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ORIGINAL PAPER

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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Abstract The barley (*Hordeum vulgare* L.) stem rust (*Puccinia graminis* f. sp. *tritici)* resistance gene *Rpg1* encodes a serine/threonine protein kinase with two tandem kinase domains. The *Rpg1* gene family was identified from the cv. Morex and consists of five additional members with divergent homology to *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 *Rpg1*, ABC1037, is located on chromosome 1(7H) bin 01, very near (~50 kb) but not co-segregating with *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 *rpg4* and *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 *Rpg1* allele that appears to be the result of unequal recombination between *Rpg1* and ABC1037 was characterized. No known resistance loci cosegregated with any family members, however characterization of the *Rpg1* family has provided insight into the evolution of this novel gene family and may present tools for understanding the functional domains of *Rpg1*. The genetic mapping, gene structures, and analysis of amino-acid sequences of the *Rpg1* gene family members are presented.

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Introduction

The barley stem rust resistance gene *Rpg1* is a dominant gene that confers resistance to most, but not all, pathotypes of the stem rust fungus *Puccinia graminis* f. sp. *tritici*. *Rpg1* was cloned by a map-based approach from the resistant cv. Morex (Brueggeman et al. 2002). A genomic fragment containing the complete *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 *Rpg1* gene (Horvath et al. 2003). In silico translation of the *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 *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 *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) (Cabrillac 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, fenthion (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 ^{32}P dCTP (New England Nuclear) using the RTS RadPrime DNA labeling system (Gibco BRL) according to manufacturers instructions.

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 *Aci*I 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 *Hind*III, Southern-blotted and hybridized with the *Rpg1* gene family cDNA probes. Representative BAC clones for each gene were detected, digested with *Hind*III, *Eag*I, *Sac*I and *Xba*I and shotgun-cloned into the plasmid pBluescript II KS

(Stratagene) by standard techniques (Sambrook et al. 1989). Plasmid clones containing the *Rpg1* family members were confirmed by Southern blot analysis and sequenced using the BigDye terminator system on an ABI Prizm 377 DNA sequencer (PE Biosystems) at the Bioanalytical Center, Washington State University, PullmanWA. The larger subclones, >3 kb, were sequenced using the EZ:TN <KAN-2> Transposon Insertion Kit (Epicenter) and in some cases primers were designed and the BAC clone DNA and large insert subclone DNA was sequenced directly. Primers were designed across restriction enzyme junctions to confirm that the sequence across these junctions was properly assembled. For a complete list of plasmids used for sequencing see Electronic Supplementary Material (S2).

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 ug of total leaf RNA was used to isolate polyA mRNA using the oligotex 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 cm^2 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 *Dpn*I (New England BioLabs). The second round of amplification contained 2.5 μl of *Dpn*I 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 Triticeae 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 (BI956515) 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

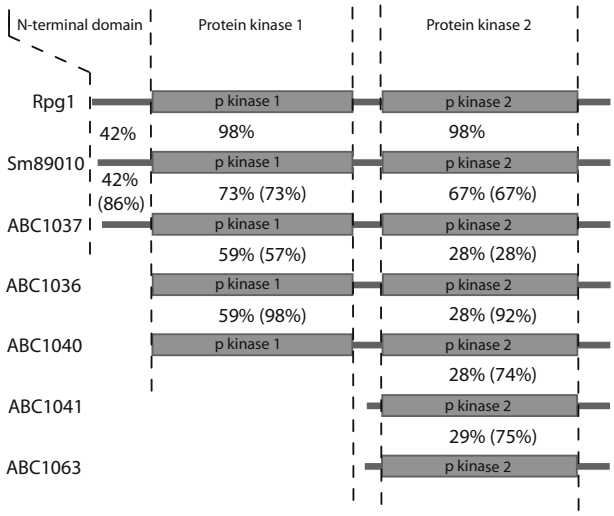

Fig. 1 Diagram of the predicted Rpg1 protein domains and alignment with family members. The cv. Morex Rpg1 protein predicted functional domains were aligned with the five Rpg1 family members. The Sm89010 line is included to help visualize the unequal recombination that appears to have taken place between *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*

the different two tandem kinase domain genes might have arisen by different evolutionary pathways.

