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Isolation and mapping of resistance gene analogs from the *Avena strigosa* genome

Abstract Degenerate primers based on conserved regions of the nucleotide binding site (NBS) domain (encoded by the largest group of cloned plant disease resistance genes) were used to isolate a set of 15 resistance gene analogs (RGA) from the diploid species *Avena strigosa* Schreb. These were grouped into seven classes on the basis of 60% or greater nucleic acid sequence identity. Representative clones were used for genetic mapping in diploid and hexaploid oats. Two RGAs were mapped at two loci of the linkage group *Asw*BF belonging to the *A. strigosa* × *A. wiestii* Steud map, and ten RGAs were mapped at 15 loci in eight linkage groups belonging to the *A. byzantina* C. Koch cv. Kanota × *A. sativa* L. cv. Ogle map. A similar approach was used for targeting genes encoding receptor-like kinases. Three different sequences were obtained and mapped to two linkage groups of the hexaploid oat map. Associations were explored between already known disease resistance loci mapped in different populations and the RGAs. Molecular markers previously linked to crown rust and barley yellow dwarf resistance genes or quantitative trait loci were found in the Kanota × Ogle map linked to RGAs at a distance ranging from 0 cM to 20 cM. Homoeologous RGAs were found to be linked to loci either conferring resistance to different isolates of the same pathogen or to different pathogens. This suggests that these RGAs identify genome regions containing resistance gene clusters.

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Introduction

Avena sativa (cultivated oats) is susceptible to diseases such as crown rust, stem rust and barley yellow dwarf (BYD). Crown rust is caused by the fungus *Puccinia coronata* f. sp. *avenae* Eriks. and is the most widespread and damaging disease of oats (Harder and Haber 1992). Stem rust, caused by the fungus *P. graminis* f. sp. *avenae* Eriks. and Henn, is a serious disease, especially on the great plains of North America and in Australia. Outbreaks may occur, however, wherever oats are cultivated (Harder 1994). BYD is caused by a group of related luteoviruses collectively referred to as the BYD viruses. This disease occurs all over the world and may account for reductions in annual grain yield in cereals of up to 30% (Burnett 1983). Breeding for resistance to these important diseases has been one of the top priorities in oat cultivar improvement programs. Several major resistance genes for either crown rust (*Pc* genes) or stem rust (*Pg* genes), which can provide complete host-plant resistance to a specific subset of races through a hypersensitive reaction, have been employed by oat breeders (reviewed by Sebesta et al. 2000). In addition to complete resistance of the simply inherited hypersensitive type, partial resistance to crown rust (Chen et al. 2000; Holland and Munkvald 2001; Zhu and Kaeppler 2003) and various levels of tolerance to BYD (Barbosa-Neto et al. 2000; Zhu et al. 2003) have also been identified.

The availability of molecular markers linked to major resistance loci and QTLs has greatly facilitated genetic analysis, gene characterization and the transfer of genes into desirable cultivars. In hexaploid oats, several dominant rust resistance genes have been localized in relation to molecular markers. The most developed oat map, based on a cross between *A. byzantina* cv. Kanota and *A. sativa* cv. Ogle (O'Donoughue et al. 1995; Wight et al. 2003), is used as a base for integrating data from various sources by comparing the positions of markers in different maps. Direct mapping and, more frequently, comparative mapping have allowed an unknown gene providing resistance to *P. coronata* (R203) (Bush et al. 1994), *Pc91* and *Pc92*

(Rooney et al. 1994), *Pc71* (Bush and Wise 1998), the *Pg9/PcX* cluster (O'Donoughue et al. 1996) and *Pg4/Pg13* (O'Donoughue et al. 1996) to be localized. Moreover, several quantitative trait loci (QTLs) for partial resistance to crown rust (Bush and Wise 1996; Zhu and Kaeppler 2003; H.W. Rines, personal communication) and for BYD (Barbosa-Neto et al. 2000; Zhu et al. 2003) have also been mapped in relation to molecular markers.

Resistance genes have also been identified in the wild diploid species *A. strigosa*—probably one of the ancestors of the hexaploids (Rajhathy and Thomas 1974). This species is an important source of resistance; genes for crown rust resistance have been transferred to the cultivated hexaploid using a series of bridging crosses (Forsberg 1990). Crown resistance genes have been mapped on the *A. strigosa* (CI 3825) × *A. wiestii* (CI 1994) diploid map (Rayapati et al. 1994; Kremer et al. 2001; Yu and Wise 2001). A gene for partial resistance to race R264B is found in linkage group B equivalent to A*sw*BF (Yu and Wise 2001; Portyanko et al. 2001). The important *Pca* cluster, which consists of several distinct genes responsible for different resistance specificities, is located at the end of group *Asw*AC (Rayapati et al. 1994; Kremer et al. 2001; Portyanko et al. 2001; Yu and Wise 2001).

A new PCR-based strategy for the rapid generation of genetic markers linked to possible resistance loci has recently been developed. This strategy is based on the observation that genes conferring resistance to a range of plant pathogens encode proteins with a high degree of structural and amino-acid sequence conservation. Most of these proteins have domains involved in signal transduction or protein-protein interactions (Backer et al. 1997; Hammond-Kosack and Jones 1997). In particular, the majority of cloned resistance genes (R genes) encode cytoplasmic receptor-like proteins characterized by the presence of an N-terminal nucleotide binding site (NBS) and a leucine-rich repeat (LRR) domain (Hammond-Kosack and Jones 1997). The NBS sequences of R genes are characterized by the presence of up to eight conserved motifs (Meyers et al. 1999; Pan et al. 2000). Using PCR and degenerate oligonucleotide primers, these have facilitated the cloning of resistance gene analogs (RGAs) in different species (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996). Several of the identified RGAs have been mapped to positions of previously identified loci that confer resistance to viruses, bacteria, fungi and nematodes in soybean, Arabidopsis, rice, barley, wheat, maize and potato. Genetic and physical mapping of RGAs has shown them to be frequently organized in clusters, as are many of the NBS-LRR cloned genes. This is consistent with the results of classic genetic studies on the organization of R genes (see reviews by Hulbert et al. 2001; Meyers et al. 2003).

