INVESTIGATION OF *Puccinia graminis* RESISTANCE GENES IN BARLEY

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INVESTIGATION OF THE *Puccinia graminis* RESISTANCE GENES IN BARLEY

Abstract

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The barley stem rust resistance gene *Rpg1* was cloned and characterized by a map-based approach. It was shown to have a novel *R*-gene structure containing tandem kinase domains. To determine the origin of *Rpg1* we analyzed alleles from several barley land races (*Hordeum vulgare*) and wild barley (*H. vulgare* subsp. *spontaneum*) accessions. Our allele analysis determined that the functional *Rpg1* gene is rare among the landraces and was absent from the wild accessions examined. During the 1990s two new races of *Puccinia graminis* f. sp. *tritici* (*Pgt*) designated QCCJ and TTKSK, virulent on *Rpg1*, emerged in North America and Eastern Africa, respectively. Analysis of barley accessions for QCCJ and TTKSK reaction identified the barley line Q21861 as the best resistance source and was designated the *rpg4* gene. Q21861 also contains resistance against *Puccinia graminis* f. sp. *secalis* (*Pgs*) designated the *Rpg5* gene. The two resistance genes initially co-segregated to a region on barley chromosome 5H(7). In an attempt to quickly identify an *rpg4/Rpg5* candidate gene, five *Rpg1* gene family members were identified and mapped. One was tightly linked to the *rpg4/Rpg5* locus but segregated away in a high-resolution mapping population. Recombinant analysis of the high-resolution population revealed that *rpg4* and *Rpg5* were distinct genes. We cloned and partially characterized a 70 kbp genomic region from barley containing the *rpg4* and *Rpg5* genes. An *Rpg5* candidate gene was
identified and confirmed by allele sequencing. The predicted RPG5 protein from the resistant line Q21861 contains a Nucleotide-Binding-Site, Leucine-Rich-Repeat region and a protein kinase domain. A reverse genetics approach, Virus Induced Gene Silencing was used to confirm the Rpg5 gene. A gene encoding an actin depolymerizing factor-like protein (ADF2) was identified as rpg4. The Adf2 gene (rpg4), in combination with Rpg5, appears to be essential for resistance against the Pgt races, but not for the Pgs isolate. One possible hypothesis for the Adf2 gene function is that it might be activated or inactivated by RPG5 upon pathogen recognition. Thus making the ADF2 protein inaccessible for the fungus to redirect actin networks to establish a haustoria-plant interface to obtain nutrients from the plant.
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Preface

This thesis is based on the following published manuscripts, which are referred to by the respective chapters. Research from chapter 1 was carried out while I was an undergraduate. Chapter 2 is reflective of my masters thesis work. Chapters 3-5 are reflective of my doctoral work.

Chapter 1 (R. Brueggeman is the main contributor)

Chapter 2 (R. Brueggeman is the main contributor)

Chapter 3 (A. Mirlohi and R. Brueggeman contributed equally to this work)
Chapter 4 (R. Brueggeman is the main contributor)


Chapter 5 (R. Brueggeman is the main contributor)

DEDICATION

I dedicate this thesis to my loving wife Leah

Thank you!

and our three beautiful boys

Max, Saul and Gus
A. DEVELOPMENT OF STEM RUST RESEARCH

Rust fungi infect and damage most crop plants but those infecting the small grain crops
wheat, barley, rye and oats have caused the largest economic losses and pose the biggest threat to
world food security. One of the all time most devastating diseases of the small grains is stem rust
also known as black rust caused by the biotrophic fungal pathogen *Puccinia graminis*.

Historically and prehistorically dating as far back as the beginning of cultivating grain crops in
the Fertile Crescent region of the Middle East, stem rust epidemics have probably been one of
the major factors contributing to crop failure and famine. It is believed that stem rust has been a
problem since the beginning of cultivating grain crops in the Fertile Crescent region. The earliest
record of plant diseases is found in the Bible. It is written that while still in the desert of Sinai,
some thirty-five centuries ago, Moses had warned the people of Israel that if they failed to
observe the commandments of Jehovah, the Lord would punish them by destroying their field
crops with smut and with rust (Deut, 28:22) indicating that rust was a major concern at the time.
Now it is assumed that, in part, it was due to stem rust (Chester, 1946). *Puccinia graminis* spores
have been identified in archeological sites in Israel containing grain bins that date as far back as
1300 BC (Kislev, 1982), approximately the same period as this first historic record of rust on
wheat.

Wheat stem rust was also a serious disease in ancient Greece and during the Roman
Empire. It was observed and recognized as early as the times of Aristotle (384-322 B.C.).
Aristotle documented that the rust was brought on by the warm vapors indicating that ancient
scientists made the correlation between a warm moist environment and severe disease onset
(Chester, 1946). The ancient Romans also sacrificed red animals to the rust god Robigo in the
spring hoping that they could appease the god and he would spare their wheat crops of the devastating red rust (Chester, 1946). Historical weather records, indicating a string of unusually moist years, suggest that rust epidemics may have caused a food crisis contributing to the fall of the Roman Empire.

1. *Puccinia graminis*

The Italian Scientist Felice Fontana was the first to describe wheat stem rust in detail in the 1760s. Then in 1797 Christian Hendrik Persoon named it *Puccinia graminis*. By the 1850s the Tulasne brothers, Charles and Louis, had discovered that a number of autoacious (single host) rust fungi produced up to five different spore types or stages during their lifecycle. They also observed and described that the red (urediniospores) and black (teliospores) were different spore stages of *Puccinia graminis*, but the reason basidiospores of *Puccinia graminis* could not infect its grass host remained a mystery until Anton deBarry’s work in 1865. Anton deBarry was interested in the observation that the basidiospores of *Puccinia graminis* were unable to infect wheat plants. He was aware of the farmer’s beliefs that barberry increased the onset of wheat rust early in the season and greatly affected the severity of the disease. Using this knowledge he successfully inoculated barberry with *Puccinia graminis* basidiospores and observed the remaining spore stages, which occur on the alternate host (Chester, 1946). This heteroecious nature of stem rust was a very important discovery in phytopathology and once the heteroecious nature of rusts was described in stem rust it was established in many other known rust fungi and their secondary hosts were identified.

The function of the spore stages were not completely understood until John Craige’s studies in 1927 (Chester, 1946). *Puccinia graminis* was determined to be a heteroecious, macrocyclic rust with a life cycle involving five spore stages and two hosts. The two most
important spore stages concerning disease management are the asexual urediniospore (dikaryotic n+n) and the sexual aeciospore (n+n). The uredinium is produced on the grass host after infection resulting from inoculation by either a urediniospore or aeciospore. The urediniospore is very stable in the environment and can be wind disseminated over long distances. Urediniospores then act as secondary inoculum reinfecting the grass host species cycling as secondary inoculum during the summer on the primary hosts including barley and wheat. When environmental conditions are favorable, the generation time from spore to secondary inoculum is approximately 10-15 days. The rapid production of inoculum, coupled with the long distances the spores can be disseminated, makes stem rust a very explosive and destructive disease where sufficient inoculum, the right environmental conditions and a susceptible host is grown over large tracts of land. These three components make up the disease triangle in epidemiological terms and dictate the severity of an epidemic.

Late in the growing season, the stem rust fungus begins producing teliospores on the stems of maturing grass host. Teliospores lay dormant in the straw during the winter providing the only means for long term survival of the obligate biotroph in the absence of a living host. In the spring, the teliospores germinate and produce four haploid basidiospores, the result of karyogamy and meiosis. Basidiospores can not infect the primary grass hosts but rather infect the alternate host *Berberis* (barberry) or, *Mahonia* spp. The infection on the alternate host results in the development of pycnia, occurring with the fusion between a pycniospore and receptive hyphae of opposite mating types. Plasmogamy results in a dikaryotic hyphae which produce aecium with dikaryotic aeciospores. Although new races can emerge by mutation events in the absence of barberry, the secondary host functions to produce greater diversity of new races because the pycnial stage preceding aecial development allows for genetic recombination.
Aeciospores are then wind disseminated back to the grass host, thus completing the sexual stages of the life cycle. In order to stop the early season inoculum coming from the secondary host, barberry eradication programs began as early as the 1600s in France and the 18th century in the United States.

2. Stem Rust in North America

Stem rust caused devastating epidemics to wheat and barley production in the Midwestern United States and Prairie Provinces of Canada until the mid twentieth century. Efforts to develop rust resistance in wheat varieties were initiated in Kansas in 1911 soon followed by numerous other states, beginning the so called “Boom and Bust” cycles of wheat breeding and production in the Midwest. R-genes deployed in the new wheat varieties would protect the crop (the Boom) until races virulent on these R-genes became the predominant races causing large-scale stem rust epidemics again (the Bust). Stem rust epidemics prior to the 1950s during a single growing season resulted in wheat yield losses as high as 200 million bushels in the US alone. Then in the 1950s wheat breeders began pyramiding several resistance genes into their varieties. This practice provided more durable resistance ending the occurrence of the major stem rust epidemics (Peterson, 2001). The losses on barley are not well documented but the epidemics were similar to that found on wheat (Steffenson, 1991). Stem rust was also managed using genetic resistance in barley, but only a single source of resistance was deployed and has protected midwestern barley cultivars since 1942.

The small grain hosts of stem rust, barley, oats, rye and wheat are all non-native crops brought to North America by the European colonists. Barberry was also brought to North America for its practical uses including production of yellow dye and edible berries used in jams.
and wine. The fast growing thorny hedges were also used to coral domestic animals. Farmers made the connection between the alternate host barberry and the early and more severe onset of rust within their wheat and barley fields much earlier than the scientific community. Farmers urged the passage of laws as early as the 1660’s requiring the destruction and banning of barberry in wheat growing regions of Rouen, France (Chester, 1946).

In the US barberry bans were enacted as early as 1726 in Connecticut and soon after in other states (Chester, 1946). Then after the severe stem rust epidemic of 1916 the US implemented the barberry eradication program in 1918. This impacted the control efforts of rust in two major ways. First it reduced the amount of early season inoculum coming in from the barberry plants found in fence rows effectively delaying disease onset by 10 days. Second, it removed the sexual stage of the rusts lifecycle thus limiting its genetic diversity making recombinant forms and new races less likely giving breeders more time because $R$-genes deployed were effective for longer periods (Roelfs, 1982).

After over half a century of managing stem rust through genetic resistance it was believed by many that stem rust was a thing of the past. Norman Borlaug’s statement sums this up quite well “Isn’t it quite possible that the greatest ally of the pathogen is our short memory of the disastrous stem rust epidemics from 1951-1954 across much of the wheat producing regions of North America” (Peterson, 2001). The misconception that stem rust has been defeated has fostered complacency and currently restricts research on this shifty enemy. Stem rust research has received less and less funding by the powers that be, leading to the dependency on too few resistant sources in the US and the major wheat and barley producing regions worldwide. The recent emergence of a new highly virulent race of stem rust in Eastern Africa and its quick
migration through Middle Eastern Asia has sent plant scientist scrambling to identify and deploy new sources of resistance in both wheat and barley.

3. Identification of Barley Stem Rust Resistance Genes

Barley is an important crop in the Upper Midwestern region of the US and Prairie Provinces of Canada with over 15 million metric tons produced annually. However, barley has a much lower rank than wheat as a food crop for human consumption thus justifiably gets substantially less attention than wheat in the field of stem rust research. Stem rust was historically as much a problem on Midwestern barley as wheat, but has not caused a widespread epidemic on barley for over 70 years due to the fortuitous deployment of a single durable resistance gene, \textit{Rpg1} (Roelfs, 1978). \textit{Rpg1} was the first stem rust resistance gene identified and was first described from an unimproved line imported by the USDA from the Canton of Lucerne in Switzerland in 1914. It was shown to be a single dominant gene originally designated the \textit{T} gene (Powers and Hines, 1933), later changed to \textit{Rpg1} for Reaction to \textit{Puccinia graminis} 1 (Sogaard and von Wettstein-Knowles, 1987). However, it was a different source of resistance that gave rise to the first released barley cultivar containing the \textit{Rpg1} gene. This source of resistance originated from a farmer named Sam Lykken from Kindred, North Dakota. During the severe stem rust epidemic year of 1935 Sam was surveying his severely rusted barley field planted with the susceptible variety Wisconsin 37, when among the entirely rusted and shriveled field he observed a single green healthy plant. He harvested seed from this plant and increased the seed for several generations. By 1941 Sam had enough seed for his own planting and 1,200 bushels to sell. This single plant selection was released as the cultivar (cv.) Kindred in 1942 and remained the dominant cultivar in the Red River valley for over a decade (Steffenson, 1991).
Kindred together with the two barley selections, Chevron and Peatland, made from the unimproved line obtained from the 1914 USDA imported germplasm from Switzerland were the only sources of stem rust resistance used in barley breeding in the Midwestern USA and Canada. With the cloning of the barley \textit{Rpg1} gene, it was shown that all three cultivars, Kindred, Chevron and Peatland, possess an identical \textit{Rpg1} allele (Brueggeman \textit{et al}., 2002 [chapter 1]). Wisconsin 37, the cultivar planted in the field from which Sam Lykken recovered the progenitor of Kindred, contains an allele that could not have given rise to the resistant allele by a spontaneous mutation event. This molecular evidence confirmed that the source of Kindred barley probably came from a seed contamination as proposed earlier by Steffenson (Steffenson, 1992).

\textit{Rpg1} resistance is classified as durable due to its successful control of stem rust since the release of Kindred in 1942. This remarkable durability held up in the 40s and 50s even through epidemics that devastated the wheat crop (Steffenson, 1992). Considering the potential variability of the fungus and the vast hectares (~ 1.8 million) over which cultivars with this resistance gene are grown (Steffenson, 1992), \textit{Rpg1} fulfills the requirements to be defined as durable (Johnson, 1984). In the field \textit{Rpg1} has conferred resistance to most pathotypes of \textit{Puccinia graminis} f. sp. \textit{tritici} (\textit{Pgt}), but there are exceptions. The genes conferring resistance to these exceptional stem rust races virulent on \textit{Rpg1} was the focus of my doctoral research.

Aside from \textit{Rpg1} there are seven other identified sources of resistance against \textit{P. graminis} from barley, but none of these have been deployed in commercial cultivars. A complete list of \textit{Rpg} genes includes \textit{Rpg1}, \textit{Rpg2}, \textit{Rpg3}, \textit{rpg4}, \textit{Rpg5}, \textit{rpg6}, \textit{rpgBH}, and \textit{RpgU}. Only \textit{Rpg1}, \textit{rpg4} and \textit{Rpg5} have been cloned (Brueggeman \textit{et al}., 2002 [chapter 1]; Brueggeman \textit{et al}., 2008 [chapter 4]) and investigated at the molecular level. \textit{Rpg2}, identified from Hietpas-5 (CIho 7124) (Patterson \textit{et al}., 1957), \textit{Rpg3}, from PI382313 (Jedel, 1990), \textit{rpgBH} from Black Hulless (CIho...
666) (Steffenson et al., 1984) and RpgU from Peatland (Fox and Harder, 1995) all confer low levels of stem rust resistance making these genes difficult to phenotype therefore non of these resistance loci have been mapped (Sun and Steffenson, 2005).

A new pathotype of Pgt, designated race QCCJ, was identified on Midwestern barley cultivars containing Rpg1 during the 1989 growing season (Roelfs et al., 1991). This race increased in prevalence and became one of the most common virulence types in the US causing minor epidemics (Roelfs et al., 1993). The widespread distribution of race QCCJ and the susceptibility of barley grown on the Northern Great Plains warranted expectations of a coming large epidemic, but it has never materialized. Barley production was threatened by race QCCJ prompting the evaluation of over 18,000 barley accessions from the USDA National Small Grains collection for sources of resistance. The best source of resistance against race QCCJ was discovered in the unimproved barley line Q21861 from the International Maize and Wheat Improvement Center (CMMYT) (Jin et al., 1994a). Genetic studies revealed that resistance to race QCCJ in line Q21861 was conferred by a single recessive gene given the designation rpg4. A recessive resistance gene is unusual among disease resistance genes suggesting that resistance may be the result of a non-functional gene or null allele. The rpg4 gene is also temperature sensitive providing resistance at lower temperatures (17-22°C), but completely ineffective at temperatures above 27°C (Jin et al., 1994b). The rpg4 gene was genetically mapped to the long arm of barley chromosome 5H(7) (Borovkova et al., 1995).

A new source of resistance to Pgt race QCCJ was recently discovered from a Hordeum bulbosum introgression into the barley cv. Golden Promise (susceptible) background. (Fetch et al., 2009) The rpg6 gene was identified in the translocation from H. bulbosum and maps to chromosome 6H. Similar to rpg4, the rpg6 gene is also recessive.
Line Q21861 also contains resistant to an isolate of rye stem rust, *Puccinia graminis* f. sp. *secalis* (*Pgs*) isolate 92-MN-90. Although *Pgs* 92-MN-90 was first isolated from rye hence the name *f. sp. secalis*, it is closely related to *Pgt* and this forma specialis of stem rust infects barley causing damage to the crop in some years (Steffenson, 1992). The single dominant resistant gene was originally designated *RpgQ*, later changed to *Rpg5* and was genetically mapped to the long arm of chromosome 5H(7) very closely linked to *rpg4* (Druka *et al*., 2000). Initially it was believed that *rpg4* and *Rpg5* could be the same gene despite the different inheritance, recessive vs dominant respectively.

A new threat to barley production worldwide is stem rust race TTKSK (original isolate synonym Ug99), a pathotype first characterized from Uganda in 1999 (Pretorius *et al*., 2000). Race TTKSK is considered highly virulent because it carries a unique combination of virulence genes and is capable of attacking most barley cultivars, including those having *Rpg1* (Steffenson and Jin, 2006), and over 70% of the world’s wheat cultivars (Singh *et al*., 2008). Race TTKSK has not reached North America but poses a threat to wheat and barley production in the US and Canada because of its wide range of virulence and the ability of stem rust to spread rapidly over long distances. Since the time it was initially discovered in 1999 (Pretorius *et al*., 2000), TTKSK has spread in Eastern Africa, then disseminated over the Red Sea into Yemen, and has been confirmed in Iran and possibly Pakistan (Nazari *et al*., 2009). It will be a short time before it spreads into the major cereal production areas of southern Asia and ultimately will find its way to North America.

A major global effort through the Borlaug Global Rust Initiative (BGRI) has been launched to breed wheat varieties resistance to pathotype TTKSK and great progress has been made (http://www.globalrust.org/about_us.cfm?m=2). However, relative to wheat very little
funding has been allocated to the barley breeding efforts due to its lower rank as a human food crop. It has been overlooked by the BGRI that in countries where TTKSK now exists (Ethiopia, Eritrea, and Yemen), barley is one of the most important food crops (Zemede, 1996) and these resource poor farmers subsisting on barley should be a focus of the initiative. To effectively control stem rust in these regions and elsewhere, it is important that both wheat and barley crops are resistant since they are often cultivated in close proximity. This practice is important to keep the Pgt population in check, otherwise the probability of virulent mutants arising on the susceptible crop with the ability to start an epidemic on the previously resistant crop is greatly increased (Schafer and Roelfs, 1985).

The barley line Q21861 was found to be one of the most resistant barleys to race TTKSK at both the seedling stage in greenhouse and at the adult plant stage in the field (Steffenson, personal communication). Understanding the genetics of race TTKSK resistance in Q21861 is important for developing the most efficient strategy for deploying this source of resistance. It has been determine that race TTKSK resistance in Q21861 is conferred by a single locus cosegregating with rpg4 and Rpg5 (Steffenson, personal communication; Brueggeman et al., unpublished).

Line Q21861 is a good source of stem rust resistance but other sources of resistance especially against race TTKSK need to be identified and transferred into adapted breeding lines. Evaluation of *Hordeum* germplasm identified several sources of resistance to race TTKSK in various landraces (*H. vulgare*) and wild barley (*H. vulgare* subsp. *spontaneum*) accessions (Steffenson and Jin 2006). Sequence analysis of rpg4 and Rpg5 alleles from many of these lines indicated that the majority contain functional alleles, however there is polymorphism among the Rpg5 alleles. The rpg4/Rpg5 locus probably confers the race TTKSK resistance in these
landraces and wild barley accessions. Some *Hordeum* germplasm with resistance to race TTKSK and a predicted non-functional *Rpg5* allele were identified, suggesting that these lines may contain novel sources of resistance (Brueggeman *et al*., unpublished). Allelism and mapping studies are underway to confirm and characterize these new sources of stem rust resistance (Brian Steffenson, personal communication).

It has been fortunate for Midwestern barley growers and breeders that the first barley stem rust resistance source discovered was so remarkably durable. However, unfortunate that so much reliance was placed on this single gene. It has long been known that reliance on a single or limited numbers of stem rust resistance genes is dangerous because of the high probability of a pathogen shift or the introduction of a virulent race. After 70+ years of exclusive use the new virulent race TTKSK may bring the *Rpg1* era to an end and barley production may once again be threatened in the Midwestern United States and Prairie Provinces of Canada.

4. Resistance Genes

Plant *R*-genes mediate resistance to a diverse variety of pathogens including fungi, bacteria, nematodes, viruses, insects and oomycetes. In 1955 Flor postulated that for each *R*-gene there is a matching avirulence (*Avr*) gene in the pathogen, and that the presence of both is required to elicit a resistance response in the plant. This gene-for-gene hypothesis was later translated into a receptor-ligand model where the *R*-protein functions as the receptor that recognizes or interacts with the corresponding *Avr* protein ligand. The protein-protein interaction acts as the trigger initiating the signaling cascades resulting in defense responses leading to resistance.

The lack of data showing that *R*-proteins directly interact with their corresponding *Avr* ligands coupled with the identification of plant proteins that directly interact with both *Avr* and
R-proteins has led to an alternate hypothesis: the guard hypothesis (Van der Biezen and Jones, 1998). In this model the R-proteins (‘the guards’) monitor other plant proteins (‘the guardees’) that are the target of modifications by the pathogen’s Avr effector proteins. The modification of the guardee by the effector is detected by the R-protein triggering the defense response. This model suggests that R-proteins maybe present in complexes containing the guardee and may not have a direct protein-protein interaction with the Avr effector protein.

Advances in molecular techniques and tools have facilitated the cloning of ~ seventy plant disease R-genes in the past three decades. These R-genes have been grouped into different classes according to their protein domain structure (Martin et al., 2003). The majority of R-genes belong to the NBS-LRR family of genes characterized by a N-terminal nucleotide-binding site (NBS) and a C-terminal leucine rich region (LRR). Many of the NBS-LRR R-proteins have different N-terminal domains. Most fall into one of two classes, the Toll and interleukin-1 receptors (TIR) or the putative leucine-zipper (LZ) motifs (Hammond-Kosack and Jones, 1997).

The only known function for this class of genes in plants is involvement in disease resistance. However, in Arabidopsis a NBS-LRR gene confers susceptibility to the fungus Cochliobolus victoriae (Lorang et al., 2007). Some NBS-LRR R-proteins have been shown to interact directly with pathogen Avr proteins and the LRR is the critical component determining pathogen specificity (Dodds et al., 2006). NBS-LRR genes also function to guard the effector targets detecting modification by pathogen Avr proteins (Jones and Dangle, 2006).

The proteins encoded by the second major class of R-genes all contain at least one serine/threonine protein kinase (STPK) domain. The STPK domain suggests that these proteins function in plant signal transduction pathways. The seven members of the STPK group confer resistance to bacterial and fungal pathogens and have been cloned from wide taxa of plant
species. This class of $R$-genes include the tomato $Pto$ gene, which confers resistance to the bacterial pathogen $Pseudomonas syringae$ (Martin et al., 1993), the Arabidopsis $PBS1$ gene conferring resistance to the bacterial pathogen $Pseudomonas syringae$ (Swiderski and Innes 2001), the rice $Xa21$ and $Xa26$ genes which confer resistance to the bacterial pathogen $Xanthomonas oryzae$ (Song et al., 1995; Sun et al., 2004), the recently cloned wheat $Yr36$ gene conferring resistance to $Puccinia striiformis$ (Fu et al., 2009) and the barley $Rpg1$ and $Rpg5$ genes (Brueggeman et al., 2002 [chapter 1]; Brueggeman et al., 2008 [chapter 4]). $Rpg1$ has a novel $R$-gene structure containing two tandem STPK domains whereas the other STPK $R$-genes contain a single protein kinase domain that can be connected to other protein domains characteristic of $R$-genes; $Xa21$ and $Xa26$ have LRR-STPK structures (Song et al., 1995; Sun et al., 2004) and $Rpg5$ has NBS-LRR-STPK structure (Brueggeman et al., 2008 [chapter 4]). The recently cloned $Yr36$ gene has a novel structure with a lipid binding domain attached to the STPK domain (Fu et al., 2009).

5. Rust Resistance Genes; Cloning and Characterization

Sixty years ago J.B.S. Haldane predicted that polymorphisms in proteins would govern host-pathogen interaction and this variability was the result of the rapid rate of microbial evolution. His knowledge of the variability of stem rust resistance in wheat spurred his suggestion that host plants could only react by generating high levels of polymorphism in their immune system genes (Haldane J.B.S., 1949). Harold Flor was a pioneer of rust resistance gene research and provided evidence to support these speculations. In the 1940s he crossed many flax rust races then determined the disease reaction on a large number of flax lines when infected with the progeny of his crosses. Flor’s flax rust research determined that the rust pathogen
Melampsori lini pathogenicity was genetically inherited as single dominant genes (Flor H.H, 1946). Later Flor proposed the gene for gene hypothesis discussed earlier (Flor H.H, 1956).

Moving into the molecular genetics era flax rust resistance has maintained its status as the most extensively studied rust resistance systems. Five resistance loci from Flax have been mapped (L, M, P, N, and K) (Ellis et al., 1988) and four R-genes have been cloned and characterized. (L, M, P and N) (Anderson et al., 1997; Dodds et al., 2001; Lawrence et al., 1995). All four characterized Flax R-genes code for TIR-NBS-LRR domain proteins. It was determined that the race specificity between the different L alleles was determined by polymorphisms within the LRR domain suggesting that the LRR domain could be responsible for Avr binding/detection (Ayliffe et al., 1999).

The subject of R-genes and their underlying mechanisms can not be fully understood without the identification and characterization of the pathogens Avr protein; proteins that directly or indirectly interact with the plant R-gene to illicit the resistance response. Many Avr proteins have been identified from a large taxonomy of pathogens and most encode small peptides from very diverse classes of proteins (Abramovitch and Martin, 2004; Catanzanti et al., 2007). The first rust Avr gene was cloned from flax rust by a map-based cloning approach (Dodds et al., 2004). This Avr gene was designated the flax AvrL567 gene because its protein product is recognized by L5, L6 and L7 and was later determined to interact directly with all three corresponding R-proteins in vivo and in yeast two-hybrid assays (Dodds et al., 2006). AvrL567 was shown to be expressed in the haustoria and encodes a 127 aa secreted protein that is delivered to the host cells across the plasma membrane (Dodds et al., 2004).

Approximately 10% of the R-genes cloned are rust resistance genes from the small cereal grains. These cloned R-genes are the maize Rp1 gene conferring resistance against P. sorghi
(Collins et al., 1999), the wheat leaf rust resistance genes \textit{Lr10, Lr21} and \textit{Lr34} (Feuillet et al., 2003; Huang et al., 2003; Krattinger et al., 2009) conferring resistance against \textit{Puccinia triticina}, the wheat stripe rust resistance gene \textit{Yr36} (Fu et al., 2009) conferring resistance against \textit{Puccinia striiformis} and the barley stem rust resistance genes \textit{Rpg1, rpg4} and \textit{Rpg5} described here.