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

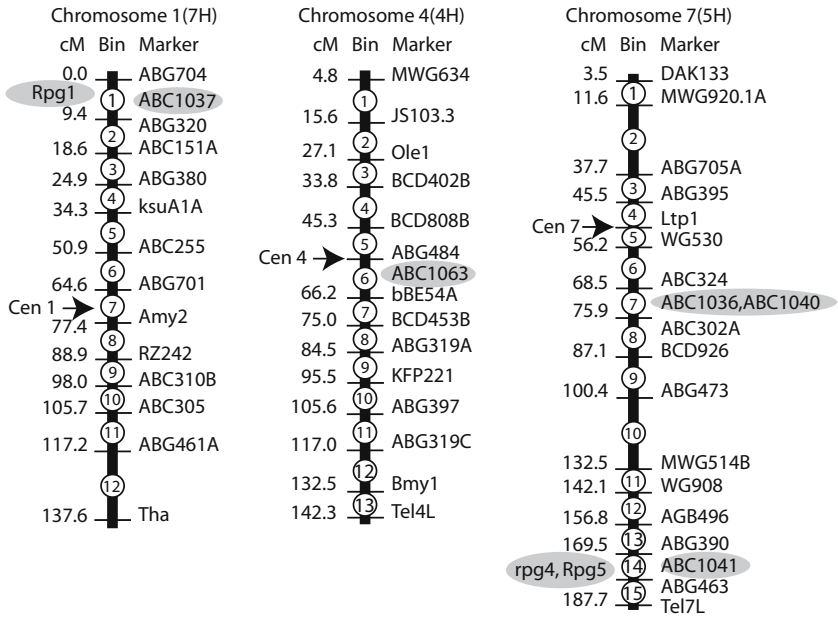

Fig. 2 Genetic map of the *Rpg1* gene family members. The barley Steptoe × Morex minimapper population was used to locate the genes on the genetic linkage BIN map. The position of the *Rpg1* family members, *Rpg1*, *rpg4* and *Rpg5* are *highlighted*. Distances between bin markers (on the *right*) are shown in cM (on the *left*). The BIN numbers are in the *circles*. BIN locations and markers are described in Kleinhofs and Graner (2001)

clones were selected for all five family members (S3) and comparison with genomic southerns 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).

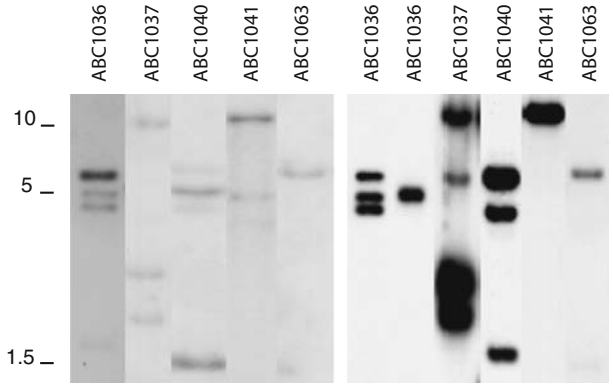

Fig. 3 Southern blots of genomic and BAC DNA hybridized with the *Rpg1* gene family member cDNA probes. **a** Genomic DNA was digested with HindIII, separated on gels and hybridized with cDNA probes from the family members indicated. **b** BAC DNA from clones used for sequencing hybridized with the cDNA probe as above. ABC1036 is represented by two BAC clones because the entire genomic equivalent of the gene was not found on a single BAC clone. Some of the faint bands are due to cross hybridization between family members. The approximate size in kilobases is shown to the *left*

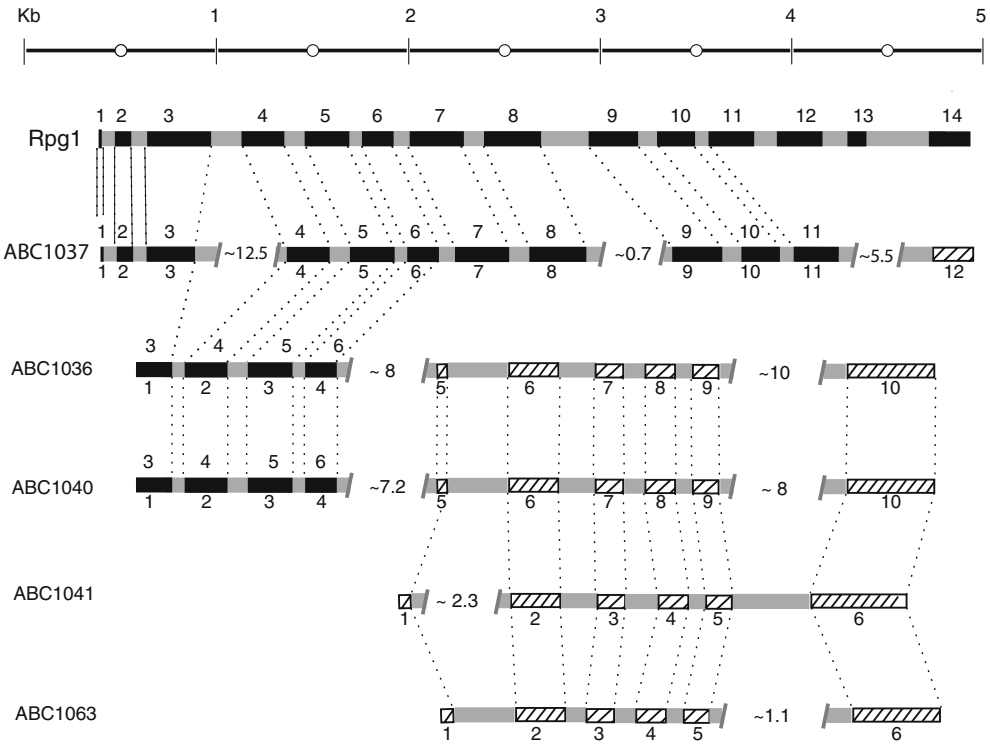

Fig. 4 *Rpg1* gene family member exon/intron structures. The introns (*gray*) and exons (*black* or *striped*) are shown to scale. Exons with sequence conservation as compared to *Rpg1* are in *black* and are *numbered* above the drawing according to the *Rpg1* gene exons. Exons with low homology to *Rpg1* are *striped*. The exon numbering specific for each gene is shown below the gene. *Small dashed lines* joining intron/exon junctions indicates conservation of these exon splice sites. Large introns are indicated by *interrupted back slashes* and approximate sizes are shown in kilobases

