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ORIGINAL PAPER

Rpr1, a gene required for *Rpg1*-dependent resistance to stem rust in barley

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Abstract *Rpg1* is a stem rust resistance gene that has protected barley from severe losses for over 60 years in the US and Canada. It confers resistance to many, but not all, pathotypes of the stem rust fungus *Puccinia graminis* f. sp. *tritici*. A fast neutron induced deletion mutant, showing susceptibility to stem rust pathotype Pgt-MCC, was identified in barley cv. Morex, which carries *Rpg1*. Genetic and *Rpg1* mRNA and protein expression level analyses showed that the mutation was a suppressor of *Rpg1* and was designated *Rpr1* (Required for *P. graminis* resistance). Genome-wide expression profiling, using the Affymetrix Barley1 GeneChip containing ~22,840 probe sets, was conducted with Morex and the *rpr1* mutant. Of the genes represented on the Barley1 microarray, 20 were up-regulated and 33 were down-regulated by greater than twofold in the mutant, while the *Rpg1* mRNA level remained constant. Among the highly down-regulated genes (greater than fourfold), genomic PCR, RT-PCR and Southern analyses identified that three genes (Contig4901_s_at, HU03D17U_s_at, and Contig 7061_s_at), were deleted in the *rpr1* mutant. These three genes mapped to chromosome 4(4H) bin 5 and co-segregated with the *rpr1*-mediated susceptible phenotype. The loss of resistance was presumed to be due to a mutation in one or more of these genes. However, the possibility exists that there are other genes within the deletions, which are not represented on the Barley1 GeneChip. The *Rpr1* gene was not required for *Rpg5*- and *rpg4*-mediated stem rust resistance, indicating that it shows specificity to the *Rpg1*-mediated resistance pathway.

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Introduction

Mutants with increased disease susceptibility are useful for understanding plant disease resistance gene function. Stem rust, caused by *Puccinia graminis* f. sp. *tritici*, can cause severe epidemics in wheat and barley. The disease has been effectively controlled in barley by a single dominant gene *Rpg1* first released with in cv. Kindred in 1942. *Rpg1* confers resistance to many, but not all, pathotypes of the stem rust fungus *P. graminis* f. sp. *tritici* (Steffenson 1992; Sun and Steffenson 2005). The remarkable durability of this gene makes it particularly interesting to study its mode of action. A better understanding of the mode of action of *Rpg1* might lead to the engineering of improved disease resistance and durability in crop plants. Towards this end, we

have cloned *Rpg1* (Brueggeman et al. 2002) and initiated its characterization (Horvath et al. 2003; Rostoks et al. 2004; Nirmala et al. 2006).

Genes required for function of qualitative disease resistance (*R*) genes have been previously isolated from barley and other species. Torp and Jorgensen (1986) and Jorgensen (1988) reported 25 barley mutants with increased susceptibility to powdery mildew. Three mutants representing two genes were identified as suppressors and designated *Rar1* and *Rar2* (Required for *Mla* resistance; initially designated *Nar1* and *Nar2*, but changed to avoid confusion with the previously named nitrate reductase deficient mutant genes in barley). It has been demonstrated that *Rar1* is required for resistance conferred by some, but not all, powdery mildew resistance genes (Jorgensen 1996). The *Rar1* gene is not required for *Rpg1* function (B. Steffenson unpublished data). The *Rar1* gene was mapped to chromosome 2(2H) bin 11 and has been cloned. It encodes a 25.5 kDa protein consisting of two tandem CHORDs (cysteine- and histidine-rich domains), which bind Zn^{2+} ions (Shirasu et al. 1999). The *Rar1* gene and mutants have been isolated from *Arabidopsis* and shown to be required for some, but not all, *R* gene function (Muskett et al. 2002; Tornero et al. 2002). The susceptibility of *rar2* mutant was later found to be caused by single amino acid substitution in MLA12 (Shen et al. 2003). Two genes required for *mlo*-mediated resistance to powdery mildew (*Ror1* and *Ror2*) have been identified (Freialdenhoven et al. 1996). *Ror1* was mapped to chromosome 5(1H) bin 7 (Collins et al. 2001), and *Ror2* was mapped to chromosome 7(5H) bin 11. *Ror2* has been cloned and encodes a syntaxin protein required for non-race specific penetration of the cell wall, but not for race specific resistance to powdery mildew (Collins et al. 2003).