\textit{Rp1} and \textit{Rp3} of maize are complex loci consisting of clusters of closely related NBS-LRR genes (Collins et al., 1999; Webb et al., 2003). The \textit{Rp3} gene has been delimited to a complex locus containing a NBS-LRR gene family consisting of nine genes but the specific gene conferring the resistance has not been identified (Webb et al., 2003). These complex loci have a tendency to mispair and recombine in meiosis creating haplotypes that are structurally variable. For example, \textit{Rp1} haplotypes in different maize lines vary in the number of \textit{Rp1} genes they carry from a single gene to greater than 50 (Smith et al., 2004). Recombinant genes and haplotypes have been identified that have different reaction to pathotypes than the original parental types (Hulbert, 1997). In addition to typical race specific resistance genes like \textit{Rp1-D}, recombinant genes have been identified that give an extensive hypersensitive reaction to inoculation with any rust isolate, including non-host species like the wheat rusts (Hu et al., 1996). Unequal recombination within these complex loci appears to be an important process to generate new combinations of NBS-LRR genes resulting in more diversity and new pathotype specificities.

The wheat \textit{R}-genes \textit{Lr10} and \textit{Lr21} confer race specific resistance to \textit{Puccinia triticina}. Both genes were cloned by map-based cloning strategies and determined to code for typical NBS-LRR \textit{R}-genes. These rust resistance loci also appear to be part of complex loci containing several copies of NBS-LRR genes from the same and different families (Huang et al. 2001) similar to the \textit{Mla} resistance locus in barley which contains three distinct NBS-LRR gene families, cosegregating with \textit{Mla} (Wei et al., 1999). The recently cloned wheat leaf rust
Resistance gene \textit{Lr34} has been shown to code for a putative ABC transporter. The current model for the function of the transporters is to translocate toxic compounds into the apoplast (Krattinger \textit{et al.}, 2009). \textit{Lr34} has conferred durable resistance against leaf rust for 50 years but is also associated with resistance against stripe rust and powdery mildew. This source of durable adult resistance when deployed with other adult plant resistance genes provides a resistance level near immunity and has been deployed in wheat cultivars worldwide.

Map-based cloning was also used to recently clone the \textit{Yr36} gene that confers resistance to wheat stripe rust (\textit{Puccinia striiformis}) (Fu \textit{et al.}, 2009). \textit{Yr36} confers broad-race resistance (slow rusting) to stripe rust at relatively high temperatures (25-35°C). New races of stripe rust are virulent on most of the known race-specific resistance sources due to the rapid pathogen evolution but the slow rusting genes providing partial adult resistance are generally durable. The \textit{Yr36} gene has been identified and contains two protein domains, a STPK and a START lipid binding domain. The authors proposed the possibility that the START domain may bind a lipid from or redirected by the pathogen which then initiates the signaling cascade via the protein kinase domain resulting in resistance (Fu \textit{et al.}, 2009).

It is important to note that no rust resistance genes are listed here from rice, the model cereal grain. Rice is not afflicted by rust thus is a non-host species. This has been the focus of controversial molecular genetics research of rust, initiated by the nobel laureate Norman Borlaug. It is his belief or as he stated “his dream” that the mechanisms that underlie the immunity in rice could be determined then transferred to wheat (Borlaug, 2000). A substantial amount of research funding maybe directed toward this line of research which could probably be better directed at the understanding of the mechanisms that underlay resistance in barley and wheat, the major cereals actually afflicted by the disease. It should be kept in mind that \textit{R}-gene
complexes or mechanisms although made up of many similar components and classes of proteins may react to the pathogen in different ways, especially across species that behave as host and non-host.

B. **Rpg1 CLONING AND CHARACTERIZATION**

In order to characterize the barley rust resistance genes and the underlying mechanisms involved in resistance first they must be identified and cloned. Dr. Andris Kleinhofs initiated the cloning of stem rust resistance genes in 1995 soon after his lab developed an extensive genetic map of barley. The development of the Steptoe/Morex map (Kleinhofs *et al.*, 1993) prompted the desire to utilize this resource to clone an agronomically important gene by a map-based approach. At this time map-based cloning was in its fledgling stages especially in a large grass genome such as barley. The *Rpg1* gene was chosen as a target for several reasons most importantly that it was a remarkably durable resistance gene present in the cv. Morex, one of the parents in the mapping population. It was also a quantitatively or simply inherited gene easily phenotyped and had recently been mapped to the telomeric end of barley chromosome 7H(1). This was fortunate because the syntenic region in rice was also being sequenced and prompted the hope of exploiting rice/barley synteny to identify the gene quickly. While synteny with rice did provide valuable molecular markers, which advanced the fine mapping in barley (Kilian *et al.*, 1997), a candidate *Rpg1* gene was not found in the rice chromosome syntenic region (Han *et al.*, 1999). This intensified the effort in Dr. Kleinhofs’ lab and after the development of many molecular tools the arduous task of cloning the first stem rust resistance gene was completed in 2002 (Brueggeman *et al.*, 2002 [paper I])
The large (>5,000 Mb), repetitive (>80%) barley genome makes map-based cloning very labor intensive and time consuming yet has been proven as the most effective strategy for identifying barley R-genes. Mlo was the first barley R-gene cloned by map-based cloning using a yeast artificial chromosome (YAC), library (Buschges et al., 1997). The subsequent construction of a bacterial artificial chromosome (BAC) library from the barley cv. Morex (Yu et al., 2000) was the pivotal tool in the more recent cloning of the barley powdery mildew resistance genes Mla1 and Mla6 (Haltermann et al., 2001; Zhou et al., 2001) and the stem rust resistance gene Rpg1, rpg4 and Rpg5 (Brueggeman et al., 2002 [chapter 1]; Brueggeman et al., 2008 [chapter 4]). The development of the barley Steptoe/Morex Bin maps in conjunction with molecular tools (EST libraries (Close et al., 2001), BAC clone libraries (Yu et al., 2000), and the sequence of related organisms (Sasaki and Burr, 2000)) has made map-based cloning in barley a much more efficient strategy. Utilizing syntenous regions of the sequenced genomes of rice and Brachypodium for molecular marker development, high quality genetic maps and the cv. Morex BAC library we have developed physical contigs across the rust resistance regions of Rpg1 (Brueggeman et al., 2002 [paper 1]), rpg4/Rpg5 (Druka et al., 2002; Brueggeman et al., 2008 [paper 4]) and the spot blotch resistance locus Rcs5 (Drader et al., personal communications).

1. **Rpg1 Map-Based Cloning**

A high resolution genetic map was constructed using 17 critical recombinants selected from 8,518 gametes by genotyping with co-dominant flanking markers. A 330 kilobase BAC contig was developed across the locus and the physical map positioned the gene between two cross overs a genetic distance of .21 cM and a physical distance of 110 kb (Brueggeman et al., 2002 [chapter 1; Fig 1]). Two STPK genes were identified in the region by low copy probe development and sequencing. Allele sequencing from resistant and susceptible lines and a
fortuitous recombinant (AMS170) combining parts of resistant and susceptible alleles of Rpg1 confirmed the identity of the correct candidate gene (Brueggeman et al., 2002 [chapter 1; Fig 3]).

The Rpg1 gene has a unique gene structure encoding two tandem kinase domains within a genomic sequence of 4,466 bp including 14 exons producing an in silico translated protein of 837 aa (~94.5 kDa) (Brueggeman et al., 2002 [chapter 1; Fig 2]). The RPG1 protein appears to be a typical kinase except for the tandem kinase domains, a structure not previously reported in plant disease resistance genes. The only report of structural and perhaps functional similarity comes from the animal Janus Kinases (JAKs), which consists of two tandem kinase domains and function in signal transduction pathways activated by diverse cytokine receptors (O’Shea and Leonard, 1998).

2. Gene Family Characterization

It has been reported that resistance to specific races of a pathogen can be conferred by different alleles at the same locus as with the Flax L locus (Dodds et al., 2004) or diverse members of resistance gene families at different loci as is the situation with Mla1 and Mla6 (Halterman et al., 2001; Zhou et al., 2001, Huang et al., 2001). In an attempt to quickly identify other candidate stem rust resistance genes that confer different resistance specificity than Rpg1, particularly candidate rpg4 or Rpg5 genes, we analyzed the cv. Morex genome for Rpg1 gene family members by nucleotide hybridization assays with a Rpg1 specific probe (Brueggeman et al., 2006 [chapter 2]). The initial hybridizations to cv. Morex BAC and cDNA libraries identified three additional genes belonging to the Rpg1 gene family. The Rpg1 structure containing two tandem kinase domains had not been previously reported, but all three initially identified Rpg1 gene family members, designated ABC1036, ABC1037 and ABC1040, contained the dual kinase domain structure (Brueggeman et al., 2006 [chapter 2; Fig. 1]). Of these, ABC1037 had the
highest homology with *Rpg1* and was very closely linked to *Rpg1* (ca. 50 kb) suggesting the locus was the result of unequal recombination giving rise to a tandem duplication. ABC1037 is expressed and contains high homology to *Rpg1* at the kinase domains but diverges at the 5’ end and has no known function.

ABC1036 and ABC1040 are highly homologous one to another and tightly linked on chromosome 5H(7) probably due to another tandem duplication, but they are highly diverged from *Rpg1* at the kinase 2 domain. The two loci at 7H(1) and 5H(7) suggest an ancient duplication followed by a translocation event then divergence leading to the two loci containing the tandem kinase genes.

Hybridization with ABC1036 and ABC1040 specific probes identified two additional genes, ABC1041 and ABC1063. These genes had relatively low homology to *Rpg1*, but were highly homologous to the protein kinase domain 2 of ABC1036 and ABC1040 and contain only a single kinase domain (Brueggeman et al. 2006 [chapter 2; Fig 1 and 4]). ABC1041 mapped very close to the *rpg4/Rpg5* genes but segregate away in the high-resolution population used to clone *rpg4* and *Rpg5*.

The presence of three tandem kinase domain genes and their scattered location in the cv. Morex genome suggest that this tandem kinase structure has an ancient origin. A search of the sequenced plant genomes *Aabidopsis, Oryzae*, and draft sequence of *Brachypodium* did not identify any *Rpg1* orthologs with this dual kinase structure, but genes with high amino acid homology to the individual kinase domains were identified. PCR using barley *Rpg1* specific primers on wheat genomic DNA produced sequences with high homology to *Rpg1*, but the PCR bands were very heterogeneous and assembling the ~1kb overlapping PCR fragment into contiguous unigene sequences was impossible (The PCR primers used are described in Mirlohi
et al., 2008 [Chapter 3; Table 2]. We cloned the heterogeneous PCR products from wheat and sequenced many independent clones. Single nucleotide polymorphism (SNP) analysis of the PCR derived clones identified at least eight and possibly more copies of Rpg1 in wheat (Brueggeman et al., unpublished). A Triticum monococcum BAC library was screened using a barley Rpg1 specific probe and a BAC contig was assembled containing three tandem repeats of dual kinase Rpg1 homologous genes. Mapping of these Rpg1 orthologs placed them in the syntenic region of the wheat genome. Therefore, the tandem kinase gene structure in plants is not unique to Hordeum. The detection of Rpg1 orthologs in wheat (Brueggeman et al., unpublished) shows that the Rpg1 gene has an ancient evolution occurring before the wheat barley split ~13 mya (Wolfe et al., 1989).

Our attempts at identifying the rpg4/Rpg5 genes or other candidate resistance genes were initially unsuccessful, but we did identify a family of genes with homology to Rpg1. Genetic mapping did not identify a putative function for any of the genes, however one family member, ABC1041, was tightly linked to the rpg4/Rpg5 locus. After cloning Rpg5 by a map-based approach amino acid comparisons revealed that the Rpg1 gene family members ABC1041 and ABC1063 were highly homologous to the Rpg5 kinase domain (60% and 61% aa identity, respectively, and 76% aa similarity). The nucleic acid homology between ABC1063 and Rpg5 is sufficiently high that cross hybridization could occur. We certainly could have identified Rpg5 with our assays, screening BAC and cDNA libraries, however the Rpg5 allele from cv. Morex is missing the kinase domain. Thus in hind sight, screening the susceptible cv. Morex cDNA and BAC libraries to identify Rpg5 was a futile effort.
3. *Rpg1* Allele Characterization

Sequence analysis of the *Rpg1* gene from seven stem rust resistant North American barley cultivars yielded no polymorphism at the nucleotide level suggesting that North American germplasm contains a single source of *Rpg1* probably originating from the unimproved bulked seed lot obtained by the USDA from Switzerland in 1914 (Brueggeman *et al*., 2002 [chapter 1]). In order to trace the origin of the *Rpg1* resistance gene from this bulked seed lot we analyzed the *Rpg1* alleles from eight land races (*Hordeum vulgare*) from Switzerland. We also analyzed alleles from eight wild barley (*H. vulgare ssp. spontaneum*) accessions originating from diverse geographic regions to attempt to gain insight into the origin of the *Rpg1* gene.

A set of 74 barley landraces from Switzerland and 318 ecogeographically diverse *H. v. spontaneum* wild barley accessions were tested with *Pgt* race MCCF. Eight barley lines, four resistant and four susceptible to race MCCF, from each of the landraces and wild barleys were initially selected. Then a comprehensive evaluation of known stem rust resistance was brought to our attention. This study revealed that *Pgt* race HKHJ was specific for *Rpg1*-mediated stem rust resistance while race MCCF was shown to be avirulent on barley containing the *rpg4/Rpg5* locus (Sun and Steffenson, 2005). Only one Swiss landrace and none of the *H. v. spontaneum* lines used in this study showed resistance to race HKHJ. The Swiss line resistant to race HKHJ proved to have an *Rpg1* gene identical to that previously sequenced from North American barley cultivars (Mirlohi *et al*., 2008 [chapter 3; Fig. 3]) and probably comes from the same source as the one imported to USA in 1914. As expected by the HKHJ phenotypes none of the other lines had a functional *Rpg1* gene. The lines that were resistant to race MCCF and susceptible to race HKHJ were subsequently genotyped at the *rpg4/Rpg5* locus. The resistance correlates with functional *rpg4/Rpg5* alleles suggesting it is due to the *rpg4/Rpg5* genes (Brueggeman *et al.*).
unpublished). These data indicate that a functional Rpg1 gene is rare in the *H.v. spontaneum* and Swiss land race populations, however a larger collection need to be characterized to confirm this.

A GTT insertion was present in all seven barley (landrace or wild) accessions containing a defective Rpg1-like gene making it a common allelic variation. This insertion results in an S to R substitution at amino acid position 319 and an F insertion at position 320 leading to virulence by both races HKHJ and MCCF (Mirlohi *et al*., 2008 [chapter 3; Fig 3]). We do not know if the Rpg1 gene with a GTT insertion carries out some other function or confers resistance to other *Pgt* races, but its presence in a diverse population and its status as an apparently functional gene in the wild barley population suggests that its function possibly has a selective advantage in the wild. The wild barleys had Rpg1 alleles that encoded intact proteins with the GTT insertion although they are non-functional in known stem rust resistance, but still have autophosphorylation activity confirming previous observations that autophosphorylation is necessary but not sufficient for resistance (Nirmala *et al*., 2006). Most of the cultivated barleys with the GTT insertion have acquired mutations that produce a truncated protein thus appear to be pseudogenes.

Recent *in vitro* assays using the Yeast-two-Hybrid system in our lab has demonstrated that the GTT insertion within the pseudokinase domain does cause interference with protein-protein interaction between RPG1 and at least three proteins identified as interactors (Nirmala, personal communication). This exemplifies the importance of identifying natural allelic variation and the information that these alleles can provide in determining amino acids or domains that are functionally important. These allelic variations can be as valuable as induced mutants when elucidating the molecular function of a gene.
4. *Rpg1* Functional Analysis

The cloning and characterization of the durable stem rust resistance gene *Rpg1* has greatly enhanced the understanding of the underlying mechanisms of stem rust resistance. In the years following the cloning of *Rpg1* in 2002, we have come a long way in elucidating the *Rpg1* mediated mechanisms of resistance. However, a great deal of work still remains.

The validation of the *Rpg1* gene (Brueggeman *et al.*, 2002 [chapter 1]) was subsequently confirmed by *Agrobacterium* mediated stable transformation of the susceptible cv. Golden Promise (Horvath *et al.*, 2003). Golden Promise had been shown to be missing the *Rpg1* gene presumed to be either deleted or highly diverged (Brueggeman *et al.*, 2002 [chapter I]). Several independent transformants reacted to the pathogen with a much higher resistance than the cv. Morex from which the gene originated. Quantitation of the *Rpg1* mRNA levels from transgenic plants containing different copy numbers of *Rpg1* (1-5 copies), showed various levels of *Rpg1* mRNA, but there was no correlation between *Rpg1* mRNA levels and resistance to *Pgt* race MCCF (Horvath *et al.* 2003). The potential for other genes within the background of various cultivars even the susceptible cultivars that affect the level of resistance conferred by a race specific *R*-gene may have utility when breeding for resistance. It is also important to identify this factor or factors to gain a further understanding of why or how they are enhancing the *Rpg1* mediated resistance. These genes also may provide further insight into elucidating the resistance mechanisms.

Expression analysis of *Rpg1* mRNA showed that it is constitutively and uniformly expressed in all plant organs and developmental stages except the leaf epidermis where it exhibited a 30-fold higher level than in the whole leaf (Rostoks *et al.*, 2004). *P graminis* penetrates the barley leaf through the stomata so the elevated *Rpg1* expression in the leaf
epidermis possibly indicates that the fungus is detected at the leaf surface. It was determined that the RPG1 protein is mostly cytoplasmic with small but significant amounts associated with membranes (Nirmala et al., 2006). This membrane association is similar to the animal janice kinases (JAKs), which share the dual kinase structure with Rpg1 and provide a model for the Rpg1 mechanism. The JAKs were originally determined to be cytosolic proteins, but recent analysis has shown that they are loosely bound to the plasma membrane (Behrmann et al., 2004) and associate with transmembrane cytokine receptors (Aaronson and Horvath, 2002). The function of the membrane bound fraction of RPG1 protein maybe similar to JAKs by associating with a transmembrane receptor (Aaronson and Horvath, 2002). Recent data using a technique for detection of in vivo RPG1 phosphorylation may substantiate this speculation (Nirmala, personal communication).

Although the structural similarity of the RPG1 protein and the animal JAKs is probably the result of convergent evolution and the Rpg1 association with a surface receptor is speculation there are other similarities shared between the two systems. Both RPG1 and the JAKS have a functional kinase domain and a nonfunctional pseudokinase (Nirmala et al., 2006). The role of the pseudokinase domain in RPG1 function appears to be important in protein-protein interaction. Mutating two adjacent lysine’s (K152 and K153) of the pseudokinase domain did not inactivate the RPG1 protein kinase auto catalytic activity, but did result in a susceptible disease reaction of the stable transformant (Nirmala et al., 2006). The wild-type RPG1 interacts with ten different proteins in the yeast two-hybrid system, but the K152 and K153 pseudokinase mutant is known to lose interaction with at least three of these proteins (Nirmala, personal communication). When the RPG1 pseudokinase was completely deleted it also abolished all interactions with proteins that interacted with the wild type RPG1.
Similarity between the two systems is also evident by the degradation of the RPG1 protein approximately 24 hours after avirulent pathogen challenge (Nirmala et al., 2007). This degradation was determined to occur via the ubiquitin-proteasome pathway and it was also shown that JAK2 also degrades after activation via the ubiquitin-proteasome pathway (Ungureanu et al., 2002).

Another similarity between the RPG1 kinase and JAKs is the interaction with transcription factors, but this similarity is also shared with resistance pathways activated by an R-gene with only a single kinase domain. Four genes identified as downstream Pto interactors by the yeast two-hybrid system were also identified to interact with RPG1. These genes were Pti1, a serine/threonine protein kinase involved in the HR response, and the transcription factors Pti4, Pti5, and Pti6 that function in the expression of pathogenesis-related (PR) genes (Zhou et al., 1997). The Pti4/5/6 transcription factors are related to the ethylene-response-element binding proteins and bind the GCC box found in the promoter regions of many pathogenesis related genes (Zhou et al., 1997). The JAKs interact and phosphorylate the Signal Transducers and Activators of Transcription (STAT) factors (Leonard, 2001).

C. MAP-BASED CLONING OF rpg4 AND Rpg5

The Rpg1 gene has conferred remarkable durability against stem rust but the new races that have emerged, QCCJ and TTKSK, are highly virulent on barley containing Rpg1. The rpg4/Rpg5 locus has been shown to confer resistance to both of these exceptional races warranting the cloning and characterization of this locus and the genes underlying this important source of stem rust resistance. We have cloned the rpg4/Rpg5 locus and shown that these two genes are distinct and both code for novel resistance proteins. The Rpg5 gene confers resistance to an isolate of Pgs and rpg4 confers resistance to Pgt. We have discovered that while resistance to Pgs isolate
92-MN-90 is conferred by \textit{Rpg5} alone, resistance against the \textit{Pgt} races QCCJ, MCCF and TTKSK requires both \textit{Rpg5} and \textit{rpg4}.

1. Cloning and Characterization of \textit{Rpg5}

High-resolution genetic mapping provided initial localization of the \textit{rpg4/Rpg5} locus to a small region of chromosome 5H(7) (Druka \textit{et al}., 2000; Han \textit{et al}., 1997; Kilian \textit{et al}., 1997). A 70 kb region between markers ARD5016 and ARD5112 was cloned and sequenced (Brueggeman \textit{et al}., 2008 [chapter 4; Fig. 1]). Sequence annotation revealed five candidate genes, two encoding predicted NBS-LRR proteins (\textit{HvRGA1} and \textit{HvRGA2}), two actin depolymerizing-like factors (\textit{HvADF2} and \textit{HvADF3}) and a protein phosphatase 2C-like protein (\textit{HvPP2C}) (Brueggeman \textit{et al}., 2008 [chapter 4; Fig. 1]). The sequence was derived from the susceptible cv. Morex BAC clones therefore the equivalent sequence was needed from the resistant line Q21861. The Q21861 sequence was generated by PCR approaches utilizing the cv. Morex sequence and development of a Q21861 lambda phage library. Annotation of the Q21861 sequence revealed that the \textit{HvPP2C} gene was missing and replaced by a STPK gene just 3 prime of the \textit{HvRGA2} gene. Allele sequencing of the candidate genes \textit{HvRGA1}, \textit{HvADF2} and \textit{HvADF3} from susceptible barley lines showed that they were essentially identical to those found in the resistant line Q21861 eliminating them as candidate \textit{Rpg5} genes. The \textit{HvRGA2} allele from Q21861 was left as the only candidate \textit{Rpg5} gene conferring resistance to \textit{Pgs} isolate 92-MN-90 and was confirmed by sequencing alleles from several susceptible genotypes.

It was determined by RT-PCR and northern blot analysis that the NBS-LRR and STPK domains were transcribed in a single mRNA, a unique gene structure among plant disease resistance genes (Brueggeman \textit{et al}., 2008 [chapter 4; Fig 3]). Annotation using cDNA to gDNA comparisons predicted the \textit{Rpg5} gene to contain seven exons in a total genomic sequence of
8,504 bp coding for an 1,378-aa (151.6-kDa) protein (Brueggeman \textit{et al.}, 2008 [chapter 4; Fig. 2]).

A technique optimized for the study of barley functional genomics is Viral Induced Gene Silencing (VIGS). VIGS is a form of post translational gene silencing (PTGS) utilizing infectious virus as a vector to introduce dsRNA into a host. VIGS is used to silence or reduce the transcript of an endogenous plant gene by use of a virus genomic RNA with an inserted anti-sense or inverted repeat with homology to the target gene. The viral infection induces the formation of dsRNA triggering the plant defense response and the degradation of the target gene transcript resulting in a loss of function. VIGS has been developed for barley using the Barley Stripe Mosaic Virus (BSMV) as a vector (Holzberg \textit{et al.}, 2002). We utilized this method for reverse genetics to confirm the NBS-LRR-STPK gene as \textit{Rpg5}. Using QRT-PCR we demonstrated a correlation between silencing the candidate \textit{Rpg5} gene and the conversion of the resistant line Q21861 to a susceptible phenotype when inoculated with \textit{Pgs} isolate 92-MN-90 (Brueggeman \textit{et al.}, 2008 [chapter 4; Fig 3 and 4]).

Present work in our lab has identified parallels between the \textit{Rpg1} mechanism of resistance and the \textit{Pto} mechanism. The \textit{Pto} gene of tomato conferring resistance to races of \textit{P. syringae} carrying the \textit{avrPto} or \textit{AvrPtoB} genes (Martin \textit{et al.}, 1993; Kim \textit{et al.}, 2003) contains a single kinase domain with homology to the PKDs of \textit{Rpg1} (29\% a.a. identity and 48\% a.a. similarity) to PKD2 of \textit{Rpg1}) and \textit{Rpg5} (36\% aa identity and 53\% aa similarity). The \textit{Pto} resistance function requires the presence of a second gene \textit{Prf}, which is a classic NBS-LRR gene (Salmeron \textit{et al.}, 1996). Elucidation of the barley stem rust \textit{Rpg1} mediated resistance pathway is underway yet \textit{Rpg1} has not been shown to require an NBS-LRR gene for resistance. However, \textit{in-vivo} co-immuno precipitation experiments using a full length \textit{Rpg1} specific antibody have
identified a putative NBS-LRR gene by MASS Spec analysis (Nirmala, personal communication). Interestingly, the \textit{Rpg5} gene contains all three domains and has aa homology to \textit{Pto} and \textit{Prf} (the NBS-LRR homology to \textit{PRF} is 26\% aa identity and 42\% aa similarity). \textit{Pto} requires two genes, an NBS-LRR and protein kinase, for resistance and \textit{Rpg1} probably does as well, but the \textit{Rpg5} gene is a novel \textit{R}-gene containing the components of both genes in a single transcript. It may be possible that having all three domains in a single transcript could facilitate the identification of not only the rust \textit{Avr} protein or proteins in this system by yeast-two hybrid or co-immuno precipitation but also could be valuable in identifying components down stream of \textit{Rpg5} in the resistance pathway or components of the resistance complex. \textit{Rpg5} maybe used as a tool to better understand these mechanisms with all three components present in a single protein. This may not only facilitate the understanding of stem rust resistance pathways but resistance pathways in general.

2. \textbf{Interaction of \textit{rpg4} and \textit{Rpg5} for \textit{P.g. f. sp. tritici} Resistance}

Recombinant analysis of the 70 kb \textit{rpg4/Rpg5} region delimited the \textit{rpg4} gene to a 1kb interval encoding only the \textit{HvAdf2} gene (Brueggeman \textit{et al.}, 2008 [chapter 4; Fig. 1]). \textit{HvAdf2} (\textit{rpg4}) encodes an apparently functional Adf gene containing three exons in a total sequence of 906 bp coding for an 147 amino acid (16.17-kDa) protein. Current evidence suggests that the \textit{rpg4} gene does not act independently, but rather that it is dependent on the \textit{Rpg5} gene for function (Brueggeman \textit{et al.}, 2009 [chapter 5]). The \textit{Pgt} QCCJ and TTKSK susceptible barley cv. Harrington and line Sm89010 contain an \textit{rpg4} allele identical at the amino acid level to the \textit{rpg4} allele in the resistant line Q21861. This initially suggested that \textit{HvAdf2} was not the \textit{rpg4} gene. However, analysis of allele sequences of \textit{rpg4} and \textit{Rpg5} coupled with SNP analysis of the critical recombinants showing that the genes were distinct, suggested that the susceptibility of
these barleys was due to a non-functional \textit{Rpg5} gene. The susceptible cv. Harrington and line Sm89010 contain truncated \textit{Rpg5} alleles missing the protein kinase domain. The Harrington/Q21861 recombinant #18 (HQ18) combines an apparently functional cv. Harrington \textit{rpg4} allele (which is identical at the amino acid level to the Q21861 \textit{rpg4} allele) with the functional Q21861 \textit{Rpg5} gene. HQ18 is resistant to \textit{Pgt} QCCJ and TTKSK, indicating that Harrington is susceptible to the \textit{Pgt} races due to a lack of a functional \textit{Rpg5} gene (Brueggeman \textit{et al}., 2009 [chapter 5]).