Table 1 *Rpg1* gene family cDNA and genomic plasmids and GenBank accession numbers

Rpg1 family member	cDNA clone used for mapping	cDNA Genbank accession number	BACclones used for sucloning	gDNA Genbank accession number
ABC1037	HVSMEn0003o03	DQ469714	290m12	DQ351213
ABC1036	HVSMEg0085c17	DQ469715	666a11701k24	DQ469712
ABC1040	HVSMEi0028b15	DQ469716	623E8	DQ469713
ABC1041	HVSMEb0017j15	DQ469717	572b18	DQ352178
ABC1063	HVSMEa0001a21	DQ469718	435B24	DQ386610

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.

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).

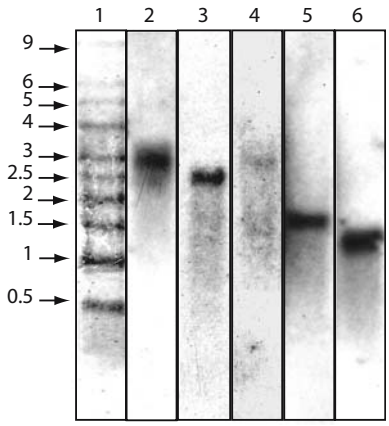

Fig. 5 Northern blot of the *Rpg1* gene family members. *Lane 1* is Millenium Marker (Ambion) stained with methylene blue. *Lanes 2–6* are cv. Morex mRNA hybridized with *Rpg1* family member cDNA probes (*lane 2* is ABC1036, *lane 3* is ABC1037, *lane 4* is ABC1040, *lane 5* is ABC1041 and *lane 6* is ABC1063). Marker band sizes are in kilobases to the *left*

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 "thalos 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 polyprotein 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

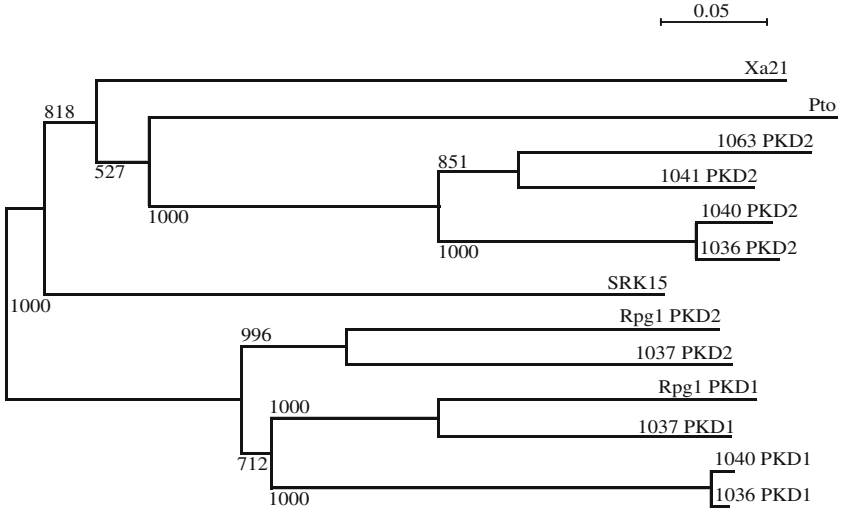

Fig. 6 Phylogenetic tree of the barley *Rpg1* gene family member protein kinase domains (PKDs) based on predicted amino acid sequence and related protein kinases. Rpg1 (acc. # AAM81972), Pto (acc. # A49332), Xa21 (acc. # 220341A) and SRK 15 (acc. # CAB41879). The dendogram was created using ClustalX program by neighbor-joining method (Saitou and Nei 1987) and was bootstrapped 1,000 times. The *number values* represent the best branch location based on the 1,000 matrices

a 372 a.a. predicted protein. These genes lack the *Rpg1* N-terminal domain and PKD1 (Fig. 1). The ABC1041 and ABC1063 homology to *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 *Rpg1* family member PKDs suggested that the ABC1037 and *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 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.

Oryza sativa protein kinase homologs

A tandem kinase gene (ABA92216.1) with homology to *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 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 Rpg1 gene family.

An example of unequal recombination

Sequence analysis of the *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 *Rpg1* indicating that an unequal recombination event between *Rpg1* and ABC1037 probably gave rise to the Sm89010 allele. The predicted unequal recombination event occurred within the third intron of *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 *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 co-segregate, 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 resistance to 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 *Rp1* 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 retrotranspon 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 PKDI. 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 Dusanter-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* Hop^{T42} mutant. This residue occurs in the JH2domain and is conserved among all JAK family members. The Hop^{T42} 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 Dusanter-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 exibit 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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