A second R-gene class has also been the target of similar PCR-based approaches for obtaining new types of RGAs. Although this class has only one recognized member, *Xa21* from rice (Song et al. 1995), another possible resistance gene—*Lrk10* from wheat—has been identified (Feuillet et al. 1997). In addition, sequences homologous to *Lrk10* map close to the *Pg9/PcX* locus in the Kanota × Ogle oat map (Cheng et al. 2002). These genes belong to a large class referred to as the receptor-like kinases (RLKs) and encode a protein containing an extracellular domain, a transmembrane segment and an intracellular catalytic serine/threonine kinase domain. Plant RLKs have been categorized into three groups based on the protein motif in the presumed extracellular domain (review by Zhang 1998). The main group is the LRR group (LRR-RLKs), to which *Xa21* belongs. Using degenerate oligonucleotides to conserved kinase motifs, Deng and Gmitter (2003) obtained RGA sequences with identity to *Xa21* in *Citrus*.

The identification of RGAs has proven to be a good way of generating markers useful in marker-assisted selection (MAS) (Melotto and Kelly 2001; Seah et al. 2001; Mohler et al. 2002; Madsen et al. 2003). Moreover, several RGAs have contributed to the cloning of full-length functional R genes (Aarts et al. 1998; Meyers et al. 1998), and they might help in the identification of R genes (Huettel et al. 2002).

This paper describes the isolation and characterization of RGAs from *A. strigosa* and their localization in the diploid and hexaploid maps of *Avena* species. *A. strigosa* was selected as the DNA source for two reasons: (1) the accession used is highly resistant to *P. coronata*, and five *Pc* genes have been mapped in a complex loci named *Pca*; (2) the diploid nature of *A. strigosa* should produce a smaller and more amenable RGA pool than that of *A. sativa* and be free of redundant homoeologous sequences.

Materials and methods

Plant material

Avena strigosa line CI 3815, which is resistant to forty isolates of *Puccinia coronata* f. sp. *avenae* maintained in the ISU (Iowa State University, Ames, Iowa, USA) collection, was used as the main source of genomic DNA for PCR amplification of RGA sequences. For the genetic mapping of RGAs, 100 $F_{6:8}$ recombinant inbred lines (RILs) from a cross between diploid *A. strigosa* (CI 3815) and *A. wiestii* (CI 1994) were used (Kremer et al. 2001). Mapping in the hexaploids was performed using 76 $F_{9:10}$ RILs from a cross between *A. byzantina* cv. Kanota and *A. sativa* cv. Ogle (O'Donoughue et al. 1995; Wight et al. 2003). Total DNA was extracted from young leaves following the method of Sharp et al. (1988).

Oligonucleotide primers and PCR strategy

A large set of degenerate primers was used to amplify NBS-containing sequences from *A. strigosa* DNA. Firstly, sequences were produced with the highly degenerate primers designed by Leister et al. (1996) based on the conserved P-loop and GLPL motifs of the NBS domain of known resistance proteins. Secondly, sequences were obtained with primers designed by Collins et al. (1998), based on four conserved motifs of that NBS domain. Four primers, P-loop, corresponded to the motif GVGKTT; four primers, kinase-2, corresponded to the motif LVLDDV; four primers, GLPL, corresponded to the GLPL motif; another four primers corresponded to the MHD motif. The level of degeneracy of all these primers was

lower than that described by Leister et al. (1996). All cloned PCR fragments were obtained using a nested primer approach. PCR reactions were first performed with all possible combinations of primers based on the use of P-loop and MHD motifs. The products of these reactions were used as templates in a second round of amplification with the kinase-2/GLPL primer combination.

The degenerate primers described by Baudino et al. (2001), based on conserved LRR-RLK motifs, were used to amplify the RLK sequences of *A. strigosa*. The forward primers S3, S4 and S5 in the putative extracellular domains were combined with the reverse primer S1 in kinase domain VII or S2 in kinase domain III.

PCR amplification conditions

PCR reactions were performed in a 25-µl volume containing 50 ng of genomic DNA, 0.2 m*M* dNTPs, 0.4 pmol each primer, 10 m*M* Tris-HCl, pH 8.3, 1.5 m*M* MgCl$_2$, 50 m*M* KCl, and 1 U *Taq* polymerase (Sigma-Aldrich, St. Louis, Mo.). When degenerate primers containing P-loop and GLPL motifs were employed, the cycle conditions used were 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 45°C for 30 s, 72°C for 50 s, and a final cycle of 7 min at 72°C. Cycling conditions for the first round of PCR using degenerate primers based on the P-loop and MHD motifs were the same as above, but the annealing temperature was 40°C. For the second-round nested PCR, the genomic DNA template was replaced by 1 µl of first-round PCR mix. Cycling conditions were 94°C for 2 min, followed by ten cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 50 s and 25 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 50 s, and a final extension of 72°C for 7 min. Thermal cycling conditions for the amplification of RLK sequences were 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 45°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 5 min.

Cloning and analysis of PCR products

PCR products of the expected size were cloned into the pBluescript vector (Stratagene, La Jolla, Calif.) using standard procedures (Sambrook et al. 1989). To differentiate between individual clones, we amplified inserts from at least ten recombinant clones by PCR directly from bacterial cultures using the SK and KS primers. Fragments of the expected size were separately subjected to digestion with the restriction enzymes *Hae*III and *Taq*I in order to select clones with different inserts. A 5-µl aliquot of the PCR reaction product was digested with 5 U of the enzyme for 2 h following the manufacturer's instructions. Restriction fragments were separated on 1.8% agarose gels. Cloned fragments that revealed a unique restriction pattern were sequenced using the BigDye terminator system and an ABI Prism 377 (Applied Biosystems, Foster City, Calif.).

Sequence analysis

Sequences of the cloned PCR products were compared to those in the EMBL and GenBank databases using the BLASTX algorithm (Altschul et al. 1997). Pairwise comparisons and multiple alignment were performed using the ALIGN program (Myers and Miller 1988) and CLUSTAL W (Higgins and Sharp 1988), respectively. For NBS sequences, a neighbor-joining tree (Saitou and Nei 1987) was generated from sequence alignments. Bootstrapping was used to provide an estimate of the confidence for each branch point. The tree was rooted using the L7M4.9 sequence, which showed the lowest level of identity with any of the oat NBS obtained.

