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ORIGINAL PAPER

Identification of RFLP and NBS/PK profiling markers for disease resistance loci in genetic maps of oats

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Abstract Two of the domains most widely shared among *R* genes are the nucleotide binding site (NBS) and protein kinase (PK) domains. The present study describes and maps a number of new oat resistance gene analogues (RGAs) with two purposes in mind: (1) to identify genetic regions that contain *R* genes and (2) to determine whether RGAs can be used as molecular markers for qualitative loci and for QTLs affording resistance to *Puccinia coronata*. Such genes have been mapped in the diploid *A. strigosa* × *A. wiestii* (Asw map) and the hexaploid MN841801-1 × Noble-2 (MN map). Genomic and cDNA NBS-RGA probes from oat, barley and wheat were used to produce RFLPs and to obtain markers by motif-directed profiling based on the NBS (NBS profiling) and PK (PK profiling) domains. The efficiency of primers used in NBS/PK profiling to amplify RGA fragments was assessed by sequencing individual marker bands derived from genomic and cDNA fragments. The positions of 184 markers were identified in the Asw map, while those for 99 were identified in the MN map. Large numbers of NBS and PK profiling markers were found in clusters across different linkage groups, with the PK profiling markers more evenly distributed. The location of markers throughout the genetic maps and the composition of marker clusters indicate that NBS- and PK-based markers cover partly complementary regions of oat genomes. Markers of the different classes obtained were found associated with the two resistance loci, *PcA* and *R-284B-2*, mapped on Asw, and with five out of eight QTLs for partial resistance in the MN map. 53 RGA-RFLPs and 187 NBS/PK profiling markers were also mapped on the hexaploid map *A. byzantina* cv. Kanota × *A. sativa* cv. Ogle. Significant co-localization was seen between the RGA markers in the KO map and other markers closely linked to resistance loci, such as those for *P. coronata* and barley yellow dwarf virus (Bydv) that were previously mapped in other segregating populations.

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Introduction

Cultivated oats (*Avena sativa*) is an economically important crop, the grain yield and grain quality of which can be severely reduced by pathogens. Barley yellow dwarf virus (Bydv) can cause important losses, as can fungal diseases such as those caused by *Puccinia graminis* and *Blumeria graminis*. The most important of all, however, is crown rust, caused by *P. coronata*, a disease that strikes oats worldwide. The deployment of disease-resistant varieties remains the most economically effective and environmentally safe method for controlling these diseases. Several genes conferring resistance to *P. coronata* and the other fungal diseases are present in cultivated oat genotypes. However, the protection offered by some fungal resistance (*R*) genes has been overcome by newly emerging pathogen races; new sources of resistance are therefore

needed for breeding programs. Besides single inherited genes conferring complete race-specific resistance, partial resistance to *P. coronata* has also been recognized. Such resistance is usually quantitatively inherited and appears to be less affected by shifts in pathogen virulence than race-specific resistance. It is, therefore, considered durable in the field (Simons 1972).

The genetic mapping of monogenic *R* genes and quantitative trait loci (QTLs) can identify closely linked molecular markers of use for marker-assisted selection resistance in breeding programs, potentially accelerating the production of new resistant genotypes. More than 100 genes conferring resistance to pathogens have been identified in oats, though only a handful have been genetically mapped. This information is documented in the Oat Database (http://avena.agr.gc.ca/oatgenes/traits_all.php?map=All&trait=Crown+rust+resistance) and reported by Yu and Herrmann (2006), Chen et al. (2007), Jackson et al. (2008), McCartney et al. (2011), Satheeskumar et al. (2011). The mapping of a number of QTLs conferring resistance to *P. coronata* and Bydv has allowed the identification of a small number of oat genetic regions involved in resistance to these diseases Oat Database (http://avena.agr.gc.ca/oatgenes/traits_all.php?map=All&trait=BYDV+resistance/) and reported by Portyanko et al. (2005) and Acevedo et al. (2010). The increasing efficiency of molecular marker technologies, plus our improving understanding of the molecular basis involved in disease resistance, offers the possibility of generating new markers inside functional genes. The integration of these putative functional markers in framework genetic maps constructed with anonymous markers might help in the identification of the genetic regions responsible for phenotypic resistance. Moreover, the positioning of functional markers in maps in which major genes or QTLs for resistance have previously been identified would help to characterize these loci in relation to the molecular basis of resistance.