Other mutations that suppress resistance genes include *eds1* (enhanced disease susceptibility) and *ndr1* (non-race-specific disease resistance). All compromise resistance to pathogens. The *Arabidopsis* EDS1 and PAD4 lipase-like proteins interact with each other and mediate the downstream signaling of Toll Interleukin1 Receptor (TIR)-type, but not coiled coil (CC)-type of Nucleotide Binding Site–Leucine Rich Repeat (NBS–LRR) resistance genes (Feys et al. 2001). The presumed membrane-bound protein encoded by the *NDR1* gene is required for many, but not all, CC-type genes (Century et al. 1995; Aarts et al. 1998). The CC-type resistance genes *RPP8* and *RPP13* appear to function independently of EDS1 and NDR1, indicating other resistance signaling pathways also exist (McDowell et al. 2000; Bittner-Eddy and Beynon 2001).

Here we report the isolation of a fast-neutron induced *rpr1* mutant from barley that suppresses the function of *Rpg1*, rendering plants susceptible to stem rust. We also present the mapping and initial characterization of a gene, *Rpr1* (Required for *P. graminis* resistance), involved in the *Rpg1*-mediated pathway for disease resistance.

Materials and methods

Plant material, irradiation treatment, and identification of mutants

The barley cv. Morex was selected for use in this study because it is known to carry *Rpg1* and is a widely used model cultivar in barley genetics (Kleinhofs et al. 1993; Yu et al. 2000). It was also the source plant of the cloned *Rpg1* gene (Brueggeman et al. 2002). Seeds of cv. Morex were provided by Patrick Hayes at Oregon State University (Corvallis, OR, USA) and was the original source used to develop the Steptoe/Morex mapping population (Kleinhofs et al. 1993). Twenty seedlings of Morex were inoculated in the greenhouse with Pgt-MCC, a stem rust pathotype that differentiates barley plants with and without *Rpg1* (Steffenson et al. 1993). From this inoculation test, a highly resistant plant was selected for seed increase in an isolated greenhouse where no other barley plants were present. This selected Morex seed source (~3 kg) was irradiated with fast neutrons (FN) (3.5 or 4.0 Gy using protocol 563) at the FAO/IAEA Seibersdof SNIF facility near Vienna, Austria. Morex M_1 seed was increased in an isolated fallow field in Langdon, ND in 1993, and ~6,000 spikes were collected from individual plants at harvest. The remaining seed was harvested as M_2 bulk seed. In the greenhouse, 20–50 M_2 seeds from individual spikes were planted in cones filled with a peat moss:perlite (3:1 v/v) potting mix (#1 Sunshine Mix, Fisons, Vancouver, Canada). Plants were inoculated with pathotype Pgt-MCC when the first leaves were fully expanded and assessed for their infection type 12–14 days later using the protocols of Steffenson et al. (1993). When segregation for stem rust reaction was observed within an individual M_2 spike, all plants were kept as putative mutants and increased to the M_3 generation for retesting to pathotype Pgt-MCC.

Plant material and RNA preparation

The cv. Morex and the *rpr1* mutant induced from Morex were grown in a growth chamber maintained at 21°C (16 h light) and 16°C (8 h dark). The primary

leaves from 10-day-old uninoculated seedlings were pooled, frozen in liquid nitrogen, and stored at −80°C until RNA isolation was performed. Total RNA was isolated using the hot (60°C) phenol/guanidinium thiocyanate method. Trizol-like reagent contained 38% saturated phenol, pH 4.3 (Fisher Scientific, Pittsburg, PA, USA), 1 M guanidine thiocyanate (Fisher Scientific), 0.1 M sodium acetate, pH 5.0 and 5% glycerol (Fisher Scientific). RNA was further purified using the RNeasy Midi kit (Qiagen, Valencia, CA, USA).