Restriction fragment length polymorphism mapping

Restriction fragment length polymorphism (RFLP) was investigated for parentals of the mapping populations (*A. strigosa* × *A. wiestii* or *A. byzantina* cv. Kanota × *A. sativa* cv. Ogle) using the *A. strigosa* RGA clones. Diploid parental DNA (10 µg/sample) was digested with each of six different restriction enzymes: *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Sst*I. Hexaploid parental DNA (15 µg/sample) was also digested with these same enzymes except for *Sst*I, which was substituted by *Xba*I. The methods of Kremer et al. (2001) were followed for Southern blotting, probe labeling and hybridization for the analysis of the diploid mapping population. For the hexaploid mapping, digested DNA was electrophoresed on a 1% agarose gel, transferred to positively charged nylon membranes (Roche Molecular Biochemicals, Mannheim, Germany) and hybridized to radio-

Table 1 Primers used in the cloning of *Avena strigosa* NBS (nucleotide-binding site) and RLK (receptor-like kinase) sequences, name of the RGA (resistance gene analog) obtained and classification of the NBS clones based on nucleotide sequence identity

RGA sequence	Primer combination	First-round primers		Second-round primers		RGA clones	Class
		Forward	Reverse	Forward	Reverse		
NBS	II	S2	AS2	–	–	II2.3	D
						II2.17	G
	III	S2	AS3	–	–	III2.2	G
						III2.4[a]	E
						III2.18	E
	L7-M2	P-loop7	MHD2	Kinase2G	GLPL5	L7M2.1	B
						L7M2.2	B
						L7M2.3	B
	L7-M4	P-loop7	MHD4	Kinase2G	GLPL6	L7M4.9[a]	A
	L8-M1	P-loop8	MHD1	Kinase2G	GLPL5	L8M1.5	B
						L8M1.7	C
						L8M1.9	C
	L8-M3	P-loop8	MHD3	Kinase2G	GLPL5	L8M3.5[a]	C
						L8M3.8	F
						L8M3.11[a]	E
RLK	S3-S1	S3	S1	–	–	S3S1.24	–
	S5-S1	S5	S1	–	–	S5S1.1.6	–
	S5-S2	S5	S2	–	–	S5S2.2.8	–
						S5S2.2.11	–

[a]Sequences with at least one stop codon

active probes made by random priming with the High Prime labeling kit (Roche Molecular Biochemicals). Following hybridization at 65°, blots were treated with 2× SSC, 0.1% sodium dodecyl sulfate (SDS) at room temperature for 30 min, twice with 1× SSC, 0.1% SDS at 65°C for 20 min each, followed by a stringent wash in

0.1× SSC, 0.1%SDS at 65°C for 30 min. The blots were briefly dried and exposed to X-Omat AR films (Kodak, Rochester, USA).

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                     P-loop                                                                                    Kin-2
III2.4*     GLGKTTLAQLVYNDRRIKNHFQLRVWLCVSQNFDQMKLTRETIESAASEFESVVSGVSSVTTNMNLLQEDLSNKLKGKKFLLVLD
L8M3.11*                                                                                              LLVLD
III2.18     GLGKTTLAQLVYNDTRIKNHFQLRVWLCVSQNFDQMKLTRETIESVASEFESVVSGVSSVTTNMNLLQEDLSNKLKGKRFLLVLD
L8M1.9                                                                                               -LMLD
L8M3.5*                                                                                              -LVLD
L7M2.1                                                                                               LLLLD
L7M2.2                                                                                               LLVLD
L7M2.3                                                                                               LLVLD
L8M1.7                                                                                               LLLLD
L8M1.5                                                                                               LLVLD
II2.17      GIGKTTLAQNIYNDHKIKGAFSNQAWICVSHEYSEVSILKEVLRNFGVHQD-----QG---ETVGELSSKLAAVVQEKSFFLVLD
III2.2      GLGKTTLAQKIYNDQKIKGNFNKQAWICVSQEYSEVALLKEVLRKIGVHYE-----AD---ENVGELSGKLEAAMENKSLFLVLD
L8M3.8                                                                                               LLFLD
II2.3       GIGKTTLARFVYRDQRIRDYFDLQIWVCVSTKFNEVRLTLEILEHVCEDKQ-----EYENISNFNVLQEILMRNIRNKRFLLVLD
             ____                                RNBS-B
III2.4*     DVWNE-DPEKWDTYRRSLVTGGKGSR-IVVTTRKKNVG-KLMGGMDPYCLNQLSDSDCWYLFRSYAFVGGNSSARPK--LEIIGM
L8M3.11*    DVWNE-DPEKWDTYRRSLVTGGKGSR-IVVTTRKKMVG-KLMGGMDPYCLNQLSDSDCWYLFRSYAFVGGNSSARPK--LEIIGM
III2.18     DVWNE-DPEKWDTYRRSLVTGGKGSR-IVVTTRNKNVG-KLMGGMDPYYLNQLSDSDCWYLFRSYAFVGGNSNARAN--LEIIGM
L8M1.9      DVWEK-NPNTLKNLKAMLRLGVGSMVRVIVTTRDEAIAREICHSVEPYKLEILTNEMCWTIIKEKTGFKDRVNKKQ---LKHIGR
L8M3.5*     DVWEK-NPNTLKNLKAMLRLGVCSMVRVIVTTRDEAIAREICHSVEPYELEILTNEMCWTIIKEKTGFKDRVNKKQ---LKHIGR
L7M2.1      DVWTR-EDRKWGQLKARLEHGGMGSV-ILTTTRDKVVA-EIMGTVEAYNLEALGGLYLKEIIETTTFSRLKVEEERPTVLVNMVG
L7M2.2      DVWTR-EDHKWEQLKARFEHGGMGSV-VLTTTRDNVVA-KIIGTVEAYNLEGLGGQYITEIIETTAFSRLKKEEEERPSVLVNMVG
L7M2.3      DVWKR-EVDKWEQLKSRLQHGGMGSV-VLTTTRDEGVA-KIMGTVKAYNLTALEDEFIKEIIESRAFGHLHKEEKRPDLLVGMVD
L8M1.7      DVWKR-EVDKWEQLKSRLQHGGMGSV-VLTTTRDEGVA-KIMGTVKAYNLSALEDEFIKEIIESRAFGHLHKEEKRPDLLVGMVD
L8M1.5      DVWKR-EVDKWEQLKSRLQHGGMGSV-VLTTTRDEGVA-KIMGTVKAYNLSALEDEFIKEIIESRAFGHLHKEEKRPDLLVGMVD
II2.17      DVWQ---PEVWINLLRIPLHSAATGV-IIVTTRHDIVA-HAIGMEHVHRVDLMAAAVGWELLCKSMNINEEKDVEN---LRNIGL
III2.2      DVWQ---PEVWINVLRTPLHAAASVM-ILVTTRHDKVA-LAIGAKHMHRVDLMSEDVGLELLWKSMNINEVEEVHG---LWDIGM
L8M3.8      DVWH---SNVWTDLLRPPLHATNTGV-ILVTTRDDQVA-LRIGVQHTHQVDLMSLEVGWELLWKSMNIDEEKDVQN---LRSIGI
II2.3       DMWEDKDRNGWTKLLAPLKSNQVNGCMVLATTRSTSVA-KMIGTMDEVRLSGLDEGFWLFFKGCAFGNENYEGDPS---LQSIGK
             ____  GLPL                                                                        RNBS-D
III2.4*     EIVKKL-KGLPLAL
L8M3.11*    EIVKKL-KGLPLAAKAIGSLLCSQDTEE-LEKCIEEEI-WELPSDK---NN---VLPALRLSYNHLPAILKRCFAFCSVFHKDYV
III2.18     EIVKKL-KGLPLAL
L8M1.9      EIATKC-GGVALAAQSLGYMLNNMKHSD-EWESVRDSYIWNLSTSEGSSSGNHEVLASLLLSYSHMPDWLKLCFSYCAVFPKGHL
L8M3.5*     EIATKC-GGVALAAQSLGYMLNNIKHSD-ERESVRDSYIWNLSTSEGSSSGNHEVLASLLLSYSHMPDWLKLCFSYCAVFPKGHL
L7M2.1      EIMERC-AGSPLAAIALGSILRNKASEE-EWKAVSRRS--NICTEE---SG---ILPILKLSYNDLPSHMKQCFSFCAIFPKDYD
L7M2.2      EIAARC-VGSPLAAVALGSILRNKTSEE-EWKAVSTRS--NICTGE---TG---ILPILELSYNDLQPHMKQCFAFCAIFPKDYK
L7M2.3      EIVKRC-VGSPLAATALGSVLRTKTSEE-EWKALSSRS--NICTEE---SG---ILPILNLSYNDLPSHMKQCFAFCAIFPKGYE
L8M1.7      EIVKRC-VGSPLAATALGSVLRTKTSEE-EWKALSSRS--NIRTEE---SG---ILPILNLSYDDLPSHMKQCFAFCAIFPKGYE
L8M1.5      EIVKRC-VGSPLAATALGSVLRTKTSEE-EWKALSSRS--NIRTEE---SG---ILPILNLSYDDLPSHMKQCFAFCAIFPKGYE
II2.17      DIVRKC-GGLPPR-
III2.2      EIVRKCKGGLPLA-
L8M3.8      EIVKRC-GRLPLA-RVTASALASRYLTKNEWKKFLGKY---VGLQNILLDG---IEGALYLSYSELPHRLKQCFLYCALYIEDSV
II2.3       HIAKTLKGFSPR--
             W(M/I)AxG
III2.4*
L8M3.11*    FEKDRLVQIWIAVGVLE
III2.18
L8M1.9      IVKYDLIHQWIPLAFSS
L8M3.5*     IVKYDLIHQWIALAFSS
L7M2.1      IDVDKLIQLWIALGLLE
L7M2.2      IDVDKLIQLWIALGILE
L7M2.3      IDVDKLIQLWIALGVLE
L8M1.7      IDVDKLIQLWIAIGFLE
L8M1.5      IDVDKLIQLWIALAFSS
II2.17
III2.2
L8M3.8      IGLD-VTKLWIAFGLLE
II2.3
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Fig. 1 Multiple alignment of the predicted amino-acid sequences of the NBSs from *A. strigosa*. The regions conserved in the NBS-LRR genes are in *dark gray*. Regions characteristic of the nonTIR class are in *bold* and *underlined*. Names of the different motifs are indicated at the *top* of the sequence