The conserved domains in most *R* genes that have so far been cloned have facilitated the use of PCR-based strategies for isolating and cloning new resistance gene family members or resistance gene analogues (RGAs) in numerous plant species. The NBS-LRR gene family represents the largest class of currently identified plant resistance genes. These genes code for proteins containing a nucleotide binding site (NBS) and a leucine-rich repeat (LRR) domain. Other *R* gene classes encode protein kinase (PK) domains either in the form of cytoplasmic proteins such as serine/threonine kinases or transmembrane proteins (Tameling and Takken 2008). The genetic and physical mapping of RGAs, the isolation of which was based on conserved motifs in the NBS domain, has been reported for a number of plant species (McHale et al. 2009 and references cited therein). These reports frequently mention the cosegregation of RGA markers and resistance loci. In some instances, efficient markers for disease resistance were found using strategies that did not involve *R* genes. These markers showed the structural characteristics of RGAs (Zhang et al. 2010; Biruma et al. 2012). The reported co-segregation between RGAs and resistance loci is likely based on the organization of plant disease resistance genes in clusters that contain both functional and non-functional members. This organization increases the probability of RGAs serving as efficient markers of resistance phenotypes and provides a sequence resource for cloning unknown *R* genes.

The spectrum of RGAs isolated from oats using degenerate primer strategies has been limited and only a few have been genetically mapped (Cheng et al. 2002; Irigoyen et al. 2004). Advances in genomics have led to a growing list of expressed sequence tags (ESTs) for some plant species, thus allowing RGAs in EST databases to be identified via data mining. In species such as oats, in which the number of ESTs is still relatively low and of limited use for checking gene predictions given their short sequence length, cDNA sequences with characteristics of RGAs isolated from related species with larger databases could provide useful hybridization probes for mapping RFLPs. In barley, Madsen et al. (2003) isolated and mapped RGA-cDNA, and in wheat, McFadden et al. (2006) mapped a set of 49 RGAs out of 211 retrieved from the EST databases for these two species. Recently, Satheeskumar et al. (2011) showed the use of wheat RGAs for obtaining markers linked to *Pc68,* an oat *P. coronata* resistance locus. The use of clones from these collections for detecting RGAs in oat might contribute towards the establishment of homology relationships among linkage groups of different oat maps and possibly with those of wheat and barley.

Fingerprinting techniques and other AFLP-like methodologies have been developed to increase the number of potentially functional molecular markers by means less laborious than RFLP. One of these, known as motif-directed profiling (van der Linden et al. 2005), targets the conserved motifs in functional domains of gene family members. It therefore samples variation within the members of a particular gene family (González et al. 2010). NBS profiling, which uses degenerate oligonucleotides directed towards the NBS domain of *R* genes, has been successfully used with different species. For example, RGA fragments generated by this method have been efficiently mapped in apple, lettuce, peanut, common bean and potato (Calenge et al. 2005; Syed et al. 2006; Leal-Bertioli et al. 2009; Jacobs et al. 2010; Bakker et al. 2011). On genetic maps, these markers show similar characteristics to RGAs isolated by other methods, such as their organization in clusters and their genetic proximity to major genes and QTLs for resistance. Motif-directed profiling has also been

successful in the generation of markers using other conserved motifs, for example the LRR domain of *R genes* (Whitaker et al. 2010) and the conserved domains of peroxidases (González et al. 2010). Recently, primers based on PK conserved motifs have been used in PK profiling. The profiles obtained were composed of large numbers of bands that, when mapped on potato, showed good genome coverage (Vossen et al., in preparation).