Microarray analysis

Three independent biological replicates of Morex and *rpr1* mutant were subjected to microarray analysis. Target synthesis and GeneChip hybridization, washing, staining, and scanning were performed at the Molecular Biology Core at Washington State University. Microarray output was examined visually for excessive background noise and physical anomalies. The default MAS statistical values were used for all analyses. All probe sets on each array were scaled to a mean target signal intensity of 125, with the signal correlating to the amount of transcript in the sample. An absolute analysis using MAS was performed to assess the relative abundance of the 22,792 represented transcripts based on signal and detection (present, absent, or marginal). The resulting data from the absolute analysis were exported into Microsoft EXCEL and then imported into GeneSifter software (GeneSifter.net, Seattle, WA, USA). Transcripts expressed differentially at a statistically significant level were determined using the Welch *t*-test (variances not assumed equal) with a *P*-value cutoff of 0.05. This list of genes was further narrowed by selecting those showing twofold or greater suppression in the *rpr1* mutant.

PCR, RT-PCR, and quantitative real-time PCR

Total RNA samples generated for the microarray experiment as described above, were also used for RT-PCR after DNase I digestion (Ambion, Austin, TX, USA). Single-strand cDNA was synthesized using the Superscript First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) using oligo$(dT)_{12–18}$ as primer. PCR was performed subsequently using RedTaq DNA polymerase (Sigma, St. Louis, MO, USA) as described with primers listed in Table 1. PCR also was carried out with 20–50 ng genomic DNA using the same sets of primers. An *Rpg1* gene fragment was amplified from genomic DNA using primers 228F1 (5′-GCCGGGGCTGGACGATGAG GAATTC-3′) and 228R1 (5′-GAACTCGAATGCA AACTCCCTTGTC-3′) as a control. RT-PCR and Quantitative real-time PCR reactions for the *Rpg1* gene were carried out using the gene specific primers *Rpg1*_Ex3_cw2 (5′-GCCGGTGTACTATCCCTTTC-3′) and *Rpg1*_Ex4_ccw2 (5′-TGTCGGACCCTCATAA GATT-3′).

Southern analysis and genetic mapping

Plant genomic DNA was extracted as previously described (Kleinhofs et al. 1993). DNA probes were labeled with [α-^{32}P] dCTP (New England Nuclear) using the All-in-one Random Labeling System (Sigma) and hybridized to barley genomic DNA blots.

The Steptoe × Morex "minimapper" population consisting of 35 selected doubled-haploid lines (DHL), was used to map the molecular markers to the barley Bin map (Kleinhofs and Graner 2001). The *rpr1* phenotype mapping and co-segregation analysis with molecular markers was carried out in Q21861 × *rpr1*

Table 1 Primer sequences used in this study

Affymetrix probe set ID	Primer name	Sequence
Contig4901_s_at	4901F	5′-ATTTCACAAGGACGGATGGCAATTT-3′
	4901R	5′-AAGCACAGCTGATCGCAGCCGGAGA-3′
HU03D17u_s_at	17uF	5′-GTTATCAGCACGCTCACGCTGAGCG-3′
	17uR	5′-TGAGTCGCGGCCGTGTCTACCTCGT-3′
HVSMEm0005P05r2_at	05r2F	5′-CAGTCGGCTCCGGTGACGGCAGCCT-3′
	05r2R	5′-GCTGCCGCTCGTACTTAAAATAGAC-3′
Contig7061_s_at	7061F	5′-ATTATGATGAAGAGATATGCGGAGT-3′
	7061R	5′-AGGCCTACTAATCTTGGAGACCACC-3′
Contig14769_at	14769F	5′-AAATGGCAGACCTCCTCCTAAAGGT-3′
	14769R	5′-TGTGTAAGATGAAGAAAGCTAACTC-3′
Contig6699_s_at	6699F	5′-TCTTCTTCTTCATCAGTCCACACGA-3′
	6699R	5′-TCCAACTCCAAATAACATGGATCCA-3′
Contig13681_s_at	13681F	5′-GGGAAGTTGTTTGGCTTCGCTTCGA-3′
	13681R	5′-ACATGTAGTGCAAAGTGTTAATTAC-3′
Contig13680_s_at	13680F	5′-ATCGCTGGTATGAAGCTACAACTCA-3′
	13680R	5′-AAGTCATTGCCGTTTCTGTTCAAGA-3′

mutant in Morex background cross consisting of 108 F_2-derived F_3 families.