RGA markers were placed on the existing diploid *A. strigosa* × *A. wiestii* (A*sw*) map (Kremer et al. 2001; Portyanko et al. 2001) and hexaploid *A. byzantina* cv. Kanota x *A. sativa* cv. Ogle (KO) map (O'Donoughue et al. 1995; Wight et al. 2003) using the computer package MAPMAKER 3.0 (Lander et al. 1987). The KO map was a framework map of 286 uniformly spaced markers. The two-point GROUP command with an LOD threshold of 3.0 and a recombination fraction of 0.35 was used to assign markers to existing linkage groups. Marker order within the linkage groups was determined using the BUILD and TRY commands, with the previously published order (O'Donoughue et al. 1995; Wight et al. 2003) used as the starting order. The marker orders obtained were verified by multipoint analysis using the RIPPLE function. The map distances were calculated using the MAP command and converted to centiMorgans using the Kosambi function (Kosambi 1944). Once RGAs were assigned to specific intervals in the KO framework linkage groups, attempts were made to more precisely map the RGA loci for some linkage groups using a large number of markers from the complete set of 1,166.

Results

PCR amplification and molecular cloning of RGA sequences

Table 1 shows the primers that successfully rendered RGA sequences. Two out of the three S2/As combinations (Leister et al. 1996) (named II and III) produced a major band of around 500 bp—which was expected based on the sequences of the NBS-LRR genes. Cloning of the PCR products and characterization of 10 to 15 clones from each ligation by restriction fragment analysis with the four-cutter enzymes *Hae*III and *Taq*I revealed that each initial PCR fragment was comprised of a mix of different sequences. A sample of 20 representative clones were sequenced and compared to the GenBank database using BLASTX. Five RGA sequences were identified.

To increase the number of NBS-sequences isolated, the method proposed by Collins et al. (1998) was tried. Most of the P-loop/MHD combinations produced either DNA smears without clear bands or fragment patterns without the band of the expected size. Amplification products of the expected size—850 bp—were obtained with 4 of the 16 primer combinations. When these were subjected to a second round of amplification with nested kinase-2/GLPL primers, poor amplifications were obtained with all of the primer combinations. However, in some, a fragment of about 550 bp was produced instead of the expected 350-bp fragment. This band was selected since sequences containing an intron in different positions of the cereal NBS have been described (Bai et al. 2002). Cloning, insert characterization, sequencing of representative clones and comparison to database information identified ten different RGA sequences (Table 1). Together with the first isolation approach, 50 *A. strigosa* clones were sequenced, and 15 (GenBank accession numbers AJ437568–AJ437575 and AJ295998–AJ296002) showed similarity to cloned disease resistance genes.

