 still retained a high number of transgene copies, and the transcript levels were three times higher than the positive control of cv. Morex. Based on these results, there appeared to be no strong correlation between stem rust resistance level and *Rpg1* copy number or transcript level (Horvath *et al.*, 2003).

In the northern Great Plains region of the United States and Canada, it is important to have resistance gene expression at the adult plant stage due to the late arrival of stem rust inoculum from the southern United States (Steffenson, 1992). As a follow-

up investigation to the seedling test, T_1 transgenic lines were increased over several successive generations (to T_2, T_3 and T_4) and evaluated for adult plant resistance in the field. Transgenic lines with different *Rpg1* copy numbers continued to exhibit different stem rust phenotypes at the adult plant stage. One particularly intriguing result was that one transgene copy of *Rpg1* was sufficient to confer a high level of stem rust resistance, whereas five transgene copies resulted in susceptibility at both seedling and adult plant stages (Horvath *et al.*, 2003; B. Steffenson, unpublished). Thus, to investigate the relationship between *Rpg1* transgene copy number and stem rust resistance level, we selected five transgenic lines containing different copy numbers of *Rpg1* and tested them for stability of transgene inheritance, level of *Rpg1* mRNA transcript, level of RPG1 protein expression, and degradation of RPG1 protein upon stem rust infection.

Materials and methods

Transgenic plant generations T_0 to T_5

Cultivar Golden Promise was transformed with the *Rpg1* genomic clone as described by Horvath *et al.* (2003). Originally, 42 primary transgenic (T_0) lines were produced. T_1 progenies from 23 T_0 lines were initially tested for stem rust resistance at the seedling stage. Segregation for stem rust resistance was observed within most T_1 lines, although two lines (H228.5 and H228.3) gave rise to all resistant progeny (Horvath *et al.*, 2003). T_2 seed was then harvested from single T_1 plants based on their rust phenotype. In 2003, the T_2 lines were sown in the field and evaluated for adult plant stem

rust resistance. A total of 30 T_2 lines from three different primary transgenic plants (H228.2c, H228.5 and H228.19) were again selected for further study based on their rust phenotype. Five T_3 seeds derived from each selected T_2 line were grown in the greenhouse to produce T_4 seeds. In 2004 and 2006, T_4 lines were tested in the field for adult plant stem rust resistance. T_5 seed was harvested from the T_4 generation in the field in 2006 and used for all subsequent seedling stem rust resistance assays in 2007, 2008, and 2009. Transgenic line selections and stem rust tests on seedling and adult plant were conducted collaboratively by the laboratories of Dr. Brian Steffenson from the University of Minnesota and Dr. Andris Kleinhofs from Washington State University.

Stem rust inoculation and disease assessment

Seedling stem rust assays were conducted on T_5 plants in two separate experiments. Seeds were sown in cones (20 cm high and 4 cm in diameter) filled with 50% soil/50% Metro Mix 200 (Vermiculite, peat moss, perlite, and sand mix, Green Island Distributors, Inc., Riverhead, NY) and grown in a growth chamber set at 22-25°C with a 14-hour photoperiod provided by 160 W VHO fluorescent and 60 W incandescent lamps (525 mmol photon/m^2/s). Seven days after planting, plants were inoculated with urediniospores of pathotype MCCJ suspended in a lightweight mineral oil (Soltrol 170, Phillips Petroleum, Bartlesville, Oklahoma). Inoculum (3.7 mg urediniospores/0.7 ml oil) was applied at a rate of ~0.025 mg per plant using rust inoculators pressurized by an air pump (27.5 kPa). Then, plants were placed in mist chambers and subjected to 30 minutes of continuous misting by ultrasonic humidifiers followed by 16 hours of periodic misting

(2 minutes of misting every 60 minutes) in the dark. Next, the mist chamber doors were opened and lights (150-250 mmol photon/m^2/s provided by 400 W sodium vapor lamps) were turned on with the misters set to run for 2 minutes every 15 minutes for the next 2 hours. Finally, the misters were turned off, facilitating the slow-drying of the plant surfaces over the next 3-4 hours. When the plants were completely dry, they were moved back to the growth chamber under the conditions previously described. Eleven to twelve days after inoculation, plants were scored for their infection types (ITs). The IT scale used for barley is based on uredinial size as described by Miller and Lambert (1955), and is patterned after the original 0-4 scale developed for wheat by Stakman *et al.* (1962).

Adult plant stem rust resistance was assayed in the field at the T_2 and T_4 generations. Barley lines were planted in mid- to late-April at the Minnesota Agricultural Experiment Station in St. Paul, MN. The test entries were planted as paired 1.0 m long rows within two outside "spreader" rows consisting of a 30:70% mix of the stem rust susceptible cv. Steptoe (CIho15229) and line 80-tt-30 (CIho16130). Standard agronomic practices were used to ensure proper fertility and weed control in the plots. To initiate stem rust infections in the field, freshly collected urediniospores of pathotype MCCJ were suspended in sterile distilled water (0.3 g spores/300 ml water with ~200 µl of Tween 20 added) and then injected into the stems of spreader row plants prior to flag leaf emergence in early June. Injections were made into 4-5 plants per meter of row using a 2 ml self-refilling syringe (Wheaton Science Products, Millville, NJ) with 0.5 cc needle (B-D Cornwall, Franklin Lakes, NJ). If the needle-inoculation failed to initiate sufficient

levels of infection, additional inoculations were made by applying a foliar application of urediniospores (1.4~3 g spores /1400 ml Soltrol oil) directly onto test and spreader rows using a low volume applicator (Ulva+, Micron Sprayers Ltd., Bromyard Industrial Estate Bromyard, UK). In mid-July, stem rust severity (visual percentage of tissue covered by uredinia on a 0-100% scale) was estimated using the modified Cobb scale (Peterson *et al.*, 1948). Additionally, infection responses (IRs) (i.e. size and type of uredinia) were assessed following the descriptions of Roelfs *et al.* (1992), with categories as R (resistant), MR (moderately resistant), MS (moderately susceptible), and S (susceptible). An additional category of highly resistant (HR) was added for "fleck" reactions, i.e. obvious stem rust infection sites with no sporulation.

T_5 transgenic line selection

For this study, a total of five T_5 transgenic lines (G04-271, G04-273, G04-288, G04-266, and G03-210) were selected for tests of stem rust resistance, transgene copy number, *Rpg1* transcript level and RPG1 protein level (Figure 1.1). The selection of these lines was based on their reported transgene copy number and also previous stem rust phenotype (Tables 1.1 and 1.2). Previously, Southern blot analyses were performed on eight T_0 lines and revealed a range from one to five copies of *Rpg1* (Horvath *et al.*, 2003). T_1 progeny from three T_0 lines (H228.5, H228.19, H228.2c) also were analyzed for their transgene copy numbers (Horvath *et al.*, 2003). The five T_5 lines derived from the assayed T_1 lines were ultimately selected for this study because they exhibited a range of *Rpg1* copy numbers (putative copy numbers from one to five) and stem rust reactions

(from highly resistant to susceptible) (Tables 1.1 and 1.2). Barley cvs. Morex (CIho15773) with *Rpg1* and Golden Promise (PI343079) with no known resistance gene were included as the controls.

Southern blot analysis on T_5 plants

To compare the transgene copy number obtained on plants from the T_0 and T_1 generations (Horvath *et al.*, 2003), Southern blot analysis was performed on five T_5 lines in this study. Three arbitrarily selected plants from each T_5 line were sampled at day 17 after planting. For each plant, a total of one gram of leaf tissue (fresh weight) was collected for genomic DNA isolation using the MIDI method as previously described (Kleinhofs *et al.*, 1993). Ten micrograms of genomic DNA was digested with *HindIII* at 37°C overnight and then separated by electrophoresis in a 1% agarose gel. Digested genomic DNA was then blotted onto nylon membranes, hybridized to the *Rpg1* probe, and labeled with [α-32P]dCTP using the All-in-one Random Labeling System (Sigma, St. Louis, MO). The *Rpg1* probe was developed and described by Horvath *et al.* (2003).

Quantitative real-time PCR

For *Rpg1* transgene mRNA quantification, the same three arbitrarily selected plants from each T_5 line were used. The second leaves (~100-200 mg) of plants were harvested for total RNA extraction using the modified hot (60°C) phenol/guanidinium thiocyanate method developed by Chirgwin *et al.* (1979). Trizol-like reagent contained 38% saturated phenol (pH 4.3), 1 M guanidine thiocyanate, 0.1 M sodium acetate (pH

5.0), and 5% glycerol. One microgram of total RNA was used for single-strand cDNA synthesis, using the Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and oligo (dT) as the primer. Quantitative real-time PCR reactions were carried out on the Rotor-Gene 2000 real-time PCR cycler (Corbett Research, Mortlake, New South Wales, Australia). Primers for *Rpg1* and reference gene *GAPDH* were described in Horvath *et al.* (2003). PCR amplification was performed at 50°C for 2 min; hot start at 95°C for 15 min; 50 cycles of 95°C for 15 s, 60°C for 20 s, 72°C for 30 s, and data collection at 72°C for 60 s.

ELISA

RPG1 protein level from the same three arbitrarily selected plants of each T_5 line was quantified by ELISA using RPG1-specific polyclonal antibodies. For RPG1 protein isolation, approximately 400 μg of leaf tissue was ground with a mortar and pestle in 1 ml of ice-cold extraction buffer [0.5 M sorbitol, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM Dithiothreitol(DTT)]. Cell debris was removed by centrifugation at 13,000 rpm for 10 min at 4°C. Approximately 100 μl of the crude protein extract were coated onto ELISA plates and incubated at 4°C for 12 hr. The supernatant was discarded, and the wells were washed three times with PBST [PBS (Phosphate buffered saline) containing Tween 20] and refilled with ~200 mg of the cross-absorption antiserum buffer prepared from cv. Golden Promise. The plates were incubated for 4 hr at room temperature on moist paper towels. Wells were washed three times with PBST, and then 200 μl of the goat anti-rabbit IgG-horseradish peroxidase conjugate was added. This

reaction was allowed to incubate for 2 hr before the wells were washed five times with PBST and allowed to dry on clean paper towels. One hundred microliters of TMB (3,3´,5,5´-tetramethylbenzidine) substrate was added per well, and the color development measured at 405 nm using an ELISA plate reader (Bio-Rad, Hercules, CA). Purified RPG1 protein or the peptide used to develop the antibody was used to construct a standard curve for ELISA.

Western blot

For Western blot assays, a different set of arbitrarily selected plants from each T_5 line was used. Eleven-day-old plants were inoculated with urediniospores of pathotype *Pgt*-MCCF by gently rubbing spores onto both the first and second leaves at a rate of 0.25 mg urediniospores per leaf using talc (Sigma, St. Louis, MO) as a carrier. Controls were mock-inoculated with talc only. After inoculation, plants were misted and then placed in a covered plastic bin filled at the bottom with water to maintain saturated conditions for the infection period. Plants were kept in this environment in the dark for 20 hr. Then, both rust-inoculated and mock-inoculated plants were sampled at 0, 4, 20, 28, 36, and 48 hrs after inoculation. Three plants for each treatment were sampled at each time point. Leaves from three plants subjected to the same treatment were pooled together and immediately frozen in liquid nitrogen and stored at -80°C until used for protein isolation.