Quantification of Rpg1 protein

Uninoculated ten-day-old barley cv. Morex and *rpr1* mutant seedling leaf tissues were ground separately with a mortar and pestle in an extraction buffer containing 0.5 M sorbitol, 50 mM Tris–Cl (pH 7.5), 10 mM $MgCl_2$ and 1 mM DTT and Plant Protease Inhibitor Cocktail from Sigma. Rpg1 protein concentration was quantified using a specific peptide antibody (Nirmala et al. 2006) by a modified ELISA method (Li et al. 2001b) from a standard curve constructed from either the peptide or His-tag Rpg1 protein. The stem rust susceptible cv. Golden Promise was used to prepare the cross absorption antisera.

Data access

All detailed data and protocols from these experiments have been deposited in BarleyBase (http://www.barleybase.org/). Files are categorized under accession number BB8 and can be downloaded at the Download Center as batch files in MAGE-ML, CSV, CEL, DAT, or expression data formats, or as individual CEL, CHP, DAT, or EXP files under "browse experiments."

Results

Mutant induction, selection, and characterization

Barley seeds irradiated with 3.5 and 4.0 Gy at the FAO/IAEA Seiberdorf SNIF facility were grown in the field in Langdon, ND, USA in 1993. M_1 spikes were picked at random and remaining seed harvested as M_2 bulk seed. Chlorophyll-deficient seedling mutation rates were observed at a frequency of 2.2 and 2.6% for M_2 spikes at 3.5 and 4.0 Gy treatments, respectively. The observed mutation rates are similar to what we have previously observed with gamma irradiation, although much lower than the rates observed with the chemical mutagen sodium azide (Kleinhofs et al. 1978). Since we were primarily interested in deletion mutations, these rates were adequate to proceed with screening for stem rust susceptible mutants.

Of the ~2,900 M_2 spikes evaluated, only one bona fide mutant for stem rust susceptibility was identified after retesting with Pgt-MCC and diagnostic molecular markers for the Morex genotype background. The resistant plants from this mutant M_1 spike (designated $M \times M_2$ 97–100) exhibited infection types of 0;1 to 10; similar to Morex, whereas the susceptible plants exhibited infection types of 3 to 3+ similar to the susceptible control of Steptoe (Fig. 1). Resistant plants had very small uredinia that were surrounded by distinct necrosis, whereas susceptible plants had large uredinia without necrosis. Nineteen resistant and seven susceptible plants were observed in spike $M \times M_2$ 97–100. This approximated a 3:1 ratio ($\chi^2 = 0.05$, $P = 0.82$), suggesting the segregation of a single gene with resistance being dominant.

A susceptible plant, named *rpr1* mutant was selected from spike $M \times M_2$ 97–100. Then the *rpr1* mutant was crossed to the parent cv. Morex and susceptible cv. Steptoe. Segregation data showed that the mutation was not in *Rpg1* as indicated by the approximately 3:1 ratio of resistant: susceptible plants found in the cross with Morex and the presence of resistant lines in the cross with cv. Steptoe (data not shown). RFLP analysis with an *Rpg1* specific probe also showed that the DNA from the mutant had all the same bands as in Morex, ruling out any major deletions or DNA rearrangements (data not shown). These results suggested that the phenotype was due to a suppressor of *Rpg1*. This was confirmed by mapping. Since the functional gene is required for *Rpg1*-mediated resistance, it was designated *Rpr1* (Required for *P. graminis* resistance). In

Fig. 1 Barley seedling leaf disease reaction to stem rust pathotype MCC. *Left*: Morex (resistant); *Right*: *rpr1* mutant in cv. Morex genomic background (highly susceptible)

the field, the mutant does not have an obvious phenotype difference from its Morex source plant in the absence of the pathogen. Real-time PCR showed that the *Rpg1* gene is expressed at the same level in both Morex wild type plant and *rpr1* mutant (Fig. 2). Protein analysis confirmed that the *Rpg1* protein is present in *rpr1* mutant as in the wild type but at a somewhat lower level (Fig. 2).