Avena strigosa RLK sequences were amplified from genomic DNA using six primer combinations targeting five conserved domains of the RLK-LRR proteins. Three combinations gave bands of the expected size and were cloned (Table 1). Thirty-five inserts were sequenced and compared to GenBank database information using BLASTX. Four different sequences were obtained with a similarity to RLK proteins (Table 1, GenBank accessions AJ629309– AJ629311). Three showed the greatest similarity to somatic embryogenesis RLKs of *Arabidopsis*, carrot and maize, and the fourth (S3-S1.24) to the resistance gene *Xa*21 of rice.

Sequence analysis

Avena strigosa NBS sequences were translated to look for characteristic motifs of plant NBS regions (Fig. 1). Stop codons were detected in four sequences: III2.4, L7M4.9, L8M3.5, and L8M3.11. Sequence L7M4.9 contained multiple stop codons and, consequently, could not be aligned with the remaining sequences at the amino acid level. It is therefore omitted in Fig. 1. For the remaining three sequences, either one (in III2.4 and L8M3.5) or two (in L8M3.11) stop codons were found. The kinase-2 and RNBS-B sites were present in the sequences amplified with primer combinations II and III. Sequences amplified using the nested primer approach showed RNBS-B, GLPL and RNBS-D motifs. This result indicates that the second-round primers unexpectedly failed to anneal with the GLPL motif but were able to anneal at the end of an additional motif (W(M/I)A×G), characteristic of nonTIR (Toll-interleukin receptor-like region)-LRR sequences and separated from RNBS-D by 14–15 amino acids (Collins et al. 1998; Pan et al. 2000; Cannon et al. 2002). The annealing of the nested primers to this motif explains the length of the fragment obtained. Consequently, it corresponded to sequences with no intron. An additional motif characteristic of the NBS region of the nonTIR gene superfamily, the tryptophan residue (W) at the end of the kinase-2 motif (described by Pan et al. 2000; Meyers et al. 2003), was also found in the *A. strigosa* sequences. Sequence analysis of the GLPL motif in the fragments first amplified with the P-loop/MHD primer combinations were different to the consensus GLPLAL sequence. For example, GSPLAA, GVALAA or GLPPRL were present, which helps to explain the poor amplification obtained in the second-round PCR with the low-degenerate primers based on the consensus sequence of the GLPL motif.

To investigate identity among *A. strigosa* NBS sequences, we performed pair-wise comparisons of all the sequences at the nucleotide level using the ALIGN program. In general, stronger identities were noticed among sequences amplified with the same primer combination than among sequences amplified with different primer combinations. For example, L8M1.7 and L8M1.9 showed the greatest identity at 98.9%, while II2.3 and L7M2.1 showed the least at 46%. The regions common for all sequences, i.e. from kinase-2 to GLPL,

were subjected to a multiple sequence alignment using the CLUSTAL W program. Figure 2 shows the neighbor-joining tree generated. Using a identity threshold value of 60%, we tentatively grouped these sequences into seven classes, designated A to G. This classification has to be taken carefully since the sequence lengths compared were only around 300 bp long. Most of the classes have several members, although three orphan classes—A, D and F— were found. The average percentage identity within each of the groups ranged from 98.4% to 77.1%, while identities between groups were less than 53%. Although most of the NBS sequences in each group were related to the primer combination used (Table 1), exceptions were observed. For all combinations but L8M3, the sequences isolated belonged to only one or two families. This indicates that using a number of different primers might be useful for isolating sequences from different families, whereas sequencing a large number of fragments obtained from the same primer combination increases the number of sequences obtained from a single family.

Genetic mapping of RGA sequences

NBS sequences were used as probes to produce RFLPs whose map locations were determined by segregation analysis both in a diploid *Avena* population of 100 RILs (*Asw* map) (Kremer et al. 2001; Portyanko et al. 2001) and in a hexaploid *Avena* population of 76 RILs (KO map) (O'Donoughue et al. 1995; Wight et al. 2003). Based on
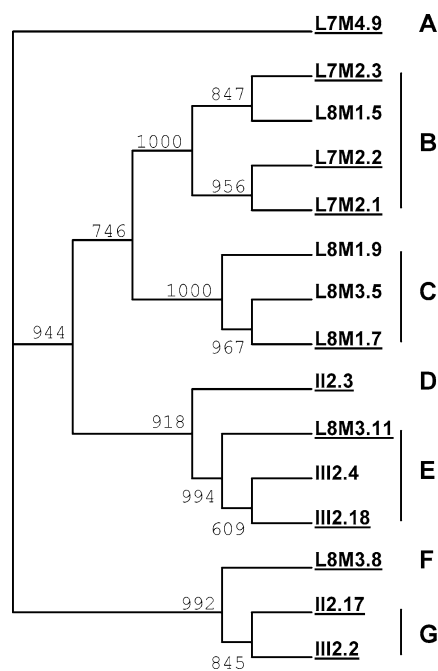
Fig. 2 Neighbor-joining tree based on the CLUSTAL W alignment of the nucleic acid NBS sequences. Sequences from kinase-2 to the GLPL were used for the alignment. The *numbers below* the branches indicate the percentages of 1,000 bootstrap replications supporting the particular nodes. Sequences *underlined* correspond to those used in the RFLP analysis

sequence identities, representative NBS sequences were selected as probes. Thus, within each class (Fig. 2), sequences with less than 70% identity were selected except for two members of class B, L7M2.1 and L7M2.2, which showed 82% identity. Table 2 summarizes the information on fragment patterns detected by each probe as well as the linkage data obtained by placing each RGA in an interval between two markers previously localized to either of the two reference maps. For the KO map, the markers defining the interval were framework markers. The map location of the RGA markers is illustrated in Fig. 3.

To detect polymorphism between each pair of parent lines, we tested several probe/enzyme combinations. The screening of diploid parental lines was performed with six different restriction enzymes and 11 probes. The complexity of the hybridization patterns was generally low. The highest number of bands detected by a single probe was three. Five probes detected single bands. Only probes II2.3 and L8M3.8 identified polymorphism between the parents of this population and were using for mapping. A single locus was detected by each probe; these were integrated into the *Asw*BF linkage group (Fig. 3).