Crude protein was extracted as described above. To partially purify the RPG1 protein from crude protein samples, immunoprecipitation was performed: 500 μl of crude protein extract was combined with RPG1 antibodies in the extraction buffer and 500 μl of 2× immunoprecipitation buffer (1 M KCl/0.02 M EDTA/2 mM PMSF) and rotated end-over-end at 4°C for 12 hr. Protein A-agarose (30 μl) was then added and incubated on ice for 1 h to preclear the immunocomplexes, which were collected at 13,000 rpm. Immunocomplexes were washed three times with 1 ml of ice-cold immunoprecipitation buffer, resuspended in 50 μl of Laemmli sample buffer boiled at 95°C for 3 min, and analyzed by SDS/PAGE.

After being separated on an SDS/PAGE gel, protein samples were electroblotted to PVDF (Polyvinylidene fluoride) membranes and blocked in TBST [20 mM Tris/500 mM NaCl/0.1% Tween-20 (pH 7.5)] containing 10% nonfat dry milk. The blots were reacted with the RPG1-specific polyclonal antibodies for 12 hr at room temperature and then with horseradish peroxidase-conjugated secondary antibodies (Alpha Diagnostics, Austin, TX). Bands were visualized with the Nu Glo-chemiluminescent detection system according to the manufacturer's instructions (Alpha Diagnostics, Austin, TX).

Results

Stem rust phenotypes of *Rpg1*-transgenic lines

Seedling tests for stem rust reaction were conducted on transgenic plants at the T_5 generation (Table 1.1). The resistant and susceptible controls reacted as expected based on previous studies with pathotype *Pgt*-MCCF: cv. Morex gave low ITs (12 with range of 0;1 to an occasional 3-), whereas cv. Golden Promise gave a high IT (3 with range of 2 to 33+). Plants from the selected T_5 lines exhibited consistent reactions over repeated experiments. Transgenic lines G04-271, G04-266, and G03-210 exhibited very low ITs (00;) with no sporulating uredinia. Of note, the resistance level of these three lines was higher than that exhibited by Morex, the cv. from which *Rpg1* was cloned. Although lines G04-271 and G04-273 shared a very similar lineage (Figure 1.1), the latter consistently exhibited slightly higher ITs (i.e. sporulating "1" type uredinia) than the former. Interestingly, line G04-288 exhibited high ITs (3 with range of 1 to 3) to stem rust even though it presumably contains five copies of *Rpg1*.

Adult stage stem rust resistance tests were conducted on plants at the T_2 and T_4 generations (Table 1.1). Resistant and susceptible controls reacted similar to previous stem rust experiments: cv. Morex was highly resistant exhibiting a stem rust severity of 0% and highly resistant to resistant IRs, whereas cv. Golden Promise was susceptible exhibiting a severity of 20-25% and moderately susceptible to susceptible IRs. Transgenic lines G04-271, G04-266, and G03-210 exhibited a severity score of 0% and highly resistant IRs as no sporulating uredinia were observed. Line G04-273, derived

from the same resistant T_2 plant as G04-271 (Fig. 1.1), exhibited a slightly higher rust severity (3% to 4%) and more compatible IRs than G04-271 (0%) in 2004 and 2006. As in the seedling tests, line G04-288 was susceptible to moderately susceptible, exhibiting stem rust severities ranging from 30 to 35%.

Transgene copy numbers as assessed by Southern blot analysis

Since transgene copy number can vary through generations of selfing, Southern blot analysis was performed on T_5 plants to estimate *Rpg1* copy number. Genomic DNA from three individual plants of each line were extracted and analyzed using the *Rpg1*-specific probe. *Rpg1* copy numbers estimated by Southern blot analysis (Figure 1.2) are listed in Table 1.2 in comparison with the original estimates made at the T_0 or T_1 generation by Horvath *et al.* (2003). Of the five transgenic lines investigated, two lines (G04-288 and G04-266) retained the same copy numbers (five and two, respectively) as found earlier in the T_0 and T_1 generations for all three individual plants investigated. Line G03-210 showed one transgene copy for two plants (#2 and #3), which is consistent with the copy number found in the T_0 and T_1 generations. However, one plant (#1) from this line showed no band in the Southern blot, a result likely due to improper DNA preparation since the mRNA and protein quantification tests conducted on the same plant revealed the presence of *Rpg1* transcript and protein (see below). The same result likely occurred with the missing *Rpg1* band in plant #1 of line G04-273 as the other two plants (#2 and #3) clearly showed one copy of *Rpg1*. All three plants of line G04-271 showed only one copy of *Rpg1*. Of note, these two lines (G04-271 and G04-273) came from the

same five-copy T_0 plant; however, at the T_1 generation, the *Rpg1* transgene copy number was reduced to three copies and at the T_5 generation to only one copy. No strong correlation was found between *Rpg1* copy number and the stem rust phenotype (Tables 1.1 & 1.2). Lines with the same copy number exhibited, in some cases, very similar rust reactions (G04-271 vs. G03-210) and in other cases different reactions (G04-271 vs. G04-273), whereas lines with different copy numbers exhibited either similar (G04-271 vs. G04-266) or different rust reactions (G04-271 vs. G04-288).

Rpg1 transcript levels

Quantitative real-time PCR was used to measure *Rpg1* mRNA levels in the transgenic lines. Mean values of three samples from each line were used to estimate *Rpg1* transcript levels (shown in Figure 1.3) in comparison with Golden Promise (0%) and Morex (100%). All transgenic lines had higher levels of *Rpg1* transcript than Morex. Transgenic lines G04-271, G04-273 and G03-210, all with one copy of *Rpg1*, exhibited mRNA levels that were about 9×, 5× and 2× greater than Morex, respectively. Transgenic line G04-266, with two copies of *Rpg1*, had an mRNA level about 2× greater than Morex. Finally, the five-copy transgenic line G04-288 showed the highest level of *Rpg1* mRNA—about 29× greater than Morex. The association between *Rpg1* transgene copy number and transcript level was not particularly strong in this small sample of transgenic lines (Table 1.2 and Figure 1.3). Yet given that the five-copy line G04-288 had a much higher *Rpg1* transcript level than the other lines, it appears that higher copy numbers generally have a positive effect on transcript level. Variation in transcript level was not

strongly correlated with stem rust resistance (Table 1.1 and Figure 1.3). This was especially true for line G04-288, which exhibited by far the highest *Rpg1* mRNA transcript level, but showed moderately susceptible to susceptible reactions to stem rust.

RPG1 protein levels

Using quantitative ELISA, RPG1 protein expression levels were assayed in 10-day old seedlings of the transgenic lines (Figure 1.4). ELISA revealed that the RPG1 protein was expressed in all transgenic lines. The five-copy transgenic line G04-288 exhibited RPG1 protein levels nearly 3× higher than cv. Morex and about 2× higher than other transgenic lines. Compared to the quantitative real-time PCR result, line G04-288 exhibited both the highest transcript and protein level; however, the protein levels assayed by ELISA were not proportional with the transcript levels. Also, differences in RPG1 protein levels were not strongly correlated with stem rust resistance (Table 1.1 and Figure 1.4). This was especially true for line G04-288, which had the highest RPG1 protein level, but was susceptible to stem rust.

Degradation of RPG1 protein

Degradation of RPG1 protein is critical for resistance after stem rust infection in cv. Morex (Nirmala *et al.*, 2007). In *Rpg1*-transgenic lines with various copy numbers, it is important to ascertain whether the degradation of RPG1 protein is essential for resistance. Thus, Western blot analysis was used to assay the level of RPG1 protein up to

48 hr post-inoculation (Figure 1.5). Western blots showed that in all the resistant transgenic lines and Morex control, RPG1 protein was degraded to undetectable levels between 20 and 28 hr post-inoculation. However, in the moderately susceptible to susceptible line G04-288, RPG1 protein remained at high levels, even 48 hr after inoculation. Thus, the susceptibility of line G04-288 appears to be due to the failure of RPG1 to degrade.

Discussion

In this study, we characterized the stem rust reactions of five selected *Rpg1* transgenic barley lines at both the seedling and adult plant stages over several generations and their relationship to transgene copy number, transgene expression level, RPG1 protein level, and RPG1 protein degradation. Stem rust phenotype data showed that a single copy of *Rpg1* (as in line G03-210) is sufficient to confer resistance against stem rust (Table 1.1). That *Rpg1* can confer resistance at the seedling stage was previously reported (Horvath *et al.*, 2003), but in this study we also clearly demonstrated the effectiveness of a single transgene at the adult plant stage under field conditions. In general, the level of resistance found in the respective transgenic lines at the seedling stage (as assessed by infection types) was very similar to the level found in the adult plant stage (as assessed by severity). This result corroborates the all stage resistance phenotype conferred by *Rpg1* in different cultivars as reported previously (Steffenson, 1992). Adult plant resistance is essential for the control of stem rust because the pathogen usually infects the crop after the heading stage. Furthermore, if barley cultivars genetically

modified for stem rust resistance are to ever be accepted by growers, they must perform well under field conditions. Our field tests clearly showed that the transgenic lines provided a high level of stem rust resistance under high inoculum pressure in the field.

To investigate the stability of different transgenic barley lines carrying *Rpg1*, assays for stem rust resistance (phenotype) as well as *Rpg1* copy number (genotype) were made over several generations (T_0, T_1 and T_5). Based on the phenotype data of plants from the T_4 and T_5 generations, all transgenic lines gave consistent stem rust reactions in several repeated seedling stage and adult stage tests, suggesting a high level of phenotypic homogeneity and consistency (Table 1.1). Southern blot analysis was subsequently used to estimate *Rpg1* copy numbers in three individual plants at the T_5 generation from each transgenic line showing stable phenotypic expression. Lines G04-288, G04-266 and G03-210 remained stable across generations for *Rpg1* copy number at two, five and one, respectively (Table 1.2). However, lines G04-271 and G04-273, which were reported to contain three copies of *Rpg1* at the T_1 generation, retained only one copy at the T_5 generation. As shown in the lineage diagram of selected transgenic lines (Figure 1.1), both G04-271 and G04-273 were derived from the same five-copy T_0 line (H228.19) and subsequently the same three-copy T_1 line (G02-448L-5R). These results clearly demonstrate the instability of *Rpg1* transmission across generations in certain lineages. The site of integration, known as the "positional effect", can play a major role in transgene stability, resulting in deletions, duplications, rearrangements, epistatic interactions, and mitotic/meiotic recombinations of the transgenes (review, Yin *et al.*,

2004). A study on successive generations of six transformed wheat lines by Srivastava *et al.* (1996) revealed the deletion of *bar* and *gus* transgenes in R_3 plants, and this instability was speculated to be due to the characteristics of the integration site. Unstable inheritance of the transgene *bar* also was reported in a maize transgenic line (Spencer *et al.*, 1992). For the three primary transgenic lines selected in this study, Horvath *et al.* (2003) studied the sites of *Rpg1* transgene insertion and found that the one-copy line H228.2c was flanked by 48-bp repetitive DNA; the two-copy line H228.5 had one copy linked to 209-bp repetitive DNA and the other copy to a flanking sequence with 190-bp of the *Rpg1* transgene DNA; and the five-copy line H228.19 had transgene copies integrated at four independent locations with two copies inserted in a tandem direct repeat configuration. To resolve the genetic basis of variable *Rpg1* transgene inheritance at the T_5 generation, additional research should be undertaken to characterize the left and right border flanking sequences of the T-DNA integration sites.