Phenotype analysis of three populations (Morex, Steptoe, and Robust/Q21861) revealed 1:3 segregation for resistance:susceptibility, demonstrating the recessive nature of \textit{rpg4} (Jin \textit{et al}., 1994a). The three \textit{Pgt} susceptible barley cultivars (Morex, Steptoe, and Robust) have identical \textit{Adf2} alleles at the amino acid level and differ from the resistant Q21861 allele by three amino acids (Q39H, A101T, S135G). Three \textit{Pgt} susceptible progeny from the Steptoe/Q21861 population have recombination within the \textit{Adf2} gene and contain a functional \textit{Rpg5} allele. These recombinant \textit{Adf2} genes contain Q39 (Q21861-like) with T101 and G135 (Steptoe-like), showing that the amino acids at positions 101 and/or 135 are important for reaction to \textit{Pgt} QCCJ and TTKSK. All \textit{Adf2} alleles tested were expressed at the transcription level, indicating that these two amino acid polymorphisms determine susceptibility/resistance to \textit{Pgt} QCCJ and TTKSK.

These data suggest that \textit{Pgt} resistance should behave as a single dominant gene in populations where only the \textit{Rpg5} gene is segregating in the presence of a non-polymorphic \textit{rpg4} gene (Harrington/Q21861 and Sm89010/Q21861). This hypothesis is currently being tested in the Harrington/Q21861 and Sm89010/Q21861 F\textsubscript{1} and F\textsubscript{2} populations and by down-regulating the two alternative \textit{rpg4} alleles using the BSMV-VIGS method (Brueggeman \textit{et al}., unpublished).
The *rpg4* gene is genetically recessive, suggesting that it may have a negative or neutral function on the fungus’ ability to establish itself within the plant. One hypothesis is that the fungus captures the dominant ADF2 protein function to facilitate feeding itself via the haustoria and that the fungus is unable to utilize the *rpg4* encoded ADF2 protein to establish or maintain its feeding structure. Establishment of the fungal haustorial apparatus and/or haustorium function probably has a dependency on ADFs. Stem rusts are biotropic fungi that invade plants in non-destructive manner developing the haustoria after penetration of mesophyll cell wall. The haustoria is associated with host plasma membrane by invagination of the plasma membrane creating the haustoria-plant cell interface that consists of the haustorial membrane, an extra-haustorial matrix, and the host plasma membrane (Schulze-Lefert and Panstruga 2003). It has been shown that in the compatible interaction between barley and powdery mildew the haustorium is associated with actin filament rings and actin filaments closely follow the haustorium when invaginating the plasma membrane (Opalski *et al.*, 2005). This is similar to what occurs in the symbiotic interaction between a mycorrhizal fungus and tobacco root cells (Genre and Bonfante, 1998), suggesting similarity between how pathogenic and symbiotic fungi may establish the fungus-host interface. All these processes are dependant on cytoskeletal restructuring which is an ADF dependant process.

The *rpg4* gene by itself is not sufficient to confer resistance, perhaps due to failure to detect the fungus via *Rpg5*. An appealing hypothesis is that the RPG4 protein function in compatible or incompatible situations can be explained by differential phosphorylation at the two important amino acids T101 in the Q21861 ADF protein or S135 in the Steptoe ADF protein. In either case it suggests that *rpg4* may act downstream of *Rpg5* and is possibly activated or deactivated by direct or indirect phosphorylation of RPG4 by the RPG5 kinase activity. If the
ADF protein is present from the \textit{Rpg4} susceptible allele in the heterozygous or homozygous state, RPG5 may be unable to activate or deactivate the protein thus the fungus can utilize it to establish itself within the plant. This hypothesis explains \textit{rpg4} as a recessive gene.

RPG5 could also be the guard protein monitoring the ADF2 protein for any modifications by the fungus effector; however, this would not explain why \textit{rpg4} is recessive. RPG5 should still be able to guard the ADF2 protein even when the \textit{rpg4} gene is present in a heterozygous state.

The temperature sensitivity of the \textit{rpg4} mediated resistance must also be addressed (Sun \textit{et al}., 1994b). The classic interpretation of temperature sensitivity is that the protein structure is stable and functional at the lower temperature, but may become unstable at higher temperatures. It is possible that the ADF protein is temperature sensitive and the elevated temperatures affect the actin depolymerizing or polymerizing process. However, it could also be other proteins or components within the pathway that are temperature dependent. Since \textit{Rpg5} is required for the \textit{rpg4} mediated resistance response it could possibly be the RPG5 protein acting in a temperature dependant manner. It has been demonstrated that an ADF is required for cold hardiness in wheat and seems to be required for the cytoskeleton rearrangement that under lays this cold tolerance (Ouellet \textit{et al}., 2001). This ADF protein appears to be phosphorylated by a 52kDa protein kinase activity which is temperature dependent suggesting the kinase protein in this system acts in a temperature dependant manner. Post pathogen recognition, RPG5 may act upon the ADF for activation or deactivation via phosphorylation and RPG5 phosphorylation activity maybe temperature dependant when phosphorylating ADF2 or other substrates.

Plant genomes have limited numbers of \textit{Adf} genes as demonstrated by 12 Adf genes present in the sequenced genomes of \textit{Arabidopsis} and rice (Feng \textit{et al}., 2006). This suggests that ADF proteins have several different functions and this may also be true of \textit{rpg4} (\textit{HvAdf2}). It is
reasonable to speculate that biotrophic fungi evolved to utilize one or more of these limited number of ADF proteins to establish or maintain their haustorial feeding apparatus. It may become possible to analyze the limited number of ADF proteins present in plants to specifically detect which ADF proteins the biotrophic fungi are utilizing to establish themselves within the host. This makes the *rpg4/Rpg5* resistance mechanism of particular research interest because it maybe a very important system to begin understanding how the fungus establishes and maintains its biotrophic life style. This scientific knowledge could be invaluable in our efforts to manage not only stem rust, but also many diseases caused by biotrophic fungal pathogens.
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CHAPTER ONE

The barley stem rust resistance gene *Rpg1* is a novel disease resistance gene with homology to receptor kinases.

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Stem rust caused by *Puccinia graminis* f. sp. *tritici* was among the most devastating diseases of barley in the northern Great Plains of the U.S. and Canada before the deployment of the stem rust-resistance gene *Rpg1* in 1942. Since then, *Rpg1* has provided durable protection against stem rust losses in widely grown barley cultivars (cv.s.). Extensive efforts to clone *Rpg1* by synteny with rice provided excellent flanking markers but failed to yield the gene because it does not seem to exist in rice. Here we report the map-based cloning and characterization of *Rpg1*. A high-resolution genetic recombination map was constructed with 8,518 gametes and a 330-kb bacterial artificial chromosome contig physical map positioned the gene between two crossovers ~0.21 centimorgan and 110 kb apart. The region including *Rpg1* was searched for potential candidate genes by sequencing low-copy probes. Two receptor kinase-like genes were identified. The candidate gene alleles were sequenced from resistant and susceptible cv.s. Only one of the candidate genes showed a pattern of apparently functional gene structure in the resistant cv.s. and defective gene structure in the susceptible cv.s. identifying it as the *Rpg1* gene. *Rpg1* encodes a receptor kinase-like protein with two tandem protein kinase domains, a novel structure for a plant disease-resistance gene. Thus, it may represent a new class of plant resistance genes.

The barley stem rust-resistance gene *Rpg1* confers resistance to many pathotypes of the stem rust fungus *Puccinia graminis* f. sp. *tritici*. North American barley cultivars (cv.s.) with *Rpg1* were first released in 1942 and since that time there have been no significant losses as a result of stem rust except for a minor epidemic caused by pathotype QCC in 1989–91 (1). The *Rpg1* resistance is dominant and considered durable (2), because it has remained effective for a long time in widely grown cv.s. across the stem rust-prone northern Great Plains (1). The earliest known source of stem rust resistance in the U.S. was derived from an unimproved cv. imported by the U.S. Department of Agriculture from Canton Lucerne, Switzerland, in 1914. Two selections from this seed lot became the cv.s. Cheveron and Peatland. Peatland has been the main source of stem rust resistance in most Canadian cv.s. since 1947 (1). A potentially different source of resistance was discovered during the severe stem rust epidemic of 1935 by Sam Lykken, a farmer from Kindred. North Dakota, who identified a single healthy plant in a heavily rusted field of Wisconsin 37 barley (3). This plant was saved and gave rise to the cv. Kindred. Kindred and cv.s. derived from it have dominated the barley hectarage in the northern Great Plains of the U.S. from 1942 to the present (1). Both sources of barley stem rust resistance are the results of the *Rpg1* gene (4, 5).

The *Rpg1* gene was mapped to the short arm of barley chromosome 1(7H) with morphological and molecular markers (6, 7). Our initial efforts at map-based cloning of the *Rpg1* gene were focused on using rice as an intergenomic cloning vehicle (8, 9). Despite the excellent synteny between barley chromosome 1(7H) short arm and rice chromosome 6 short arm and the excellent alignment of flanking markers, the *Rpg1* gene was not found in the syntenous position in rice (10). The development of a barley bacterial artificial chromosome (BAC) library allowed us to initiate a chromosome walk toward *Rpg1* (11). A resistance gene analog (RGA), B9 (12), and barley homologues of the maize rust-resistance gene *Rp1-D* (pic20) (13) were proposed as the barley stem rust-resistance gene *Rpg1*. We mapped both B9 and the pic20 homologues at high resolution and found that they did not cosegregate with *Rpg1*. The pic20 probe, however, did identify a complex RGA locus near *Rpg1* and helped to extend the BAC contig (14).

Numerous genes that confer resistance to a variety of plant pathogens have been cloned and characterized (15–17). The genes that seem to be involved in signal transduction and behave in a gene for gene manner (18) have been classified into four groups. The majority resembles intracellular receptors and contains a predicted nucleotide binding site/leucine-rich repeat (NBS/LRR) structure. Another group encodes an LRR putative extracellular receptor and a membrane anchor. Two additional groups contain serine/threonine kinase domain without any obvious ligand-binding domain (i.e., Pto) and receptor kinases with LRR extracellular ligand-binding, transmembrane, and serine/threonine kinase domains (i.e., *Xa21*) (16). A recently reported tomato *Verticillium* wilt-resistance gene *Ve* seems to encode a new group with cell-surface glycoprotein, signals for receptor-mediated endocytosis, and leucine zipper or PEST sequences (19, 20). Numerous genes with LRR extracellular ligand-binding, transmembrane, and serine/threonine kinase domains (i.e., *Xa21*) (18) have been classified into four groups. The majority resembles intracellular receptors and contains a predicted nucleotide binding site/leucine-rich repeat (NBS/LRR) structure. Another group encodes an LRR putative extracellular receptor and a membrane anchor. Two additional groups contain serine/threonine kinase domain without any obvious ligand-binding domain (i.e., *Pto*) and receptor kinases with LRR extracellular ligand-binding, transmembrane, and serine/threonine kinase domains (i.e., *Xa21*) (19). Here we report map-based cloning and partial characterization of the barley stem rust-resistance gene *Rpg1*. The *Rpg1* gene identity was confirmed by high-resolution genetic and physical mapping, sequencing of multiple alleles from resistant and susceptible lines, a fortuitous recombination that combined parts of the resistant and susceptible parents, and exclusion of other potential candidate genes by sequence analysis. The *Rpg1* gene encodes a receptor kinase-like protein with two tandem protein

Abbreviations: BAC, bacterial artificial chromosome; cv., cultivar; LRR, leucine-rich repeat; RT, reverse transcription; cm, centimorgan.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF509747 (Rpg1 cdna); AF509748–AF509765 (Rpg1 genomic sequences from barley lines Morex, OSU6, Q21861, 80-TT, Bowman, Chevron, Leger, Kindred, Peatland, Steptoe, Lion, Wisconsin 38, Moravian III, OSU15, SM8910, Dicktao, Gobernadora, and AMS1770, respectively); AF509744–AF509746 (Rpg1 genomic sequence from barley lines Morex, Steptoe, and OSU6, respectively); AF509766–AF509779 (genomic sequences from low copy probes isolated from the Rpg1 genomic region NR.G021, NR.G026, NR.G027, NR.G046, NR.G048, NR.G050, RS.B347, RS.B409A, RS.B409B, RS.B410, RS.B414, and RS.B416, respectively).

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kinase domains, which is a novel structure for a plant disease-resistance gene. It does not contain a strong membrane-targeting motif and known receptor sequences, suggesting a mechanism of action similar to the tomato Pto gene (20, 21). The barley Rpg1 gene may be useful for durable stem rust-resistance in other species.

Materials and Methods

Materials. Barley crosses used to produce recombinant gametes are given in Table 1, which is published as supporting information on the PNAS web site, www.pnas.org. Most of the crosses were produced by, and for, the North American Barley Genome Project and are described at http://www.css.orst.edu/barley/nabmp/nabmp.htm. The F2 materials were generated in this study. FN53 is a fast neutron-induced brachytic dwarf (brh1.ae) derived from cv. Steptoe (22). MD2 is a barley genetic stock carrying multiple dominant morphological markers (23). Resistant (24) and susceptible (25) barley lines used are described in supporting information on the PNAS web site. Libraries used include the cv. Morex BAC library (11) and cDNA libraries made from various tissues and with different biotic and abiotic stresses (26) (http://wheat.pl.usda.gov/ggpages/bgn/31/close.htm).

Molecular Marker Development. Low-copy probes suitable for genomic mapping and hybridization-based BAC library searching were identified from small insert libraries developed from individual BAC clones. The BAC clone DNA was digested with four- or six-base recognition restriction enzymes and subcloned into a plasmid vector. The subclones were hybridized with 32P-labeled barley total genomic DNA probe. Subclones that did not show any or very little hybridization signal were selected and tested further by hybridization to Southern blots of HindIII-digested barley genomic DNA. The clones generated with six-base recognition enzymes were further digested with several restriction enzymes to develop small inserts. The digested DNA was electrophoresed, blotted, and transferred to filters for hybridization with barley total genomic DNA probe (27). DNA fragments that failed to hybridize were selected as low-copy probes.

Genetic Mapping. Recombinants between the Rpg1 flanking markers ABG704 and ABG077, derived from 8,518 gametes (Table 1), were used for high-resolution mapping. The genetic distances are expressed in number of crossovers. The stem rust resistance gene may be useful for durable stem rust-resistance in other species.

Fig. 1. A genetic and physical map of barley stem rust-resistance gene Rpg1 region on chromosome 17H. (A) High-resolution genetic map based on 8,518 gametes showing critical markers and the number of total crossovers (c.o.) separating them. (B) Physical map encompassing Rpg1 based on BAC clone contig. The distance from Pic20A to RSB308 was estimated to be ∼270 kb.

Results

High Resolution Genetic Mapping of the Rpg1 Gene. The Rpg1 locus was previously mapped to the telomeric end of barley chromosome 17HS (9, 10). A high-resolution genetic map, based on 8,518 gametes (Table 1), was developed with recombinants from
the region between the \textit{Rpg1} flanking markers ABG704 and ABG707. The interval between MWG035540 (BB172#13) and the barley homologue of the maize \textit{Rp1-D} gene (pic20A) delimited the \textit{Rpg1} region, contained 17 recombinants, and represented a genetic distance of \(~0.21\) centimorgan (cM) (Fig. 1).}

\textbf{Physical Mapping of the \textit{Rpg1} Region.} A BAC clone contig was established for the \textit{Rpg1} region by chromosome walks from pic20A and 19j10LRR loci (Fig. 1). The probe 19j10LRR was obtained from L. D. Holappa (M. K. Walker-Simmons Laboratory, Pullman, WA). A BLASTX search revealed it to be “similar to a non-LTR [long terminal repeat] retroelement reverse transcriptase” \((S = 170, 3e-41)\). Hybridization to barley polymorphism filters revealed many bands, some polymorphic, which were mapped. A 2.1-kb band, generated with the restriction enzyme \textit{NcoI}, mapped one crossover distal to \textit{Rpg1} and cosegregated with MWG035540 and other markers. The probe was used to isolate \(~2,000\) BAC clones from the cv. Morex library. A BAC clone, 022c13, containing the mapped band was identified after digestion with \textit{NcoI} and hybridization with 19j10LRR. A chromosome walk was initiated from this BAC clone and led to an overlap with BAC clones isolated from the pic20A walk.

\textbf{Identification of Candidate Genes.} Three putative candidate genes were identified by draft sequencing in the region between the two closest \textit{Rpg1} flanking crossovers (Fig. 1). Two were receptor kinase-like genes and one was a truncated phosphoenolpyruvate kinase-like gene that was not considered further. The first kinase-like gene and one were a truncated phosphoenolpyruvate kinase-like proteins from \textit{Arabidopsis} and the SRK receptor kinase-like gene family. Thus, this gene was a strong candidate to be \textit{Rpg1}, and subsequent sequence analysis of stem rust-resistant (\textit{Rpg1}) and susceptible (\textit{rpg1}) cv. alleles confirmed this conclusion (data presented below). A second receptor kinase-like gene, \textit{NRG031}, was identified and sequenced from Morex, OSU6 (both resistant), and Steptoe (susceptible). Alleles from resistant and susceptible lines were very similar, excluding \textit{NRG031} from \textit{Rpg1} candidacy.

The candidate genes were identified by sequencing of the approximately \(35\) kb immediately distal to \textit{Rpg1}. This sequenced region consisted primarily of long terminal repeat retrotransposons that are a common feature of the intergenic space in barley \((32)\) and other grass species, such as maize \((33)\). For the about \(70\) kb remaining, sublibraries were constructed with the \(48\) bp recognition endonucleases \textit{SacI} and \textit{TaqI} and the \(6\)-bp recognition restriction endonucleases \textit{HindIII} and \textit{EagI}. Approximately \(18\)× coverage of this region was screened for low-copy clones, sequenced, and analyzed with the BLASTX and BLASTN algorithms. Sequences with putative ORFs were hybridized to Southern blots of \textit{HindIII}-digested genomic DNA. Putative genes were completely sequenced from cv. Morex BAC DNA. In total, \(80\) kb of nonredundant putatively low-copy sequence was generated and examined with BLAST searches as well as with the gene prediction software \textsc{genemark.hmm}. No other putative genes were identified.

\textbf{Characterization of the Candidate Genes.} The Morex (resistant) allele of candidate gene 1 (RSB228) seemed to encode a functional gene. The Steptoe (susceptible) allele contained 40-bp differences, three of which introduced stop codons in the 4th, 8th, and 13th exons, indicating a defective gene. The Steptoe × Morex recombinant \textit{AMS170} allele had a crossover within the gene in the 436–524-aa region including parts of exons 7 and 8. The distal part of the \textit{AMS170} allele contained the two stop codons in the 8th and 13th exons from Steptoe, explaining the stem rust-susceptible phenotype (\textit{rpg1}). The other part of the \textit{AMS170} gene had the Morex haplotype and eliminated all proximal genes from \textit{Rpg1} candidacy (Fig. 1).

The genomic and cDNA sequence comparisons predicted the gene to contain 14 exons in a total sequence of \(4,466\) bp coding for an \(837\)-aa (\(94.5\) kDa) protein (Fig. 2). A BLASTP search of the nonredundant protein database showed the best homology to numerous hypothetical receptor kinase-like proteins. The best hit to characterized function proteins was to the SRK family of self-incompatibility S-receptor kinases. The highest homology was to the kinase domain regions. The \(N\)-terminal part of the protein sequence exhibited low homology to several predicted \textit{Arabidopsis} proteins with unknown function. A BLASTN search with the \textit{Rpg1} coding sequence of the nonredundant database revealed no significant homologies. The deduced protein sequence contained two tandem \textit{pfam00069} protein kinase domains. Program \textsc{signalp version 2.0} (http://www.cbs.dtu.dk/services/\textsc{signalp}/) indicated that the predicted protein sequence does not contain a signal
peptide. The PREDICTPROTEIN tool at the European Bioinformatics Institute (http://www.ebi.ac.uk/~rost/predictprotein) identified a putative transmembrane domain from amino acid 321 to 331, although with low probability. Alternatively, the TMPRED program at http://www.ch.embnet.org/software/TMPRED.form.html found 3 putative transmembrane domains, amino acids 23–47, amino acids 316–334, and amino acids 626–643, although only the 316–334-aa domain was predicted with a score higher than threshold.

Future work will be required to determine whether the predicted RPG1 protein is membrane-bound or intracellular.

Candidate gene 2 (NRG031) was homologous to putative receptor kinase-like genes. Eukaryotic GENEMARK.HMM version 2.2a (http://opal.biology.gatech.edu/GeneMark/eukhmcmg.html) gene prediction program predicted 2 exons in a total sequence of 981 bp coding for a 327-aa protein. Sequence analysis of alleles from resistant (Morex and OSU6) and susceptible (Steptoe) lines showed differences at only two amino acid positions among the three alleles sequenced. Morex and Steptoe had isoleucine, whereas OSU6 had threonine in position 4. At position 241, OSU6 and Steptoe had glycine, but Morex had alanine. Because all amino acid differences found in the susceptible allele were also present in one or the other of the resistant alleles, this gene was removed from further candidacy. Analysis of Rpg1 cDNA. Hybridization of the RSB228 probe to 30 arrayed cDNA filters representing 10 different tissue libraries and ~500,000 clones identified four cDNA clones. Sequence analysis revealed three different expressed genes homologous to Rpg1. One partial cDNA clone, HVSM2055g12, was identical to the genomic sequence, whereas another came from a very similar member of the same gene family (ABC1037) located proximal to Rpg1 and excluded from candidacy by genetic mapping. The third clone came from a more distantly related member of the Rpg1 gene family and mapped to chromosome 7(5H). The HVSM2055g12 cDNA was used to predict the 3’ end of the Rpg1 gene and intron–exon structure for exons 9±14. The homologous, nearly full-length cDNA ABC1037 was used to predict a preliminary intron–exon structure of the 5’ part of the Rpg1 gene. Sequences of RT-PCR products generated from cv. Morex leaf total RNA with primers corresponding to predicted exons allowed for the construction of apparently the complete coding sequence of the Rpg1 gene. Even though the region from exon 1 to 3 forms an uninterrupted ORF, RT-PCR products indicated the presence of two introns. Translation of the reconstructed cDNA sequence suggested that the coding sequence was complete, because an in-frame stop codon was detected four codons upstream of the first predicted methionine. The gene prediction programs GENSCAN (http://genes.mit.edu/GENSCAN.html) and FGENESH (http://www.softberry.com), as well as NEURAL NETWORK PROMOTER PREDICTION (http://www.fruitfly.org/seq_tools/promoter.html) localized the putative transcription start site of the gene about 400 bp upstream of the translation start site.

Northern blots of Morex, Steptoe, and AMS170 total or poly(A) RNA failed to yield positive bands with Rpg1 probe although positive results were obtained with an actin probe (data not shown). This finding suggested that the gene was expressed at low levels.

Comparison of Rpg1 Alleles from Resistant and Susceptible Barley Lines. Sequence and RFLP analysis of 19 susceptible and 9 resistant barley lines confirmed that the RSB228 probe was the Rpg1 gene. The Morex gene sequence was compared with resistant cv. alleles from Kindred, Chevron, Peatland, Q21861, Leger, Bowman, 80-FT-29, and OSU6. All sequences, except OSU6, were identical, suggesting that the candidate gene was functional and probably from the same source (see Discussion). The resistant Hordeum vulgare subsp. spontaneum line OSU6 allele had a 3-bp insertion that resulted in a serine to arginine conversion and an insertion of a phenylalanine in the 6th exon at amino acid position 320 (Fig. 3 and Fig. 5, which are published as supporting information on the PNAS web site). This was the only nucleic acid difference between OSU6 and Morex that resulted in an amino acid difference. Other transitions occurred in the 7th exon and 8th and 13th introns, and a 30-bp deletion was observed in the 13th intron. Thus, the Rpg1 gene seemed to be very highly conserved and functional in all resistant lines analyzed.

In addition to Steptoe and AMS170 discussed above, the alleles from susceptible barley lines Lion, MoravianIII, Wiscon38, SM99010, Dicktoo, Gobernadora, and OSU15 were sequenced. The susceptible allele sequences formed three groups, all seeming to encode defective proteins (Figs. 3 and 5). Group I cvs. Lion, MoravianIII, Steptoe, and Wisconsin38 alleles, compared with Morex, had 42 bp differences translating into three stop codons and 8-aa changes. The 5’ end, including all of the first exon (6 bp), the first intron, and the first 38 bp of exon 2, of the alleles sequenced from Group II cvs. Dicktoo, Gobernadora, and SM99010 were very similar among themselves but were not homologous to the Morex allele. The sequence after this point to amino acid 160 is still quite diverged with several bp substitutions resulting in amino acid differences and a 96-bp deletion in exon 3. These differences indicated that the gene would not be functional. The allele from OSU15, the only representative of Group III, was very similar to the OSU6 sequence. However, it contained a single bp deletion in the 7th exon resulting in a frame shift that introduced a stop codon at amino acid position 358 and a predicted truncated protein.

Other susceptible barley lines (Baroness, Betzes, Golden Promise, Harrington, Klages, Oderbrucker, OWB-D, OWB-R, and 80-tt-30) were analyzed and failed to hybridize with the RSB228 probe. PCR amplification, with the primers described previously, also failed. These lines seemed to have a highly diverged or deleted Rpg1 gene.

Synteny with Rice and Other Cereals. Hybridization of the RSB228 probe with rice cv. Nipponbare and M201 genomic DNA was
negative. Searching two rice cv. Nipponbare BAC libraries representing 11X and 15X genomes yielded no positive clones, suggesting there are no homologues detectable by hybridization present in rice. A BLASTX search of the Chinese rice genome database (http://www.sorghumdb.org) of rice representing the rice sub-species indica genome sequence also failed to uncover homologous regions. A tblastx search of the “other” expressed sequence tag (EST) database yielded positive hits with wheat in addition to barley. Other than within the Triticeae, fair homology was observed with ESTs from Sorghum propinquum (S = 179, 4e-46) and Sorghum bicolor (S = 93, 9e-45), Medicago truncatula (S = 80, 3e-40), Lycopersicon esculentum (S = 75, 4e-37), and Zea mays (S = 99, 3e-35).

Discussion

A candidate gene for the barley stem rust-resistance gene Rpg1 was identified by map-based cloning. The Rpg1 gene identity was confirmed by high-resolution genetic and physical mapping, sequencing of multiple alleles from resistant and susceptible lines, a fortuitous recombination that combined parts from the resistant and susceptible parents, and exclusion of other potential candidate genes by sequence analysis. Conceptual translation of the sequence showed amino acid homology to the S receptor kinase gene SRK protein family. SRK (S locus receptor kinase) encodes a plasma membrane-spanning receptor serine/threonine kinase specific to the stigma epidermis (34, 35). It is presumed to be the determinant of self-incompatibility specificity in the stigma (36). Another interesting homology was to the maize kinase interacting kinase KIK1 gene predicted to encode a receptor kinase-like protein with an N-terminal signal peptide, an extracellular ligand-binding domain, a membrane-spanning domain, and a cytoplasmic serine/threonine protein kinase domain (37). The KIK1 protein was suggested to transfer a signal downstream by means of a kinase-associated protein phosphatase (KAPP) (37). Another protein with homology to the Rpg1 gene product was the GA-induced LRR receptor kinase-like protein OsTMK from rice, which was also suggested to interact with a KAPP protein (38).

Receptor kinase-like gene products are defined by the presence of a potential N-terminal hydrophobic signal peptide, an N-terminal domain with a transmembrane region, and a C-terminal intracellular protein kinase domain (39, 40). The two largest subclasses of plant receptor kinases are those whose extracellular domain contains LRRs or similarity to the S-locus glycoprotein. Some extracellular domains of plant receptor kinases are unique. The cloned rice Xa21 gene (41), which confers resistance to Xanthomonas oryzae pv. oryzae and belongs to a receptor kinase-like gene family, showed homology to Rpg1 in the kinase domain. However, the Xa21 gene contains the LRR extracellular domain whereas the Rpg1 gene has an N-terminal domain that does not resemble any previously described receptor. In that sense, Rpg1 might be more similar to the tomato Pto gene (20), which confers resistance to Pseudomonas syringae pv. tomato. The Pto gene, also showing similarity to Rpg1 in the kinase domain, is a serine/threonine kinase without an identifiable ligand-binding domain (21). However, the Pto gene product and the corresponding avirulence gene product of the pathogen, avrPto, have been shown to interact directly (42). The Pto threonine 204 residue implicated in interaction with avrPto (43) is conserved in both kinase domains of the RPG1 protein (positions 302 and 612) as it is in a number of other kinases including Xa21, KIK1, SRK, and OsTMK. Thus, the significance of this observation is not clear.