Polymorphism between the parents of the hexaploid KO map was detected with 10 of the 11 NBS probes assayed with at least one of the six restriction enzymes used. Different probes gave different RFLP patterns, although L7M2.1 and L7M2.2 showed bands in common (but with differences in intensity). In agreement with this species' ploidy number, the complexity of the hybridization patterns was greater than that observed for the diploid. The number of bands ranged from three to ten, and good correspondence was observed between the real number of bands in the hexaploid and that expected (three times the number of bands in the diploid) (Table 2). Most of the clones showed more than one polymorphic band that allowed up to four loci to be identified per NBS clone— for example, L8M3.11. The total number of scored loci was 19. The 1:5 ratio of codominant to dominant NBS markers observed was higher than that in the first KO map for anonymous markers (O'Donoughue et al. 1995). This might be influenced by the larger average number of bands detected by the NBS probes. Six NBS loci showed significantly distorted segregation ($P<0.05$ based on χ^2). Two of them could not be assigned to any linkage group and the locus on KO13, L8M3.8, was placed in a previously identified distorted region (O'Donoughue et al. 1995; Wight et al. 2003).

Most of the scored loci were unequivocally assigned to a KO linkage group. As a whole,15 were fitted into eight established linkage groups (Fig. 3). Both KO7_10_28 (Sect. 7) and KO17 presented markers that showed a certain degree of clustering. In KO17, two markers (L7M2.2a and III2.18b) were located in a region described as either duplicate or homoeologous to a region in KO7 (Wight et al. 2003), where a cluster with the markers L7M2.2b, L7M2.2c and L7M2.1 (with 9.9-cM space interval) were found. Moreover, two markers in KO17, L7M2.3a and L8M3.11b, were mapped at one end of the

Table 2 RFLPs produced by NBS and RLK cloned sequences, number of hybridizing bands in the diploid and hexaploid species, number and type of loci scored, assigned linkage groups and map intervals where loci were placed

RGA clone	Number of bands (diploid/hexaploid)	Loci	Locus type	Linkage group	Map interval
II2.3	1/5	II2.3	Codominant	AswBF	Cdo400-cdo1508
		II2.3a	Dominant*	KO14	Cdo400-bcd1184
		II2.3b	Dominant	KO2	Umn5846-end
II2.17	2/4	II2.17	Dominant	KO24_26_34	Isu1651-end
III2.2	1/3	III2.2	Dominant	Unlinked	
III2.18	3/8	III2.18a	Codominant	KO6	Isu1755a-isu1146
		III2.18b	Codominant	KO17	Isu1736-cdo53
L7M2.1	2/5	L7M2.1	Dominant	KO7_10_28	Cdo962b-bcd1280a
L7M2.2	2/7	L7M2.2a	Dominant	KO17	Isu1736-cdo53
		L7M2.2b	Dominant	KO7_10_28	Bcd1280a-phytoA
		L7M2.2c	Dominant*	KO7_10_28	Cdo962b-bcd1280a
L7M2.3	2/5	L7M2.3a	Dominant	KO17	Umn5078-end
		L7M2.3b	Dominant	KO7_10_28	Bcd1280a-phytoA
L7M4.9	1/5	L7M4.9a	Dominant	KO22_44+18	Csu25-end
		L7M4.9b	Dominant	KO24_26_34	Bcd961-cdo373
L8M1.7	1/3	Non polymorphic			
L8M3.8	1/3	L8M3.8	Codominant	AswBF	Pic20-bcd1278
		L8M3.8	Codominant*	KO13	Cdo460a-isu2064b
L8M3.11	3/10	L8M3.11a	Dominant*	Unlinked	
		L8M3.11b	Dominant*	KO17	Umn5078-end
		L8M3.11c	Dominant	Unlinked	
		L8M3.11d	Dominant*	Unlinked	
S3S1.24	1/3	S3S1.24a	Dominant*	KO11_41+20	Umn5112-cdo1436c
		S3S1.24b	Dominant	Unlinked	
S5S1.1.6	2/3	S5S1.1.6	Dominant	KO11_41+20	Cdo1090c-isu543a
S5S2.2.11	1/3	S5S2.2.11	Codominant	KO8	Bcd1950b-end

*$P \leq 0.05$

group with a 6.3-cM interval. The NBS sequences that detected these markers did not necessarily belong to the same sequence class (Fig. 2). Except for L7M2.1 and L7M2.2—both of class B and both of which showed 82% identity—low levels of identity were found for the other linked sequences (i.e. L7M2.3 and L8M3.11 with 53.8% identity, and III2.18 and L7M2.2 with 47% identity). The remaining markers were dispersed among different linkage groups.

The NBS markers revealed by the same probe were assigned to linkage group regions where homoeologous RFLPs have previously been identified. This was particularly evident for the groups KO6, KO7_10_28 (Sect. 7) and KO17, and for KO22_44+18 (Sect. 22) and KO24_26_34 (Sect. 26). This reinforces the proposed homoeology of these chromosomal regions (Wight et al. 2003).

Two probes detected markers common to the diploid and hexaploid maps. Probe II2.3 produced a marker in AswBF as well as two markers in the hexaploid map, one unequivocally located in KO2 and the other closely linked to two markers in KO14: cdo400 and wg719. However these were loosely linked to the remaining markers of this linkage group. Since a homoeologous relationship has

been previously established between AswBF and KO14 in this region, we tentatively assigned II2.3a to KO14.

Three RLK sequences were scored as RFLPs in the KO mapping population. Hybridization patterns were very simple, and either three or four bands were observed in the corresponding blots (Table 2). These probes produced four polymorphic bands, three of which were placed in linkage groups. Two loci were assigned to KO11_41+20 (Sects. 11 and 20, respectively) and the third to KO8 (Fig. 3).

Discussion

Although different strategies were used and a large number of primer combinations assayed, relatively few oat NBS sequences were identified in our investigation. Nested PCR gave poorer results than have been obtained with species such as maize (Collins et al. 1998) and grapes (Donald et al. 2002). For the first PCR reaction with primers based on P-loop and MHD motifs, only one-quarter of the primer combinations yielded *A. strigosa* fragments of the expected size (taking into account the consensus spacing of NBS motifs used for priming). Moreover, the second amplification round based on kinase-2 and GLPL primers failed to produce any