Expression levels of transgenes can vary considerably, depending on the copy numbers integrated, insertion sites, genome background, and even the environment (review, Zhong, 2001). In this study, we found one susceptible transgenic line (G04-288), and it had five copies of *Rpg1*. When two or more copies of a transgene are integrated into a plant genome, a phenomenon called transgene silencing may occur. There are two types of transgene silencing: transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). Since PTGS was frequently reported in high copy number transgenic plants, the susceptibility of line G04-288 was thought to be due to this

phenomenon. However, assays for both mRNA and protein levels showed that *Rpg1* was highly expressed and that transgene silencing was not the cause for susceptibility. In line G04-288, the high transgene copy number actually had a positive effect on expression as shown by a 29× greater mRNA level and 3× greater protein level compared to cv. Morex (Figures 1.2 and 1.3). According to the RNA threshold model, PTGS would occur when RNA produced by a transgene exceeds a critical threshold, thereby triggering a silencing mechanism that removes all homologous RNAs (review, Stam *et al.*, 1997). This hypothesis was supported by a systematic transgene expression study in *Arabidopsis* conducted by Schubert *et al.* (2004) where they reported that RNA silencing was triggered if the transcript level of a transgene surpassed a gene-specific threshold. For example, using reporter gene *GFP* under the control of promoter CaMV 35S, Schubert *et al.* (2004) found that high *GFP* transcript levels were observed for plants harboring up to four copies, but the same transcripts were barely detectable in plants with five or more transgene copies. Additionally, the threshold can be gene-specific: for transgene *SPT*, nine or 12 copies severely reduced transcript levels, while for transgene *GUS*, three or more copies resulted in pronounced reductions in transcript levels (Schubert *et al.*, 2004). In our study, it is possible that the transcript level for line G04-288 was still under the threshold and therefore PTGS was not triggered. Also, no PTGS was triggered in any of the other transgenic lines even though they exhibited markedly higher levels of *Rpg1* transcript than Morex (Figure 1.3). The higher expression levels observed were not due to an enhanced promoter region since these *Rpg1*-transgenic lines were developed using the native promoter regions of *Rpg1* (Horvath *et al.*, 2003). Although the five-copy line G04-

288 exhibited higher transcript and protein levels than lines with fewer copies, gene dosage (i.e. copy number) is not the only cause for enhanced expression since even one-copy transgenic lines showed higher expression levels than one-copy cv. Morex. Thus, it is conceivable that the higher expression levels of *Rpg1* in transgenic lines were largely due to positional effects, where integration occurred in transcriptionally active areas and transgene expression was influenced by proximal enhancer sequences. Transgene integration may not be strictly random. Rather, it can preferentially occur in transcriptionally active regions of the genome (Topping *et al.*, 1991). In barley, Stahl *et al.* (2002) investigated the patterns and sites of T-DNA integrations into the barley genome and found that twelve of the 46 integrations were within actively transcribed *BARE*-1 retrotransposons (Stahl *et al.*, 2002). Isolation of the flanking sequences of T-DNA insertion sites in these transgenic lines would help elucidate the influence of regulatory elements upon *Rpg1* transgene expression levels.

Without challenge from the stem rust pathogen, *Rpg1*-transgenic lines showed high but different *Rpg1* mRNA and protein levels compared to cv. Morex. Previous studies revealed in Morex, and several other resistant barley cultivars carrying *Rpg1*, that the RPG1 protein disappears between 20 to 24 hr in response to avirulent stem rust pathotypes such as *Pgt*-MCCF (Nirmala *et al.*, 2007). Thus, it is important to investigate whether the variation observed in transcript and protein levels caused the differences in RPG1 protein degradation and consequently the different responses to stem rust infection. Western blot degradation assays revealed that RPG1 protein level in line G04-288

remained high 48 hr after inoculation with pathotype *Pgt*-MCCF. In contrast, with all other resistant transgenic lines, RPG1 disappeared to undetectable levels within 28 hr after inoculation. This sequence of events is consistent with previous studies that the degradation occurred rapidly between 22-23 hr post-inoculation (Nirmala *et al.*, 2007). The stem rust infection period from inoculation to the time when the fungus begins to form a penetration peg and enter stoma is about 22 hr (Roelfs *et al.*, 1992). It is interesting that the degradation of RPG1 occurred about the same time period, indicating a role of the RPG1 protein in the early stages of the stem rust defense response. A recent study revealed that the RPG1 protein became phosphorylated within five minutes after inoculation with urediniospores of an avirulent stem rust pathotype (Nirmala *et al.*, 2010), suggesting a role of RPG1 as a rapid responding protein during the very early stages of the resistance signaling pathway. Phosphorylation is required for the degradation of RPG1 protein through the proteasome pathway, and the degradation of RPG1 protein is correlated with stem rust resistance (Nirmala *et al.*, 2007). Our study confirmed that, in *Rpg1*-transgenic lines, stem rust resistance would not be triggered without degradation of RPG1 protein.

One possible role of protein degradation in plant disease resistance is removal of negative regulators of plant defense responses (Martin *et al.*, 2003). For example, the degradation of *Arabidopsis RPM1* gene product was coincident with the hypersensitive response, suggesting negative regulation controlling cell death (Boyes *et al.*, 1998). However, RPG1 may not be the factor that negatively limits hypersensitivity, because the

absence of RPG1 is not sufficient for resistance in cv. Golden Promise as *Rpg1* needs to be introduced into this cultivar in order to activate resistance. Observations about the dynamics of RPG1 protein are consistent with the guard hypothesis for plant disease resistance (review, Jones and Dangl, 2006). One possible mechanism proposed by Nirmala *et al.* (2007) is that, upon infection by the stem rust pathogen, the RPG1 protein either directly or indirectly recognizes the pathogen elicitors and then is degraded to an undetectable level. Either the degradation process or some breakdown products would then be perceived by the plant cell, which subsequently activates the signaling pathway for resistance (Nirmala *et al.*, 2007). In *Arabidopsis*, Shao *et al.* (2003) reported a similar mechanism involving the cleavage of a protein kinase PBS1 during *RPS5*-mediated resistance to *Pseudomonas syringae* bacteria expressing the AvrPphB protein. It was speculated that the cleavage of PBS1 by AvrPphB and the phosphorylation of the cleavage product activates RPS5. However, unlike PBS1, no degradation product from RPG1 protein has been detected yet (Nirmala *et al.*, 2007). For *Rpg1*-mediated resistance, other genes are required: a fast neutron-induced deletion mutant from barley cv. Morex showed susceptibility to stem rust pathotype *Pgt*-MCCF, and the loss of resistance was presumed to be due to a mutation in the *Rpr1* (Required for *Puccinia* resistance) gene (Zhang *et al.*, 2006). On the pathogen side, the genome of *P. graminis* f. sp. *tritici* has been sequenced, and corresponding stem rust virulence effectors are actively being pursued based on bioinformatic analysis (Les Szabo, personal communication). Further studies about other genes that may directly or indirectly interact with RPG1 and the roles

of the corresponding AvrRPG1 protein from *P. graminis* f. sp. *tritici* will help elucidate the mechanism of *Rpg1*-mediated resistance to stem rust.

Another interesting result found in this study was the recovery of transgenic lines with enhanced stem rust resistance. We found several Golden Promise transgenic lines (G04-271, G04-266 and G03-210) that exhibited a distinctly higher level of seedling resistance than Morex, the cultivar from which *Rpg1* gene was cloned. The reason for the enhanced resistance over that conferred by Morex could be due to the presence of additional or more highly inducible genes for the *Rpg1*-mediated resistance pathway in the recipient cv. Golden Promise. Genome-wide parallel transcript profiling is now available for barley after the development of the 22K Barley1 GeneChip (Close *et al.*, 2004). Using this gene array platform, Zhang *et al.* (2008) conducted transcriptome comparisons between cv. Golden Promise and an *Rpg1* transgenic line G02-448F-3R in response to stem rust. A total of 15 upregulated and 9 downregulated genes were identified without challenge by stem rust, and a total of 34 probe sets were found to have different expression between cv. Golden Promise and transgenic line G02-448F-3R after challenge with pathotype *Pgt*-MCCF (Zhang *et al.*, 2008). The majority of these differentially expressed genes have predicted functions in cellular metabolism, signal transduction, regulation of gene expression, and plant defense (Zhang *et al.*, 2008). These genes could be candidates for the enhanced stem rust resistance observed in some transgenic lines. Phenotype analysis revealed that the two single copy transgenic lines G04-271 and G04-273, which share a very similar lineage, exhibited slightly different

phenotypes at both seedling and adult plant stages (Table 1.1). In both lines, the *Rpg1* transgene had high expression levels as revealed by qRT-PCR (Figure 1.3) and ELISA (Figure 1.4). Also, Western blot showed degradation of RPG1 in both lines between 20 and 28 hr post-inoculation (Figure 1.5). Considering the close lineage of these two lines, the phenotype differences might be caused by integration site shifts or gene mutations occurring independently that affect the signaling pathways for *Rpg1*-mediated resistance. Transcriptome analysis, using either the barley mRNA profiling microarray or next-generation whole transcriptome sequencing, would be a great tool to help elucidate the phenotype differences between lines G04-271 and G04-273.

Transfer of the cloned stem rust resistance gene *Rpg1* into Golden Promise resulted in the conversion of the highly susceptible cultivar into a resistant one. A single copy of *Rpg1* is sufficient to confer resistance, and the resistance is effective not only at the seedling stage (Horvath *et al.*, 2003), but also at the adult plant stage in the field as shown in this study. Although stem rust has not caused major losses since the 1930s in North America due to the protection offered by *Rpg1*, a recently emerging pathotype called *Pgt*-TTKSK poses a great threat to global cereal production because the vast majority of wheat and barley cultivars are susceptible. Pathotype *Pgt*-TTKSK has already spread throughout East Africa (Singh *et al.*, 2008) and is now present in the Middle East (Nazari *et al.*, 2009). Resistance to *Pgt*-TTKSK has been found in barley line Q21861 (Steffenson *et al.*, 2009), but it does not possess sufficient quality for malting purposes. The most rapid means of developing a *Pgt*-TTKSK-resistant malting barley is to

transform an already industry acceptable cultivar with resistance genes derived from Q21861. The success of *Rpg1*-transformation in barley demonstrates the great potential for improving disease resistance levels of current barley cultivars by transferring available resistance genes. However, developing a successful transformation program requires reliable information on the stability and expression of the transgene. As shown in this study through repeated phenotyping and genotyping tests, transgenes may not be inherited stably over generations, and copy number could affect the function of the transgene. Specifically, in the case of *Rpg1*, introducing one copy can achieve a high level of stem rust resistance, while over-expressing *Rpg1* in high copy number transgenic lines can have a negative effect on resistance. Studying the molecular mechanisms during host-pathogen interactions is of great importance for successfully developing disease resistant transformants, as the stable inheritance and expression of transgenes depend on the interactions between the transgene, the recipient genome, and the pathogen.