The present study examines the feasibility of different techniques for obtaining new RGA markers in oats. The specific aims of this work were (1) to increase the number of markers potentially related to *R* genes, (2) to identify putative genomic regions involved in resistance in oats and (3) to search for genetic co-localization with previously phenotypically defined disease resistance loci in different oat maps. The results (1) identify RFLP markers derived from *R* gene-like ESTs from barley and wheat, plus RGA genomic sequences previously obtained from different *Avena* species; (2) identify markers using NBS/PK profiling, employing both genomic and cDNA as templates; and (3) map the newly obtained markers on three maps: (a) the diploid map *A. strigosa* × *A. wiestti*, in which two loci for resistance to *P. coronata* have been identified (Yu and Wise 2000; Portyanko et al. 2001), (b) the *A. sativa* hexaploid map MN841801-1 × Noble-2, in which eight QTLs for partial resistance to *P. coronata* have been identified (Portyanko et al. 2005; Acevedo et al. 2010) and (c) the hexaploid reference map *A. byzantina* cv Kanota × *A. sativa* cv Ogle (Wight et al. 2003) in which resistance loci previously mapped in other segregating populations can be located by map comparisons.

Materials and methods

Plant material

Recombinant inbred lines (RILs) of three mapping populations and their corresponding parents were used in this study. The Asw population—provided by Dr. R. Wise, Iowa University, USA—was formed by 100 $F_{6:8}$ RILs derived from a cross between the diploid species *A. strigosa* (CI 3815) and *A. wiestii* (CI 1994). The *A. strigosa* parental is resistant to 40 isolates of *P. coronata* in the Iowa State University collection, whereas *A. wiestii* is susceptible to all of them (Yu and Wise 2000). The MN population—provided by Dr. H. R. Rines, Minnesota University, USA—consisted of 158 $F_{6:8}$ RILs from the cross between the hexaploid lines MN841801-1, which has shown partial and durable resistance to different populations of crown rust for more than 30 years, and Noble-2, which is a cultivar susceptible to these isolates (Portyanko et al. 2005). The KO population was formed by 76 $F_{9:10}$ RILs—provided by Dr. N. Tinker, Agriculture and Agri-Food Canada, Ottawa, Canada—from the cross between the hexaploid species *A. byzantina* cv. Kanota and *A. sativa* cv. Ogle (O'Donoughue et al. 1995).

RFLP analysis

Thirty-one RGA clones were used as probes in RFLP experiments. These included (a) ten DNA clones isolated from *Hordeum vulgare* cv. Regatta (Madsen et al. 2003), out of which eight correspond to expressed sequences; (b) a set of RGA-ESTs clones including six from barley and eight from wheat; and (c) seven genomic RGA clones from different *Avena* species (Irigoyen et al. 2004). cDNA barley clones were obtained from the Research Institute for Bioresources (Okayama University, Japan) and from the Institut für Planzengenetik und Kulturpflanzenforschung (Gatersleben, Germany). cDNA wheat clones were obtained from the Kihara Institute for Biological Research (Yokohama City University, Japan). The RGA names and accession numbers are indicated in Supplementary Table S1. The barley and wheat clones were: (1) RGAs previously located in different linkage groups on barley and wheat maps (Madsen et al. 2003; McFadden et al. 2006), and/or (2) RGAs previously mapped near resistance loci on those barley and wheat maps, and/or (3) RGAs with nucleotide sequence identities of under 70 %, and hence likely members of different RGA families. DNA was extracted from young leaves of the parents and RILs as described by Irigoyen et al. (2004). DNA samples were digested for polymorphism surveys with three restriction enzymes: *Dra*I, *Eco*RI and *Hind*III. Restriction digestion, Southern blotting, labelling of probes by non-radioactive procedures and filter hybridization were performed as described by Loarce et al. (1996). The hybridization temperatures were 65 °C for the *Avena* probes and 60 °C for heterologous probes. RFLP markers were designated by the name of the RGA clone followed by a letter indicating the polymorphic band.