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Fig. 3 Molecular marker map of the linkage groups in which the different RGAs isolated were mapped. *Asw*BF corresponds to the diploid map from the cross between *A. strigosa* × *A. wiestii* (following Portyanko et al. 2001). The *slanted bar* represents a break to show the two portions of this group relevant to this work. The KO groups correspond to the framework hexaploid map from the cross between *A. byzantina* cv Kanota × *A. sativa* cv. Ogle (following Wight et al. 2003). *Slanted bars* on several groups indicate that the complete linkage group is not represented. Markers are indicated at the *right* of the vertical lines. Markers in *bold* are the RGAs of this study. Markers in *italics* are non-framework markers. Deduced positions of resistance disease genes or QTLs are indicated

within boxes at the *left* of the *vertical lines*. The description of the R genes and QTLs are as follows: resistance to *P. coronata*: *Pc38*, *Pc39* and *Pc48* (Wight et al. 2004), *Pc71* (Bush and Wise 1998), *Pca* (Kremer et al. 2001), *PcX* (O'Donoughue et al. 1996), R264B (Yu and Wise 2001); resistance to *P. graminis*: *Pg9* (O'Donoughue et al. 1996); QTLs for resistance to *P. coronata*: PC54 and PC59 (Bush and Wise 1996), PrPcq1, PrPcq3, PrPcq4 and PrPcq5 (H.W. Rines, personal communication), Pcq2 (Zhu and Kaeppler 2003); QTLs for resistance to barley yellow dwarf virus, Bydq1 and Bydq2 (Zhu et al. 2003), BydqA and BydqE (Jin et al. 1998), Bydq Pav-129, Bydq Pav-IL and Bydq Pav-NY (Barbosa-Neto et al. 2000). *Bar* indicates a genetic distance of 10 cM

fragments of the desired size, and only the selection of fragments of sizes larger than expected rendered NBS sequences. In contrast, 26 out of the 68 maize clones sequenced were RGAs (Collins et al. 1998). Similarly, 50% of the sequenced clones of grapevine have been thus identified (Donald et al. 2002). This unexpected result might be caused by the large departure from the consensus GLPL seen in the *A. strigosa* sequences obtained by the nested approach (Fig. 1) together with the low degeneracy

of the primers used. Peñuela et al. (2002) indicate that primers based on the consensus P-loop and GLPL motifs, such as those described by Collins et al. (1998) and used in the present work, resulted in a bias towards the isolation of TIR-NBS sequences in species harboring both TIR and nonTIR sequences. In *A. strigosa*, a cereal which has only nonTIR sequences (Pan et al. 2000; Bai et al. 2002), the use of these primers seems to greatly influence the overall yield of NBS sequences. To increase this number,

additional strategies need to be employed, such as those founded on primers based on the characteristic oat GLPL motif, the use of primers based on conserved regions of known cereal NBS-LRR genes (Bai et al. 2002) and the design of primers based on different NBS motifs specific for nonTIR sequences (Peñuela et al. 2002; Huettel et al. 2002). A second reason for the low yield of NBS sequences could be the strict selection of the fragment length in both amplification rounds (the aim of which was to reduce the sequencing of spurious sequences). This might leave out a number of NBS sequences, mainly those with introns. These form an important fraction of the NBS-LRR family [one-half of the nonTIR-NBS sequences of *Arabidopsis* (Meyers et al. 2003) and one quarter of those of rice (Bai et al. 2002)]. Introducing more relaxed criteria with respect to fragment size should increase the number of NBS sequences obtained.

Even though there is general agreement that the use of larger combinations of primers with low degeneracy is more fruitful than the use of just a few combinations of primers with high degeneracy (Shen et al. 1998; Noir et al. 2001), it is unlikely that sequencing efforts can be reduced if large samples of NBS sequences are to be obtained. It has been suggested that the screening of clones using restriction fragments comparisons should be avoided given the high identity of most paralogous sequences (Fourmann et al. 2001). However, restriction analysis of clones appears to be useful for obtaining a representative set of sequences from the different RGA loci of a species. In our case, nucleotide sequences with levels of identity up to 82% revealed different RFLP patterns in oats, indicating that most of the isolated sequences have a low global sequence identity and have specific genome locations. Accordingly, genetic analysis showed a lack of marker cosegregation, except for two markers in KO7_10_28, and genetic distances between RGA neighbor markers were greater than 5 cM (Fig. 3). These results might indicate that closely clustered and highly identical sequences— whatever these may be in this genome—have likely been missed by restriction enzyme clone screening.

The above observations do not preclude that several of the *A. strigosa* NBS sequences might be members of complex loci. Previous reports on the organization of R genes and RGA sequences indicate there to be large spaces between cluster members. For example, in the *Dm3* region of lettuce, an R gene is found every 120 kb on average (Meyers et al. 1998); in the *Mla* region in barley, 11 gene copies are distributed over a 240-kb region (Wei et al. 1999); in soybean, six RGAs are found over 300 kb (Peñuela et al. 2002). In terms of genetic distances, RGA clusters have been identified whose different members map at a 6- to 8-cM intervals (Madsen et al. 2003; Radwan et al. 2003). Furthermore, it has been reported that resistance genes and RGA clusters contain both closely related genes and members of other gene families (Leister et al. 1996; Madsen et al. 2003). The existence of a cluster formed by at least three related sequences (two identified by probe L7M2.2 and one identified by probe L7M2.1) in linkage group KO7_10_28 cannot therefore be ruled out.

We identified homoeologous clusters in linkage group KO17 formed by sequences L7M2.2 and III2.18. A second complex loci was also found in homoeologous KO17 and KO7_10_28 represented by sequences L7M2.3 and L8M3.11.

Association of RGAs with disease resistance loci

The linkage group positions of the RGAs localized in the present experiment were compared with the positions of known loci for inherited qualitative and quantitative disease resistance in oats. These positions were inferred from previous published maps, as displayed and described in Fig. 3. Comparisons among maps was facilitated by using the Gnomad database (Tinker 2003).

The ten KO linkage groups in which RGAs were mapped showed markers which have been reported to be linked to resistance loci using different oat segregations. In the KO map, genetic distances between these markers and the RGAs ranged from 0 cM to 20 cM. Given the relatively large confidence intervals for the localization of QTLs and the level of uncertainty introduced by comparative mapping, the associations found should be taken as a preliminary indication of the useful role RGAs might play in the identification of resistance loci in oats. This is especially important in a polyploid species like cultivated oat where the homoeology among certain chromosomes could cause uncertainty in the identification of true homologs in different maps. However, some of the associations observed in this work are reinforced by other observations, such as the association of an RGA with more than one resistance locus; this indicates that all of them might belong to the same resistance cluster. For instance, marker S3S1.24a maps in KO11_41+20 (Sect. 11) close to the position of two major loci for resistance to *P. coronata*, *Pc71* (Bush and Wise 1998) and *Pc39* (Wight et al. 2004) as well as a QTL (Prpcq4) for resistance to the same pathogen (H.W. Rines, personal communication) (Fig. 3).