Table 1.1. Reaction of Golden Promise *Rpg1*-transformed lines and controls to wheat stem rust pathotype *Pgt*-MCCF at the seedling stage and adult plant stage

Lines and Controls	Adult Stage Evaluation			Seedling Stage Evaluation	
	Stem rust severity (0-100% scale)[1] and infection response (IR)[2]			Infection types (ITs) (0-4 scale) [3]	
				2007/2008/2009 (T_5) [4]	
	2003 (T_2) [4]	2004 (T_4) [4]	2006 (T_4) [4]	Most Common	Range
G04-271	0 HR	0 HR	0 HR	00;	0 to 00;
G04-273	0 HR	3 MS-MR	4 MS-S	0;1	00; to 20;
G04-288	30 MS-S	30 S-MS	35 S	3	1 to 3
G04-266	0 HR	0 HR	0 HR	00;	0 to 00;
G03-210	0 HR	--[5]	--[5]	00;	00; to 0;
Morex	0 HR	0 HR	0 HR	12	0;1 to 3-
Golden Promise	25 MS-S	20 S-MS	20 MS-S	3	2 to 33+

[1] Stem rust severity is the visual percentage of stem and leaf sheath tissue covered by uredinia on a 0%-100% basis. The mean severity of two replicates is given here.
[2] Infection responses (IRs) at the adult plant stage were assessed based on the size and type of uredinia observed following the description of Roelfs *et al.* (1992), where R=resistant, MR=moderately resistant, MS=moderately susceptible, and S=susceptible. An additional category of highly resistant (HR) was added for "fleck" reactions, i.e. obvious stem rust infection sites with no sporulation.
[3] Seedling infection types (ITs) were assessed based on the size and type of uredinia observed according to the 0-4 scale developed for wheat by Stakman *et al.* (1962) as modified by Miller and Lambert (1955) for barley. For each accession, the one or two most common ITs observed are given in addition to the range of ITs observed (lowest and highest types).
[4] Year experiment was conducted and transgenic generation tested.
[5] Not tested.

Table 1.2. Transgene copy numbers in Golden Promise *Rpg1*-transformed lines based on Southern blot analysis[1]

Lines and Controls	T_0 copy number	T_1 copy number	T_5 copy number[2]
G04-271	5	3	1
G04-273	5	3	1
G04-288	5	5	5
G04-266	2	2	2
G03-210	1	1	1
Morex	1	1	1
Golden Promise	0	0	0

[1] The copy numbers for the T_0 and T_1 generations were given by Horvath *et al.* (2003); whereas copy numbers for theT_5 generation were estimated by Southern blot analysis in this study.
[2]Southern blot assays were done on three individual plants per line. Copy number estimates were consistent for all plants within a line, except for G04-273 and G03-210 where one plant each showed no *Rpg1* band (See Figure 1.2).

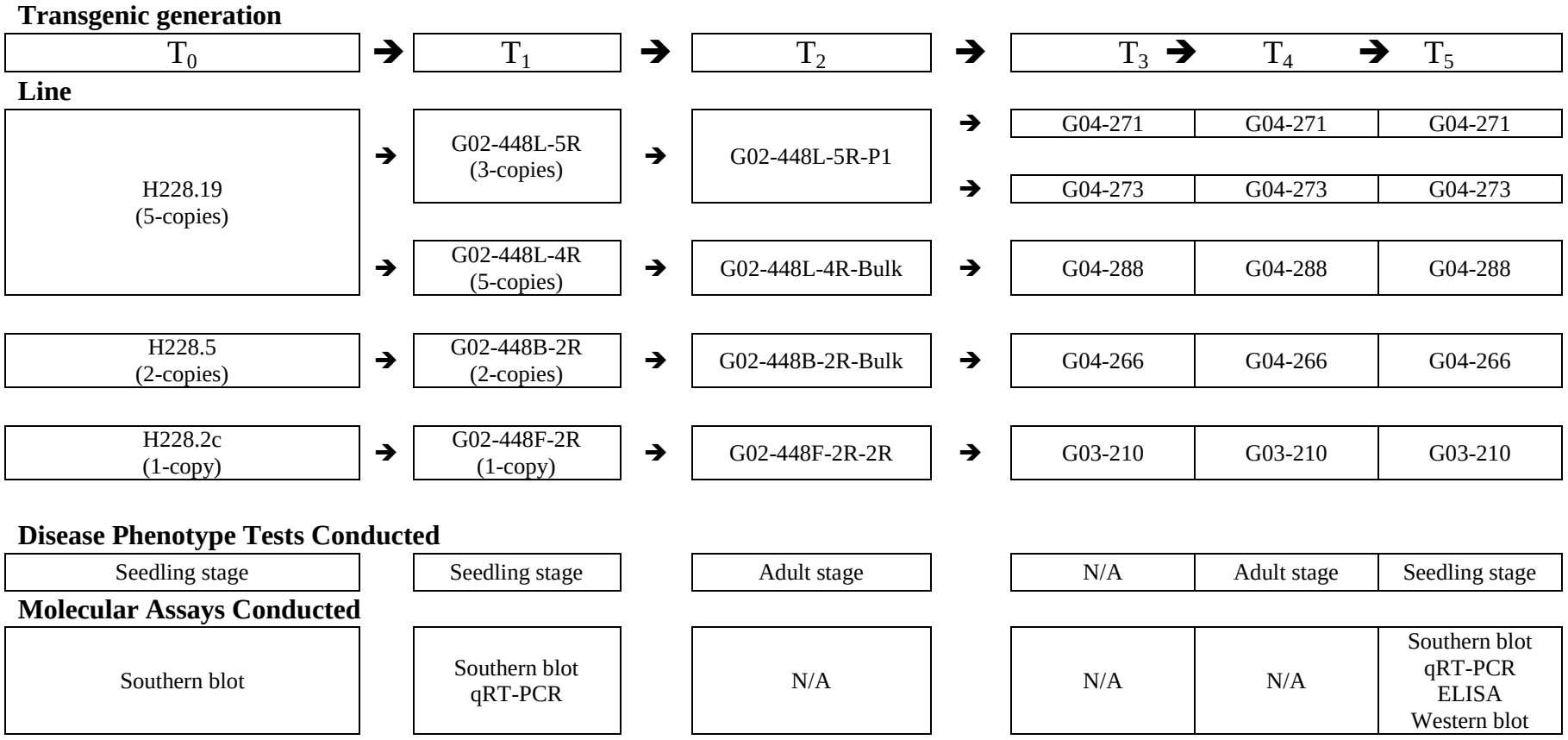

Figure 1.1. Ancestry of Golden Promise *Rpg1*-transgenic lines used in this study and the disease phenotype and molecular assays performed during each transgenic generation, T_0 to T_5. The five transgenic lines used in this study were originally selected based on copy number and a consistent stem rust phenotype.

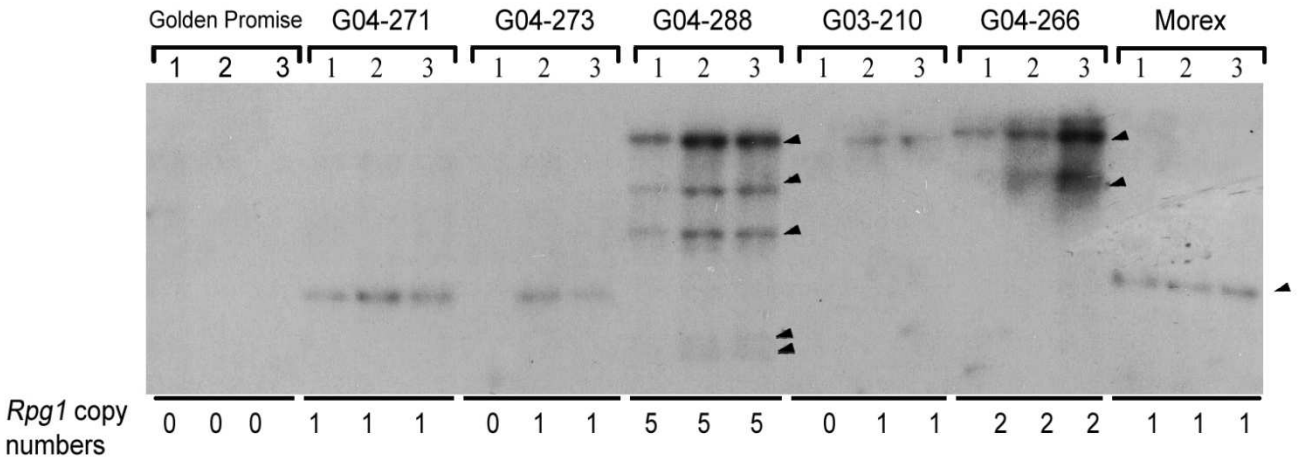

Figure 1.2. Southern blot analysis of *Rpg1* copy number in Golden Promise *Rpg1*-transformed lines. For each line, three individual plants (marked as 1, 2, 3 at top) were investigated. Control cv. Morex showed one band and cv. Golden Promise showed no band. The estimated *Rpg1* copy numbers for transgenic lines are given at the bottom of each lane. Copy number estimates were consistent for all plants within a line, except for G04-273 and G03-210 where one plant each showed no *Rpg1* band.

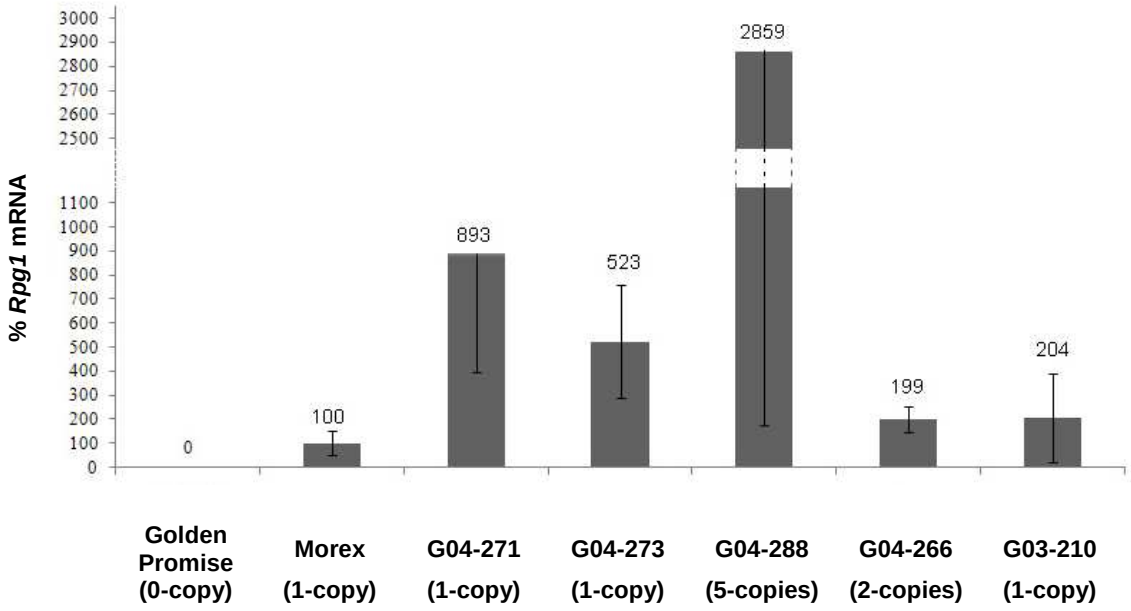

Figure 1.3. *Rpg1* transcript levels in Golden Promise *Rpg1*-transformed lines containing different copy numbers. *Rpg1* mRNA levels were normalized against *GAPDH* as a reference gene. The *Rpg1* mRNA level in Morex was considered as 100% and all other transgenic lines were compared with Morex mRNA level. Numbers given above each column are the mean values of *Rpg1* mRNA level (as a percentage of Morex) from three individual plants per line. Error bars represent the standard deviations.