To facilitate mapping, the mutant carrying *rpr1* in a Morex background was crossed with Q21861. The Q21861 line, which carries the *Rpg1* gene, was chosen to provide molecular marker polymorphism and it eliminated *Rpg1* polymorphism. Thus, only the *Rpr1* gene would be segregating. The number of homozygous resistant, heterozygous, and homozygous susceptible plants in F_2-derived F_3 families was 30, 59, and 18, respectively. This fits a 1:2:1 ratio ($\chi^2 = 3.8$ and $P = 0.15$). Molecular mapping placed the gene on chromosome 4(4H) bin 5 between the markers Adh4 and ABA003.

Effect on other stem rust resistance genes

To determine if the *Rpr1* gene is also required for *rpg4* and *Rpg5* mediated stem rust resistance, the *rpr1* mutant × Q21861 F_2-derived F_3 families were also evaluated for resistance to pathotype QCC (identifies *rpg4*) and isolate 92-MN-90 (identifies *Rpg5*). The Q21861 line carries the genes *rpg4* providing resistance to stem rust pathotype QCC and *Rpg5* providing resistance to stem rust isolate 92-MN-90, while Morex is susceptible to both pathotypes. The number of homozygous resistant, segregating, and homozygous susceptible $F_{2,3}$ families identified in response to pathotype QCC and isolate 92-MN-90 was 21:53:33 ($\chi^2 = 2.68$ and $P = 0.3$) and 27:48:28 ($\chi^2 = 0.5$ and $P = 0.85$), respectively. These data were consistent with the 1:2:1 ratio expected if *rpr1* is not required for *rpg4* and *Rpg5* gene function. A markedly different ratio of 1 (resistant): 8 (segregating): 7 (susceptible) ratio would be expected if either *rpg4* or *Rpg5* require an unlinked functional *Rpr1* gene. *Rpr1* is located on chromosome 4(4H), whereas *rpg4* and *Rpg5* are closely linked and located on chromosome 7(5H). Thus, the expected segregation ratio for unlinked genes is valid.

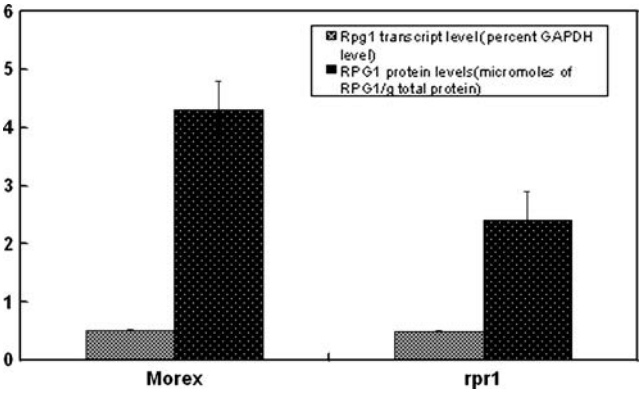

Fig. 2 *Rpg1* mRNA and Rpg1 protein levels in the wild-type Morex and the *rpr1* mutant demonstrate that the *Rpg1* gene is functional in the *rpr1* mutant. Transcript levels were measured by quantitative real-time PCR and protein levels were measured by ELISA using a specific peptide antibody as described in Materials and methods

Identification of candidate *Rpr1* genes by microarray analysis

Microarray analysis was used to identify genes whose expression at the mRNA level was eliminated in the *rpr1* mutant. The FN bombardment is known to induce deletion mutations (Li et al. 2001a). Since the *rpr1* mutant was induced with FN, we expected that it would be due to a deletion. Such deletions may be identified by microarray analysis, a method that examines transcript abundance, as they would abolish transcript production. We compared gene transcript levels in the wild-type cv. Morex and the *rpr1* mutant plant by using the Affymetrix Barley1 GeneChip that contains > 22,000 expressed genes (Close et al. 2004). Based on comparison of the wild-type versus *rpr1* mutant transcript abundance ratio, a value of > 2.00 identified 33 genes with reduced transcript levels and 20 genes with increased transcript levels in the *rpr1* mutant. However, in this study, we were primarily interested in deleted genes, which would be expected to be highly down-regulated. Therefore, we identified eight genes with a wild-type to mutant transcript abundance ratio level of > 4.00. These highly down-regulated candidate genes were further analyzed (Table 2).