The Rpg1 gene product is unique because it contains two tandem protein kinase domains. This finding has not been previously reported in cloned plant pathogen-resistance genes and seems to be uncommon. Some Arabidopsis-predicted genes encode two kinase domains, e.g., AC007234.4 and AC069159.10.

Examples of characterized genes encoding tandem kinase domains are the mammalian Janus kinases that, in addition to two kinase domains, also have plasma membrane-binding and SH2 domains (44). In the case of the mammalian ribosomal protein S6 kinase 3 domains, which are involved in activation of mitogen-activated kinase cascade, both of the kinase domains are catalytically active although they exhibit different phosphotransferase activities (45). Analysis of the protein kinase catalytic domains from 65 different kinases found 9 invariant amino acid residues (46). The most conserved amino acid residues found in the core of the kinase catalytic domain were also conserved in RPG1 kinase domain 2 but not in kinase domain 1, indicating that this domain may not be active.

The origin of the Rpg1 gene bred into barley cvs. in the U.S. and Canada can be traced to two apparently different sources, one an introduction of an unimproved line from Switzerland, which yielded the resistant cvs. Chevron and Peatland, and another from a single resistant-plant selection made by a farmer from Wisconsin 37 barley that became the cv. Kindred. Our sequencing results suggested that both sources of Rpg1 are the same. Wisconsin 37 seed was no longer available, but analysis of Wisconsin 37 cv. subsp. spontaneum and the parents Oderbrucker (Wisconsin Pedigree 5) and Lion showed that even a rare recombinant could not account for the resistant allele in Kindred, because Oderbrucker had an apparent deletion and Lion a susceptible allele nearly identical to the Steptoe allele. These results suggested that the resistant plant identified in the farmer’s field of Wisconsin 37 was most probably the result of an admixture derived from Peatland or Chevron.

Other possible different sources of resistance were from the cv. Q21861 and the H. vulgare subsp. spontaneum line OSU6. The pedigree of Q21861 is not known, but the allele sequence was identical to Morex and thus, probably from the same source. The OSU6 line probably represented a unique source of the resistance gene, but still only had minor nucleotide substitutions and a 3-bp insertion. Interestingly, the 3-bp insertion is also seen in all of the susceptible alleles sequenced, suggesting that they arose from this type of resistance gene rather than from the one that gave rise to the Morex-type gene. Thus, the Rpg1 gene in all resistant lines seemed to be highly conserved. On the other hand, the rpg1 alleles from susceptible cvs. varied from highly conserved to highly diverged or even absent. Thus, the gene does not seem to be essential in absence of the disease.

Physical and genetic mapping of the Rpg1 region indicated a region of high recombination proximal to the Rpg1 gene and a region of low recombination distal to the gene (Fig. 1). Approximate calculations suggested that the region between markers pic20A and ABC1037 had a physical/genetic distance ratio of approximately 350 kb/cM similar to that calculated for the adjoining proximal pic20 region (14). The physical/genetic distance ratio for the ABC1037 to 19j10LRR region was ~4 Mb/cM, the predicted barley genome average (30). The region defined by the markers MUG035540, BB172#13, and 19j10LRR showed even higher physical/genetic distance ratio, because these three probes cosegregated in the 8,518 gamete population but did not form overlapping BAC clone contigs. Thus, the physical/genetic ratio cannot be calculated but must be in excess of 25 Mb/cM based on 1 recombinant and a minimum physical distance of 300 kb. Similar divergent ratios from 0.18 to 5 Mb/cM of physical to genetic distance were observed in the Mla cluster on chromosome 5(1H) (47).

We have suggested that map-based cloning in large grass genomes such as barley could be facilitated by using rice as an intergenomic cloning vehicle (9). Despite the excellent gene colinearity with the Rpg1 flanking markers, we did not find a candidate gene in rice (10). Screening of two rice cv. Nipponbare BAC libraries representing 11X and 15X genome coverage failed to find positive clones. A BLASTN search of the
Chinese rice subspecies indica genome database was also negative. These results suggest that a homologous gene detectable by Southern hybridization does not exist in the rice genomes tested. Expressed sequence tags with good homology to the Rpg1 coding sequence were found in wheat and with fair homology in sorghum, maize, barley, medic, and tomato. These data suggest that Rpg1 homologues may have a function in other species.

In summary, we cloned the barley stem rust-resistance gene Rpg1 by map-based cloning. The gene seems to encode a receptor kinase-like protein with two tandem kinase domains but no recognizable receptor and membrane anchor domains. Tandem arrangement of kinase domains is a novel feature of plant disease-resistance genes. Structural similarity to tomato Pto protein supports the hypothesis that the RPG1 protein could potentially recognize the avirulence gene product of the *Puccinia graminis* f. sp. *tritici* pathotype MCC and function in a signal transduction pathway.

**Note.** Preliminary evidence indicates that the Rpg1 genomic fragment transformed into the susceptible cv. Golden Promise confers resistance to the barley stem rust pathotype MCC. Data were based on the analysis of 12 different transgenic plant families, 10 of which produced resistant progeny.

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CHAPTER TWO

The barley serine/threonine kinase gene *Rpg1* providing resistance to stem rust belongs to a gene family with five other members encoding kinase domains

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The barley serine/threonine kinase gene \textit{Rpg1} providing resistance to stem rust belongs to a gene family with five other members encoding kinase domains

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Abstract The barley (\textit{Hordeum vulgare} L.) stem rust (\textit{Puccinia graminis} f. sp. \textit{tritici}) resistance gene \textit{Rpg1} encodes a serine/threonine protein kinase with two tandem kinase domains. The \textit{Rpg1} gene family was identified from the cv. Morex and consists of five additional members with divergent homology to \textit{Rpg1}. All family members encode serine/threonine kinase-like proteins with at least one predicted catalytically active kinase domain. The five family members were sequenced from cDNA and genomic DNA and genetically mapped. The family member most closely related to \textit{Rpg1}, ABC1037, is located on chromosome 1(7H) bin 01, very near (~50 kb) but not co-segregating with \textit{Rpg1}. Two others, ABC1036 and ABC1040, are closely related to each other and tightly linked on chromosome 7(5H) bin 07. ABC1041 mapped to chromosome 7(5H) bin 13, tightly linked to the rust resistance genes \textit{rpg4} and \textit{Rpg5} providing resistance to barley stem rust pathotype QCC and rye stem rust pathotype 92-MN-90, respectively, but segregated away in a high-resolution population. ABC1063 was localized to chromosome 4(4H) bin 6. An interesting \textit{Rpg1} allele that appears to be the result of unequal recombination between \textit{Rpg1} and ABC1037 was characterized. No known resistance loci cosegregated with any family members, however characterization of the \textit{Rpg1} family has provided insight into the evolution of this novel gene family and may present tools for understanding the functional domains of \textit{Rpg1}. The genetic mapping, gene structures, and analysis of amino-acid sequences of the \textit{Rpg1} gene family members are presented.

Introduction

The barley stem rust resistance gene \textit{Rpg1} is a dominant gene that confers resistance to most, but not all, pathotypes of the stem rust fungus \textit{Puccinia graminis} f. sp. \textit{tritici}. \textit{Rpg1} was cloned by a map-based approach from the resistant cv. Morex (Brueggeman et al. 2002). A genomic fragment containing the complete \textit{Rpg1} gene transformed into the highly susceptible cv. Golden Promise conferred resistance to the barley stem rust fungus pathotype MCC, demonstrating that the gene isolated was the functional \textit{Rpg1} gene (Horvath et al. 2003). In silico translation of the \textit{Rpg1} sequence revealed an 837 amino acid (a.a.) protein with tandem protein kinase domains (PKDs), a novel resistance gene structure (Brueggeman et al. 2002). Analysis of the two \textit{Rpg1} kinase domains suggested that PKD2 was catalytically active while the PKD1 may not function in autophosphorylation but may be important for resistance. This was confirmed by site-directed mutagenesis and kinase activity assays (Nirmala et al. 2006). Analysis of \textit{Rpg1} alternate splice...
forms identified one predicted protein with a putative transmembrane domain and signal peptide (Rostoks et al. 2004); however, the majority of the Rpg1 protein is cytoplasmic (Nirmala et al. 2006).

Rpg1 has significant a.a. homology (NCBI BLASTP) to the PKD of the *Brassica napus* S-receptor kinase family with the highest homology to the gene family member *Srk15* (35% a.a. identity with Rpg1 PKD1 and 34% a.a. identity with PKD2) (Cabriillac et al. 1999). *Srk15* encodes a plasma membrane-spanning receptor kinase that is specific to the stigma epidermis (Stein et al. 1991, 1996) and presumed to be the determinant of self-incompatibility (Takasaki et al. 2000).

The Rpg1 serine/threonine (S/T) protein kinase (PK) belongs to the cd00180 domain family (http://www.ncbi.nlm.gov/structure/cdd/cddsrv.cgi?uid=cd00180&version=v2.06) placing Rpg1 into the RLK/Pelle super family of S/T PKs. The RLK/Pelle family includes receptor-like kinases (RLKs) and receptor-like cytoplasmic kinases (RLCKs) (Shiu and Bleeker 2003). Alignment of characterized plant S/T PKDs showed that they range in size from 250–300 amino acid residues and are not conserved uniformly but are made up of alternating regions of high and low conservation. Eleven major conserved subdomains (I–XI) are evident and important for catalytic activity (S1). Nine highly conserved a.a. in kinase catalytic domains are expected to play important roles in adenosine triphosphate (ATP) binding and phosphotransfer, involved in signal transduction (Hanks et al. 1988). The nine invariant a.a. indicative of a catalytically active PK, were identified and used to predict the inactive PKD1 and active PKD2 present in Rpg1. These predictions were confirmed by autophosphorylation assays (Nirmala et al. 2006).

The past two decades has culminated in the cloning and partial characterization of more than 40 disease resistance (*R*) genes (Martin et al. 2003). The majority of *R*-genes belong to a class of proteins containing a nucleotide-binding site and leucine rich repeats (NBS-LRR) whereas only four have been characterized as PKs. Disease resistance systems have been identified that require both PK and NBS-LRR genes. The tomato *Pto* gene confers resistance to races of *Pseudomonas syringae* pv. *tomato* that carry the *avrPto* or *AvrPtoB* gene (Martin et al. 1993; Kim et al. 2002). The *Pto* resistance function requires the presence of the NBS-LRR gene *Prf* (Salmeron et al. 1996). Pto is located in the cytoplasm and contains no recognizable receptor domain placing it into the RLCK class of genes. The *Arabidopsis PBS1* gene, which confers resistance to the bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola* (Swiderski and Innes 2001), is another RLCK whose resistance function requires a second gene, *RPS5*, which encodes an NBS-LRR gene. It was shown that the *Arabidopsis PBS1* is cleaved following infection by the pathogen effector protein AvrPphB protease and RPS5 may act as the guard for PBS1 (Shao et al. 2003).

RLKs and RLCKs are abundant in the *Arabidopsis* genome, which contains more than 610 family members (Shiu and Bleeker 2003). Many of the *Arabidopsis* RLK/RLCK subfamilies are present in clusters. The evolution of plant genomes is shaped by the occurrence of multiple gene duplication events that may occur through unequal recombination. Like many plant genes, most of the cloned plant *R* genes are members of families of related genes, indicating that gene duplication and subsequent diversification are common processes in plant *R* gene evolution (Richter and Roland 2000). Previously identified *R* genes related to *Rpg1*, *Pto* and *Xa21*, are part of complex gene loci containing tandem arrays of closely related genes, some of which confer different or altered resistance specificities (Martin et al. 1994; Song et al. 1997). Genetic studies of the *Xa21* and *Pto* gene families have provided insight into the function and evolution of *R* genes.

The *Pto* gene is part of a small gene family consisting of at least six members located on tomato chromosome 5 (Martin et al. 1993). It is presumed that duplication and diversification of the *Pto* gene family has led to the generation of alternative recognition specificities. The *Pto* gene family member *Fen* encodes a 318 a.a. S/T PK sharing 87% a.a. identity with *Pto*. *Fen* does not confer resistance to any tested pathogens, but mediates a response similar to HR in tomato plants treated with the organophosphorous insecticide, fenithion (Martin et al. 1994).

The cloned rice *Xa21* gene (Song et al. 1995), which confers resistance to *Xanthomonas oryzae* pv. *oryzae* belongs to a RLK gene family and has homology to *Rpg1* at the kinase domains (32% a.a. identity and 52% a.a. similarity to Rpg1 PKD2 and 28% a.a. identity and 47% a.a. similarity to *Rpg1* PKD1). The *Xa21* multigene family contains at least eight members (Ronald et al. 1992; Song et al. 1995). Most of these members were mapped to a single complex locus on rice chromosome 11 that contains at least nine major resistance genes (Ronald et al. 1992). Analysis of the coding and flanking sequences of seven *Xa21* gene family members identified recombination, duplication, and transposition as the major events contributing to the evolution of this complex resistance locus (Song et al. 1997). It has been shown that recombination between separate *Xa21* family members has occurred during the evolution and that transposon insertions resulted in
truncated genes with alternative gene structure and differing resistance specificity (Song et al. 1997).

In addition to Rpg1 our laboratory has been working on the map-based cloning of two other barley stem rust resistance genes, rpg4 and Rpg5 (Druka et al. 2000). In this study we identified and partially characterized the Rpg1 barley stem rust resistance gene family with the goal of identifying other rust resistance genes particularly the rpg4 and Rpg5 genes. The Rpg1 gene family members in cv. Morex were identified by utilizing the Rpg1 gDNA probe to isolate homologous barley sequences by cross-hybridization and by using in silico database mining. The Rpg1 gene family members were mapped and each was sequenced from both genomic and cDNA sources to determine their gene structure. One member was closely linked to Rpg1 and another was closely linked to the rpg4/Rpg5 locus. The Rpg1 barley gene family members did not co-segregate with any other mapped barley disease resistance loci but they could function in disease resistance and possibly rust resistance pathways yet to be discovered. This work also uncovered and partially characterized an apparent unequal recombination event that produced a chimeric gene between Rpg1 and its closely linked family member ABC1037.

Materials and methods

Southern analysis

Barley genomic DNA isolation, Southern blotting and hybridization were as described (Kleinhofs et al. 1993). Approximately 25 ng of cDNA insert was labeled with

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32P \text{dCTP (New England Nuclear) using the RTS RadPrime DNA labeling system (Gibco BRL) according to manufacturers instructions.}
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Library screening

Arrayed barley cv. Morex cDNA libraries or the barley cv. Morex BAC library (Yu et al. 2000) were initially screened using the Rpg1 gDNA probe RSB228 (Brueggeman et al. 2002). Clones identified from the cDNA libraries were used as new probes to re-screen the cDNA libraries to identify further diverged family members and to hybridize the Morex BAC library to identify genomic clone equivalents for each cDNA. For BAC confirmation blots, DNA was digested with restriction enzymes following the manufacturer’s recommendations (New England Biolabs, MBI Fermentas, Gibco BRL) and transferred to nylon membranes (New England Nuclear) by the alkaline transfer procedure. Hybridizations were at 65°C and the final wash was at 65°C with 0.5 SSC 0.1% SDS.

Genetic mapping

Low resolution genetic mapping used the Steptoe × Morex “minimapper” population consisting of 35 lines selected from the original USBGP (US Barley Genome Project) 150 Hordeum bulbosum-derived doubled-haploid line population (Kleinhofs et al. 1993; Kleinhofs and Graner 2001). The “minimapper” lines were selected to allow placement of probes to specific bins on the Steptoe × Morex map (http://wheat.pw.usda.gov/cgi-bin/graingenes/report.cgi?class = mapdata;query = Barley*;name = Barley,+BinMap + 2005). High resolution mapping at the Rpg1 locus utilized 33 recombinants selected from approximately 8,000 gametes between the Rpg1 flanking markers ABG704 and ABG077 as described in Brueggeman et al. (2002). High resolution mapping at the rpg4/Rpg5 locus utilized 55 recombinants selected from approximately 5,000 gametes between the rpg4/Rpg5 flanking markers Aga5 and ABG391 as described in Druka et al. (2000).

CAPS marker development

The ABC1063 gene was non-polymorphic using RFLP markers therefore Cleaved Amplified Polymorphic Sequence (CAPS) markers were developed. The Morex cDNA sequence was utilized to design primers to amplify ~1 kb overlapping fragments of the gene from Morex and Steptoe. The PCR fragments were separated on a 1 × TE, 1% agarose gel with low EDTA (0.002 M) and run at 100 V. The PCR fragments were excised from the gel and placed in trimmed filter tips (Rainin RT-L22F) inside 1.5 ml tubes and frozen. After thawing, the DNA was eluted by centrifuging at 5,000 rpm for 10 min. The fragments were directly sequenced and compared to the Morex sequence in order to identify single nucleotide polymorphisms (SNPs) that could be utilized for CAPS marker development. An AciI SNP was identified, primers developed and used to map the gene.

Cloning and sequencing

The Morex BAC clones detected by hybridization with the Rpg1 gene family cDNAs were isolated. DNA extracted, digested with HindIII, Southern-blotted and hybridized with the Rpg1 gene family cDNA probes. Representative BAC clones for each gene were detected, digested with HindIII, EagI, SacI and XbaI and shotgun-cloned into the plasmid pBluescript II KS
RNA isolation and RT-PCR

Total leaf RNA from 14-day-old Morex plants was isolated using a Trizol RNA method (Chomczynski and Sacchi 1987) with modifications (R. Wise, Iowa State University, personal communication). Integrity and quantity of the RNA samples were checked by formaldehyde denaturing agarose gel electrophoresis. Approximately 1 μg of total RNA was used for RT-PCR reactions using M-MLV Reverse Transcriptase (Promega) under the manufacturer’s recommended standard conditions. RT-PCR fragments were directly sequenced using the low EDTA elution method described above.

Northern analysis

About 500 μg of total leaf RNA was used to isolate polyA mRNA using the oligoT mRNA Mini kit (Qiagen). Three to five micrograms of polyA mRNA were denatured in 50% v/v formamide, 6.1% formaldehyde, 1 × MOPS buffer (40 mM MOPS, pH 7.0, 10 mM sodium acetate, 1 mM EDTA) for 15 min at 55°C immediately before loading. Samples were resolved with a 1% w/v agarose, 6.7% v/v formaldehyde gel in 1 × MOPS buffer for 2 h at 4 V/cm. Northern transfer was performed following the manufacturer’s recommendations (Perkin Elmer Genescreen Plus). Membrane was soaked in 2 × SSC and prehybridized (5 × SSC, 5 × Denhardts, 1% SDS, 10% Dextran sulfate, 50% Formamide) for 3 h at 42°C in a hybridization tube using ~3 ml solution per 10 cm² of membrane. The cDNAs were labeled using [α-32P]-dCTP and ALL-IN-ONE Random Prime DNA Labeling Mix (Sigma-Aldrich). Unincorporated nucleotides were removed using a performa DTR gel filtration cartridge (Edge Biosystems). Hybridization (5 × SSC, 5 × Denhardts, 1% SDS, 10% Dextran sulfate, 50% Formamide) was done at 42°C overnight. Filters were rinsed twice in 2 × SSC/0.1% SDS, 5 min at room temp once in 0.2 × SSC/0.1% SDS, 10 min at room temp, once in 0.2 × SSC/0.1% SDS, 30 min at 42°C and twice in 0.2 × SSC/0.1% SDS, 30 min at 65°C. Autoradiography was at ~80°C for 7 days.

5’ RACE

5’ RACE was performed using three methods: (1) 5’ RACE System for Rapid Amplification of cDNA Ends (Invitrogen); (2) Primer switching method; and (3) Nested PCR/DpnI method. The 5’ RACE for Rapid Amplification of cDNA Ends kit was used under Invitrogen’s recommended standard conditions. Primer switching method was performed as described by Rosenberg et al. (2003). The PCR/DpnI method was performed using two gene-specific nested reverse primers designed from the most extending 5’ cDNA sequence of each gene. DNA was isolated from a library of phagemid particles containing cDNA derived from cv. Morex seedling shoots cloned into the Lambda Uni-ZAP XR vector (Stratagene) (provided by T. J. Close, UC Riverside, CA, USA). The first round of amplification contained 20 ng of lambda DNA, 0.2 mM dNTP mix, 25 pmol of T7 primer, 25 pmol of gene-specific reverse primer, 2.5 μl of RedTaq DNA polymerase (Sigma), and 5 μl of 10× RedTaq reaction buffer in 50 μl volume. Amplifications were performed in a PTC-1000 programmable thermocycler (MJ Research) at 95°C for 4 min, followed by 10 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min; followed by 60°C for 3 min and 72°C for 5 min. The amplified DNA was then digested with 10 U of the Dam methylation-dependent restriction enzyme DpnI (New England BioLabs). The second round of amplification contained 2.5 μl of DpnI digested first round PCR reaction product, 0.2 mM dNTP mix, 25 pmol of SK primer, 25 pmol of gene-specific nested reverse primer, 2.5 μl of RedTaq DNA polymerase (Sigma), and 5 μl of 10× RedTaq reaction buffer in a 50 μl volume. Second round amplifications were at 95°C for 4 min, followed by 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min followed by 72°C for 5 min. The PCR reaction was then loaded onto a low EDTA 1% agarose gel and directly sequenced using the second round primers as described previously.

Phylogenetic analysis

A phylogenetic tree (CLUSTALX Phyllip NJ plot bootstrapped 1,000×) of the PK was developed using...
the derived a.a. sequences from the Rpg1 gene family PKDs and published a.a. sequences of Rpg1, Pto, Xa21 and SRK 15.

Results

Identification of Rpg1 gene family members

Hybridization of the the Rpg1 gDNA probe RSB228 (Brueggeman et al. 2002) to ten-arrayed barley cDNA libraries (first 10 listed at http://www.genome.arizona.edu/genome/barley.html) representing approximately 500,000 clones identified one Rpg1 cDNA (acc. # AY115550) (Brueggeman et al. 2002), and two other cDNA clones designated ABC1036 (acc. # DQ469715) and ABC1037 (acc. # DQ469714). Sequence analysis of these cDNA clones revealed that ABC1037 was closely related to Rpg1 and ABC1036 was more diverged from Rpg1. Utilizing the ABC1036 and ABC1037 nucleotide sequences, a BLASTN search of the Triticaceae EST database (NCBI) identified two new barley cv. Morex ESTs (BG366053, BG300587). Sequencing of the EST cDNA clones revealed two new genes, ABC1040 and ABC1041. An additional ABC1037 EST (B1956515) was also identified. Using ABC1036, ABC1037, ABC1040, and ABC1041 as hybridization probes, 21 cDNA clones were identified from the ten cDNA libraries (S3). A BLASTN search of the barley EST database (NCBI) with the ABC1041 sequence identified two additional ESTs (BF621830 and 628226) representing one more gene, ABC1063. Hybridization of the original ABC1063 cDNA clone to the 10 cDNA libraries identified 20 cDNAs all representing ABC1063 transcripts (S3).

The Rpg1 family of genes were separated into three homology-based groups: (1) Rpg1 and ABC1037; (2) ABC1036 and ABC1040; (3) ABC1041 and ABC1063. The gene structures and homology relationships are shown in Fig. 1. The group 1 gene, ABC1037, contains two tandem protein kinase domains and a N-terminal domain with good homology to Rpg1. ABC1036 and ABC1040 are very closely related to each other, but further diverged from Rpg1. Both ABC1036 and ABC1040 contain the two tandem PKDs, but not the N-terminal region homologous to the 110 a.a. N-terminus of Rpg1. ABC1041 and ABC1063 contain only the PKD2 and are moderately homologous to each other and quite diverged from Rpg1 (Fig. 1). ABC1041 and ABC1063 are included as Rpg1 gene family members due to their homology with the ABC1036 and ABC1040 PKD2, which is quite diverged from the Rpg1 and ABC1037 pKD2. These results suggest that

[Fig. 1 Diagram of the predicted Rpg1 protein domains and alignment with family members. The cv. Morex Rpg1 protein predicted functional domains were aligned with the five Rpg1 family members. The Sm89010 line is included to help visualize the unequal recombination that appears to have taken place between Rpg1 and ABC1037. The boundaries of the three protein domains are indicated on Rpg1. The a.a. identity between protein domains of different members and Rpg1 is presented as % identity. The % a.a. identity between adjacent family protein domains is in parentheses.]

Genetic and physical mapping

The Rpg1 family members mapped to three of the seven barley chromosomes (Fig. 2). ABC1037 mapped on chromosome 1(7H) bin 01 and co-segregated with Rpg1 in a low-resolution mapping population, but was proximal to Rpg1 by two crossovers out of 8,518 gametes in a high-resolution Rpg1 mapping population (Brueggeman et al. 2002). Rpg1 and ABC1037 reside on the same Morex BAC clone 607p19 and are separated by ~50 kb. ABC1036 and ABC1040 co-segregated at low resolution and mapped to chromosome 7(5H) bin 07. They have not been mapped at high resolution, but hybridization analysis showed they were not located on overlapping BAC clones. ABC1041 mapped to chromosome 7(5H) bin 13 and co-segregated with the barley stem rust resistance genes rpg4 and rpg5 at low resolution, but in a high-resolution mapping population (Druka et al. 2000) segregated distal of the rpg4/Rpg5 locus. ABC1063 mapped to chromosome 4(4H) bin 06.

The barley cv. Morex BAC library (Yu et al. 2000) was screened with the Rpg1 family cDNA probes described above and a total of 28 BAC clones were identified. Southern blot analysis confirmed that BAC
clones were selected for all five family members (S3) and comparison with genomic southers revealed that all hybridizing fragments were identified (Fig. 3).

cDNA sequence analysis

The cDNA clones identified by database mining and hybridization (S3) were sequenced from 3' and 5' ends. The end sequences were placed into contigs representing five unigene sequences. A clone from each of the gene contigs representing the most full-length cDNA was sequenced (Table 1). Clones that did not appear to represent full-length cDNAs were extended using RT-PCR facilitated by predicted gene structure deduced from the genomic sequence. The genomic gene structures were predicted by sequence comparison with the Rpg1 cDNA sequence and using the FGENESH gene prediction program (http://www.softberry.com). The equivalent genomic sequences were obtained from BAC subclone libraries (Table 1). Analysis of the cDNA clone sequences indicated that all contained uninterrupted open reading frames and may represent functional genes. The cDNA sequences were aligned with their respective genomic sequences to identify the exon/intron junctions (Fig. 4). The exon/intron junctions were highly conserved between Rpg1 and ABC1037 and between Rpg1 and ABC1036 and ABC1040 PKD1. The PKD2 exon/intron junctions were conserved among the ABC1036, ABC1040, ABC1041 and ABC1063 genes, but not with Rpg1. These results are in line with expectations based on the homology analysis (Fig. 1).

Comparison of the most 5' ABC1037 cDNA sequence with the genomic sequence suggested that it did not represent a full length mRNA. We were not able to extend this sequence using several 5'-RACE procedures. However, a probable full-length mRNA sequence was predicted using RT-PCR and primers designed from the genomic sequence. This procedure identified the putative transcription start site (TSS) to position −52 to −161 based on the predicted AUG start methionine for ABC1037. The predicted size of the ABC1037 transcript was confirmed by Northern blot analysis (Fig. 5).
The ABC1036 and ABC1040 cDNAs (acc. #s DQ469715 and DQ469716, respectively) also appeared to be incomplete. 5‘/H11032 RACE identified the TSS at position +551 bp relative to the Rpg1 genomic sequence. This placed the TSS of ABC1036 and ABC1040 at the beginning of subdomain I of the PKD1. This result seemed improbable since it would result in the translation start site 5‘/H11032 of subdomain III of the PKD1 and a truncated PKD1. Several attempts to identify additional 5‘ cDNA sequences using primers based on genomic sequences failed. Northern blot analysis predicted mRNAs of ~2.9 kb (Fig. 5) while the cDNA sequence analysis predicted a ~2.57 kb transcript.