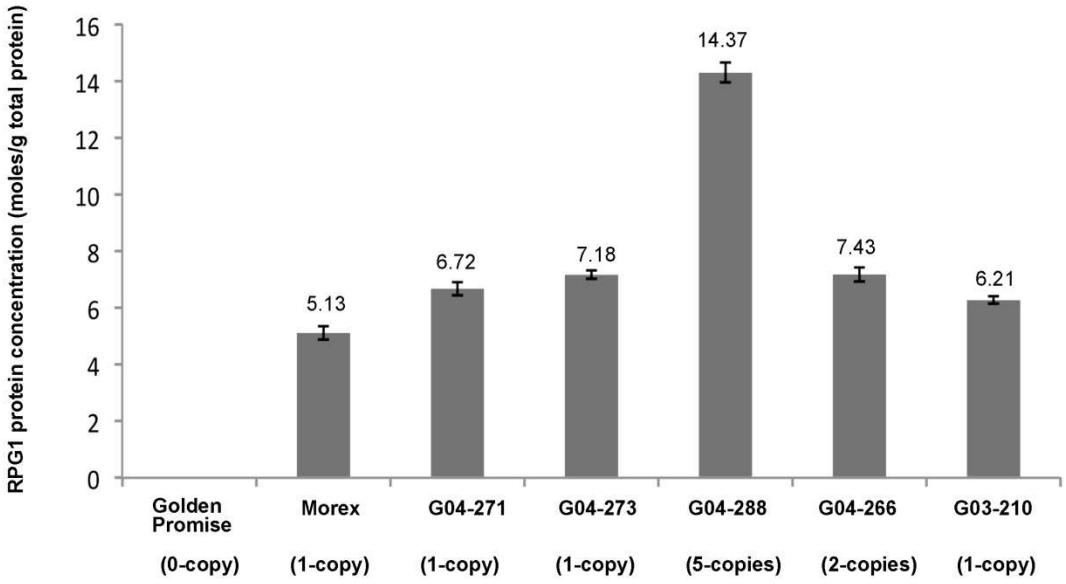

Figure 1.4. Results of ELISA for RPG1 protein expression in 10-day-old Golden Promise *Rpg1*-transformed lines. RPG1 protein concentration was measured as moles per gram of total protein. For each barley line, three plants were sampled individually and the mean RPG1 protein levels are given above the bars. Error bars represent the standard deviations.

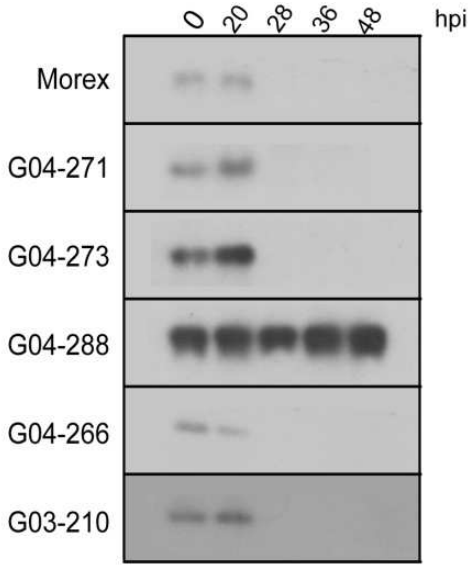

Figure 1.5. Western blot assays for RPG1 protein after infection by wheat stem rust pathotype *Pgt*-MCCF in Golden Promise *Rpg1*-transformed lines. The first leaves were inoculated and, for each time point, the inoculated tissue from three plants was pooled for protein samples. Time point 0 was sampled right before inoculation. Other time points were 20 hr, 28 hr, 36 hr, and 48 hr post-inoculation (hpi).

Chapter 2

Stem rust resistance genes *Rpg5* and *HvAdf2* (*rpg4*) are both required for resistance to wheat stem rust pathotypes *Pgt*-TTKSK and *Pgt*-QCCJ

Introduction

In the Upper Midwest region of the United States, stem rust has been an important disease threatening barley (*Hordeum vulgare* L.) production. There are two different stem rust pathogens that can attack barley: the wheat stem rust pathogen (*Puccinia graminis* Pers.:Pers. f. sp. *tritici* Eriks. & E. Henn. or *Pgt*) and the rye stem rust pathogen (*Puccinia graminis* Pers.:Pers. f. sp. *secalis* Eriks. & E. Henn. *Pgs*). Historically, wheat stem rust has been the most important of the two pathogens on barley in North America, causing frequent yield losses during epidemic years prior to the 1940s (Roelfs, 1978). In 1942, the first barley cultivar with stem rust resistance was released to farmers in the United States (Steffenson, 1992). The stem rust resistance of this and all subsequent barley cultivars released for the northern Great Plains of the United States is conferred by a single gene, *Rpg1* (Steffenson, 1992). The emergence of a virulent wheat stem rust pathotype (*Pgt*-QCCJ) in 1989 rendered *Rpg1* ineffective and caused minor stem rust epidemics in 1990 and 1991 in North America (Roelfs *et al.*, 1991; Steffenson, 1992). A newly emerging wheat stem rust pathotype (*Pgt*-TTKSK, original described race designation TTKS with isolate synonym of Ug99) first detected in Uganda in 1999 (Pretorius *et al.*, 2000) poses a great threat to both barley and wheat production worldwide because of its wide virulence on many cultivars (Singh *et al.*, 2006; Steffenson *et al.*, 2009). Pathotype *Pgt*-TTKSK has spread throughout eastern Africa and has subsequently been reported in Yemen (Singh *et al.*, 2008) and Iran (Nazari *et al.*, 2009). New variants within the original Ug99 lineage (i.e. pathotypes *Pgt*-TTKST and *Pgt*-TTTSK) were detected in a rust screening nursery at Njoro, Kenya from 2006 to

2007 (Jin *et al.*, 2008; Jin *et al.*, 2009). In 2009, another pathotype (PTKST) within the Ug99 lineage was detected in South Africa and exhibits a virulence pattern similar to pathotype *Pgt*-TTKST with the exception of *Sr21* avirulence (Pretorius *et al.*, 2010). The virulence variation reported within the African stem rust population poses an even greater challenge to the world's barley and wheat crops.

In Minnesota and North Dakota, rye stem rust is sporadically found on commercially grown barley, although it is not as serious a problem as wheat stem rust (Steffenson *et al.*, 1984). However, the rye stem rust pathogen is still a potential threat to barley production because some isolates are virulent on many barley cultivars, including those carrying the widely used gene *Rpg1* (Steffenson *et al.*, 1984; Sun and Steffenson, 2005).

Developing resistant cultivars is an effective way to control stem rust in barley. Seven major stem rust resistance genes have been identified in barley: *Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, *rpgBH*, and *rpg6*. *Rpg1* was the first stem rust resistance gene identified in barley (Powers and Hines, 1933), but it is ineffective against wheat stem rust pathotypes *Pgt*-QCCJ and *Pgt*-TTKSK as well as rye stem rust isolate *Pgs*-92-MN-90. After a preliminary screening of 18,000 barley accessions against pathotype *Pgt*-QCCJ, Jin *et al.* (1994a) identified only 13 with a useful level of resistance. Of these 13 accessions, line Q21861 exhibited the highest level of resistance at both the seedling and adult stages. Line Q21861 originated from the barley breeding program at the International Maize and

Wheat Improvement Center (Centro Internacional de Mejoramiento de Maíz y Trigo, CIMMYT) in Mexico and was later selected and found to be resistant to stem rust in Australia (Dill-Macky *et al.*, 1992). To study the inheritance of resistance to *Pgt*-QCCJ in this line, Jin *at al.* (1994b) crossed Q21861 with several barley cultivars and identified a recessive gene (named *rpg4*) conferring resistance at low incubation temperatures (18-20°C). In addition to conferring resistance to *Pgt*-QCCJ, line Q21861 also is resistant to rye stem rust (Sun *et al.*, 1996). Genetic studies involving Q21861 revealed that a single dominant gene at the same locus as *rpg4* was responsible for conferring resistance to the rye stem rust pathogen (Sun *et al.*, 1996). High resolution mapping and sequencing of candidate genes in the region revealed that the gene conferring rye stem rust resistance is not *rpg4*, but rather a closely linked gene (now named *Rpg5*) acting with dominant gene action (Brueggeman *et al.*, 2008). Initially, *rpg4* was mapped to the long arm of chromosome 7 (5H) using RAPD and RFLP markers (Borokova *et al.*, 1995). More detailed high-resolution genetic and physical mapping positioned the *rpg4*/*Rpg5* locus to a 70-kb region of this chromosome (Brueggeman *et al.*, 2008). Sequence analysis indicates that the actin depolymerizing factor-like gene *HvAdf2* is the probable *rpg4* gene and *Rpg5* encodes an R protein with a nucleotide binding site (NBS) domain, a leucine-rich repeat (LRR) domain and a serine/threonine protein kinase (S/TPK) domain (Brueggeman *et al.*, 2008).

Line Q21861 is one of the most resistant barley lines identified against *Pgt*-TTKSK (B. Steffenson, unpublished). Genetic studies revealed that this resistance is

conferred by a single gene at the *rpg4/Rpg5* locus within the resolution of the small population used in the investigation (Steffenson *et al.*, 2009). Further characterization of recombinants at the *rpg4/Rpg5* locus revealed that resistance to rye stem rust (isolate *Pgs*-92-MN-90) is conferred by *Rpg5* alone, independent of *rpg4*; however, resistance to wheat stem rust pathotype *Pgt*-QCCJ appears to require both *rpg4* and *Rpg5* (Brueggeman *et al.*, 2008; Brueggeman *et al.*, 2009). Due to the close linkage between *rpg4* and *Rpg5* (physical distance of ~40kbp), it is uncertain which gene confers resistance against *Pgt*-TTKSK in line Q21861 based on available barley cultivars and recombinants. As a highly resistant barley line carrying both *rpg4* and *Rpg5* as well as *Rpg1*, Q21861 is a very valuable source of stem rust resistance that can be utilized in barley breeding programs. Thus, resolving the functional resistance genes in Q21861 against the virulent wheat stem rust pathotype *Pgt*-TTKSK is of great significance.

For gene function analysis, virus-induced gene silencing (VIGS) can be used to rapidly silence target genes in plants. VIGS does not rely on the often difficult and laborious transformation process or time-consuming population development schemes. VIGS is a virus vector technology that harnesses the plant's RNA-mediated anti-viral defense mechanism. After infecting plants with a virus carrying target sequences with homology to a host gene, the virus triggers the host plant's defenses, degrading any RNA with sufficient homology to the target sequences (review, Vance and Vaucheret, 2001). VIGS has been successfully applied on both dicots (such as *Nicotiana benthamiana* (Kumagai *et al.*, 1995) and *Arabidopsis* (Burch-Smith *et al.*, 2006)) and monocots (such

as barley (Holzberg *et al.*, 2002) and wheat (Scofield *et al.*, 2005)). *Barley Stripe Mosaic Virus* (*BSMV*) is a tripartite, positive-sense RNA virus that was developed as a vector for VIGS on monocot hosts (Holzberg *et al.*, 2002). For example, by infecting barley with *BSMV* containing a phytoene desaturase (PDS) gene insertion in the γRNA, host plants will show a photo-bleached symptom--a result of PDS gene silencing (Holzberg *et al.*, 2002). Using *BSMV*-mediated VIGS, Brueggeman *et al.* (2008) showed that silencing *Rpg5* rendered line Q21861 susceptible to rye stem rust isolate *Pgs*-92-MN-90, confirming the gene's role in conferring resistance to this stem rust pathogen. Resistance to both *Pgt*-TTKSK and *Pgt*-QCCJ in line Q21861 was mapped to the *rpg4*/*Rpg5* locus. The aim of this study was to determine the role of these two genes in conferring resistance to wheat stem rust pathotypes *Pgt*-TTKSK and *Pgt*-QCCJ. VIGS was used to individually knock down the expression of *HvAdf2* (*rpg4*) and *Rpg5*. Then, assessments were made to determine whether the silencing of each gene resulted in phenotypic changes in response to stem rust infection.