NBS/PK profiling using genomic DNA

DNA of parent and RIL lines was isolated from young leaves using the DNeasy 96 Plant Kit (Qiagen Inc., California, USA). NBS/PK profiling was performed as described by van der Linden et al. (2004) with some modifications. The restriction enzymes, *Alu*I, *Hae*III, *Mse*I and *Rsa*I, were used for digestion of 400 ng of genomic DNA. Adapters were ligated to the restriction sites. The digestion and ligation of adapters was performed in a single reaction at 37 °C. The amplification of NBS- or PK-specific fragments was performed by PCR using NBS- or PK-specific primers in combination with an adapter primer.

The annealing temperature was 55 °C. The NBS-specific primers were NBS1, NBS2 and NBS5A described by van der Linden et al. (2004), the NBS3 primer described by Calenge et al. (2005) and the NBS6 primer described by Brugmans et al. (2008). The PK primers used were those described in Vossen et al. (in preparation): PK1Fa, PK1Fb, PK1F2a, PK3Fa, PK3Fb, PK4R1a and PK4r1b targeting different regions of the conserved PK domain. The PCR products were reamplified using the adapter primer labelled with the fluorochrome IRDye700 for the visualization of individual fragments in denaturing polyacrylamide gels using a LI-COR 4300 DNA Analysis System (LI-COR Biosciences, Lincoln, NE, USA). Polymorphic markers obtained by NBS/PK profiling were designated with the name of the restriction enzyme and the degenerated primer used, followed by a number or letter indicating the relative position of the polymorphic band from the top of the gel (e.g., *Alu*I in combination with primer PK1Fa scored at position 2: AluI/PK1Fa_2).

NBS/PK profiling using cDNA

Total RNA of young leaves from *A. strigosa*, *A. wiestii*, MN841801-1 and Noble-2 was extracted with Tripure Isolation Reagent (Roche, Germany) according to the manufacture's instructions. Total RNA was treated with Turbo RNase-free DNase (Ambion, Life Technologies, USA) to remove all contaminating DNA from the samples. Poly-A^+ RNA was subsequently extracted from the total RNA using the Dynabeads Oligo $(dT)_{25}$ (Invitrogen, Life Technologies, USA). cDNA synthesis was performed using 40 ng of mRNA, employing the SMARTer PCR cDNA Synthesis Kit (Clontech, Takara Bio, Japan) according to the manufacturer's instructions. NBS/PK profiling was carried out as previously described for genomic DNA using 100 ng of cDNA digested with the restriction enzymes *Alu*I, *Mse*I or *Rsa*I.

RGA cloning and sequence analysis

Several NBS and PK markers from NBS/PK profiling were excised from the gel to determine their sequences using an LI-COR Odyssey scanner (LI-COR Biosciences, Lincoln, NE, USA). The fragments were gel purified using the QIAEXII Gel Extraction Kit (Qiagen, USA) and reamplified using identical conditions to the second PCR run of the NBS/PK protocol. PCR products were checked on agarose gels and purified with the Qiaquick Gel Extraction Kit (Qiagen, USA) and cloned in the pGEM-T-vector (Promega, USA). Three clones of the right size were selected for sequencing using the BigDye Terminator system and an ABI automated sequencer from Applied Biosystems (USA). Sequences were analysed using CodonCode Aligner v.3.7.1 software (CodonCode Corporation, Dedham, MA, USA) and compared with entries in the NCBI nucleotide database using the BLASTX and BLASTN programs. Those with significant sequence similarity to resistance genes of the NBS-LRR class or PKs were classified as RGAs. These were translated into protein sequences and aligned using the ClustalX programme with the default options. Several positive RGAs were used as probes in RFLP analysis and the markers obtained were bin mapped on the reference maps (see below). These RFLP markers were named with the initial letters of the restriction enzyme and primer used in the NBS/PK profiling which generated the excised band, followed by the number of the band and the number of the positive clone (e.g. the probe derived from clone 4 from the Alu/PK4R1b_1 band would take the name AP4b14).