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The ABC1041 and ABC1063 cDNAs (acc. #s DQ469717 and DQ469718, respectively) appeared to be full-length messages containing a putative TSS and a full length 5‘ UTR. In silico translation of the cDNA sequences suggested that the coding sequences were complete since in-frame stop codons were present upstream of the first predicted start methionine indicating a 5‘ UTR. This data was supported by Northern blot analysis showing that the mRNAs were ~1.7 kb and ~1.5 kb, respectively, as predicted by the sequences (Fig. 5).

<table>
<thead>
<tr>
<th>Rpg1 family member</th>
<th>cDNA clone used for mapping</th>
<th>cDNA Genbank accession number</th>
<th>BACclones used for subcloning</th>
<th>gDNA Genbank accession number</th>
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</tr>
</tbody>
</table>

CDNA plasmid clones are designated with library they were isolated from and the address. All BAC clones are from the cv. Morex BAC library. The ABC1036 and ABC1040 full-length cDNA sequences were completed by RT-PCR fragment sequencing.

The ABC1036 and ABC1040 cDNAs (acc. # s DQ469715 and DQ469716, respectively) also appeared to be incomplete. 5’ RACE identified the TSS at position +551 bp relative to the Rpg1 genomic sequence. This placed the TSS of ABC1036 and ABC1040 at the beginning of subdomain I of the PKD1. This result seemed improbable since it would result in the translation start site 5’ of subdomain III of the PKD1 and a truncated PKD1. Several attempts to identify additional 5’ cDNA sequences using primers based on genomic sequences failed. Northern blot analysis predicted mRNAs of ~2.9 kb (Fig. 5) while the cDNA sequence analysis predicted a ~2.57 kb transcript.

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Genomic sequence analysis

A 2 kb sequence 5' of the ABC1037 TSS was analyzed and compared with the Rpg1 promoter sequence using NCBI BLAST 2 program. No apparent or significant similarity between the two gene promoter regions was detected. A search for small almost identical stretches of nucleotides was also negative.

The ABC1037, ABC1036 and ABC1040 genes contained very large introns from 8,057–12,516 bp (Fig. 4). BLAST searches of the ABC1037 genomic sequence revealed four transposon-like elements, two within the 3rd intron and two within the 11th intron (Fig. 4). The first transposon-like element in the third intron had 92% nucleic acid (n.a.) homology to a transposon “thalo MITE” element annotated from Hordeum vulgare (acc. # AY146587). The second transposon-like element in the third intron had 84% n.a. identity to an unclassified class II DNA transposon from Triticum aestivum “George_1611A10_1.1” (acc. # CR626934). Transposon like elements present in the 11th intron had ~92% n.a. identity to transposon “Xobar” and 92% n.a. identity to transposon “Sherlock” respectively from Hordeum vulgare (acc. # AY661558). BLASTX and BLASTN searches of the fourth intron of ABC1036 and both the fourth and ninth introns of ABC1040 revealed insertions of retrotransposon-like elements with high (82–84%) n.a. homology to a LTR retrotransposon Leojyg-184G9-1 from Hordeum vulgare, (acc. # AY268139.1) (Fig. 4). The Leojyg-184G9-1-like retro element found in the ninth intron of ABC1040 had a second retrotransposon-like element nested within it with 95% n.a. homology to the Copia/Ty1-like retrotransposon “Horpia-2 from Hordeum vulgare, (acc. # AF427791). A BLASTX search of the 10th intron of ABC1036 also revealed a polypeptide like sequence representing a possibly uncharacterized barley retroelement with 55% a.a. identity to a reverse transcriptase from a retrotransposable-like element of Medicago truncatula (acc. # ABD28426.1) (Fig. 4).

Characterization of the Rpg1 family gene structure

The Rpg1 gene structure was divided into three probable functional domains: (1) the N-terminal domain from a.a. 1 to 129, (2) PKD1 from 130 to 393 a.a., and (3) PKD2 from 438 to 710 a.a. (cv. Morex Rpg1 numbering, acc. # AF509748). Amino acid sequence homology within these functional domains was compared between different Rpg1 family members and Rpg1 (Fig. 1). The ABC1037 gene was most closely related to Rpg1, sharing 64% overall a.a. identity. ABC1037 has a gene structure similar to Rpg1, with two tandem S/T kinase domains that share 73 and 67% a.a. identity with Rpg1, respectively, and a 98 a.a. N-terminal region with 49% homology to the N-terminus of Rpg1. Genomic and cDNA sequence analysis of ABC1037 indicated a functional gene predicted to contain 12 exons in a total genomic sequence of 22,183 bp encoding a 694 a.a. protein (Fig. 4). A BLASTN and BLASTX search with the ABC1037 N-terminal coding sequence of the non-redundant database (NCBI) revealed no other significant homologies besides the Rpg1 homology.

Two other Rpg1 family members, ABC1036 and ABC1040, were very closely related to one another, 91% overall a.a. identity, and have two protein kinase domains, but were only moderately homologous to Rpg1, ~59% a.a. identity at PKD1 and ~28% identity at PKD2 (Fig. 1). ABC1036 and ABC1040 have conserved intron/exon junctions that are conserved with Rpg1 only in the PKD1 domain. Genomic and cDNA sequence analysis indicated that ABC1036 and ABC1040 may not be functional and could represent transcribed pseudogenes. ABC1036 and ABC1040 contain ten exons in a total sequence of 21,924 and 18,253 bp (Fig. 4), respectively, coding for similar 642 and 632 a.a. predicted proteins.

More distantly related Rpg1 family members included ABC1041 and ABC1063. These genes are closely related (75% overall a.a. identity), but map to different chromosomes. ABC1041 contains six exons in a total sequence of 4,562 bp (Fig. 4) coding for a 425 a.a. predicted protein. ABC1063 also contains six exons in a total sequence of 2,934 bp (Fig. 4) coding for
a 372 a.a. predicted protein. These genes lack the \textit{Rpg1} N-terminal domain and PKD1 (Fig. 1). The ABC1041 and ABC1063 homology to \textit{Rpg1} in the PKD2 was low (28 and 29% a.a. identity, respectively), but they appeared to be functional as judged by the presence of all nine a.a. conserved in functional PKs (S1).

Phylogenetic relationships of the PK domains

Phylogenetic analysis of the \textit{Rpg1} family member PKDs suggested that the ABC1037 and \textit{Rpg1} tandem kinase domains arose by duplication of a single progenitor PKD. The resulting gene with two tandem PKDs was then duplicated giving rise to the two genes (Fig. 6). The ABC1036 and ABC1040 PKD1 is closely related to the \textit{Rpg1} and ABC1037 PKD1, but the PKD2 is not, suggesting that the two PKDs evolved separately and were brought together by an unknown transposition event. This tandem kinase progenitor gene then presumably underwent a localized duplication, giving rise to ABC1036 and ABC1040.

\textit{Oryza sativa} protein kinase homologs

A tandem kinase gene (ABA92216.1) with homology to \textit{Rpg1} and ABC1037 was identified using BLASTP search NCBI (39 and 38% a.a. identity, respectively). However, there is a single kinase gene annotated from rice (ABA94724.1) with higher homology to each of the kinase domains of \textit{Rpg1} (51 and 50% a.a. identity to PKD1 and PKD2, respectively) and ABC1037 (38 and 41% a.a. identity to PKD1 and PKD2, respectively). ABC1036 and ABC1040 do not have tandem kinase gene homologues from rice. However, two separate single kinase genes (ABA94724.1 and AAO18450.1) have been identified with high homology to each of the tandem kinase domains of ABC1036 and ABC1040 (61 and 60% for PKD1, respectively and 65 and 70% for the respective PKD2). The rice gene with the highest homology to ABC1041 (90% a.a. identity) is the same single kinase gene (AAO18450.1) previously identified with the highest homology to the PKD2 of both ABC1036 and ABC1040, which is consistent with the phylogenetic analysis and a.a. alignments. The best hit with ABC1063 is a unique rice kinase (NP 914370.1) with 76% a.a. identity. There is no known function for the rice PK genes with homology to the \textit{Rpg1} gene family.

An example of unequal recombination

Sequence analysis of the \textit{Rpg1} alleles from the susceptible cultivars Sm89010, Dicktoo, and Gobernadora were previously characterized as having a highly diverged 5’ coding region (Brueggeman et al. 2002). The N-terminal region of this allele was highly homologous (86% a.a. identity) to the 5’ region of ABC1037 while the 3’ region including PKD1 and PKD2 was highly homologous (98%) to \textit{Rpg1} indicating that an unequal recombination event between \textit{Rpg1} and ABC1037 probably gave rise to the Sm89010 allele. The predicted unequal recombination event occurred within the third intron of \textit{Rpg1} and the third intron of ABC1037. The recombinant gene is spliced together at the PKD1 subdomain I (S1). RT-PCR, using primers that span the chimeric junction, and subsequent sequence analysis indicated that the gene is transcribed and the recombinant intron is spliced out to form a probable functional gene (data not shown). The function of the Sm89010 \textit{Rpg1} allele is unknown, but it does not function in stem rust pathotype MCC resistance as indicated by susceptibility of the Sm89010 line.
Discussion

Our goal was to characterize the Rpg1 gene family members in order to determine if family members were responsible for resistance against other rust diseases and to evaluate their evolutionary relationships. One member, ABC1041, mapped very close, but did not cosegregate, with the rpg4/Rpg5 genes. Although ABC1041 was not rpg4 or Rpg5, we cannot exclude its participation with these resistance genes or pathways since kinases are sometimes required together with NBS-LRR genes to provide disease resistance as demonstrated by the Pto/Prf and PBS1/RPS5 gene pairs described in the Introduction.

The ABC1037 gene, closely linked to and very homologous to Rpg1, probably arose by a recent duplication event. Duplication and subsequent divergence is the major theme believed to have shaped the evolution of plant disease resistance genes (Richter and Roland 2000). We are not aware of any disease resistance function for ABC1037 although it appears to be a functional gene. ABC1037 was apparently involved in a fairly recent unequal recombination event giving rise to a chimeric gene with an ABC1037 N-terminal region (a.a. 1–110) and a C-terminal region from Rpg1 (a.a. 140–837). The chimeric gene was not functional in the barley stem rust pathotype MCC and its function, if any, is unknown although it is expressed at the mRNA level. It could possibly function to provide resistance to as yet unidentified rust pathotypes or other pathogens. Evidence exists that recombination between gene family members or different alleles of the same gene may be involved in the creation of novel resistance specificities (Richter et al. 1995; Dodds et al. 2001).

It is tempting to speculate that the N-terminal regions of the Rpg1 and ABC1037 genes are important for R gene specificity, possibly representing a novel receptor domain or a domain responsible for interaction with a receptor. BLASTN and BLASTX searches using the N-terminal domains of ABC1037 and Rpg1 did not reveal homology to any known receptors. A domain swapping study is underway to further explore the N-terminal receptor hypothesis.

Some of the Rpg1 family members appeared to be the products of recent duplication events and subsequent divergence suggesting that the newly emerged or duplicated genes may have taken on a subset of the original function. The new genes may have other rust resistance specificity as described for the M and N loci of flax and the Rpl locus in Maize (Richter et al. 1995; Dodds et al. 2001) or resistance against other pathogens or completely novel functions. However, genetic mapping did not associate the Rpg1 family members with any known functions. Although all of the genes were transcribed, their translation was not tested and possible functions, if any, are unknown.

The Rpg1 family members ABC1036 and ABC1040 are diverged from Rpg1, but very closely related to each other indicating a recent duplication event. Both ABC1036 and ABC1040 have uncommonly large fourth introns, containing sequences with high homology to Leojyg-like retrotransposon inserted at the same region suggesting that the duplication occurred after the retroelement insertion. The ninth intron of ABC1040 contains another Leojyg-like retrotransposon element with a Horpia 2-like retrotransposon element nested within it that is not present within the ninth intron of ABC1036 suggesting this insertion event occurred after duplication or the element was deleted from ABC1036 after duplication.

The PKD2 containing Rpg1 family members, ABC1041 and ABC1063, had relatively little homology to Rpg1 (Fig. 1). These two genes were placed into the Rpg1 gene family based on their high homology to the PKD2 of the family members ABC1036 and ABC1040. ABC1041 and ABC1063 are also diverged from one another and mapped to different chromosomes. They are expressed at the transcription level and contain probable functional kinases, but their function, if any, is not known.

Most of the Rpg1 family members (ABC1036, ABC1037, ABC1040, and ABC1041) contained unusually large introns ranging from ~3 to ~12.6 kb in length that are not present in Rpg1 (Fig. 3). These are among the largest plant introns reported to date. cDNA sequences show that these large introns are spliced out during mRNA maturation. The largest introns contained repetitive retrotransposon- and transposon-like sequences. These repetitive element insertions are probably responsible for the expansion of these large introns. Repetitive element duplication and insertion has been implicated for the expansion of large grass genomes (Bennetzen 2000). In rice at the Xa21 locus, transposable elements and retrotransposable elements are a major source of variability and can even create novel resistance functions as demonstrated with Xa21D. Transposable elements may also influence the rate of recombination (Richter and Ronald 2000) or facilitate duplication events by serving as the site for homologous unequal recombination (Ellis et al. 1995).

Analysis of the nine highly conserved a.a. in S/T PKs (as reviewed in Introduction) showed that the Rpg1 PKD1 had four a.a. and ABC1037 PKD1 had three a.a. that were not conserved. Otherwise, all the other
PKDs had all nine a.a. conserved suggesting that they are functional PKs and could play a role in signal transduction.

The 5’ RACE products obtained for ABC1036 and ABC1040 consistently resulted with a TSS at the PKD1 subdomain I. This TSS would result in a translation start site after subdomain III and would translate a truncated PKD1. It is possible that the 5’ RACE results for ABC1036 and ABC1040 are due to a mRNA secondary structure that terminated the reverse transcription reaction at that point prematurely. On the other hand, it is possible that there are additional small exons upstream of large introns that were not detected in this study. The mRNA size estimated from Northern blot (2.9 kb) is not very different from the sequence predicted size of 2.57 kb; therefore, it is possible that this is the correct TSS. The ABC1040 Northern blot always hybridized weakly and showed considerable smearing suggesting that there could be multiple TSSs or this gene may be pseudogene with an unstable mRNA transcripts (Fig. 4).

Protein kinases with tandem kinase domains appear to be fairly rare. The RLK/Pelle is one of the largest gene families in *Arabidopsis* with >600 members yet only two tandem kinase genes have been identified (S. Shiu, personal communication) (At1g11300, acc. # NM_101003 and At2g32800, acc. # NM_128840). Both At1g11300 and At2g32800 genes are represented by full-length cDNA sequences in the NCBI database and both tandem PKDs belong to the same subfamily as *Rpg1* (cd00180 domain family). At2g32800 is a tandem kinase gene with similar structure to *Rpg1*. At1g11300 kinase domains are not adjacent to one another but have b-lectin and s-/DUF26 domains at the N-terminus as well as between the kinase domains representing a b-lectin/DUF26/kinase gene duplication expressed in a single transcript. Searching the NCBI rice genome database with the Rpg1 and ABC1037 a.a. sequences (BLASTP) identified one tandem kinase gene with homology to *Rpg1* and ABC1037. However, it seems unlikely that the rice tandem kinase gene and *Rpg1* and ABC1037 have a common tandem kinase progenitor. It has been reported that of the >1131 rice RLK/Pelle family members 27 have two kinase domains (Shiu et al. 2004). Given that tandem kinase genes are rare in plant and animal systems, it is interesting that four of the *Rpg1* family gene members have tandem PKDs that appear to have arisen by two independent means (Fig. 6).

An interesting class of proteins with tandem kinase domains has been characterized in animal systems. Janus Kinases (JAKs) appear to be similar to *Rpg1* not only with the presence of tandem PKDs, but also with one PKD functional and the other a pseudokinase (Luo et al. 1997; Pellegrini and Dusanser-Fourt 1997; Nirmala et al. 2006). There is evidence that the pseudokinase, JH2 domain, although not catalytically active, plays a role in regulating JAK activity. This is evident by the E695-K mutation in a JAK protein found in the hyperactive *Drosophila* HopT12 mutant. This residue occurs in the JH2 domain and is conserved among all JAK family members. The HopT12 mutant hyperphosphorylates and hyperactivates D-Stat transcription factors when over-expressed in *Drosophila* cells (Luo et al. 1997). The non-autophosphorylating PKD1 in *Rpg1* also appears to be essential for function since mutations within this domain abolish disease resistance (Nirmala et al. 2006).

The JAKs do not have their own receptor, but rather interact with a cytokine receptor (Behrmann et al. 2004). The N-terminal regions of the JAK proteins are variable and have been implicated in the interaction with the cytokine receptors (Pellegrini and Dusanser-Fourt 1997). Similarly *Rpg1* may interact with yet to be identified receptor(s).

In summary, five new *Rpg1* gene family members were mapped, sequenced and characterized. Two were closely linked to the rust resistance loci *Rpg1* and *Rpg5/rpg4*. An apparent unequal recombination event between *Rpg1* and ABC1037 was characterized. The resulting chimeric allele did not exhibit the *Rpg1* resistance specificity. Although our attempt at identifying other rust resistance genes in the barley genome by homology to *Rpg1* was not immediately realized, this work may facilitate the identification of resistance genes identified in the future. To determine if the *Rpg1* N-terminal domain is important for resistance specificity a reciprocal recombination (i.e. *Rpg1* N-terminal domain and ABC1037 PK domains) was constructed and is being tested.

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CHAPTER THREE

Allele sequencing of the barley stem rust resistance gene Rpg1 identifies regions relevant to disease resistance

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Identifies Regions Relevant to Disease Resistance

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ABSTRACT


The stem rust resistance gene Rpg1 has protected North American barley cultivars from significant yield losses for over 65 years. The remarkable durability of this gene warrants further study as to its possible origin and allelic variation. Eight Swiss barley (Hordeum vulgare) landraces and eight wild barley (H. vulgare subsp. spontaneum) accessions from diverse geographic regions were analyzed to uncover novel alleles of Rpg1 and learn about its possible origin. The two germplasm groups included accessions that were resistant and susceptible to Puccinia graminis f. sp. tritici pathotype MCCF. Allele-specific primers were utilized to amplify 1 kbp overlapping fragments spanning the Rpg1 gene and sequenced if a polymerase chain reaction (PCR) fragment was generated. Variation among the PCR products revealed significant polymorphisms among these Hordeum accessions. Landraces and wild barley accessions susceptible to pathotype MCCF exhibited the highest degree of Rpg1 polymorphism. One resistant landrace (Hv672) and one resistant wild barley accession (WBDC040) yielded all seven Rpg1-specific PCR fragments, but only landrace Hv672 coded for an apparently functional Rpg1 as determined by comparison to previously characterized resistant and susceptible alleles and also resistance to HKHJ, a stem rust pathotype that can specifically detect Rpg1 in the presence of other resistance genes. Accessions resistant to stem rust pathotype MCCF, but completely lacking Rpg1-specific PCR amplification and hybridization with an Rpg1-specific probe, suggested the presence of stem rust resistant gene(s) different from Rpg1 in the Hordeum germplasm pool. Some Rpg1 alleles that retained the ability to autophosphorylate did not confer resistance to Puccinia graminis f. sp. tritici pathotype MCCF, confirming our previous observations that autophosphorylation is essential, but not sufficient for disease resistance. Thus, the Rpg1 protein plays a complex role in the stem rust disease-resistance-signaling pathway.

Additional keywords: functional analysis, R genes.

Stem rust (caused by the wheat stem rust fungus Puccinia graminis f. sp. tritici) has been one of the most important pathogens of barley (Hordeum vulgare L.) in the Upper Midwest region of the United States and Prairie Provinces of Canada over the past century. A single dominant gene (Rpg1) introgressed into barley cultivars has effectively controlled the stem rust epidemics which devastated the crop in the Northern Great Plains prior to 1942 (18). Many qualitative rust resistance genes in other cereal crops have been overcome by virulent pathotypes in a relatively short period of time (12); however, Rpg1 has been durable, protecting barley from serious stem rust losses for more than 65 years. This remarkable durability warrants further characterization of Rpg1.

Rpg1 was mapped to the short arm of barley chromosome 1(7H) and cloned from cultivar Morex by construction of high-resolution genetic and physical maps (2). Highly resistant transgenic plants were obtained by transforming the susceptible barley cultivar Golden Promise, devoid of Rpg1, with the Morex Rpg1 gene, demonstrating that the gene isolated was functional in conferring stem rust resistance (9). Rpg1 contains 14 exons in 4,466 bp of genomic sequence encoding an 837 amino acid protein with two tandem kinase domains, which is a unique resistance gene structure (2).

Sequence analysis of Rpg1 from resistant North American barley cultivars revealed no amino acid sequence polymorphism (2). This suggests a singularity of the Rpg1 source that can be traced to an unimproved bulked seed lot (from which the sister selections of Chevron [PI 38061] and Peatland [PI 539109] were derived) obtained by the United States Department of Agriculture from Switzerland in 1914. Another potential source of stem rust resistance in barley was the cultivar Kindred (CIho 6969), a farmer selection of a single stem rust resistant plant from a field of susceptible Wisconsin 37 barley (18). The plant from which Kindred was derived was probably an admixture because it differed from Wisconsin 37 for many more traits than just stem rust resistance. Moreover, sequence analysis revealed that the Rpg1 in silico translated amino acid sequence in Kindred was identical to that of Chevron and Peatland (2). The source of Rpg1 in Kindred was likely Chevron because this cultivar had been used in the Wisconsin barley breeding program for a number of years prior to the release of Wisconsin 37.

During the map-based cloning effort of Rpg1, allele sequencing revealed three groups of stem rust susceptible barley lines (2). The first group contained susceptible rpg1 genes with 3 base pairs (GTT) inserted in the sixth exon at nucleotide position 1,546 relative to the Rpg1 sequence of Morex. The second group contained an Rpg1-like gene with a highly diverged 5′ region (2), and the third group had no detectable Rpg1 gene sequences. The second group, including barley line Sm99010, was recently characterized as containing a recombinant Rpg1 gene with ABC1037 and an Rpg1 gene family member located ≈50 kbp proximal to Rpg1 (1). Lines with this recombinant Rpg1 gene have a 5′ end highly homologous to ABC1037 and a 3′ end with high homology to Rpg1 (1). Reverse transcription (RT)-PCR and sequence analysis indicated that the Sm99010 recombinant Rpg1 gene is tran-
scribed, but does not occur in conferring stem rust resistance (to pathotype MCCF) because it and two other barley cultivars (Dicktoo and Gobernadora) containing the Sm90010-like Rpg1 gene were susceptible (2). It is possible that the Sm90010 Rpg1 gene expresses novel specificities to other stem rust pathotypes, as unequal exchange events have been suggested as a means for the evolution of plant disease resistance genes (7,15). In flax, 13 alleles at the L rust resistance locus have been reported to express 10 different rust resistance specificities. These resistance specificities are presumed to have evolved through various mechanisms, including single base substitutions, insertions/deletions (indels), unequal recombination, and reassortment of variable regions within the alleles by multiple intra-genic sequence exchange events (7).

Naturally occurring allelic diversity in plants has been suggested to be an important genetic component for phenotypic variation and crop improvement. It is therefore important to characterize this diversity in order to identify economically important alleles and polymorphisms (3). In addition to landraces of domesticated barley (H. vulgare), the wild progenitor H. vulgare subsp. spontaneum is known to be a valuable source for alternative forms of disease resistance genes for barley improvement (8,22). Stem rust resistance has been reported in wild barley, but the frequency of resistance is often low (<10%) (8,20). Given the recent threat of new pathotypes such as P. graminis f. sp. tritici TTKS (i.e., isolate Ug99) in the world, wild barley may become an important new source of stem rust resistance genes (20).

The lack of Rpg1 polymorphism among previously-tested stem rust resistant barley cultivars suggests either a highly conserved or single source for this gene. High conservation of a protein kinase disease resistance gene has been shown with the tomato Pto gene, which confers resistance to Pseudomonas syringae pv. tomato. The wild tomato species Lycopersicon pimpinellifolium is the source of the Pto gene in cultivated tomato (L. esculentum). A Pto ortholog, LhrPto, isolated from another wild tomato relative (L. hirsutum), shares 97% amino acid identity with the Pto from L. pimpinellifolium (16). Even more surprising, the domesticated tomato has a highly conserved, transcriptionally active pto allele that fails to confer disease resistance (10). Since the Lycopersicon species separated presumably millions of years ago, these findings suggest that the PTO kinase has been highly conserved, even when it does not retain disease resistance function.

The objectives of this study were to use molecular techniques to uncover new alleles of Rpg1 and learn about its possible origin in eight Swiss barley (H. vulgare) landraces and eight wild barley (H. vulgare subsp. spontaneum) accessions from diverse geographic regions. The presence/absence of Rpg1, its primary structure, and expression were analyzed by PCR of genomic DNA and mRNA (RT-PCR). Southern blotting, sequencing, western blotting, and autophosphorylation analyses.

**MATERIALS AND METHODS**

**Plant materials and disease phenotyping.** From a germplasm collection of 74 barley landraces from Switzerland and 318 ecogeographically diverse wild barley accessions (20), 16 (eight from each group) were selected and used in this study (Table 1). Landraces from Switzerland were chosen for analysis because they are the donors of Rpg1 (i.e., Chevron and Peatland) in North American barley cultivars and may possess additional allelic diversity for the gene. Wild barley accessions also were included because of their great allelic diversity and potential for identifying the origin of Rpg1 in cultivated barley. Within each germplasm group, four resistant and four susceptible accessions to stem rust pathotype MCCF were chosen from ecogeographically diverse locations to identify functional Rpg1 variants as well as spontaneous mutations that result in loss of function. Pathotype MCCF has been used for a number of years to select breeding lines with Rpg1, develop high resolution maps for map-based cloning of the resistance gene, and conduct functional analyses with the cloned gene (2,13,19). Recently, a comprehensive evaluation was made on the reaction of known stem rust resistance genes in barley to diverse pathotypes of P. graminis f. sp. tritici and isolates of P. graminis f. sp. secalis (the rye stem rust fungus) (21). This study revealed that P. graminis f. sp. tritici pathotype HKHJ was more effective than MCCF for detecting Rpg1 in the presence of several other known stem rust resistance genes (21). Thus, the germplasm initially selected for this study based on reaction to pathotype MCCF was subsequently evaluated to pathotype HKHJ to further resolve the functionality of Rpg1. Cultivars Morex (with Rpg1) and Steptoe (without any known resistance genes) also were included as the resistant and susceptible controls, respectively. Plants were grown and assayed for their reaction to stem rust as previously described (19).