Material and methods

Plant materials and growth conditions

Four barley accessions (Q21861, Steptoe, HQ18 and SQ41) were selected for this study because they possess different recombinations at the complex *rpg4/Rpg5* locus (Figure 2.1). Q28161 is a line of unknown parentage selected from a CIMMYT (Centro Internacional de Mejoramiento de Maíz y Trigo) barley breeding nursery. It has functional *rpg4* and *Rpg5* genes that confer resistance to both *Pgt*-TTKSK and *Pgt*-

QCCJ. Steptoe (CIho 15229) is a stem rust susceptible feed barley cultivar from Washington State University that lacks *Rpg5* and differs from Q21861 in the *HvAdf2* (*rpg4*) allele by only three amino acids (Q39H, A101T, S135G) (Brueggeman *et al.*, 2008). HQ18 is a progeny derived from the cross Harrington/Q21861 that contains the Harrington *HvAdf2* (*rpg4*) allele (which encodes the same amino acid sequence as Q21861) in combination with the Q21861 *Rpg5* allele (Brueggeman *et al.*, 2008). SQ41 is a progeny line derived from the cross Steptoe/Q21861 that contains the Q21861 *Rpg5* allele and a recombination within *HvAdf2* (*rpg4*), which produces an amino acid sequence that contains glutamine at the 39 position (Q21861-like), but with threonine and glycine at positions 101 and 135, respectively (Steptoe-like) (Brueggeman et al., 2009).

Due to the wide virulence and threat to agriculture of pathotype *Pgt*-TTKSK, all experiments were done in the Bio-Safety Level-3 (BSL3) Containment Facility on the St. Paul campus of the University of Minnesota during the winter months. Experiments with pathotype *Pgt*-QCCJ were conducted in growth chambers within the Plant Growth Facility on campus. Plants were grown in plastic pots (13 cm × 13 cm × 13 cm) filled with 50% soil/50% Metro Mix 200 (Green Island Distributors, Inc., Riverhead, N.Y.) and fertilized with Osmocote 14-14-14 at planting (Scott's Company, Marysville, OH) and Peters Dark Weather 15-0-15 every two weeks (Scott's Company). The BSL3 greenhouse was maintained at 20-22°C with a 14 hr photoperiod (supplemented by 400W high pressure sodium lamps emitting a minimum of 300 μmol photons/s/m^2) and the growth

chamber at 20-22°C with 14 hr photoperiod (400 W metal halide lamps emitting 550 μmol photons/s/m^2).

Virus-Induced Gene Silencing (VIGS)

BSMV consists of three genomic RNAs α, β and γ (Figure 2.2A). The γ genome of *BSMV* was modified to silence either *rpg4* or *Rpg5* with their respective antisense RNA, and the empty γ genome vector with only the multiple cloning site (MCS) was included as a control. Brueggeman *et al.* (2008) developed the γ-as*Rpg5* and γ-as*Adf2* constructs used in this study with a 297-bp fragment and 139-bp fragment from cDNA sequences of *Rpg5* and *HvAdf2* (*rpg4*), respectively (Figure 2.2B).

The VIGS inoculation protocol used in this study was as described by Holzberg *et al.* (2002). *BSMV* α, β and γ genomes were transcribed into RNA using mMessage mMachine T7 kit (Ambion, Austin, TX). For each plant, one micro liter of each *BSMV* RNA genome components (α, β and γ-as*Rpg5* or γ-as*Adf2* or γ-MCS) were mixed (1:1:1) with 22.5 μl inoculation buffer FES (0.1 M Glycine, 0.06 M K_2HPO_4, 1.0% Sodium Pyrophosphate, 1.0% Bentonite, 1.0% Celite) and then inoculated onto the second leaves of 10-day old barley plants by firmly rubbing the entire lamina 3-5 times according to the described procedures for *BSMV*-VIGS (Scofield *et al.*, 2005).

Stem rust inoculation

Plants were inoculated with the stem rust pathogen 10 days after *BSMV*-VIGS inoculation. Fresh urediniospores of stem rust pathotypes *Pgt*-TTKSK and *Pgt*-QCCJ were increased on a susceptible host (wheat accession McNair701), collected on the same day as the rust inoculation, suspended in a lightweight mineral oil (Soltrol 170, Phillips Petroleum, Bartlesville, Oklahoma) at a concentration of 14 mg urediniospores/0.7 ml oil, and applied at a rate of approximately 2.5 mg/plant with an atomizer pressured at 25-30 kPa. Then, plants were placed in mist chambers and subjected to 30 minutes of continuous misting by ultrasonic humidifiers followed by 16 hours of periodic misting (2 minutes of misting every 60 minutes) in the dark. Next, the mist chamber doors were opened, and lights (150-250 mmol photon/m^2/s provided by 400 W sodium vapor lamps) were turned on with the misters set to run for 2 minutes every 15 minutes for the next 2 hours. Finally, the misters were turned off, facilitating the slow-drying of the plant surfaces over the next 3-4 hours. When the plants were completely dry, they were moved back to the greenhouse or growth chamber under the conditions previously described.

Disease assessment

All four barley accessions were subjected to the following four treatments "No VIGS", "VIGS-MCS", "VIGS-as*Rpg5*" or "VIGS-as*Adf2*" and then infected with either stem rust pathotype *Pgt*-TTKSK or *Pgt*-QCCJ. Twelve days after stem rust inoculation, plants were scored for their infection types (ITs). The 0-4 IT scale used for barley is a modification of the one developed for wheat (Stakman *et al.*, 1962) and is based

primarily on uredinial size as described by Miller and Lambert (1955). The systemic spread of virus symptoms (i.e., mosaic pattern of chlorosis) was a guide to assess successful infection by *BSMV* and the potential for VIGS. For each plant, the youngest leaves (i.e. the 4^{th}, 5^{th} and 6^{th} leaves) were scored for ITs. For VIGS-treated plants, ITs were scored where virus symptoms were present.

Results and Discussion

Reaction of barley to stem rust pathotypes *Pgt*-TTKSK and *Pgt*-QCCJ without VIGS

In response to stem rust pathotypes *Pgt*-TTKSK and *Pgt*-QCCJ, accessions Q21861 and HQ18 exhibited low ITs (0;1 and 0;), whereas Steptoe and SQ41 exhibited high ITs (33+, 3 and 3-) (Table 2.1, Figures 2.3 & 2.4). In general, Steptoe was more receptive than SQ41 to stem rust infection by both pathotypes since it had higher numbers of uredinia (Figures 2.3 & 2.4). Based on the gene structure at the *rpg4/Rpg5* locus among these accessions (Figure 2.1), the susceptibility of SQ41 suggests that a functional *Rpg5* allele alone (as in SQ41) is not sufficient for conferring resistance. Also, for the *HvAdf2* (*rpg4*) allele, only two amino acids (position 101 and 135) are different in SQ41 compared to resistant line Q21861. These results indicate that these two amino acids in the *HvAdf2* (*rpg4*) allele are important for resistance to pathotypes *Pgt*-TTKSK and *Pgt*-QCCJ.

For recombinant line HQ18, it was already known that parental cultivar Harrington is susceptible to both pathotypes *Pgt*-TTKSK (preliminary data, unpublished) and *Pgt*-QCCJ (Brueggeman *et al.*, 2008). Harrington lacks the protein kinase domain in the *Rpg5* gene; however, the *HvAdf2* (*rpg4*) allele in Harrington encodes the same predicted protein product at the amino acid level as Q21861 (Brueggeman *et al.*, 2008). The susceptibility of Harrington to both pathotypes suggests that the *HvAdf2* (*rpg4*) allele in Harrington is not sufficient for resistance. Along with the *HvAdf2* (*rpg4*) allele from Harrington, recombinant line HQ18 also carries a complete *Rpg5* gene from Q21861 with all three domains (NBS-LRR-S/TPK) and expressed resistance to both *Pgt*-TTKSK and *Pgt*-QCCJ. These results indicate that both *HvAdf2* (*rpg4*) and *Rpg5* are required for resistance to pathotypes *Pgt*-TTKSK and *Pgt*-QCCJ.

Silencing of *Rpg5*

In response to *Pgt*-TTKSK infection after silencing *Rpg5* with VIGS, Steptoe and SQ41 again exhibited high ITs of 3. The other two accessions carrying *Rpg5* (Q21861 and HQ18) exhibited markedly higher ITs (12, 21 and 2) than in the No-VIGS (IT=0;1) or VIGS-MCS (IT=0;1) treatments, suggesting that *Rpg5* is critical for conferring a high level of resistance in these two accessions. A similar result was found in response to pathotype *Pgt*-QCCJ: the silencing of *Rpg5* resulted in higher ITs in Q21861 (IT=21) and HQ18 (IT=2) than observed in the control treatments of No-VIGS (IT=0;) or VIGS-MCS (IT=0;), while the ITs remained the same across treatments for Steptoe (IT=3) and SQ41 (IT=3). VIGS results confirmed the requirement of *Rpg5* for conferring resistance

to pathotypes *Pgt*-TTKSK and *Pgt*-QCCJ, because silencing the gene markedly reduced the resistance level in both Q21861 and HQ18 (Figures 2.3 & 2.4).

Silencing of *HvAdf2* (*rpg4*)

After silencing *HvAdf2* (*rpg4*), Q21861 and HQ18 continued to exhibit low ITs (0;1 and 0;) similar to the No-VIGS or VIGS-MCS treatments in response to both *Pgt*-TTKSK and *Pgt*-QCCJ. Likewise, Steptoe exhibited no marked changes in its high ITs (3) across these treatments to both pathotypes. Line SQ41 also exhibited no marked changes in response (IT=3) to *Pgt*-QCCJ after silencing *HvAdf2* (*rpg4*). However, in response to *Pgt*-TTKSK infection, SQ41 exhibited lower ITs (2) after *HvAdf2* (*rpg4*) silencing compared to the No-VIGS (IT=3) or VIGS-MCS (IT=3) controls, suggesting that the suppression of *HvAdf2* (*rpg4*) expression actually increases the resistance level in line SQ41. The recessive nature of gene *HvAdf2* (*rpg4*) suggests that its gene product might be non-functional. The non-functional *HvAdf2* (*rpg4*) in lines Q21861 and HQ18 might explain why silencing this gene resulted in no changes in the ITs of these resistant lines, since neither the expression nor silencing of this gene would trigger susceptibility. On the other hand, the Steptoe-like *HvAdf2* (*rpg4*) gene in SQ41 may act as a dominant functional gene (*Rpg4*) that encodes a susceptibility factor that is recognized by the stem rust pathogen (Brueggeman *et al.*, 2009). In response to pathotypes *Pgt*-TTKSK and *Pgt*-QCCJ, the product from the dominant *Rpg4* allele in SQ41 triggered susceptibility, and thus silencing this gene made SQ41 less susceptible to *Pgt*-TTKSK. Yet in response to *Pgt*-QCCJ infection, there was no reduction in susceptibility for line SQ41 after silencing

HvAdf2 (*rpg4*). Although pathotypes *Pgt*-QCCJ and *Pgt*-TTKSK exhibited a very similar virulence pattern against the *rpg4/Rpg5* locus for the accessions tested in this study, the difference with respect to SQ41 after silencing *HvAdf2* (*rpg4*) might be that the two pathotypes do not trigger the exact same responses in barley during the host-pathogen interaction, and there might be other susceptibility factors in barley interacting with *Pgt*-QCCJ, the latter scenario nullifying the silencing of *HvAdf2 (rpg4)* in reversing susceptibility.