Bin mapping

The data marker sets and maps of Portyanko et al. (2001), Portyanko et al. (2005) and Acevedo et al. (2010) and the framework marker data set of Wight et al. (2003) were used to select a subset of offspring genotypes for the Asw, MN and KO mapping populations, respectively, using MapPop v.1.0 software (Vision et al. 2000). Genotype selection was performed with the SAMPLEMAX command, which identifies the most informative genotypes from a mapping population based on the maximum number of recombination breakpoints distributed over the genome. The number of selected genotypes was 36 for the Asw diploid population, and 38 and 17 for the hexaploids MN and KO, respectively. The number of selected lines was higher than in other studies (Howard et al. 2005; Fernández-Silva et al. 2008) with the aim of minimizing the distribution of poorly homogeneous markers and the genetic distance between markers observed in some regions of poor resolution (mainly in the Asw and MN maps).

Markers obtained by RFLPs and NBS/PK profiling were included in the bins limited by consecutive markers in the Asw, MN and KO reference maps using MapPop v.1.0 software, employing the Bin Mapping command (Vision et al. 2000). Also included in these maps were the RFLP markers revealed by four *A. strigosa* NBS probes (L7M2.3, L8M3.8, II2.17 and III2.18) plus three *A. strigosa* probes (based on the kinase genes S3S1.24, S3S1.1.6 and S5S2.2.11) previously located in some of these maps (Irigoyen et al. 2004; Loarce et al. 2009).

Co-localization of NBS/PK profiling markers with resistance loci

The KO framework map was used to test for a possible association between the distribution of RGA markers

mapped in this study and the distribution of phenotypic loci for pathogen resistance previously located either directly in the KO mapping population or through comparative mapping. The version of the KO map used had 227 bins defined by framework markers (Wight et al. 2003). The position data for the markers closely linked to the major genes and peak markers of QTL for resistance to *P. coronata* and to Bydv are publicly available in the Oat Database (http://avena.agr.gc.ca/oatgenes/). Loci described in Zhu et al. (2003), Zhu and Kaeppler (2003), Chen et al. (2007), Jackson et al. (2008), Acevedo et al. (2010), McCartney et al. (2011), Satheeskumar et al. (2011) were also included The coincidence of a marker(s) for a major gene or QTL for resistance and NBS/PK profiling markers was analysed by counting the number of bins occupied by markers closely linked to resistance loci or by one or more peak markers for resistance QTLs, the number of bins occupied by RGAs and the number co-occupied by resistance markers and RGAs. Chi-squared analysis was used to test the null hypothesis, assuming the independent distribution of bins occupied by resistance loci and bins occupied by RGAs.

Associations among RGA markers and QTLs for agronomic traits were also examined. Information about the position of these QTLs and their closely linked markers was also taken from the Oat Database (http://avena.agr.gc.ca/oatgenes/).