DNA and RNA were isolated from a single plant of each of the 16 selected accessions based initially on their stem rust phenotype. The plants were grown in plastic pots containing potting mix in a growth chamber under cool fluorescent lights with a 16 h

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**TABLE 1. Reaction of Swiss landrace and wild barley accessions to Puccinia graminis f. sp. tritici pathotypes MCCF and HKHJ at the seedling stage**

<table>
<thead>
<tr>
<th>Line</th>
<th>Origin</th>
<th>IT range</th>
<th>General reaction</th>
<th>IT range</th>
<th>General reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swiss Hv584</td>
<td>Fuermis, Switzerland</td>
<td>0:1 to 10;</td>
<td>Resistant</td>
<td>3- to 3</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Swiss Hv587</td>
<td>Platta, Switzerland</td>
<td>0:1 to 21</td>
<td>Resistant to moderately resistant</td>
<td>3- to 3</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Swiss Hv619</td>
<td>Fetal, Switzerland</td>
<td>10;</td>
<td>Resistant</td>
<td>3- to 3*</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Swiss Hv672</td>
<td>Surava, Switzerland</td>
<td>10; to 12</td>
<td>Resistant</td>
<td>10; to 21</td>
<td>Resistant to moderately resistant</td>
</tr>
<tr>
<td>Swiss Hv489</td>
<td>Trans, Switzerland</td>
<td>3</td>
<td>Susceptible</td>
<td>3 to 3*</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Swiss Hv492</td>
<td>Scheid, Switzerland</td>
<td>3 to 3*</td>
<td>Susceptible</td>
<td>3 to 3</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Swiss Hv611</td>
<td>Plan, Switzerland</td>
<td>3 to 3</td>
<td>Susceptible</td>
<td>3 to 3</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Swiss Hv645</td>
<td>Scoul, Switzerland</td>
<td>3 to 3</td>
<td>Susceptible</td>
<td>3 to 3</td>
<td>Susceptible</td>
</tr>
<tr>
<td>WBDC040</td>
<td>Haifa, Israel</td>
<td>0:1 to 21</td>
<td>Resistant to moderately resistant</td>
<td>3 to 3</td>
<td>Susceptible</td>
</tr>
<tr>
<td>WBDC124</td>
<td>Samarkand, Uzbekistan</td>
<td>0:1 to 21*</td>
<td>Resistant to moderately resistant</td>
<td>3 to 3*</td>
<td>Susceptible</td>
</tr>
<tr>
<td>WBDC220</td>
<td>Chimkent, Kazakhstan</td>
<td>10:2 to 21*</td>
<td>Resistant to moderately resistant</td>
<td>3 to 3*</td>
<td>Susceptible</td>
</tr>
<tr>
<td>WBDC225</td>
<td>Dushanbe, Tajikistan</td>
<td>0:1 to 21</td>
<td>Resistant to moderately resistant</td>
<td>3 to 3*</td>
<td>Susceptible</td>
</tr>
<tr>
<td>WBDC019</td>
<td>West Azerbaijan, Iran</td>
<td>3 to 3*</td>
<td>Susceptible</td>
<td>3 to 3*</td>
<td>Susceptible</td>
</tr>
<tr>
<td>WBDC160</td>
<td>Sweida, Syria</td>
<td>3 to 3*</td>
<td>Susceptible</td>
<td>3 to 3*</td>
<td>Susceptible</td>
</tr>
<tr>
<td>WBDC269</td>
<td>Lebanon</td>
<td>3-2 to 33*</td>
<td>Moderately susceptible to susceptible</td>
<td>3 to 3</td>
<td>Susceptible</td>
</tr>
<tr>
<td>WBDC323</td>
<td>Alahski, Turkmenistan</td>
<td>3 to 33*</td>
<td>Susceptible</td>
<td>3 to 3</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Morex</td>
<td>Minnesota, USA</td>
<td>0:1 to 21*</td>
<td>Resistant to moderately resistant</td>
<td>12 to 21</td>
<td>Resistant to moderately resistant</td>
</tr>
<tr>
<td>Steptoe</td>
<td>Washington, USA</td>
<td>3 to 33*</td>
<td>Susceptible</td>
<td>3 to 33*</td>
<td>Susceptible</td>
</tr>
</tbody>
</table>

*IT = infection types are based on a 0 to 4 scale, + and – denote more or less sporulation of uredinia, respectively. ITs of 0, 1, 2, and combinations thereof are indicative of resistance, whereas ITs of 3, 4 and combinations thereof are indicative of susceptibility (21)."
photoperiod and day/night temperature of 21 ± 1°C/18 ± 1°C. For protein analysis by western blot and autophosphorylation, a single plant from the same seed source was grown under the above conditions.

**DNA and RNA isolation.** Genomic DNA for Southern hybridization was isolated from young barley leaves with a modified CTAB method (11). For PCR amplification, genomic DNA was isolated from young barley leaves according to Edwards et al. (6). Total RNA was isolated from young seedling leaves using Trizol reagent (4). Integrity and quantity of the RNA samples were checked by 1x TBE (0.089 M Tris-borate, 0.089 M boric acid, and 0.002 M EDTA) agarose gel electrophoresis.

**PCR and RT-PCR.** PCR primers were designed from cultivar Morex and line Sm89010 Rpg1 allele sequences (GenBank accession numbers AF509748 and AF509762, respectively) to amplify approximately 1-kbp overlapping fragments covering the entire Rpg1 gene (Fig. 1 and Table 2). Primer set 228F6/228R6 amplified a fragment from both cultivars Morex and Sm89010; however, the Sm89010 fragment is of higher molecular weight (Table 2). Primer combination 37F2/228R6 amplified only the Sm89010 Rpg1 allele and resulted in a 1,678-bp band. DGK-F1 and DGSex2 primers are internal to 37F2/228R6 and were used only in the sequencing reaction in order to cover the gap between the forward 37F2 and reverse 228R6 primers. PCR reactions and analyses were as previously described (2).

Approximately 1µg of total RNA was used for oligo dT-primed first-strand cDNA synthesis using the Reverse Transcription System (Promega, Madison, WI) according to the manufacturer’s recommended conditions followed by PCR reactions (2). Primers EX3cw2/228R6 and 37-F2/228R7 were used for RT-PCR to amplify Morex or Sm89010 Rpg1-specific cDNA, respectively.

**Southern blot analysis.** Southern blotting and hybridization were as previously described (11). Five micrograms of HindIII-digested barley genomic DNA was separated by agarose gel electrophoresis, blotted onto nylon membranes, and hybridized with the Rpg1-specific DNA probe RSB228 (2).

**Sequencing.** PCR fragments were eluted from the agarose gel, purified, concentrated by ethanol precipitation, and used as templates for sequencing reactions. Sequencing reactions were performed using the Big-Dye Terminator kit (Applied Biosystems, Foster City, CA) and sequenced at the Bioanalytical Center, Washington State University, Pullman, WA. Sequences were analyzed and contigs assembled using ContigExpress from the Vector NTI software package.

**cDNA cloning and sequencing.** Oligo dT-derived cDNA from Morex and WBDC040 was used as the template to amplify the ninth intron/exon junction of Rpg1. RT-PCR products were cloned in the pGEM-T vector (Promega) following the manufacturer’s protocol. Clones were confirmed by restriction digestion with EagI (New England Biolabs, Beverly, MA) and NcoI (Fermentas, Hanover, MD) and sequenced using T7 and SP6 universal primers.

**Protein analysis.** Polyclonal antibody was raised in New Zealand white rabbits at the Washington State University animal facility against a recombinant His-RPG1 protein purified from *Pichia pastoris* (13). Ribi adjuvant was used according to the manufacturer’s recommendation (Corixa, Hamilton, MO). Western blotting and autophosphorylation assays were performed as previously described (13).

**Rapid amplification of cDNA 3′ ends (3′ RACE).** 3′ RACE was carried out using the FirstChoice RLM-RACE Kit (Ambion, Austin, TX) and the 3′ RACE System (Invitrogen, Frederick, Maryland).

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**Fig. 1.** Schematic representation of the Rpg1 gene structure. A, The relative position of the seven cultivar Morex Rpg1-specific primer pairs and overlapping fragments generated in relation to the Rpg1 gene structure. Introns are shown in gray and exons in black shading. The 5′ and 3′ untranslated transcribed regions (UTRs) are indicated. B, Black bars indicate fragments amplified using the Morex-specific set of primer pairs, hatched bars indicate fragments amplified using the barley line Sm89010 allele-specific primers, and white bars indicates no amplification. The R or S designation within parentheses indicate resistance or susceptibility to *Puccinia graminis* f. sp. *tritici* pathotypes MCCF/HKHJ, respectively.
RESULTS

Stem rust reaction. The controls reacted as expected with Morex exhibiting resistant to moderately resistant infection types and Steptoe susceptible infection types to pathotypes MCCF and HKHJ (Table 1). Hv584, Hv587, Hv619, and Hv672 were among the most resistant accessions found to *P. graminis* f. sp. tritici pathotype MCCF in the Swiss landrace germplasm, giving infection types ranging from 0 to 21 (Table 1). In contrast, Hv489, Hv492, Hv611, and Hv645 were among the most susceptible accessions found, giving infection types of 3–33+. Resistance to wheat stem rust in wild barley is fairly rare (8,20). The resistant accessions found, giving infection types ranging from 0;1 to 213–. Accession (20) and were among the most resistant found to pathotype WBDC220, and WBDC225 (Fig. 1B), suggesting the presence of an intact *Rpg1* gene in these genotypes. Southern blots of genomic DNA digested with HindIII and hybridized with the *Rpg1*-specific probe RSB228 confirmed these results (Fig. 2). The hybridizing band was the same size (approximately 4.5 kbp) as observed in cultivar Morex and the wild barley accession OSU6 (2) containing an intact *Rpg1* gene. These data were not in agreement with the stem rust phenotypes as only Hv672 was resistant to both pathotypes MCCF and HKHJ. OSU6 was previously characterized as resistant to stem rust pathotype MCCF, but multiple subsequent assays with both pathotypes MCCF and HKHJ on the selected plants used in the molecular analysis showed that

Fig. 2. Southern blot analysis of *Hordeum* accessions. Genomic DNA of each line was digested with HindIII and hybridized with the *Rpg1*-specific probe RSB228. The Morex-specific *Rpg1* band is approximately 4.5 kbp. The Sm9010-specific *Rpg1* band is approximately 7.5 kbp (not shown) and of similar size to fragments hybridizing with DNA of Swiss landraces Hv619, Hv489, and Hv645. Harrington does not contain *Rpg1*. The R or S designation within parentheses indicate resistance or susceptibility to *Puccinia graminis* f. sp. tritici pathotypes MCCF/HKHJ, respectively.

### Table 2. Primers used to amplify Morex-like and Sm89010-like gene fragments for genotyping and sequencing

<table>
<thead>
<tr>
<th>Fragment name*</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Location and product size*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Sm</td>
<td>37F/228R6</td>
<td>cttcaaccttcctctctcagatc/ggaaggacgctaaagcaagcttc</td>
<td>–52–1,626/1,678 bp None</td>
</tr>
<tr>
<td>228F1/228R11</td>
<td>228F6/228R6</td>
<td>209–1,624/1,414 bp 130–1,090/959 bp</td>
<td></td>
</tr>
<tr>
<td>228F1/228R1</td>
<td>ccgaggctgcaatggaagacgctaaagcaagcttc</td>
<td>1,420–2,500/1,085 bp 884–1,967/1,082 bp</td>
<td></td>
</tr>
<tr>
<td>228F2/228R2</td>
<td>tgggtagctcctctctctctctcagatc/ggaaggacgctaaagcaagcttc</td>
<td>2,151–3,247/1,095 bp 1,612–2,708/1,095 bp</td>
<td></td>
</tr>
<tr>
<td>228F3/228R3</td>
<td>ccgaggctgcaatggaagacgctaaagcaagcttc</td>
<td>2,893–3,980/1,085 bp 2,353–4,410/1,086 bp</td>
<td></td>
</tr>
<tr>
<td>228F4/228R4</td>
<td>ccgaggctgcaatggaagacgctaaagcaagcttc</td>
<td>3,629–4,561/931 bp 3,090–4,020/929 bp</td>
<td></td>
</tr>
<tr>
<td>228F5/228R5</td>
<td>ccgaggctgcaatggaagacgctaaagcaagcttc</td>
<td>4,349–5,100/750 bp 3,810–4,561/750 bp</td>
<td></td>
</tr>
<tr>
<td>228F6/228R6</td>
<td>ccgaggctgcaatggaagacgctaaagcaagcttc</td>
<td>5,219–6,065/879 bp None</td>
<td></td>
</tr>
<tr>
<td>228F7/228R7</td>
<td>ccgaggctgcaatggaagacgctaaagcaagcttc</td>
<td>5,219–6,065/879 bp None</td>
<td></td>
</tr>
<tr>
<td>RT-PCR1</td>
<td>EX3cw2/228R6</td>
<td>465–1,088/396 bp</td>
<td></td>
</tr>
<tr>
<td>RT-PCR2</td>
<td>EX3cw2/228R7</td>
<td>465–1,088/396 bp</td>
<td></td>
</tr>
<tr>
<td>Seq1Sm</td>
<td>DKG-F1</td>
<td>875 NA*</td>
<td></td>
</tr>
<tr>
<td>Seq1Sm</td>
<td>DGSEX2</td>
<td>953 NA*</td>
<td></td>
</tr>
<tr>
<td>Ubiquitin</td>
<td>Hvu_Ubi1_cw1</td>
<td>219 bp 219 bp</td>
<td></td>
</tr>
</tbody>
</table>

* Fragments 1 to 7 primers were used to amplify the Morex *Rpg1* coding regions as shown in Figure 1. Fragment 1 Sm primers amplified fragment 1 only from the Sm90101-like *Rpg1* allele. Reverse transcription-polymerase chain reaction (RT-PCR) 1 and 2 primers were used to amplify a specific *Rpg1* mRNA fragment from either Morex or Sm9010, respectively. Seq1Sm primers DGK-F1 and DGSEX2 are internal to the location of primers 37F2 and 228R6, and were used to cover the sequencing gap in fragment 1Sm, which was approximately 1.7 kbp.

* Bases upstream of the start codon are designated with minus (–) values. The product size is given in base pairs and the location indicated by base pair position.

* NA, not applicable.

MD) under the manufacturer’s recommended standard conditions. The nested gene-specific primers used for both sets were 228-F3 and 228-F4 as previously described in Brueggeman et al. (2).
it is susceptible (B. Steffenson, unpublished data). Accession
WBDC040 was resistant to moderately resistant to pathotype
MCCF and susceptible to pathotype HKHJ, while WBDC323 was
susceptible to both pathotypes.

All of the PCR-amplified genomic fragments were sequenced
and aligned to resolve the phenotype versus genotype discrep-
ancy. The predicted amino acid sequence of the resistant Swiss
landrace Hv672 was identical to that of cultivar Morex Rpg1,
supporting the phenotype results to both stem rust pathotypes.

Rpg1 sequence analysis of wild barley accession WBDC040
suggested the presence of an inactive Rpg1 gene since it had a
GTT nucleotide insertion at nucleotide position 1,546 relative to
the Morex sequence, a motif indicative of a susceptible allele (5).
The GTT insertion resulted in an S to R amino acid substitution at
position 319 and an F insertion at 320 (Fig. 3). Initial RT-PCR
analysis suggested that WBDC040 produced an Rpg1 mRNA
similar to Morex (Fig. 4). However, sequencing of several cDNA
clones of the ninth exon/intron junction of Rpg1 from Morex and
the resistant wild barley accession WBDC040 showed that there
was a substitution altering the conserved 3′ AG splice site of
intron 9 to AA at position 3251 resulting in the next downstream
AG being used as the 3′ splice site. This resulted in a 17-nucleo-
tide deletion in the mRNA of the WBDC040 allele, causing a frame
shift and a stop codon at amino acid position 617 (Fig. 5). There-
fore, the resistance of WBDC040 to pathotype MCCF could not be
due to the Rpg1 gene. This was confirmed by protein analyses that
showed that the RPG1 protein failed to accumulate in WBDC040
and autophosphorylation was not detected (Fig. 6). These results
suggest the presence of a gene different from Rpg1 conferring
resistance to pathotype MCCF in this accession. The sus-
cceptibility of WBDC040 to pathotype HKHJ supports this result.

Rpg1 sequence analysis of wild barley accession WBDC323
identified a few nucleotide substitutions scattered throughout the
nucleotide sequence in addition to the GTT insertion as previ-
ously described. At the amino acid level, however, there are only
two differences in the Rpg1 sequence between WBDC323 and
Morex. The GTT insertion results in an S to R substitution at
position 319, an F insertion at position 320, and an I to F

---

**Fig. 3.** Predicted amino acid alignment of Rpg1 alleles from Swiss barley landraces and wild barley accessions compared with Morex and Sm989010. Gray boxes represent identical residues and white areas represent diverged residues compared to the resistant Morex Rpg1 allele. Dashes represent gaps in the alignment. Dots represent blocks of conserved amino acid sequences between all alleles. Asterisk indicates stop codon. The R or S designation within parentheses indicate resistance or susceptibility to *Puccinia graminis* f. sp. *tritici* pathotypes MCCF/HKHJ, respectively. Sequence information has been deposited in the GenBank database under the following accession numbers: Swiss Hv489, DQ854803; Swiss Hv619, DQ854804; Swiss Hv672, DQ854805; WBDC019, DQ854806; WBDC040, DQ854805; WBDC160, DQ854806; and WBDC323, DQ857358. The Morex and Sm989010 sequences have been previously deposited and can be found under accession numbers AF509748 and AF509762, respectively (2).
substitution at position 269 (Fig. 3). RT-PCR analysis showed that the \textit{Rpg1} gene was expressed in this accession at the mRNA level (Fig. 4), and western blot and autophosphorylation assays showed that the RPG1 protein is present and capable of autophosphorylation (Fig. 6). Though the GTT insertion and/or the I to F substitution did not affect the autophosphorylation activity, the protein was not functional in conferring resistance to either pathotype MCCF or HKHJ.

**Lines with an apparent unequal recombination.** We previously described an apparent unequal recombination event in

![Fig. 4. Reverse transcription-polymerase chain reaction showing Morex-like or Sm89010-like bands. A, Oligo dT-primed cDNA derived from total RNA was amplified with either Morex-like \textit{Rpg1}-specific primers (right lane designated M) or Sm89010-like \textit{Rpg1}-specific primers (left lane designated S). The accessions used for cDNA isolation are listed above. The Morex band is approximately 0.4 kbp and the Sm89010 band is approximately 0.9 kbp. Lines devoid of \textit{Rpg1} did not yield a band with either set of primers. B, Oligo dT-primed cDNA of each was amplified using ubiquitin-specific primers to demonstrate loading and RNA quality.](image)

![Fig. 5. Sequence comparison of Morex and WBDC040 \textit{Rpg1} intron 9/exon 10 junction and predicted amino acid sequence. The WBDC040 cDNA sequence is based on three cloned reverse transcription-polymerase chain reaction generated fragments. Note the G to A substitution at the 3' end of intron 9 (white A) in the WBDC040 genomic sequence and the subsequent use of the next AG pair (AG bold) for splicing of the mRNA. The resulting cDNA is missing 17 nucleotides from exon 10 causing a frame shift and a stop codon at position 617. The truncated translation product failed to accumulate a protein detectable by the RPG1-specific antibody.](image)

![Fig. 6. Western blot and autophosphorylation assays. A, RPG1 protein visualized with an RPG1-specific antibody. WBDC040 and GP (cultivar Golden Promise, which does not have the \textit{Rpg1} gene) failed to produce a detectable RPG1 protein. B, Autophosphorylation assay corresponding to RPG1 western blot. WBDC040 and GP did not autophosphorylate. The K2 (kinase domain 2) mutant also failed to autophosphorylate, while the K1 (kinase domain 1) mutant autophosphorylated as previously reported (13). C, Coomassie blue staining of total protein to demonstrate protein loading in each lane.](image)
barley line Sm89010, resulting in a chimeric gene between ABC1037, a closely linked Rpg1 homologue, and Rpg1 (1). In the Swiss landraces Hv619, Hv489, and Hv645, amplification of fragments 3 to 7 with the Morex-specific primers was successful and of the expected size, but fragment 1 failed to amplify (Fig. 1). Fragment 2 amplified, but was larger than from Morex (1.6 kbp versus 1.0 kbp). This was similar to the situation previously observed with the Sm89010 allele. Successful amplification of fragment 1 using Sm89010 recombinant allele-specific primers confirmed the presence of an Sm89010-like recombinant allele in these lines (2). Southern blot hybridization of HindIII digested Hv619, Hv489, and Hv645 DNA yielded a band similar to that previously observed with Sm89010 (1) and with a higher molecular weight than from the lines containing the Morex-like allele (Fig. 2). RT-PCR amplification using primers specific to Morex or Sm89010 alleles resulted only in a band similar to Sm89010 in these lines and no amplification with Morex-specific primers (Fig. 4). Nucleic acid sequence in silico translation predicted amino acid identity among the three Swiss landraces Hv619, Hv489, and Hv645 and with the Sm89010 Rpg1-like recombinant gene (Fig. 3). At the nucleotide level, there were only three differences between the entire Sm89010 Rpg1 gene sequence and the sequences of the three Swiss landraces (2). There was a G to A substitution at position 2,183 as well as single nucleotide deletions of A at 2,199 and C at 4,713, with numbering based on the Sm89010 Rpg1 sequence (data not shown). These single nucleotide polymorphisms (SNPs) did not change the amino acid sequence since the G 2,183 A substitution was in the third nucleotide in the codon and A 2,199 and C 4,713 deletions were in introns. The recombinant Rpg1-like gene is not expected to provide stem rust resistance as previously demonstrated with the Sm89010 line (1). Landraces Hv489 and Hv645 were indeed susceptible to stem rust pathotypes MCCF and HKHJ as expected; however, Hv619 was resistant to pathotype MCCF, suggesting the presence of a gene different from Rpg1 conferring the resistance. The lack of a functional Rpg1 gene in Hv619 also was confirmed by susceptibility to pathotype HKHJ. All three lines with the recombinant type Rpg1 allele produced an Rpg1 antibody positive protein and autophosphorylated (Fig. 6).

This content is a continuation of the previous text and includes information about lines with apparent 3' and 5' deletions in the Rpg1 genes. The wild barley accessions WBDC019 and WBDC160 were susceptible to stem rust pathotypes MCCF and HKHJ and had apparent 3' and 5' deletions in the Rpg1-like genes. For WBDC019, fragment 7 (the most 3' Rpg1 PCR fragment) failed to amplify and for WBDC160, fragments 1 and 2 (the most 5' Rpg1 PCR fragments) failed to amplify with either the Morex- or Sm89010-specific primers (Fig. 1B). Southern blot analysis showed that both lines had Rpg1 probe-positive hybridizing fragments; however, accession WBDC019 showed a very high molecular weight fragment, while the WBDC160 fragment was about the same size as Morex (Fig. 2). Since WBDC019, the only accession lacking amplification for fragment 7, had a unique high molecular weight fragment hybridized, we assumed that there might be a large insertion in this region. Additional efforts using amplification conditions for long range PCR (>10 kbp fragments) and fragment 7 primers (228F12/228R12) did not result in amplification. Long range PCR also was applied for amplification of fragment 1 and 2 from WBDC160 using primers 228F11/228R11 and 228F6/228R6, respectively, but without success. RT-PCR produced a band similar to Morex (Fig. 4), indicating that the gene is expressed and suggesting the possibility of an mRNA similar to the Morex-like allele. The RT-PCR was carried out with primers that amplified the internal Morex-like region of the gene. Therefore, the bands are approximately the same size as the Morex Rpg1 mRNA.

The amplified genomic DNA fragments 1 to 6 from WBDC019 and fragments 3 to 7 from WBDC160 were sequenced and aligned with the other Rpg1 derived amino acid sequences (Fig. 3). The susceptible Rpg1 allele from WBDC019 had diverged, including the GTT insertion, but still was very similar to the Morex Rpg1 protein sequence until the C terminus where it could no longer be amplified and recognized as a Morex Rpg1 homologue. All attempts at extending the sequence with 3' RACE failed.

The in silico translated sequence of the susceptible wild barley WBDC160 Rpg1 starts at amino acid position 162 and is fairly similar to the Morex sequence from then on (Fig. 3). This gene also has the GTT insertion resulting in substitution of S 319 R and insertion of F 320. Both lines produced Rpg1 antibody-positive protein and autophosphorylated (Fig. 6). Rpg1 gene PCR amplification deficient lines. Of the 16 lines analyzed, 8 failed to produce any amplification products with either Morex-specific or Sm89010-specific Rpg1 primers (Fig. 1B). The eight lines were evenly divided between the germplasm groups with four Swiss landraces and four wild barley accessions failing to yield any amplification products. To confirm the absence of Rpg1 alleles in the eight genotypes, an additional 27 combinations of primers designed from the Morex and Sm89010 nucleotide sequences were tested on this group. No amplification products were obtained with these sets of primers, confirming the absence of a Morex-like or Sm89010-like Rpg1 in these lines (data not shown). In accordance with the PCR amplification results, no hybridization to the RSB228 probe was observed with DNA from lines lacking PCR amplification (Fig. 2). To further confirm the lack of an Rpg1-like gene, two Swiss landraces resistant (Hv584 and Hv587) and one wild barley accession susceptible (WBDC269) to pathotype MCCF from this group were included in RT-PCR analysis. No amplification products were obtained using Morex or Sm89010 Rpg1 primers (Fig. 4). Five of these accessions (Hv584, Hv587, WBDC214, WBDC220, and WBDC225) were resistant to pathotype MCCF and three (Hv492, Hv611, and WBDC269) were susceptible. All of these accessions were susceptible to pathotype HKHJ. These results clearly indicate the presence of a stem rust resistance gene(s) different from Rpg1 in these Hordeum accessions.

**DISCUSSION**

To investigate the diversity and possible origin of the barley stem rust resistance gene Rpg1, we analyzed two subsets of *H. vulgare* and *H. vulgare* subsp. *spontaneum* germplasm: one comprised of eight Swiss landraces and the second of eight wild barley accessions from diverse geographic regions. Accessions resistant and susceptible to stem rust pathotype MCCF were chosen in order to identify functional Rpg1 variants as well as spontaneous mutations that result in loss of function (Table 1). Additionally, pathotype HKHJ also was used in this study because it is highly effective for detecting Rpg1 in the presence of other stem rust resistance genes.

The Swiss landraces used in this study were a potential source of stem rust resistant germplasm and possibly new Rpg1 alleles. However, only one accession (Hv672) with an intact Rpg1 gene was found to confer resistance to both *P. graminis* f. sp. *tritici* pathotypes MCCF and HKHJ (Fig. 1; Table 1). The gene in Hv672 was identical in predicted amino acid sequence to the cultivar Morex Rpg1 protein (Fig. 3). Thus, Swiss landraces appear to be the sole source for Rpg1 as both cultivars Chevon and Peatland from Vorrenwald Eich in Canton Lucerne (18) and accession Hv672 from Surava in Canton Graubünden (B. Steffenson, unpublished data) have intact Rpg1 genes that encode a protein size, but amino acid sequences identical to Morex, and are all functional in conferring resistance to pathotype HKHJ. It is interesting to note that Hv672 (together with its duplicate selection Hv671) was the only accession among 74 Swiss landraces tested that exhibited resistance to pathotype HKHJ (B. Steffenson, unpublished data). Thus, the presence of Rpg1 also is
apparently rare in Swiss landraces. The Rpg1-derived amino acid sequence in Kindred was previously shown to be identical to that of Chevron and Peatland (2). As mentioned previously, the donor of Rpg1 in Kindred was likely Chevron.

Wild barley is known to be a rich source of different disease resistance alleles for cultivated barley improvement (8). However, to stem rust, the frequency of resistance is relatively low, even in a large (318 accessions) and diverse collection (20). To identify the possible source of Rpg1 in cultivated barley, eight ecogeographically diverse wild barley accessions that were resistant or susceptible to stem rust pathotype MCCF were selected for this study. Analysis of the Rpg1 alleles from the eight wild barley accessions failed to identify any with an intact and functional Rpg1 gene as was found for the Swiss landrace accession Hv672 (Fig. 1 and Table 1). Surprisingly, most of the resistant wild barley accessions contained either a defective Rpg1 gene or did not contain a recognizable Rpg1 gene. This result indicates that other stem rust resistance genes (effective against P. graminis f. sp. tritici) pathotype MCCF exist in wild barley. The fact that defective Rpg1 alleles were found among the wild barley accessions indicates that the gene exists or has existed in these populations.