Both *Rpg5* and *HvAdf2* (*rpg4*) are required for resistance

To corroborate the silencing of *Rpg5* and *HvAdf2* (*rpg4*) in VIGS-treated lines, total RNA from each treatment was isolated and will be subjected to quantitative real-time PCR to assay the silencing levels; however, these results are not available at this time. Nonetheless, based on our phenotype results, it appears that both *Rpg5* and *HvAdf2* (*rpg4*) are required for resistance to *Pgt*-TTKSK and *Pgt*-QCCJ in barley. *Rpg5* acts as a dominant gene and requires all three domains (NBS-LRR-S/TPK) to be functional, whereas *rpg4* acts in a recessive manner and possibly encodes a non-functional protein.

The presence of all three domains in the novel *Rpg5* gene suggests its function in both pathogen recognition and signal transduction. Brueggeman *et al.* (2008) predicted that the LRR domain might serve as a pathogen receptor outside cell, while the intracellular NBS and S/TPK domains may play a role in signal transduction. The S/TPK domain of *Rpg5* shares significant similarity with the tomato resistance gene *Pto*

(Brueggeman *et al.*, 2008). Interestingly, in tomato, resistance to *Pseudomonas syringae* pv. *tomato* expressing the *avrPto* gene requires both *Pto* and another NBS-LRR class gene *Prf* (Salmeron *et al.*, 1996). Also, genetic and physical mapping revealed the close proximity of *Prf* and *Pto* within the *Pto* gene cluster in the tomato genome (Salmeron *et al.*, 1996). The combination of all three domains in *Rpg5* and the physical proximity of *Pto* and *Prf* may suggest a common strategy in plants for utilization of both NBS-LRR protein and protein kinase for disease resistance signaling.

The gene product of *HvAdf2 (rpg4)* is an actin-depolymerizing factor-like protein. Such proteins have been documented in non-host resistance, but not gene-for-gene interactions (Brueggeman *et al.*, 2008). Brueggeman *et al.* (2009) speculated that *Rpg5* may directly or indirectly interact with *rpg4* through phosphorylation and lead to activation or deactivation of the actin depolymerizing factor. There are three other genes identified in the *rpg4/Rpg5* complex, including a NBS-LRR gene *HvRGA1*, an actin depolymerizing factor gene *HvAdf3*, and a protein phosphatase 2C protein gene *HvPP2C* (Brueggeman *et al.*, 2008). The functions of these additional genes are still unknown, but it is very likely that they also play certain roles in conferring stem rust resistance. The complete model for the mechanism of *rpg4/Rpg5*-mediated stem rust resistance has yet to be discovered. Key questions regarding pathogen recognition, signal transduction, and cytoskeleton organization should be addressed in the future to elucidate the molecular basis for *rpg4/Rpg5*-mediated resistance.

Table 2.1. Infection types of selected barley accessions in response to wheat stem rust pathotypes *Pgt*-TTKSK and *Pgt*-QCCJ under different VIGS treatments. The most common ITs observed on the youngest leaves (4^{th}, 5^{th} and 6^{th} leaves) and the range of ITs observed are given.

VIGS treatment	Accession	*Pgt*-TTKSK		*Pgt*-QCCJ	
		Most Common	Range	Most Common	Range
No-VIGS	Q21861	0;1	0; to 0;1	0;	0 to 0;
	Steptoe	33+	23- to 33+	3	2 to 3
	SQ41	3	23- to 33+	3-	2 to 3
	HQ18	0;1	0; to 210;	0;	0; to 2
VIGS-MCS	Q21861	0;1	0; to 0;1	0;	0; to 0;1
	Steptoe	3	2 to 33+	3-	2 to 33+
	SQ41	3	2 to 33+	3	2 to 33+
	HQ18	0;1	0; to 10;	0;	0; to 0;1
VIGS-*asRpg5*	Q21861	12	0; to 21	21	0;1 to 2
	Steptoe	3	2 to 3	3	32 to 33+
	SQ41	3	23- to 33+	3	2 to 3+
	HQ18	21	0;1 to 2	2	10; to 3
VIGS-*asAdf2*	Q21861	0;1	0; to 0;1	0;	0; to 0;1
	Steptoe	3	23- to 33-	3	2 to 3+
	SQ41	2	2- to 3-2	3	23- to 3+
	HQ18	0;1	0; to 12	0;	0; to 12

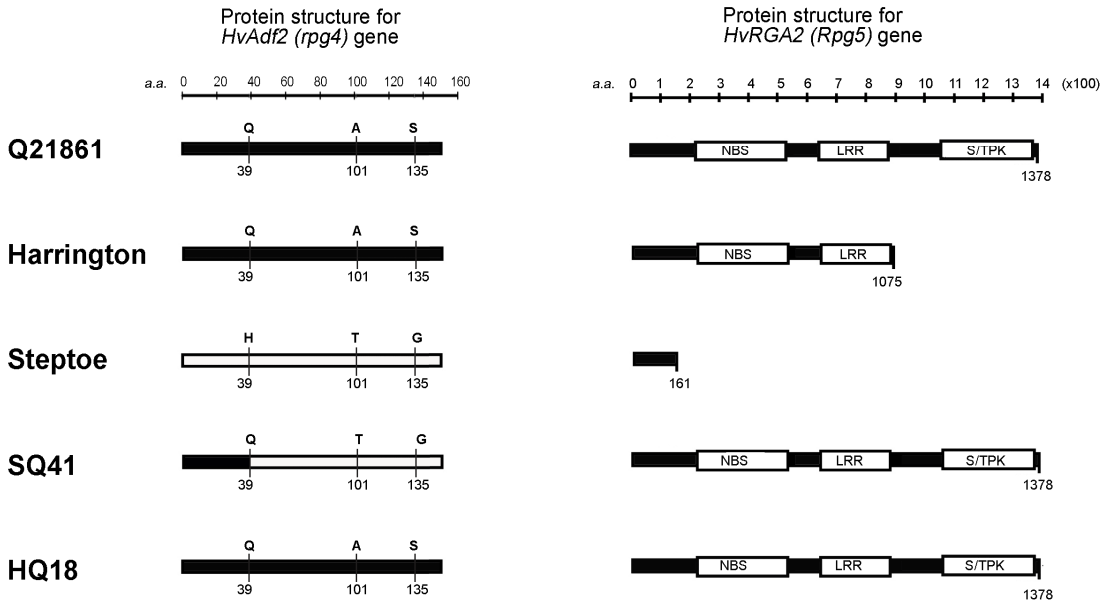

Figure 2.1. Predicted protein structures of the *Rpg5* and *HvAdf2(rpg4)* genes for barley accessions Q21861, Harrington, Steptoe, SQ41, and HQ18. The different *rpg4* alleles exhibit variation at three amino acid positions (39, 101, 135). *Rpg5* alleles differ on the basis of whether they encode functional proteins at all three domains (NBS-LRR-S/TPK) (i.e. lines Q21861, SQ41, HQ18) or truncated proteins (i.e. Harrington, Steptoe). (All protein structures and amino acid position data are based on Brueggeman *et al.* (2008))

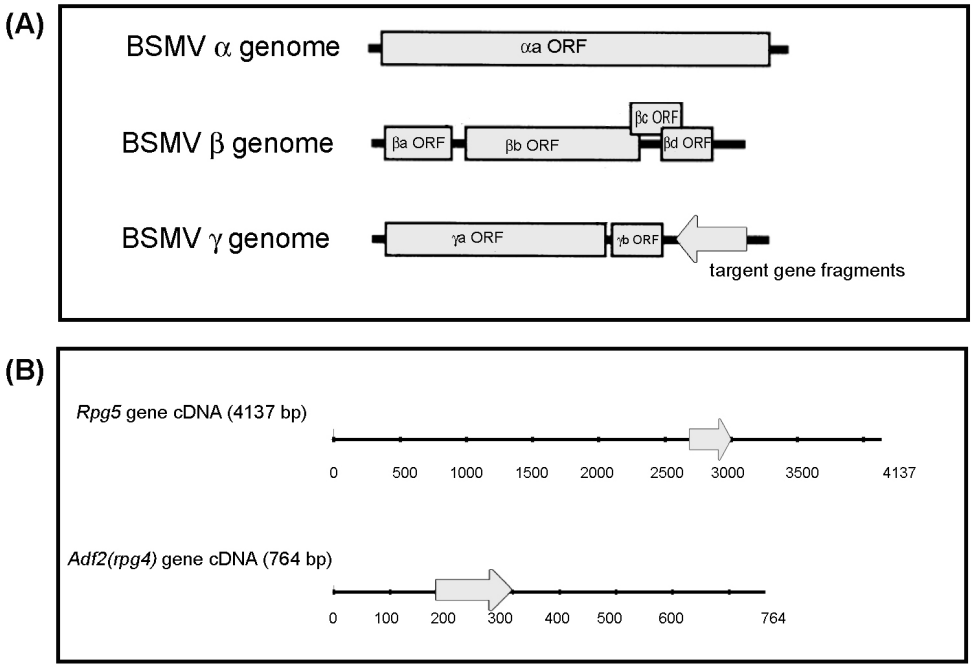

Figure 2.2. *BSMV* genomic RNAs (α, β and γ) (A) and the barley cDNA fragments (B) used for silencing *Rpg5* and *rpg4*. Grey boxes represent *BSMV* Open Reading Frames (ORFs), and the arrows represent cDNA fragments inserted into the *BSMV* γ genome vector in antisense orientation. (*BSMV* genome structures are based on Holzberg *et al.* (2002); *BSMV*-γ-*asRpg5* and *BSMV*-γ-*asAdf2* constructs were developed and provided by Robert Brueggeman, North Dakota State University.)