Three deletions are identified in the *rpr1* mutant

The highly (ratio > 4.00) down-regulated genes were tested one by one using genomic PCR, RT-PCR and Southern blot hybridization to identify those that were deleted. All data were in agreement and the RT-PCR and genomic PCR data are shown in Fig. 3. These analyses identified two genes with unknown function (Contig4901_s_at and HU03D17u_s_at) and one gene with a putative receptor-like protein kinase function (Contig 7061_s_at) that were missing in the *rpr1* mutant. Lack of PCR amplification from *rpr1* mutant genomic DNA and RNA using the primer pairs 17uF/17uR, 4901F/4901R, and 7061F/7061R (Table 1) confirmed that the genes corresponding to Contig4901_s_at,

Table 2 Highly down-regulated genes identified in the stem rust susceptible *rpr1* mutant from microarray analysis using Barley1 GeneChip

Ratio	Affymetrix probe set ID	Deleted	Descriptions	*E*-value
26.9	Contig4901_s_at	Yes	Expressed protein	2e-07
24.7	HU03D17u_s_at	Yes	None	
7.9	HVSMEm0005P05r2_at	No	Peroxidase	2e-23
7.4	Contig7061_s_at	Yes	Putative receptor-like protein kinase	2e-19
5.3	Contig14769_at	No	None	
4.9	Contig6699_s_at	No	None	
4.1	Contig13681_at	No	Histidine kinase–like protein	8e-20
3.8	Contig13680_s_at	No	Histidine kinase–like protein	4e-17

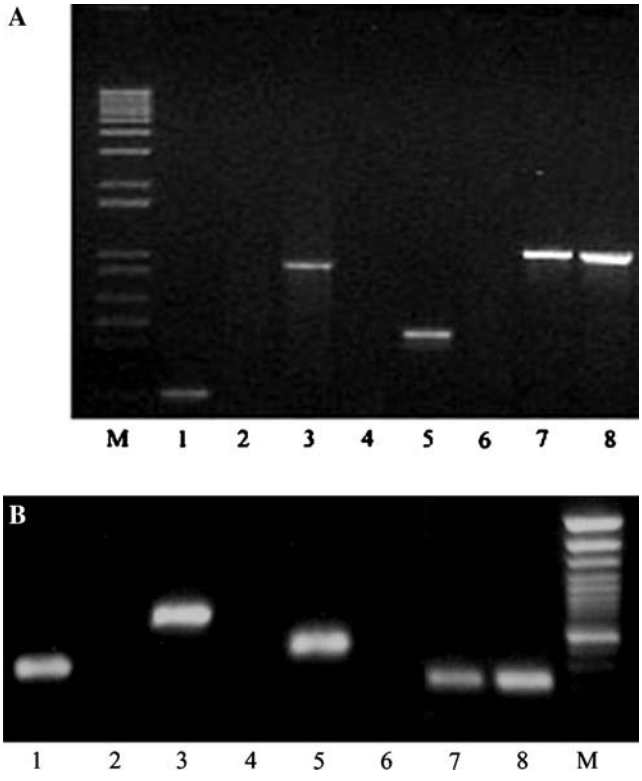

Fig. 3 Genomic-PCR and RT-PCR analysis of wild-type and mutant *rpr1* demonstrate the absence of three genes in the mutant. **a** The wild-type cv. Morex (*odd numbered lanes*) and *rpr1* mutant (*even numbered lanes*) DNA was amplified with primers for genes HU03D17u_s_at (*lanes* 1, 2), contig4901_s_at (*lanes* 3, 4), contig7061_s_at (*lanes* 5, 6) and *Rpg1* (*lanes* 7, 8) was used for DNA quality control. *M* = 1 kb plus DNA ladder from Invitrogen. **b** The wild-type cv. Morex (*odd numbered lanes*) and *rpr1* mutant (*even numbered lanes*) RNA was amplified with primers for genes HU03D17u_s_at (*lanes* 1, 2), contig4901_s_at (*lanes* 3, 4), contig7061_s_at (*lanes* 5, 6) and *Rpg1* (*lanes* 7, 8) was used for RNA quality control. *M* = 100 bp DNA ladder from Invitrogen