Similarly, we observed associations between homoeologous RGAs and different resistance genes, indicating that RGAs likely identify homoeologous resistance loci. An example of this is that observed for the homoeologous RGA markers revealed by probes L7M2.2, L7M2.1 and III2.18 located in otherwise homoeologous regions of linkage groups KO6, KO7_10_28 and KO17, and which show associations with major, quantitative resistance loci for crown rust and BYD (Fig. 3). In this same KO6 region, two other RGAs, one from *A. sativa* (Cheng et al. 2002) and another from sorghum (Portyanko et al. 2001), have been located. Together, these results show that the RGAs tagging these genetic regions seem to belong to the same clusters of R genes that contain paralogous genes responsible for specific resistance to different *P. coronata* isolates and, at least for KO17, to two different pathogens —fungi and viruses. The clustering of oat R genes conferring resistance to different races of a pathogen has been well documented by genetic analysis (Marshall and Shaner 1992; Sebesta et al. 2000). Close linkage between

the genes conferring resistance to the two main fungal diseases of oats has also been observed (Chong et al. 1994). At the molecular level, individually cloned genes belonging to the same cluster encode different resistance specificities for the same pathogen, as in locus *Mla* for powdery mildew resistance in barley (Halterman et al. 2001). Further, it has been shown that different genes physically linked in the same cluster may encode resistance specificities to two different pathogens—for example, in a cluster in the potato genome, *Rs1* codes for resistance to potato virus *X* and *Gpa2* for resistance to nematodes (van der Vossen et al. 2000).

The low level of polymorphism shown for RGAs between *A. strigosa* and *A. wiestii* limited to two the number of RGAs that could be directly mapped on the diploid segregating population, but none of these were found to be associated to any of the two resistance loci, R264B and *Pca*, mapped on that population. However, the linkage group on which R264B was located (Yu and Wise 2001) has been described as being equivalent to *Asw*BF (Portyanko et al. 2001) and homoeologous to regions of the linkage groups KO24_26_34 (Sects. 24 and 26) and KO22_44+18 (Sect. 22) (Wight et al. 2003). In these two KO regions (Fig. 3), two RGA markers (L7M4.9a and b) were positioned. This suggests these two markers are candidate loci for R264B-2. Interestingly, QTLs for partial resistance to crown rust (PrPcq3) and BYD (Bydq1 and Bydq Pav-NY) have been located on these homoeologous regions using different hexaploid segregations (H.W. Rines, personal communication; Barbosa-Neto et al. 2000; Zhu et al. 2003), indicating that all of these genes could derive from the diploid ancestor. Similar associations can be postulated for the major locus *Pca* directly mapped at the terminal portion of the *Asw*AC group of the diploid map (Portyanko et al. 2001). Five resistance genes —*Pc81*, *Pc82*, *Pc83*, *Pc84* and *Pc85*—have been found to be tightly linked at this complex locus (Kremer et al. 2001; Yu and Wise, 2001) with isu2192 the RFLP marker most closely linked to this locus, at a distance of approximately 20 cM. The present mapping results for KO7_10-28 and their homoeologs indicates that the NBS sequences L7M2.1 and L7M2.2 are candidates for the *Pca* cluster. This association is further supported by other results. Thus, after examining RFLP markers (bcd1280 and isu2192) linked to different resistance loci in different mapping populations, Zhu and Kaeppler (2003) suggested that the *Pca* locus of *A. strigosa* might belong to the same gene family as the locus responsible for partial resistance to crown rust (Pcq2) carried by the hexaploid oat line MAM17-5 believed to be derived from *A. strigosa*. However, a second RGA cluster located distally exists in linkage groups KO7_10-28 and KO17, represented by L7M2.3 and L8M3.11. Based on the previously reported genetic distance between isu2192 and *Pca* in the diploid population (Kremer et al. 2001; Yu and Wise 2001), it cannot be ruled out that L7M2.3 and L8M3.11 are true co-members of R genes in this important *Pca* cluster. PCR markers from the different NBS sequences are being currently developed for use in direct mapping on the diploid population.

Six of the RGAs described here can be correlated with QTLs for disease resistance. QTL positions in the KO reference map inferred from other oat maps could be more prone to error than positions for major genes given the large map intervals covered by each QTL. Thus, the QTL for partial resistance to crown rust, Prpcq4 (H.W. Rines, personal communication), has been tentatively assigned to either of two different loci on the reference KO map: linkage groups KO11_41+20 and KO14 (Fig. 3). RGAs belonging to different classes of resistance genes were mapped close to these positions: one NBS-LRR sequence and one RLK-LRR. There is evidence that R clusters may contain functionally related genes not necessarily similar at the sequence level. This is the case for the tomato *Pto* cluster which encodes five related kinases and an NBS-LRR protein (Salmeron et al. 1996). In sugarcane, several clusters have also been observed containing expressed RGAs with different protein domains (Rossi et al. 2003).

Other linkages between QTLs and RGAs seems to be less conflictive; for example, those discussed above that also encompass major resistance loci. These linkages are indicative of the possible involvement of RGAs with quantitative disease resistance as previously reported in other plants (Backes et al. 2003 and references therein). This supports the hypothesis that genes for QTLs may be allelic forms of major genes (Robertson 1985); R genes might act in a nonrace-specific manner or in a less evident way.

Several oat RGAs were not associated with resistance genes; this is not surprising since many more NBS-LRR and RLK sequences must exist in the oat genome compared to the few resistance loci already mapped. Moreover, not all of the disease resistance loci mapped in relation to molecular markers were associated with the RGA described in the present study (Fig. 3). We failed to identify RGAs associated with any resistance gene to *P. graminis*, which again demonstrates that the oat RGA set is far from complete. Nevertheless, the control of stem rust resistance might be exerted by a different kind of gene than that assayed in these experiments. Cheng et al. (2002) isolated an oat sequence homologous to the RLK gene *Lrk10* (Feuillet et al. 1997) which mapped closely linked to *Pg9*, a resistance gene to stem rust. The molecular characterization of RLK sequences isolated in our work indicates that they belong to a different group of protein kinases than *Lrk10* . This demonstrates that the molecular identification of R genes requires a wider screening of sequences. This should be done using information on conserved domains in the different resistance gene analogs already cloned.

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