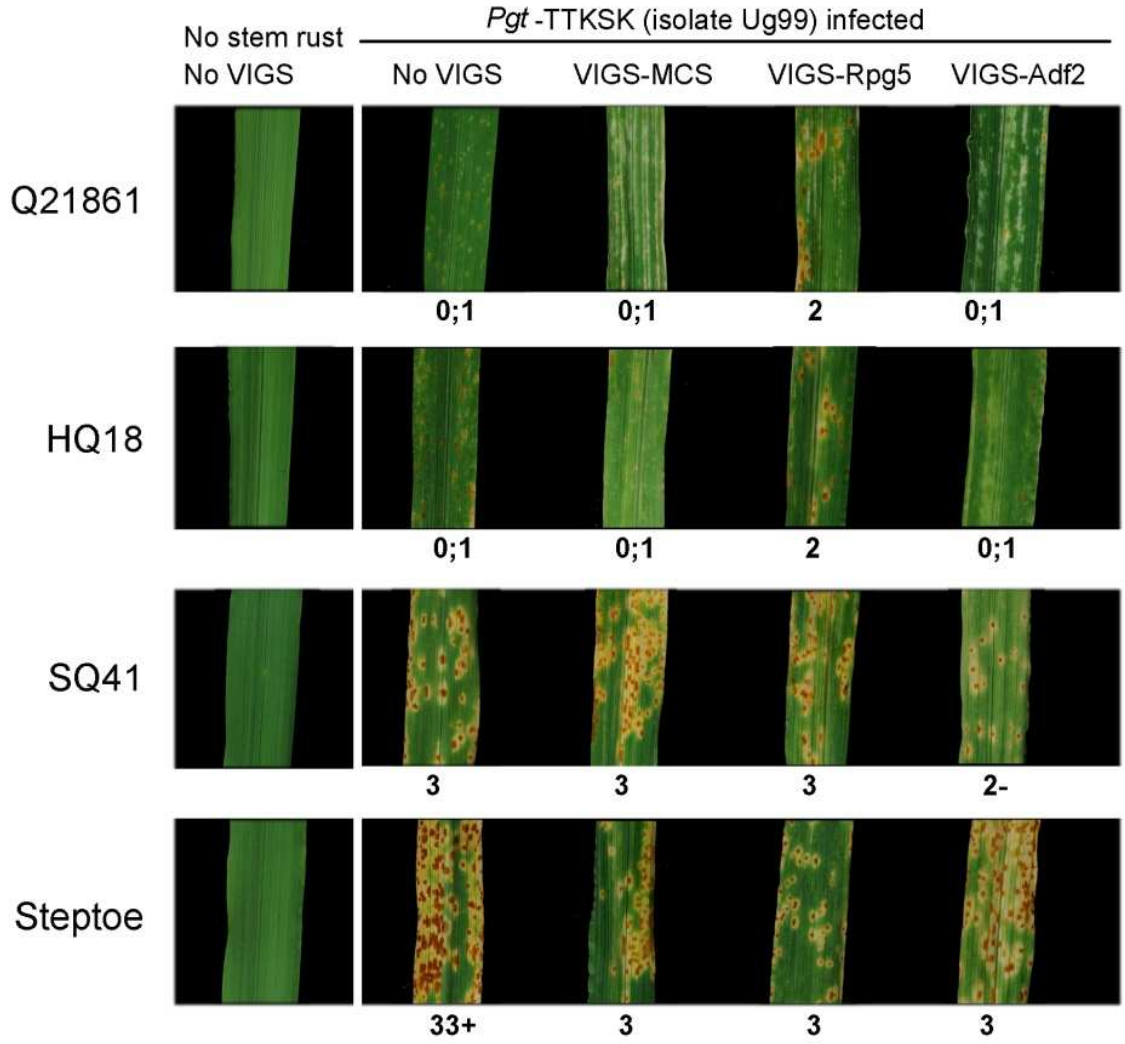

Figure 2.3 *Pgt*-TTKSK infection types (ITs) of barley accessions treated with VIGS. All accessions were subjected to the treatments of "No VIGS", "VIGS-MCS", "VIGS-as*Rpg5*" or "VIGS-as*Adf2*" and then inoculated with stem rust pathotype *Pgt*-TTKSK. The most typical ITs for each treatment are given in the figure with the corresponding ITs scores provided underneath.

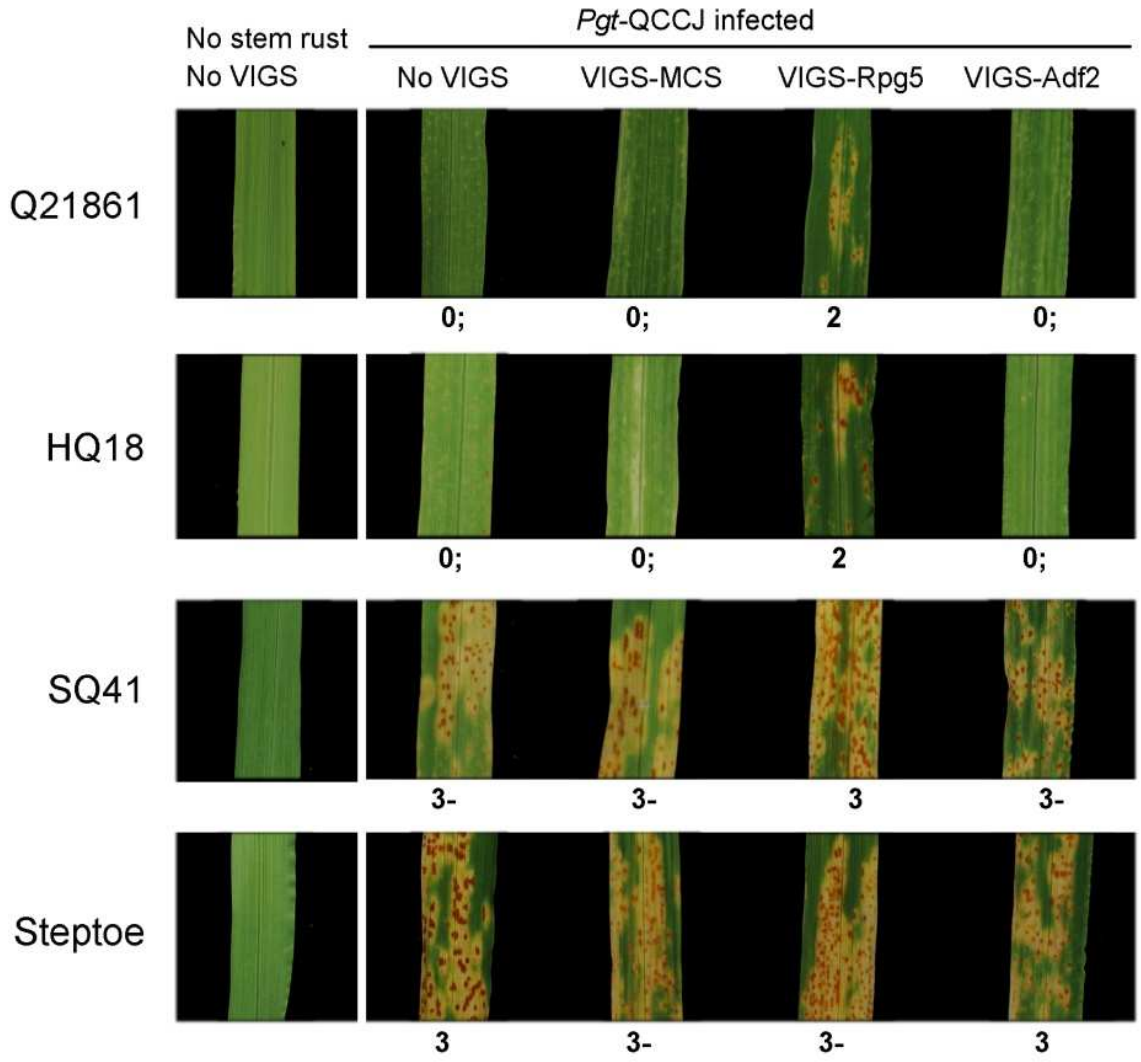

Figure 2.4 *Pgt*-QCCJ infection types (ITs) of barley accessions treated with VIGS. All accessions were subjected to the treatments of "No VIGS", "VIGS-MCS", "VIGS-as*Rpg5*" or "VIGS-as*Adf2*" and then inoculated with stem rust pathotype *Pgt*-QCCJ. The most typical ITs for each treatment are given in the figure with the corresponding ITs scores provided underneath.

Conclusions

Stem rust remains a great threat to world cereal production today, especially given the recent emergence of highly virulent pathotypes from eastern Africa. It is therefore important to study the function of known resistance genes to facilitate the development of more durably resistant cultivars. The major part of this thesis focused on three important stem rust resistance genes in barley: *Rpg1*, *rpg4*, and *Rpg5*. These three genes have great importance for barley stem rust resistance: *Rpg1* has been a durable resistance gene for over 60 years in the Upper Midwest region of the USA (Steffenson, 1992), and the *rpg4/Rpg5* complex confers resistance to the notoriously virulent pathotype *Pgt*-TTKSK (Steffenson *et al.*, 2009). These three genes are also among the most well characterized resistance genes in barley at the molecular level. *Rpg1*, *rpg4*, and *Rpg5* have all been cloned using a map-based cloning approach (Brueggeman *et al.*, 2002a; Brueggeman *et al.*, 2008). However, the mechanisms for *Rpg1*-mediated and *rpg4/Rpg5*-mediated stem rust resistance are still unknown, especially regarding the interactions between these genes and their corresponding pathogen effectors and the mechanisms for triggering resistance signaling pathways. The primary objective of this thesis was to further our understanding on the mechanisms of these genes in conferring stem rust resistance in barley.

In the first chapter of this thesis, the primary objective was to investigate the basis of phenotypic variation for stem rust resistance in transgenic lines containing different copy numbers of *Rpg1*. Five transgenic lines with 1, 2, 3, and 5 copies of *Rpg1* were investigated for stem rust resistance, stability of transgene inheritance over several

generations, the level of *Rpg1* mRNA transcript, level of RPG1 protein expression, and degradation of RPG1 protein upon stem rust infection. Southern blot analysis for transgene copy number estimation revealed the instability of transgene inheritance over several generations of selfing in some lines (i.e. lines G04-271 and G04-273 were reduced from 5 copies at T_0 to 3 copies at T_1 and only 1 copy at T_5), but stable inheritance in others (i.e. lines G03-210, G04-266 and G04-288 retained 1 copy, 2 copies and 5 copies, respectively, from T_0 to T_5). Post-transcriptional gene silencing (PTGS) was not triggered in the five-copy line G04-288 or any other transgenic line. Moreover, *Rpg1* transgene copy numbers were not strongly correlated with transcription and protein levels: e.g. the five-copy line G04-288 exhibited 29× and 3× greater transcript and protein levels than the one-copy cv. Morex, from which *Rpg1* was cloned, while all other transgenic lines (with either one or two copies) exhibited 2-9× greater transcript levels and 1.2-1.4× greater protein levels than cv. Morex. Upon stem rust infection, all transgenic lines exhibited RPG1 protein degradation between 20-28 hr post-inoculation. The one exception was susceptible line G04-288, which continued to exhibit high RPG1 protein levels even at 48 hr post inoculation. This result suggests that the failure of RPG1 protein to degrade rapidly resulted in susceptibility to stem rust.

In the second chapter of this thesis, we used VIGS to investigate the role of two closely linked resistance genes, *Rpg5* and *HvAdf2* (*rpg4*) in conferring resistance against wheat stem rust pathotypes *Pgt*-QCCJ and *Pgt*-TTKSK. Four barley accessions (Q21861, Steptoe, SQ41 and HQ18) were selected based on their genetic structure at the

rpg4/Rpg5 locus. Q21861 and HQ18 are resistant to both pathotypes, while Steptoe and SQ41 are susceptible. Silencing *Rpg5* in Q21861 and HQ18 resulted in higher infection types compared with non-silencing controls, suggesting that *Rpg5* is involved in the resistance pathway for both pathotypes. Also, in response to *Pgt*-TTKSK infection, silencing *HvAdf2* (*rpg4*) made SQ41 less susceptible, suggesting that the *Rpg4* allele in SQ41 might encode a susceptibility factor and therefore silencing this gene reduces the level of susceptibility in SQ41. *Rpg5* encodes a protein with a novel combination of three domains: NBS, LRR and S/TPK, while *HvAdf2* (*rpg4*) encodes an actin depolymerizing factor (Brueggeman *et al*. 2008). The involvement of both genes for resistance indicates a complex mechanism in the host-pathogen interaction during stem rust infection.

With the emergence and rapid spread of the virulent pathotype *Pgt*-TTKSK, breeding for resistance is of paramount importance. Studying the mechanisms of these resistance genes could help breeders incorporate novel and more durable resistance into barley cultivars. Researchers are currently making careful investigations to unlock the molecular basis of *Rpg1*- and *rpg4/Rpg5*- mediated resistance. Further studies on the role of RPG1 phosphorylation in conferring resistance, the functions of other genes in the *rpg4/Rpg5* complex, as well as the identification of corresponding stem rust effectors are under way. As our understanding of stem rust disease mechanisms in barley develops, yield losses caused by stem rust should be mitigated and the world's cereal production further secured.

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