HU03D17u_s_at, and Conting7061_s_at were deleted and transcriptionally silent in the *rpr1* mutant line (Fig. 3). The remaining highly down-regulated genes are still present in the *rpr1* mutant genome as demonstrated by PCR-amplification with the corresponding primer sets (Table 1). Probes for the three deleted genes were used to identify BAC clones from the cv. Morex BAC library (Yu et al. 2000). Preliminary analyses showed that the BAC clones were not overlapping, each group contained one complete deletion and the deletions were large (data not shown).

Genetic mapping of Contig4901_s_at, HU03D17u_s_at and Contig7061_s_at

Molecular mapping in the *rpr1* mutant × Q21861 population with probes for all three genes showed that they co-segregated with one another and with the *rpr1* phenotype and mapped to chromosome 4(4H) bin 5 between the markers Adh4 and ABA003. Thus, three gene deletions encompass the putative *Rpr1* gene. We hypothesize that one, two, or all three genes may be responsible for the stem rust susceptibility of the *rpr1* mutant. In order to determine which deletion is responsible for the *rpr1* phenotype, DNA encompassing the deletions was isolated from a BAC library of cv. Morex and is being sequenced. Preliminary data indicate that these are independent albeit closely linked deletions. In order to identify which deletion includes the *Rpr1* gene, the mutant was crossed to cv. Morex and a large population of F_2 individuals is being screened to identify recombinations among the closely linked deletions.

Discussion

The barley cv. Morex carries *Rpg1*, a resistance gene that is effective against many pathotypes of the stem rust pathogen *P. graminis* f. sp. *tritici*. To elucidate how *Rpg1* confers resistance, we irradiated Morex with FN and identified a stem rust susceptible mutant. The mutant gene, designated *Rpr1* (Required for *P. graminis* resistance) was shown to be independent of the resistance gene *Rpg1* by several methods in this study.

First, *Rpr1* was mapped to chromosome 4(4H) bin 5 different from that of *Rpg1* on chromosome 1(7H) bin 1. Second, the mutant line still has a functional *Rpg1* gene as demonstrated by expression of an *Rpg1* specific mRNA and Rpg1 protein (Fig. 2), so the *rpr1*-mediated phenotype is not due to interference with *Rpg1* gene transcription or translation. Based on the hypothesis that the FN -induced *rpr1* mutation is likely a deletion, we considered genes with highly reduced transcript abundance as candidates for the *Rpr1* gene. Microarray analysis of the mutant compared to the parent cultivar identified eight genes with greater than fourfold reduction in transcript level (Table 2). Three of these turned out to be due to deletions as demonstrated by genomic PCR, RT-PCR (Fig. 3) and Southern analyses (data not shown). The three deletions identified as Contig4901_s_at, HU03D17u_s_at, and Contig7061_s_at were mapped and co-segregated with one another and with the mutant phenotype and therefore, are *Rpr1* candidate genes. Since this analysis was done in a fairly small population (108 F_2), the mutant was crossed to cv. Morex and a large population of F_2 individuals is being screened to identify recombinants among the closely linked deletions and determine which deletion carries the *Rpr1* gene.

Isolation and preliminary characterization of BAC clones for the three deleted genes from the parent cv. Morex BAC library showed that the three deletions, although closely linked, are not overlapping and must be fairly large. Therefore, we cannot exclude the possibility that there are other candidate genes within these deletions that were not identified because they are not present on the Barley1 GeneChip. To eliminate this possibility, we are sequencing the DNA within the deleted regions to identify other possible candidate genes. This work will help to identify the *Rpr1* gene. Identification of the *Rpr1* gene will facilitate analysis of *Rpg1* gene function and the signaling pathway(s) involved in the stem rust resistance.