A curious GTT indel occurs in the Rpg1 gene of several Swiss landraces and wild barley accessions. This indel results in an S 319 R substitution and insertion of F at position 320 (Fig. 3). In the wild barley accession WDBC323, the only other amino acid change relative to the Morex Rpg1 sequence is an I 269 F substitution resulting in a stem rust susceptible phenotype. We have previously observed the GTT indel in the wild barley accession OSU6 (PBI004-7-D-015 accession no. 8321) (2). OSU6 was previously characterized as resistant to stem rust pathotype MCCF, but multiple subsequent assays with both pathotypes MCCF and HKHJ on the selected plants used in the molecular analysis showed that it is susceptible (B. Steffenson, unpublished data). Except for the amino acid changes resulting from the GTT indel, OSU6 does not have any other differences in the Rpg1 amino acid sequence compared to the Morex Rpg1 sequence. The S to RF change due to the GTT indel appears in seven out of the eight accessions containing an Rpg1-like gene analyzed here and thus is very common (Fig. 3). These seven accessions (Hv619, Hv489, Hv645, WDBC040, WDBC019, WDBC160, and WDBC323) do not show a consistent pattern of resistance or susceptibility to the stem rust pathotype MCCF, but all were susceptible to HKHJ, the best pathotype for indicating the functionality of Rpg1. Thus, it seems that the GTT insertion affects Rpg1-mediated stem rust resistance as indicated by reaction to pathotype HKHJ, but may not affect resistance conferred by other genes, i.e., those conferring resistance to pathotype MCCF. The potential role of the GTT insertion is unknown. It occurs at position 319 in the Rpg1 amino acid sequence, placing it near the end of the kinase 1 domain. This domain is not essential for Rpg1 autophosphorylation activity, but plays an important, yet undefined role in the overall stem rust resistance reaction (13). The fact that the Rpg1 protein with the GTT insertion is active in autophosphorylation suggests that the kinase 2 domain, which is essential for autophosphorylation and disease resistance, is intact and the gene is expressed and may retain some unknown function (Fig. 6).

A set of primers that can differentiate barleys with and without Rpg1 was developed based on the gene’s nucleotide sequence and the GTT insertion (5). The authors tested 42 different barley cultivars and showed that the primers correctly identified the phenotype in all cases, but one. The one exception was cultivar Maud, originally classified as resistant (with a question mark), but grouped with susceptible lines that do not contain an Rpg1 gene (5). We repeatedly tested Maud at both the seedling and adult plant stages and found it to be susceptible to stem rust pathotype MCCF (B. Steffenson, unpublished data). These results support our conclusion that the GTT indel is associated with a nonfunctional Rpg1 gene, at least in terms of P. graminis f. sp. tritici resistance to pathotypes MCCF and HKHJ.

Another interesting group of Rpg1 alleles are those represented by an apparent unequal recombination between the Rpg1 gene and a closely linked paralog designated ABC1037 (1). This recombination was observed only in the Swiss landraces and not in any of the wild barley lines tested. The gene is expressed at the mRNA level (Fig. 4). Moreover, an RPG1 antibody positive protein is produced and is active in autophosphorylation (Fig. 6). The function, if any, of this recombinant allele is unknown, but we speculate that it may involve recognition of a putative ArrRpg1 effector of the pathogen. Recombination among homologous disease resistance genes has been observed and can result in the creation of novel resistance phenotypes (14,17).

The two wild barley accessions WDBC019 and WDBC160 with apparent 3′ and 5′, respectively, incomplete Rpg1 genes were susceptible to both stem rust pathotypes as expected (Fig. 1 and Table 1). Both genes, however, are expressed at the mRNA level (Fig. 4) and produce an RPG1 antibody positive protein that undergoes autophosphorylation (Fig. 6). The genomic nucleotide sequence of the Rpg1 gene in these lines is apparently similar enough to the Morex Rpg1 gene in the interior regions to produce mRNA and protein with Rpg1 properties and an intact kinase domain 2 that is active in autophosphorylation. The failure to amplify Rpg1 fragment 7 from WDBC019 and fragments 1 and 2 from WDBC160 suggest that those regions of the Rpg1 gene may be deleted, harbor a large insertion, or are highly diverged. A 19 nucleotide insertion and 11 nucleotide deletion in intron 12, plus a 17 nucleotide deletion at the 3′ end and 25 nucleotide deletion at the 5′ end of exon 13 of the WDBC019 allele, indicated high divergence in this region of the gene. However, these changes apparently are far enough downstream of the kinase 2 domain and do not affect the protein’s autophosphorylation activity (Fig. 6).

The reason for the extensive divergence of the sequence at the 3′ end while the rest of the Rpg1 gene primary structure is mostly conserved is unknown.

The origin of the widely used and durable Rpg1 gene bred into North American barley cultivars remains a mystery since we have yet to discover a wild barley accession containing an intact and functional Rpg1 gene. Although the number of wild barley accessions examined by complete sequencing to date is small, they nevertheless represent some of the most stem rust resistant germplasm found to date in extensive evaluations of H. vulgare sp. spontaneum germplasm (8,20). In wild barley, we repeatedly found an Rpg1 gene with a very similar primary gene structure, but containing a GTT insertion that apparently renders the gene nonfunctional in providing stem rust resistance. Is this the progenitor resistance gene that later developed specificity for the stem rust pathotypes MCCF/HKHJ by deletion of the GTT triplet in the cultivated species? A similar, but inverted situation has been described with the Pto disease resistance gene (10). In that case, the wild tomato relative L. pinninellifolium contributed the functional resistance gene, but the domesticated L. esculentum has maintained highly related and expressed pto and fen alleles. These alleles encode predicted proteins of 87% and 98% identity with the PTO and FEN kinases and are active in vitro autophosphorylation, but not functional in disease resistance. The answer to the question of the Rpg1 origin will require a more extensive search of wild barley germplasm for a potential Rpg1 donor.

Eight of the 16 Hordeum accessions analyzed in this study did not have an Rpg1-like gene recognizable by multiple PCR primers or Southern blot analysis, yet five expressed some resistance to pathotype MCCF (Fig. 1 and Table 1). These results clearly indicate the presence of a gene(s) different from Rpg1 conferring resistance to stem rust pathotype MCCF. The observed resistance could be due to the rpg4/Rpg5 complex as there is some evidence that one or both of these genes confers resistance to pathotype
MCCF in addition to other pathotypes (21)(B. Steffenson, unpublished data). Additional studies are underway to elucidate the genetics of stem rust resistance in these accessions.

In summary, Rpg1 alleles with a primary structure different from the one found in cultivar Morex, but apparently functional in conferring stem rust resistance to pathotypes MCCF and HKHJ were not discovered in this study. Moreover, we failed to find a progenitor Rpg1 gene among the wild barley accessions examined. A common potential progenitor gene has a GTT indel that apparently results in failure to provide resistance to pathotype MCCF. Further, examples of an apparent unequal recombination were discovered, but only in the domesticated Hordeum group (i.e., Swiss landraces) suggesting that this may be a relatively recent event. We have previously shown that RPG1 autophosphorylation is essential, but alone is not sufficient for conferring stem rust resistance (13). The observation that all the lines examined in this study, except WBDC040, were active in autophosphorylation reinforces the previous data that it is not sufficient for resistance. Thus, RPG1 plays a complex role in the stem rust disease resistance-signaling pathway.

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LITERATURE CITED

CHAPTER FOUR

The stem rust resistance gene *Rpg5* encodes a protein with nucleotide-binding-site, leucine-rich, and protein kinase domains.

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The stem rust resistance gene Rpg5 encodes a protein with nucleotide-binding-site, leucine-rich, and protein kinase domains

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We isolated the barley stem rust resistance genes Rpg5 and rpg4 by map-based cloning. These genes are colocalized on a 70-kb genomic region that was delimited by recombination. The Rpg5 gene consists of an unusual structure encoding three typical plant disease resistance protein domains: nucleotide-binding-site, leucine-rich repeat, and serine threonine protein kinase. The predicted Rpg5 protein has two putative transmembrane sites possibly involved in membrane binding. The gene is expressed at low levels. Posttranscriptional gene silencing using VIGS resulted in a compatible reaction with a normally incompatible stem rust pathogen. Allele sequencing also validated the candidate Rpg5 gene. Allele and recombinant sequencing suggested that the probable rpg4 gene encoded an actin depolymerizing factor-like protein. Involvement of actin depolymerizing factor genes in nonhost resistance has been documented, but discovery of their role in gene-for-gene interaction would be novel and needs to be further substantiated.

*actin depolymerizing factor | barley | disease resistance domains | map-based cloning

Stem rust caused by the fungus Puccinia graminis (Pg) was historically one of the most significant foliar diseases of barley and wheat, with genetic resistance being the primary means of control. Durable resistance in barley has been achieved against many pathotypes for the past 60+ years by the widespread use of the single resistance gene, Rpg1 (1). Barley can be attacked by Pg f. sp. tritici (Pgtr), the wheat stem rust pathogen and Pg f. sp. secalis (Pgs), the rye stem rust pathogen. In cultivated barley, five genes are known to confer resistance to Pgt and three to Pgs (2, 3). Only Rpg1 has been cloned and characterized (4, 5). A virulent Pg pathotype, designated OCC, was isolated from Midwestern barley cvs. containing Rpg1 in 1989 (6). A resistance gene identified in barley line Q21861 was designated Rpg4. It acts in recessive manner to pathotype OCC and is temperature-sensitive (3, 7, 8). Besides rpg4, Q21861 carries Rpg5 providing resistance to Pgs isolate 92-MN-90 (3, 8). The Rpg5 gene, previously RpgQ, is dominant or semidominant in action and was reported to cosegregate with rpg4, although three exceptions were found in 769 F2 progeny (9). The rpg4 gene was mapped to the long arm of barley chromosome 7(5H) (10). More detailed mapping and identification of syntenic rice chromosome regions (11, 12) allowed the development of a physical map covering the presumed rpg4 gene region (13).

Advances in molecular techniques and tools have facilitated the cloning of numerous plant disease resistance genes (R genes) in the past two decades. R genes are grouped into different classes according to their protein domain structure (14). The largest group consists of the NBS-LRR family of R genes, which is characterized by an N-terminal nucleotide-binding site (NBS) and C-terminal leucine rich repeats (LRRs). Another class, with relatively few members, is the extracellular LRR and transmembrane (TM) domain containing genes conferring resistance to the fungus Cladosporium fulvum, the leaf mold pathogen of tomato. The rice genes Xa21 (15) and Xa26 (16) conferring resistance to the bacterial blight pathogen Xanthomonas oryzae are the only examples of receptor-like kinase genes consisting of an extracellular LRR, a TM, and a cytoplasmic serine/threonine protein kinase (S/TPK). Last, there is a class of R genes that consists of S/TPK domains. S/TPK R genes include the previously described barley Rpg1 gene (4); the tomato Pto gene, which confers resistance to the bacterial pathogen Pseudomonas syringae pv. tomato (17); and the Arabidopsis PBS1 gene, which confers resistance to the bacterial pathogen Pseudomonas syringae pv. phaseolica (18).

The S/TPK group of R genes is unique in that two of the members, Pto and PBS1, have been shown to require an NBS-LRR gene, Pgf and RPS5, respectively, for resistance (19, 20). This demonstrates that NBS-LRR and protein kinases sometimes work together to provide resistance to plant pathogenic organisms.

Here, we report the cloning and preliminary characterization of two unique barley stem rust resistance genes Rpg5 and rpg4. Rpg5 encodes an R gene protein containing the NBS, LRR, and S/TPK domains in a single transcript. We validated the candidate Rpg5 gene by multiple allele sequencing and VIGS (21). Available recombinants point to the rpg4 gene encoding an actin depolymerizing factor (Adf). Ads play an important role…


The authors declare no conflict of interest.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. EU883792 (Rpg5 DNA from barley line Q21861); EU878778 (genomic sequence from barley line Q21861); EU878778 (genomic sequence from barley line Morex); EUB81932-EUB81935 (HvAdf2 genomic sequence from barley lines Golden Promise, Harrington, MD2, and Stetpole, respectively); EUB85581-EUB8583 (HvAdf1 genomic sequence from barley lines Harrington, MD2, and Stetpole, respectively); EUB83787-EUB83780 (HvAdf2 genomic sequence from barley lines Golden Promise, Harrington, MD2, and Stetpole, respectively); and EUB83791 (HvAdf3 genomic sequence from barley line Stetpole)].

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in cytoskeleton organization and have been reported to be involved with nonhost disease resistance (22). Their role in gene-for-gene interaction would be novel and needs to be further substantiated.

Results

High-Resolution Genetic and Physical Mapping. High-resolution genetic mapping identified the Rpg5/pag4 locus as between ARD5112 and ARD5016. This region was delimited by 10 cross-over events, in the HvAdf2 region and 3 between ARD5112 and HvAdf3 (Fig. 1 A and D). The recombinants around the HvAdf2 region separated pag4 (resistance to Pst pathotype QCC) from Rpg5 (resistance to Pst isolate 92-MN-90) (Fig. 1D).

Physical mapping identified BAC clones 543P19 and 116G10 spanning the ARD5112 to ARD5016 region (Fig. 1B). Genetic and physical mapping confirmed that these BAC clones included the flanking markers and the Rpg5/pag4 locus.

Identification and Analysis of Candidate Rpg5 and pag4 Genes. Sequence analysis of the cv. Morex BAC clones 543P19 and 116G10 identified a 70-kb region flanked by the RFLP markers ARD5005 and ARD5112. This region was annotated, and five candidate genes were identified, two encoding predicted NBS-LRR-S/TPK genes. HvPP2C gene could not be amplified with Morex-specific PCR primers, suggesting a diverged gene or an indel event. The Q21861 noncolinearity region was amplified using PCR chromosome walking (23) and sequenced revealing a S/TPK domain associated with the Rpg5 candidate gene HvRGA2 (Fig. 2). The S/TPK domain was absent from susceptible cvs. Morex and Steptoe. They have a cysteine-to-adenine conversion that introduces a stop codon resulting in a predicted truncated protein. In addition, they are missing the kinase domain coding region. Group 3 (susceptible) contains Golden Promise and MQ27. They have a single cysteine insertion causing a frame-shift mutation that results in a stop codon and a predicted truncated protein. Group 4 (susceptible) consists of Harrington. Harrington is missing the kinase domain coding region and the second transmembrane domain.
completed the noncolinearity region except for a small retrotransposon block, estimated at ~2 kb by restriction mapping, which we could not assemble because of its repetitive nature. The S/TPK domain was the only additional gene found in the Q21861 indel region.

An approximately 18-kb region between HvRGA1 and Rpg5 contained a large retrotransposon block in Morex. We were not able to assemble Q21861 contiguous sequence from this region. The partial sequences obtained were all repetitive and/or retrotransposon-like, with no evidence of additional genes. The 57.3-kb total Q21861 sequence identified the same candidate genes as in the susceptible Morex sequence, except that the HvPP2C gene was replaced by a protein kinase domain.

Comparison of Morex and Q21861 sequences suggested that HvrRGA2 was the Rpg5 gene (nucleotide and amino acid numbers given are from the Q21861 Rpg5 sequence, starting with the first nucleotide in the transcription start codon and the first amino acid). The Morex HvrRGA2 consisted of typical NBS-LRR domains and a PP2C gene a short distance downstream (Fig. 1C). The Q21861 HvrRGA2 gene consisted of NBS-LRR-S/TPK domains (Figs. 1C and 2). Furthermore, the susceptible cv. Morex genomic and cDNA sequence revealed a nucleotide substitution at position +483, resulting in a stop codon at amino acid position 161 and a predicted truncated protein (Fig. 2B, Group 2). This correlation of an apparently nonfunctional protein and a susceptible phenotype suggested that HvrRGA2 was the Rpg5 gene. Sequence analysis of the HvrRGA2 alleles from the susceptible parents showed Steptoe to be similar to Morex, encoding a predicted truncated protein. MD2 and Golden Promise contained a single-nucleotide insertion (C337) causing a frame-shift mutation at amino acid position 114 resulting in a stop codon at aa position 217 (Fig. 2B, Group 3). The susceptible cv. Harrington HvrRGA2 allele contained an amino acid sequence very similar to Q21861, but it was lacking the PK domain (Fig. 2B).

The remaining candidate genes were eliminated based on allele sequencing. The candidate gene HvRGA1 codes for an 895-aa (98.5-kDa) predicted NBS-LRR protein with highest homology to an Oryza sativa hypothetical NBS-LRR protein (GenBank accession no. EAY80410). Sequence analysis of HvRGA1 alleles revealed that the resistant parent Q21861 and the susceptible parent MD2 had identical HvRGA1 predicted amino acid sequences. The susceptible parent Harrington differed from Q21861 by only a single amino acid (C324R). The susceptible cvs. Morex and Steptoe were identical at the amino acid level, and differed from Q21861 by five amino acids (S290A, K340N, A445G, N474D, and F586L). The single amino acid difference between Q21861 and Harrington occurred within the NBS domain but was not within one of the highly conserved subdomains. Two of the amino acid differences between Q21861 and Morex/Steptoe occurred within the NBS domain and three occurred within the predicted LRR region. The amino acid sequence identity between Q21861 and MD2 in addition to the very limited polymorphism between Q21861 and Harrington suggested that HvRGA1 was not Rpg5.

The HvAdf3 gene codes for a protein with the highest amino acid homology to a Lophopyrum elongatum actin depolymerizing factor-like gene (GenBank accession no. AAG28460). Allele sequencing from the mapping population parents (Steptoe, MD2, and Q21861) and cv. Morex showed that HvAdf3 had no polymorphism at the amino acid level, suggesting that it is a highly conserved protein and not the Rpg5 gene.

The HvPP2C gene sequence from cv. Morex codes for a protein with the highest homology to an expressed O. sativa protein phosphatase 2C family protein (GenBank accession no. ABF99721). Specific HvPP2C primers (from Morex) failed to amplify Q21861, MD2 or cv. Golden Promise (susceptible), suggesting that either the HvPP2C gene was not present or was highly diverged. Southern blot analysis confirmed that HvPP2C was absent from Q21861 genome (data not shown). Sequence analysis of the λ clone RSB762 confirmed the absence of HvPP2C gene from this region of the resistant line Q21861.

Genetic mapping identified seven recombination events between the markers ARD5016 and HvRGA1, a physical region of 12 kb containing HvAdf2 and HvRGA1 (Fig. 1D). The sites of recombination were identified to within ~200-bp intervals by sequencing and SNP analysis. Recombinants with the susceptible cv. HvAdf2 allele were resistant to the stem rust isolate 92-MN-90, eliminating HvAdf2 from consideration as the Rpg5 gene (Fig. 1D). However, the six recombinants differentiating rpg4 (Pst pathotype OCC resistance) from Rpg5 (Pst isolate 92-MN-90 resistance) and an additional recombinant occurring distal of HvAdf2 (HQ1) identified HvAdf2 as the probable rpg4 gene.

**HvAdf2** codes for a 147 aa (16.2 kDa) actin-depolymerizing factor-like protein with highest homology to the *O. sativa* actin-depolymerizing factor 4 expressed gene (GenBank accession no. ABF99587.1). Sequence analysis of the HvAdf2 alleles revealed that the resistant parent Q21861 and susceptible parent Steptoe and cv. Morex differed by three amino acids (Q39H, A445I, and S135G). However, the pathotype QCC susceptible parent Harrington had an HvAdf2 gene identical to the Q21861 allele at the amino acid level.

**Structure and Expression of the HvRGA2 (Rpg5) Gene.** RT-PCR analysis of HvRGA2 showed it was expressed as an mRNA level in all parents tested, indicating that transcription did not correlate with resistance. However, PCR primers designed to amplify the junction between the LRR domain and the S/TPK domain produced an RT-PCR product only from Q21861, MD2, and Golden Promise (supporting information (SI) Table S1). Although MD2 and Golden Promise contain the intact NBS-LRR-S/TPK transcript, both MD2 and Golden Promise alleles were shown to contain a frame-shift mutation within the N-terminal region of the gene resulting in a stop codon at amino acid position 217 and a putative truncated protein (Fig. 2B, Group 3). To obtain HvRGA2 (Rpg5) transcription start site (TSS) primers were designed from the genomic sequence in 100 bp increments. They were used in RT-PCRs to delimit the TSS to within 100 bp at position ~408 to ~346 bp.

HvRGA2 (Rpg5) encodes an apparently functional NBS-LRR-S/TPK gene containing seven exons transcribed into a predicted 4.4-kb mRNA coding for a 1,378-aa (151.6-kDa) predicted protein (Fig. 2). The transcript size was confirmed by Northern blot analysis, which showed a single hybridizing band at ~4.8 kb (Fig. 3A).

The S/TPK domain contains all nine conserved amino acids (24), suggesting a functional kinase. The kinase domain had the highest homology to an *O. sativa* putative S/TPK (GenBank accession no. EAZ08788) but also showed significant similarity to the known R gene *Pto* (36% amino acid identity and 53% amino acid similarity). The Rpg5 S/TPK domain was very homologous to the unknown function *Rpg1* gene family members ABC1042 and ABC1063 with 60% and 61% amino acid identity, respectively, and 76% amino acid similarity (25).

The NBS-LRR region contained the 4 NBS conserved subdomains and 12 imperfect LR repeats. This region had the highest homology to an *O. sativa* hypothetical protein (GenBank accession no. EAY98635). The Rpg5 NBS-LRR is very similar to the rice gene *Pt-tu* (40% amino acid identity and 54% amino acid similarity) conferring resistance to the rice blast fungus (26).

The TMPRED program (www.ch.embnet.org/software/ TMPRED.form.html) predicted two transmembrane domains, one on the C-terminal side of the NBS domain and the other on the C-terminal side of the LRR domain (Fig. 2B, Group 1).
Virus-Induced Gene Silencing of the Rpg5 Gene. The BSMV vector (21) was used to posttranscriptionally silence the Rpg5 gene with its antisense RNA. Approximately 30% (average of three independent experiments) of the Q21861 BSMV-asRpg5 treated seedlings showed a conversion from incompatible reaction to compatible, whereas all of the control BSMV-MCS-treated plants remained resistant to isolate 92-MN-90 (Fig. 3B). The BSMV-MCS control demonstrated that the BSMV-VIGS vector itself did not induce susceptibility to fungal infection.

Quantitative analysis (qRT-PCR) of the Rpg5 mRNA was carried out on samples taken at the time of fungal infection (designated TP0) and at 14 d after fungal infection (designated TP14). At TP0, the BSMV-asRpg5 construct (Fig. 4, bar 4, 1) showed significant mRNA reduction with reference to the BSMV uninoculated control (Fig. 4, bar 3) and BSMV-MCS inoculated control (Fig. 4, bar 2). At TP14, the uninoculated uninfected control (Fig. 4, bar 4) showed an Rpg5 mRNA level comparable to the TP0 control (Fig. 4, bar 3), but the TP14 BSMV uninoculated control (Fig. 4, bar 5) showed much higher mRNA levels which are comparable to the BSMV-MCS inoculated control (Fig. 4, bar 8) and the BSMV-asRpg5 inoculated plants that did not show resistance phenotype (Fig. 4, bar 7). Only the BSMV-asRpg5 inoculated seedlings showing a susceptible phenotype (Fig. 4, bar 6) had highly reduced Rpg5 mRNA levels compared with the uninoculated and MCS inoculated seedlings (Fig. 4).

The data suggested that the Rpg5 mRNA may be induced by infection with isolate 92-MN-90. A time-course experiment showed that there is a strong induction of the Rpg5 mRNA at day 1 (12.7%, relative to a GAPDH control) and a second smaller peak at days 8 (3.5%) through 11 (3.2%). This response, however, was also observed in mock and uninoculated controls and therefore is not fungus specific (data not shown). We conclude that the Rpg5 mRNA levels fluctuate because of environmental or developmental reasons, but the exact cause and pattern remain to be investigated. However, the Rpg5 mRNA silencing by the BSMV-asRpg5 VIGS inoculation must be made with a control sample taken at the same time point under identical environmental conditions.

Discussion

High-resolution mapping identified a 70-kb region on chromosome 7(SH) encompassing the Rpg5 and rp44 genes. Within this locus, we identified five candidate genes. Multiple allele sequencing and recombinant characterization eliminated four genes, leaving only the HvRGA2 as the candidate Rpg5 gene (Fig. 1). Allele sequencing and VIGS confirmed the candidate Rpg5 gene. This is the first report of an R-gene encoding a disease-resistance protein composed of NBS-LRR-S/TPK domains. A search of the Oryza and Arabidopsis genomes did not identify any genes with this novel three-domain structure. The protein also contains two predicted transmembrane domains, suggesting a potential membrane-bound protein. The location of the transmembrane domains, if functional, suggests that the RPG5 LRR domain may reside outside the cell and act as the pathogen receptor, whereas the NBS and PK domains are intracellular and propagate the disease resistance signaling.

An NBS-LRR domain is present in a majority of plant disease resistance proteins identified to date (14). Another class of resistance proteins is represented by STPKs such as Pto, PBS1, and RPM1 (14, 18). The tomato Pto and the Arabidopsis PBS1 STPKs require function of the NBS-LRR genes PRF and RPS5, respectively, for disease resistance (18, 19). The Rpg5 gene, identified here, is the only reported disease resistance gene where all three domains are present in a single protein. The S/TPK of the Rpg5 gene is clearly required for disease resistance, as indicated by the susceptible cv. Harrington allele that appears to have an intact and expressed NBS-LRR protein but lacks the protein kinase domain. The presence of all three domains in a single protein may facilitate studying their interactions.

The Pto and Prf proteins have been reported to act coincidentally with one another for detecting and eliciting a disease-resistance response to the pathogen (27). Given this close interaction between the PK and NBS-LRR proteins, it is not surprising that a gene has been found that combines the two domains in one protein.

The Rpg5/rp44 locus also confers resistance to pathotype QCC. The HvAdf2 gene was identified as the probable rp44 gene based on analysis of the genetic recombinants. Interestingly, whereas we found recombinants that expressed resistance to isolate 92-MN-90 but susceptibility to pathotype QCC, the reciprocal recombinants were not observed. In general, resistance to pathotype QCC corresponded with resistance to isolate 92-MN-90 in studies of a large number of domesticated and wild
barleys (b.s., unpublished data). The observation that the QCC-susceptible cv. Harrington has an expressed ADF gene identical to the resistant Q21861 allele at the amino acid level also suggested that additional factors may be involved. One hypothesis is that the recessive rpg4 gene functions as a pathotype OCC-specific disease-resistance gene only in the presence of another functional disease resistance gene, perhaps Rpg5. ADFs are critical in remodeling the actin cytoskeleton during normal plant development and under biotic and abiotic stress. They are typically small proteins that function in rapid recycling of actin monomers (28). Actin functions in cytoskeleton organization, which coordinates essentially all aspects of plant growth (29). Actin microfilament polymerization has been shown to be involved in nonhost disease resistance. For example, using cytochalasin E, an inhibitor of actin microfilament polymerization and Arabidopsis defense-related mutants eds1, pad4, and nab4, it was shown that nonhost resistance to the wheat powdery mildew fungus Blumeria graminis f. sp. tritici in Arabidopsis largely depends on actin cytoskeleton dynamics and function of the EDS1 gene (30). Similar observations have been documented in other systems.

The posttranscriptional gene silencing of Rpg5 by VIGS showed significant reduction of Rpg5 transcript at both sampling time points 0 d (seedlings pooled) and 14 d (susceptible and resistant plants sampled separately). Approximately 30% of the BSMV-asRpg5 products resulted in a susceptible reaction to the rust fungus. At the 14-d time point, the level of silencing observed corresponded to the phenotype observed with the susceptible plants showing significant reduction in the Rpg5 mRNA, whereas the resistant plants had mRNA level similar to the controls (Fig. 4). The time-course experiments indicated that Rpg5 mRNA levels were variable over time. However, induction by fungal infection could not be established because of similar observations in mock and uninoculated controls. The variable Rpg5 mRNA levels were surprising, because previous experiments with Rpg1 showed low but steady mRNA levels with or without fungal infection (31, 32).