Results

Development of markers

RFLPs-RGAs

Southern blot analysis using the 31 RGA probes showed a range of hybridization patterns in digested genomic DNA from the parents of the three mapping populations. Supplementary Table S2 describes the restriction enzyme selected for each probe, the hybridization patterns and the number of polymorphic bands produced by each RGA probe analysed. Eleven clones produced a smear of hybridizations with few clear bands; these were discarded from analysis. The number of bands produced by the probes in the diploid parents served to classify them as single copy sequences (1–2 bands), low copy number sequences (3–6 bands) or repeated sequences (more than 6 bands). The RFLP profiles of the hexaploids indicated that the set of NBS sequences evaluated has maintained a rather stable copy number during speciation. The diploid parents were polymorphic for 19 probes, whereas this fell to 15 and 12 for the hexaploids Kanota and Ogle and MN841801-1 and Noble-2, respectively. The genomic and cDNA clones showed similar levels of polymorphism among the three parental genotypes; the exceptions were the barley clones which showed more polymorphism between the diploids *A. strigosa* and *A. wiestii* than between the parents of the hexaploid populations. As a whole, 20 out of 31 clones gave polymorphic band(s) in at least one of the populations. Within the polymorphic clones, 25 % revealed polymorphism in two populations and 50 % produced polymorphic bands in all three populations. These types of probe helped identify regions of potential homology or homoeology among genomes, although in many instances no polymorphism was revealed by certain probes for some bands. Fifteen of the 24 barley and wheat probes showed polymorphism in at least one mapping population. Taken together, these results indicate that RGA probes from closely related species can increase the number of RGA loci detectable in *Avena*; those tested here may be expected to detect useful polymorphism in other *Avena* populations.

NBS/PK profiling

The five degenerate primers based on the NBS domain, and the seven based on the PK domain, were used in combination with four restriction enzymes (*Alu*I, *Hae*III, *Rsa*I and *Mse*I). The 48 primer-enzyme (PE) combinations were assessed in the parents of the diploid mapping population (*A. strigosa* and *A. wiestii*) and the hexaploid parents MN-841801-1 and Noble-2. The hexaploid parents Kanota and Ogle were not tested with *MseI* resulting in 36 PE combinations. For each PE combination, 40–60 distinct bands ranging from 200 to 800 bp were obtained (Supplementary Fig. 1A). Different fragment patterns were generated with primers targeting the same conserved motif, but with slightly different nucleotide compositions, indicating that different sequences were targeted by these primers. Although all the PE combinations generated clear amplification patterns, only a fraction of them showed polymorphism between the parents of each mapping population. Supplementary Table S3 shows the PE combinations that produced polymorphic fragments in each population and the number of fragments obtained from each PE combination. The percentage of NBS/PK polymorphic PE combinations was 33.3 % for the diploid parents, 22.9 % for the MN-841801-1 and Noble-2 parents, and 88.8 % for the Kanota and Ogle parents. The differences found among Kanota and Ogle and the other two pairs of parents are explained taking into account that the amplification products of Kanota and Ogle DNA were fractionated in a LI-COR analyser with a more powerful laser than the amplifications of DNA from the other parents. Despite this, differences in the level of polymorphism depending on the primer used were evident and common to the three pairs of parents. PK profiling was very effective in

the detection of polymorphism between the three pairs of parents. Indeed, PK profiling generated a larger total number of polymorphic bands than NBS profiling (318 vs. 166). Primers PK3Fa, PK3Fb, PK4R1a and PK4R1b in combination with any enzyme generated more polymorphism than primers PK1Fa or PK1Fb. PK1F2a never produced scorable polymorphisms. In NBS profiling, combinations containing primers NBS1 and NBS2 detected the highest level of polymorphism. *Mse*I was clearly the least successful enzyme in combination with any NBS or PK primer. Overall, the enzyme/primer combinations generated 151 polymorphic fragments between the diploid parents, 66 between MN-841801-1 and Noble-2, and 267 between Kanota and Ogle.

Sequence analysis of NBS and PK fragments

A number of polymorphic bands produced by NBS/PK profiling were excised from the gels, reamplified, cloned and sequenced. The excised bands were selected from among those mapping in the Asw and MN linkage groups close to previously identified resistance loci (see Bin mapping section). The bands were selected independently of their intensity and separation from other bands. Sixteen bands were obtained from PK profiling and four from NBS profiling. All 20 bands (cloned and sequenced) contained the degenerate primer used to amplify the sequences. Only one NBS fragment contained an additional sequence with significant similarity to genes containing NBS domains, whereas four fragments showed similarity to genes with PK domains (Supplementary Table S4). These five cloned bands were used as probes in RFLP experiments. Four of these were polymorphic between the parents of some of the three mapping populations and they could be incorporated in the genetic maps (see "Bin mapping").