Rpr1 is absolutely required for stem rust resistance conferred by *Rpg1*, but is not required for function of the stem rust resistance genes *rpg4* or *Rpg5*. This indicates that *Rpr1* shows specificity for the *Rpg1*-mediated resistance pathway. Interestingly, the *rpr1* mutation does not appear to have any obvious effect on the plants' phenotype in the absence of the pathogen.

Genetic screens for suppressors of *R* genes have been a common practice to identify components in resistance pathways. *Rar1* gene was identified in mutational screen for suppressor of *Mla12* resistance in barley to powdery mildew fungus *Blumeria graminis* f.sp. *hordei*. *Rar1* is required by several, but not all, barley *Mla* genes as well as other unlinked powdery mildew resistance loci. Homologs of *Rar1* in *Arabidopsis* and *Nicotiana benthamiana* play a conserved role in the function of a subset of NB-LRR R proteins that confer resistance to different pathogens (Muskett et al. 2002; Tornero et al. 2002). *Rcr3*, a tomato gene identified by mutational analysis, is required specifically for *Cf-2*-mediated resistance to leaf mold caused by *Cladosporium fulvum*. In tomato, different *Cf* genes confer resistance through recognition of cognate *C. fulvum* Avr genes. Genetic analysis demonstrated that *Rcr3* is not required for *Cf-9* and *Cf-5* function, therefore *Rcr3* is not a component of a conserved Cf signal transduction pathway (Dixon et al. 2000). Like *Rcr3*, *Rpr1* identified in our study is unique because it appears to suppress the function of a single *R* gene. These studies revealed genetic complexity of pathogen perception mechanisms in plants.

Fast neutron mutagenesis has been a useful tool for forward and reverse genetics, especially in *Arabidopsis*. Deletions identified from FN mutants range from 1 bp to greater than 30 kbp. The FN mutagenesis is also highly applicable to crop plants with large genomes because the number of plants required to give the same mutation coverage are comparable in plants with different genome size (Li and Zhang 2002). However, one challenge is to characterize deletions that knock out multiple genes spanning very large regions, which happens to be the case in our study. While whole genome sequencing can identify all deletions present in a mutant line, it is currently not practical to use sequencing for deletion identification (Li and Zhang 2005).

Transcript-based cloning (Mitra et al. 2004) is a gene cloning method based on expression-level polymorphism between wild type plant and mutant by a microarray approach. Mitra et al. applied transcript-based cloning to identify *DMI3* gene from *dmi3* mutant in *Medicago truncatula*. In barley, several studies have also demonstrated the potential of transcript-based cloning by working with known *rar1* and *xantha* mutations (Zakhrabekova et al. 2002; Gadjieva et al. 2004; Mitra et al. 2004), using Barley1 GeneChip and barley cDNA microarray, respectively. In our study, we employed this method to rapidly identify three candidate *Rpr1* genes, which are located in large deletions spanning three probe sets on the Barley1 GeneChip. This is the first demonstration of effective identification of multiple deleted genes in a FN mutant using a microarray approach in barley, a crop plant with a large and complex genome. Because gene identification is independent of gene position, this method does not require the construction of a genetic map. The result of a microarray experiment with an appropriate

mutant is a list of candidate genes that can be further analyzed to identify the genes responsible for the mutant phenotype. This technology considerably shortens the time of gene cloning compared to the laborious map-based cloning method. The significance of the highly up-regulated genes identified from microarray analysis is not clear at this time, but will be further analyzed in conjunction with pathogen infection time-course study.

In summary, we have identified three candidate genes for the *Rpr1* gene that is required for *Rpg1*-mediated stem rust resistance. Further analysis of the *rpr1* mutation, identification of other alleles and loci, and cloning of *Rpr1* will help to elucidate the mechanism of resistance to the biotrophic pathogen *P. graminis* f. sp. *tritici* conferred by the durable stem rust resistance gene *Rpg1*.

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