In summary, we have identified two types of plant disease-resistance genes. The Rpg5 gene combines features of NBS-LRR type disease-resistance gene with the S/TPK domain, suggesting that this gene may function both in pathogen perception and signal transduction. The candidate gene for rpg4 needs further verification, but, if confirmed, it would show involvement of actin cytoskeleton in race-specific disease resistance.

Materials and Methods

Plant Materials. Progeny from the crosses Steptoe/Q21861, Harrington/ Q21861, and MD2/Q21861 were used for genetic mapping. Q21861 is the source of the stem rust-resistance genes Rpg5 and rpg4 (3, 9). Steptoe and Harrington are barley cultivars, and MD2 is a genetic stock with multiple dominant mutations. All three are susceptible to stem rust. Plants were grown in the greenhouse with daylight temperatures of 18°-21°C, respectively. Metal halide lights supplemented a 16-/8-h light/dark photoperiod.

Molecular Markers. RFLP markers were generated as described (13) or by designing PCR primers based on the cv. Morex or line Q21861 sequence (this study). Primers are described in Table S1.

Genetic and Physical Mapping. High-resolution mapping at the Rpg5/Strp4 locus used 50 recombinants selected from 5,232 gametes between the flanking markers Aqa5 and ABG391. These 50 recombinants were reduced to 10 between the flanking markers AR56016 and AR05112. BAC physical maps were generated as described (13).

Sequencing and Sequence Analysis. The cv. Morex BAC clones (543P19 and 116G10) forming a contig across the Rpg5/Strp4 region were sequenced at the Institute for Genomics Research (TIGR). All other cultivar DNA was sequenced with the BigDye terminator system on an ABI 373 DNA sequencer (Applied Biosystems) at the Laboratory for Biotechnology and Bioanalysis, Washington State University, Pullman. The A clone RS8762 was subcloned into the NotI site of pBluescript (Stratagene) and sequenced by using the EZ::TNTM (Stratagene) and sequenced by using the EZ::TNTM (Stratagene) and sequenced by using the EZ::TNTM (Stratagene) and sequenced by using the EZ::TNTM (Stratagene) and sequenced by using the EZ::TNTM (Stratagene) and sequenced by using the EZ::TNTM (Stratagene) and sequenced by using the EZ::TNTM (Stratagene) and sequenced by using the EZ::TNTM (Stratagene). All PCR-generated fragments were either directly sequenced or cloned into pGEM-T Easy vector (Promega) and sequenced by using the EZ::TNTM (Stratagene). For direct sequencing, gel slices were placed in plugged tips (Rainin Instrument) and frozen at −20°C. After complete thawing, the tip was centrifuged at 4,000 × g for 10 min. The eluate was extracted two times with 24:1 chloroform/isooamy alcohol and precipitated with 0.3 M NaOAc and 2.5 volumes 95% EtOH. Primers and clones are described in Table S1.

Contigs were assembled by using Vector NTI Advance 9.0 contig express (Invitrogen). The cultivar sequence comparisons were done by using Vector NTI alignX and the National Center for Biotechnology Information blast2seq function (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). All sequences were analyzed with the BLASTX and BLASTN algorithms.

Library Construction. High-molecular-mass Q21861 genomic DNA was isolated by using a modified CTAB method (33). Genomic DNA (~20 μg) was partially digested with 0.1 units of Sau3A II for 1 h before hybridization of a majority of 15- to 23-kb fragments. DNA was dephosphorylated with 0.1 units of calf intestine alkaline phosphatase for 30 min and separated in 1.2% low melting agarose. DNA fragments 9–23 kb were excised from the gel and recovered by β-agarse treatment. Genomic fragments were ligated into the Lambda Dash II vector (Stratagene) predigested with BamHI and packaged using the Gigapack III XL packaging extract (Stratagene).

RNA Isolation and Northern Blot. Isolation of RNA, Northern blot analysis, and hybridization were as described (25).

RT-PCR. Approximately 1 μg of total RNA was used for RT-PCRs by using M-MLV Reverse Transcriptase (Promega) under the manufacturer’s recommended conditions. RT-PCR fragments were directly sequenced after elution from agarose gels as described for direct sequencing.

3′ and 5′ RACE. 3′ RACE was carried out by using the 3′ RACE System (Invitrogen) following the manufacturer’s recommended conditions. 5′ RACE was carried out by using the FirstChoice RLM-RACE Kit (Ambion) following the manufacturer’s recommended conditions and SMART technology (34). The gene specific primers used for RACE are described in Table S1.

VIGS and qRT-PCR. Barley plants for VIGS experiments were grown in the growth chamber in plastic pots with a day/night temperature of 20°C/−1°C and 18°C/−1°C, respectively. A 16-/8-h light/dark photoperiod was provided by cool fluorescent tubes (525 μE/m2s).

Rpg5 was silenced by using VIGS as described (21). A 314-bp Rpg5 cDNA fragment (~2,033 to 2,346 bp) was generated by PCR and ligated in antisense (as) orientation into BSMV-PDS4 infectious clone (pBSMV-PDS4as) digested with PacI and NotI (21). The BSMV-asRpg5 construct was cotranscribed with the α and β genomes of the tripartite BSMV virus by using the mMessage mMACHINE T7 kit (Ambion), and the T7 promoter. RNA was inoculated onto Q21861 barley plants at the two-leaf stage. The seedlings were inoculated with isolate 92-MN-90 urediniospores at 0.025 mg per plant mixed with a talc carrier (~11–12 days after virus infection. After inoculation, the plants were misted and placed in the dark under high humidity conditions for 22 h, then exposed to light and misted periodically. After 4 h, the misting was stopped, and the leaves were left to dry slowly. When the leaf surfaces were completely dry, plants were moved to the growth chambers at 20°C and 80% relative humidity. The plants were scored for compatibility or incompatibility at 14 days postfungal infection.

The negative control contained a 121-bp antisense fragment of the multi cloning site (MCS) from pBluescript KS (Stratagene). The MCS sequence did not hybridize to barley genomic DNA at low stringency conditions, indicating no homologous regions in the barley genome. Q21861 and Steptoe were used as the resistant and susceptible virus uninoculated controls. qRT-PCR was performed on Rotor-Gene 2000 thermocycler (Corbett Research) using the Quantitect SYBR green PCR system (Qiagen). The Rpg5 primers used are described in Table S1 and GAPDH primers were described (35). Tissue samples for qRT-PCR were collected at 0 or 14 d after fungal infection. Tissue samples for the time course experiment were collected at 0, 1, 3, 5, 7, 8, 11, and 14 days after fungal inoculation.

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CHAPTER FIVE

The \textit{rpg4/Rpg5} stem rust resistance locus in barley; resistance genes and cytoskeleton dynamics

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The \textit{rpg4}/\textit{Rpg5} stem rust resistance locus in barley

Resistance genes and cytoskeleton dynamics

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Two closely linked resistance genes, \textit{rpg4} and \textit{Rpg5}, conferring resistance to several races of \textit{Puccinia graminis}, were cloned and characterized. The \textit{Rpg5} gene confers resistance to an isolate of \textit{Puccinia graminis} f. sp. \textit{secalis} (\textit{Pgt}), while \textit{rpg4} confers resistance to \textit{Puccinia graminis} f. sp. \textit{tritici} (\textit{Pgt}). \textit{Rpg5} is a novel gene containing nucleotide binding site-leucine rich repeat domains in combination with a serine threonine protein kinase domain. High-resolution mapping plus allele and recombinant sequencing identified the \textit{rpg4} gene, which encodes an actin depolymerizing factor-like protein (ADF2). Resistance against the \textit{Pgt} races QCCJ, MCCJ, TTKSK (aka Ug99) and RCRS requires both \textit{Rpg5} and \textit{rpg4}, while \textit{Rpg5} alone confers resistance to \textit{Pgt} isolate 92-MN-90. The dependency on the actin modifying protein ADF2 indicates cytoskeleton reorganization or redirection plays a role in pathogen-host interactions. \textit{Rpg5} may interact with ADF2 to activate or deactivate its function in the resistance response. Alternatively, \textit{Rpg5} could initiate signal transduction leading to resistance in response to detecting ADF2 protein modification. \textit{Pgt} may redirect the actin cytoskeleton by inducing modifications of ADF2. The redirection of actin could possibly enable the pathogen to develop a haustoria-plant cell cytoskeleton interface for acquisition of nutrients.

The co-evolution of plants and pathogenic fungi has led to the development of an innate defense system in the former that activates a series of responses to resist colonization by latter. Activation of defense mechanisms begins with a receptor recognizing the threat of a potential invader. Resisting introgression at the cell periphery is the first line of defense and is dependant upon a dynamic reorganization of the challenged cell's cytoskeleton. If the initial defense strategy fails to stop penetration, a second line of defense is triggered by race specific resistance genes, resulting in a hypersensitive response (HR) and localized cell death.1,2

We recently reported the cloning and characterization of two closely linked stem rust resistance genes \textit{rpg4} and \textit{Rpg5}.3 The \textit{Rpg5} gene is predicted to encode a protein with novel structure containing three \textit{R}-gene domains: a nucleotide binding site (NBS), leucine rich repeat (LRR), and serine threonine protein kinase (STPK). The \textit{Rpg5} predicted protein structure suggests a typical \textit{R}-gene function in recognition of the pathogen. \textit{Rpg5} confers resistance to an isolate of rye stem rust independent of \textit{rpg4}, but resistance to several races of wheat stem rust requires both genes. The \textit{rpg4} gene is predicted to encode an actin depolymerizing factor-like (ADF) protein. The dependency on the actin modifying protein ADF2 indicates cytoskeleton reorganization or redirection plays a role in the pathogen-host interaction. \textit{Rpg5} could potentially interact with \textit{rpg4} to activate or deactivate its function in the resistance response. Alternatively, \textit{Rpg5} may initiate signal transduction leading to resistance in response to detecting \textit{Rpg4} (ADF2) protein modification. The pathogen may modify ADF2, via effector molecules, to interfere with the resistance reaction or redirect the actin cytoskeleton. The redirection of actin could possibly enable the pathogen to develop a haustoria-plant cell cytoskeleton interface for acquisition of nutrients.

Stem Rust Resistance Genes in Barley

Wheat stem rust, caused by the pathogen \textit{Puccinia graminis} f. sp. \textit{tritici} (\textit{Pgt}), was a devastating disease of wheat and barley prior to the 1950’s.4 Additionally, the rye stem rust pathogen, \textit{P.} \textit{secalis} (\textit{Pgs}) also can attack and cause losses in barley. In the US and Canada, durable resistance in barley was achieved through the deployment of varieties with the single resistance gene \textit{Rpg1}, while in wheat it was achieved by pyramiding several stem rust resistance genes in one cultivar. Since the deployment of this genetic resistance in barley and wheat in the early 1940s and mid-1950s, respectively, losses due to stem rust have been minimal, and widespread stem rust epidemics were considered by many to be a thing of the past until recent appearance of \textit{Pgt} race TTKSK (aka isolate Ug99) in Uganda in 1999.5

The barley \textit{Rpg1} gene is remarkably durable and confers resistance to most races of \textit{Pgt}; however, some races of \textit{Pgt} and some isolates of \textit{Pgt} are virulent on barley cultivars containing \textit{Rpg1}. A
The R-genes cloned to date have been grouped into different classes according to their protein domain structure. The major class of R-genes possess an N-terminal NBS and a C-terminal LRR and are designated NBS-LRR R-genes. In relatively few cases, NBS-LRR genes have been shown to specifically interact with pathogen avirulence (Avr) gene products. The guard hypothesis is an alternative to direct R-gene/Avr-gene interaction. In the guard hypothesis, the R-genes act as surveillance proteins detecting modification of host target proteins by pathogen effectors. The direct or indirect interaction between the host R-gene protein and pathogen AVR protein is believed to activate the signaling cascades resulting in HR and disease resistance. These signaling cascades may involve phosphorylation via the second major class of R-genes, the protein kinases.

Six R-genes containing at least one STPK domain make up the second major R-gene class. The STPK R-genes confer resistance to bacterial and fungal pathogens and have been cloned from a wide taxa of plant species. The STPK domain suggests that these proteins function in plant defense signal transduction pathways involving phosphorylation. However, STPK domain-containing genes are also known to interact directly with Avr genes, demonstrating a capacity to recognize non-self proteins through protein-protein interactions.

Tomato Pto-mediated resistance against Pseudomonas syringae is one of the most extensively studied and understood mechanisms of disease resistance. Interestingly, Pto requires the presence of a second gene Pfr (an NBS-LRR gene) for resistance. Functional analysis of the Pto/Pfr system indicated that specific mutations in the activation loop of the Pto kinase induced spontaneous symptoms similar to HR observed when plants expressing Pto are infected with AvrPto containing strains of P. syringae. The spontaneous HR response was AvrPto independent, but Pfr dependent suggesting that Pfr acts downstream or coincident with Pto. It was later demonstrated that Pto and Pfr do act coincidently in the signaling pathway, and that the relationship is dependant on a physical interaction between the two proteins. In this resistance complex, Pto acts as a regulatory subunit of Prf, and Prf contributes to the pathogen recognition specificity of Pto.

Resistance against P. syringae strains that carry AvrPphB also requires two Arabidopsis genes, RPS5, a NBS-LRR gene, and PBS1, a serine threonine protein kinase gene. These systems indicate that resistance protein complexes require both an NBS-LRR and STPK protein for pathogen recognition and resistance.

The Rpg5 mechanism of pathogen recognition and signaling pathway may parallel Pto. The Pto-mediated resistance requires both NBS-LRR and STPK genes for resistance, and the Rpg5 predicted protein structure contains all three NBS, LRR and STPK domains with significant amino acid homology to both Pto and Prf (kinase homology to Pto is 36% aa identity and 53% aa similarity and the NBS-LRR homology to PRF is 26% aa identity and 42% aa similarity). The Rpg5 protein structure suggests that the three domains act coincident with one another in barley, similar to tomato, and are required for Rpg5 mediated resistance to Pgt isolate 92-MN-90. The Rpg5 protein with all three domains in a single protein may facilitate the identification of other components
of the resistance complex or the pathogen AVR protein by the yeast two-hybrid assay that otherwise may be missed when using an STPK or NBS-LRR gene as bait separately. It is plausible that very few identified AVR proteins interact with their corresponding NBS-LRR R-genes due to incomplete interaction because the resistance complex requires both STPK and NBS-LRR proteins for complete interaction. Many of the NBS-LRR R-genes identified may have an STPK counterpart that has not been identified due to lack of polymorphism.

**The rpg4 Gene Encodes an Actin Depolymerizing Factor-Like Protein**

Genetic mapping and allele analysis using two barley populations containing HvAdf2 polymorphisms (Steptoe x Q21861 and MD2 x Q21861) identified recombinants that delimited the rpg4 region to 1kbp. This 1kbp region contains only the HvAdf2 gene, indicating that it is the rpg4 gene. However, the barley variety Harrington and line Sm89010, which are susceptible to *Pgt* QCCJ, contain an rpg4 allele identical at the amino acid level to the rpg4 allele in Q21861, which is resistant to *Pgt* QCCJ. This suggested that HvAdf2 was not the rpg4 gene. Further analysis of allele sequences of rpg4 and Rpg5 together with SNP analysis of the recombinants suggested that a functional Rpg5 may be required in addition to rpg4 for resistance to *Pgt* QCCJ. The *Pgt* QCCJ susceptible barleys Harrington and Sm89010 with rpg4 alleles identical to the resistant Q21861 rpg4 allele, do not contain a functional Rpg5 allele. The Harrington x Q21861 recombinant #18 combines the Harrington rpg4 allele with the Q21861 Rpg5 gene, and it is resistant to *Pgt* QCCJ, indicating that cultivar Harrington is susceptible to this race due to a lack of a functional Rpg5 (Brueggeman R, et al. unpublished). These data suggest that *Pgt* QCCJ resistance should behave as a single dominant gene in populations where only the Rpg5 gene is segregating in the presence of a non-polymorphic rpg4 gene. This hypothesis is being tested in the Harrington x Q21861 and Sm89010 x Q21861 F1 and F2 populations and by down-regulating the two alternative rpg4 alleles (Brueggeman R, et al. unpublished).

Phenotype analysis of three populations (Morex, Steptoe and Robust x Q21861) revealed 1:3 segregation for resistance: susceptibility, demonstrating the recessive nature of rpg4. The rpg4 and Rpg5 genes are tightly linked (~40 kbp physically) adding to the complexity of the two-gene requirement for resistance. The recessive behavior of rpg4 implies that it encodes a non-functional ADF2 protein, resulting in resistance or that the functional Rpg4 gene encoding a functional ADF2 protein acts as a susceptibility factor in the reaction with *Pgt* race QCCJ. The three QCCJ susceptible barley cultivars (Morex, Steptoe and Robust) have identical Adf2 alleles at the amino acid level and differ from the resistant Q21861 allele by only three amino acids (Q39H, A101T, S135G). Three Pgt QCCJ susceptible progeny from the Steptoe x Q21861 population have recombination within the Adf2 gene and contain a functional Rpg5 allele. These recombinant Adf2 genes contain Q39 (Q21861-like) with T101 and G135 (Steptoe-like), showing that the amino acids at positions 101 and/or 135 are important for reaction to *Pgt* QCCJ. Since both positions involve amino acids that could be phosphorylated, i.e., either T added or S replaced, it suggests that phosphorylation may be involved in regulating ADF2 function. All Adf2 alleles tested were expressed at the transcription level, indicating that these two amino acid polymorphisms determine susceptibility/resistance to *Pgt* QCCJ.

**The Role of Actin Depolymerizing Factors in Resistance**

During pathogen attack by both fungi and bacteria, the plant cytoskeleton dynamically rearranges. ADF proteins, in concert with other actin binding proteins, regulate actin filament dynamics. ADFs are believed to function by increasing the turnover of filamentous F-actin by depolymerization of monomeric G-actin from the pointed ends of the filaments. In addition, ADFs may also nucleate the assembly of new actin filaments. Many interactions between the host and pathogen may be dependent on ADF proteins, including the cytoskeletal polarization in response to non-host pathogens. Cytoskeleton polarization in response to pathogen challenge is well documented; however, very little is known concerning the signaling processes that mediate this response. Studies with the barley *mlo* non-race specific resistance system conflicting resistance to *Blumeria graminis* f. sp. *hordei* using actin cytoskeleton pharmacological inhibitors and genetic interference by overexpressing a barley actin depolymerizing factor gene (HvAdf3), demonstrated that actin cytoskeleton function was required for basal defense against an appropriate mildew pathogen and for *mlo*-mediated non-race specific resistance at the cell wall. However, actin function was not required for several race specific immune responses. It was also demonstrated that actin cytoskeletal disruption led to increased fungal penetration by non-host pathogens.

Chemical or genetic disruption of actin polymerization results in greater efficiency of non-host pathogen penetration; however, in tobacco cytoskeleton perturbation can also prime the cell for HR. It was proposed that the host detects disruption of the actin cytoskeleton similar to the guard hypothesis, thus triggering the HR response. Evidence is mounting that the actin cytoskeleton is the target of plant pathogen effectors to suppress non-race specific resistance, which may signal to induce a second line of defense mediated by the pathogen specific R-genes. There is also evidence supporting non-host pathogens that evade the first line of defense and enter the cell are met by an HR mediated resistance that could be due to several simultaneously acting R-genes.

ADF proteins possibly play an important role in response to pathogen challenge. It is plausible that ADF proteins are potential effector targets of fungi to circumvent a defense response. Previous studies have shown that cytoskeleton disruption was only compromised in race non-specific and non-host resistance. This begs the question: is rpg4-mediated resistance a form of race non-specific resistance? Our data indicate that rpg4 functions to confer resistance to a number of different *Pgt* races, but not to all. Therefore it appears to have race specificity, but only in the presence of the typical R-gene Rpg5. In the absence of Rpg5, the rpg4 gene may provide some non-host or non-race specific resistance to various pathogens.
**Rpg5 and rpg4 Interact to Confer Stem Rust Resistance**

In all known *Pgt* QCCR resistant barley varieties and recombinants, it appears that the full resistance function of *rpg4* requires the presence of a functional *Rpg5* gene. We hypothesize that *Rpg5* is the R-gene in this system that detects the *Pgt* and *Pgg* pathogens. *Rpg5* mediated resistance to *Pgg* isolate 92-MN-90 is independent of *rpg4*. This leads to the speculation that *Rpg5* may operate in two distinct resistance pathways, one that is similar to most race-specific R-gene mediated resistance mechanisms and is independent of actin reorganization events mediated by *rpg4*. The second pathway may be more like a non-race specific type of resistance mechanism dependant on *rpg4*-mediated actin reorganization. Like the *Pto/Prf* system, *Rpg5* may be capable of recognizing more than one *Puccinia graminis* derived AVR protein, leading to differential reactions and activation of different resistance pathways.

It is plausible that *Rpg5* interacts with *rpg4* directly or indirectly to activate or deactivate the actin depolymerizing factor through phosphorylation. As discussed earlier, the resistant and susceptible alleles differ by two amino acids that are both potential phosphorylation sites. It has also been shown that ADFs can be deactivated or activated by phosphorylation or dephosphorylation, respectively, via protein kinases and phosphatases. This activation or deactivation may lead to an incompatible or compatible reaction with pathogens. Where the actin reorganization occurs and how R-gene mediated resistance is dependant on this reorganization still needs to be elucidated.

An attractive hypothesis is that the invading fungus actually captures the function of the cytoskeleton to feed itself. In this case, a non-functional *adf* gene that the fungus targets may prevent the fungus from becoming established within the plant. Miklis and co-workers have suggested that although actin disruption initially enhanced pathogen entry into the cell, it may have a long-term negative effect on the compatible interaction. It has been shown that in the compatible interaction between barley and powdery mildew, the haustoria (fungal feeding organs) are associated with actin filament rings, and actin filaments closely follow haustoria when invaginating the plasma membrane. This is similar to what occurs in the symbiotic interaction between a mycorrhizal fungus and tobacco root cells, suggesting similarity between how pathogenic and symbiotic fungi may establish the fungus-host interface for nutrient acquisition. The host cytoskeleton rearrangement machinery, initially utilized for resistance, may be redirected by the pathogen to establish a feeding mechanism that is actin filament-dependent. It is possible that functional *Adf2* enables the fungus to produce or maintain this feeding structure, thus facilitating fungus growth or that non-functional or inappropriately functional *adf2* disrupts the actin filaments such that the fungus cannot maintain the feeding structure resulting in incompatibility.

A complete model for the function of *rpg4* and *Rpg5* in *Pgt* resistance must take into account the observations that these two genes work together and that the *rpg4* gene function is recessive and temperature sensitive. It is, of course, possible that each gene functions independently, but weakly and both are required for a strong resistance phenotype. It should be noted that both *Rpg4* alleles are expressed at the mRNA level, although we do not yet know if this is true at the protein level. The differential interaction between *Rpg4* or *rpg4* encoded proteins with *Rpg5* could be modulated by the fungus or *Rpg5* could modulate their interaction with the fungus. In order to satisfy the requirement that the *rpg4* gene action is recessive, the presence of *Rpg4* encoded ADF2 molecules must lead to susceptibility, while *rpg4* encoded ADF2 must have a neutral or negative function.

It is well documented in animal pathogenic bacteria that pathogens produce proteins that disrupt actin cytoskeleton function in order to become adapted to the host and thus become pathogenic. There is little evidence of plant pathogenic fungi producing effector molecules that disrupt actin dynamics; however, many genera of fungi produce cytochalasins the major chemical used for pharmacological inhibition of actin filament function. There is precedence of a bacterial plant pathogen secreting the effector, AvrPto, into the host cytoplasm, which disrupts the cytoskeleton polarization response. AvrPto protein has been shown to suppress callose deposition at the site of *Pseudomonas syringae* challenge, a cytoskeleton dependant response, leading to loss of basal resistance in Arabidopsis. *Rpg5* could also be the guard protein monitoring the ADF2 protein for any modifications by the fungus effector; however, this would not explain why *rpg4* is recessive. *Rpg5* should still be able to guard ADF2 even when the *rpg4* gene is present in a heterozygous state.

The *rpg4/Rpg5* resistance system is the first link between an R-gene that putatively recognizes a race specific pathogen and an actin binding protein, which may be a link between the cytoskeleton dynamics occurring post pathogen interaction.

**The Next Step**

The molecular basis of disease resistance appears very complex, and although progress has been made in identifying many components there are still key questions unanswered. A complete signal transduction pathway leading to fungal pathogen recognition has yet to be identified and little is understood about how resistance genes activate defense signaling. It is apparent from the R-gene literature that different resistance pathways are activated by pathogen recognition, and there is probably cross talk and common components involved among them. We are lacking answers to some of the key questions regarding the *rpg4/Rpg5*-mediated mechanism of resistance that may help answer questions regarding receptor activation and dynamic cytoskeleton rearrangement. Our future research will focus on addressing some of the questions regarding how and at what level *rpg4* and *Rpg5* interact to confer resistance to stem rust.

The *rpg4/Rpg5* locus may answer some questions about disease resistance signaling. The novel gene structure of *Rpg5* may provide answers to why NBS-LRR and STPK proteins interact and are required components of diverse R-gene receptor complexes. The interaction between *rpg4* and *Rpg5*, whether direct or indirect, may also provide answers to how pathogen recognition signals the rapid rearrangement of the actin cytoskeleton and what role this rearrangement plays in the haustoria-plant cell interface. It may also be the first system where we have a pathogen effector target
that a fungus acts upon in order to perturb cytoskeleton dependent resistance.

References

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SUMMARY AND FUTURE DIRECTION

The focus of my doctoral research was to clone and characterize the genes underlying the rpg4/Rpg5 stem rust resistance locus. This locus is very important because it is the best source of resistance conferring resistance to races virulent on Rpg1, the only source of resistance deployed in Midwestern barley cultivars. We cloned the Rpg1, rpg4 and Rpg5 genes by a map-based approach and performed functional analysis of the RPG1 protein, gene family characterization and elucidation of the resistance pathways. Rpg1 and Rpg5 both have novel R-gene structures containing at least one STPK domain with considerable homology to the Pto kinase. Therefore what is known about both the Pto and Rpg1 mediated mechanisms will be used to drive the functional analysis of the rpg4/Rpg5 mediated resistance mechanisms. The cytoskeleton rearrangement aspect of the rpg4/Rpg5 mechanism appears to have far reaching scientific importance beyond the average race specific resistance responses.

Functional analysis of the rpg4 and Rpg5 genes is in its infancy but with our Rpg1 research leading the way many techniques and tools needed are already in hand and standardized. Although the rpg4/Rpg5 resistance mechanism is probably somewhat different than Rpg1 it is currently the best model for gaining insight into the Rpg5 mechanism. We have already discovered pieces of information about the rpg4/Rpg5 mechanism that will help in the efforts to elucidate how these genes interact to confer resistance. We have interpreted rpg4 as a disease resistance gene but recent data suggests that this designation may not fit. After sequencing and analyzing the alleles from resistant and susceptible cultivars a more valid interpretation maybe that rpg4 is a susceptibility factor present within some of the susceptible cultivars. Analyzing the sequence between the different rpg4 and Rpg5 alleles along with the interpretation of the recessive heritability and the populations used to determine this have shed
some light on the possible function of rpg4 and may explain why rpg4 inheritance is recessive. Future research will be focused on how and at what level rpg4 and Rpg5 interact. I believe the answers to these questions will provide great insight into how biotrophic fungal pathogens maintain their life style within the plant host and will have great utility in future disease management strategies.

Stem rust has reemerged as an important disease that has the potential to cause severe barley and wheat yield losses and possibly could threaten world food security. In order to keep the stem rust disease under control in an effective, sustainable and environmentally friendly manner resistance genes must be deployed responsibly. Barley production should not depend on the deployment of a limited number and sources of resistance genes as is present situation with Rpg1 in the USA and Canada. Several sources of resistance should be pyramided such that the genes will operate in an effective durable manner. This depends on the identification and utilization of new sources of resistance. It also makes the understanding of the mechanisms underlying these resistance genes important so this basic scientific knowledge can be applied in the field to deploy R-genes for maximum effectiveness and durability.