NBS/PK profiling using cDNA

The proportion of sequences obtained from the NBS and PK fragments that showed significant similarity to known *R* genes and RGAs was lower than expected, given those reported for other plant species (van der Linden et al. 2004; Calenge et al. 2005) and Vossen et al. (in preparation). The explanation for this might lie in the poor recognition of target sequences by the primers in genomic DNA because of the locations of introns. To test this idea, the adequacy of the primers was examined using cDNA as a template. A total of 32 PE combinations were used on cDNA generated from leaf RNA from *A. strigosa*, *A. wiestii*, MN841801-1 and Noble-2. Fragment patterns were less complex than those obtained with genomic DNA, both in the diploid and hexaploid genotypes (Supplementary Fig. S1B). Typical profiling patterns involved from 20 to 50 bands, most showing little variation in intensity. The majority were monomorphic between both the diploid and hexaploid parents.

The molecular characterization of the fragments was performed using 20 bands from the cDNA NBS profiling gels and 26 from the cDNA PK profiling gels. Bands were selected that met the following criteria: (1) intense and well separated from neighbours; (2) longer than 200 bp; (3) generated from primers NBS5A and NBS6 (targeting the kinase-2 motif), and PK primers Pk1Fa, Pk3Fb and PK4R1b (targeting different PK motifs). For 14 (71.4 %) of the 20 bands excised from the NBS profiling gels, and for 24 (92.3 %) of 26 bands from the PK profiling gels, sequences were obtained that showed significant similarity to known proteins deposited in the GenBank database. Within each group of productive sequences, 10 (71.4 %) showed strong similarity to NBS-LRR proteins, whereas 19 (79.16 %) showed strong similarity to receptor-like serine–threonine kinases. The successful amplification seen with the cDNA showed that the primers used for NBS profiling adequately amplified the target sequences (Supplementary Table S5). Moreover, nine sequences were located inside coding sequences that had no obvious NBS or PK domains.

All the sequences obtained with NBS primers could be aligned to each other after translation (Supplementary Fig. S2A). All contained the kinase-2 and RNBS-B motifs. The larger ones also contained the GLPL motif. All were characterized as belonging to the non-TIR-NBS-LRR class of *R* genes by the presence of the W residue at the end of the kinase-2 motif. The translated PK sequences showed the characteristic amino acids that define the conserved subdomains (I to XI) of the catalytic domain of PKs. The HRD residues in the subdomain VIb of the active centre were observed in several sequences. All the sequences could be aligned to each other except for four with multiple stop codons, probably representing pseudogenes (Supplementary Fig. S2B).

Bin mapping

Bin mapping accuracy was confirmed by analysing several framework markers of each map in the selected lines. The positions obtained by MapPop for these markers were the same as shown in their respective framework maps.

The polymorphic bands obtained using the different techniques were analysed in the selected lines of each mapping population and bin mapped. Figures 1 and 2 show the resulting diploid Asw map and the hexaploid MN map. The hexaploid map KO is provided in Supplementary Fig. S3. An example of the segregation of profiling markers is shown in Supplementary Fig. S1A. The main features of the bin maps are summarized in Table 1. The RGAs mapped included RFLP-RGAs from the oat, barley and

wheat NBS probes. The RFLP-RGAs previously detected by genomic oat probes in some of the maps studied here are also included (Irigoyen et al. 2004; Loarce et al. 2009). Supplementary Table S2 summarizes the linkage data obtained and places each RFLP-RGA in an interval between the framework markers defining the bin. The RGAs mapped also included markers generated by NBS/PK profiling (Supplementary Table S3), along with RFLP-RGAs obtained from probes derived from the excised bands. The latter bands showed similarities to either NBS or PK sequences (Supplementary Table S4).

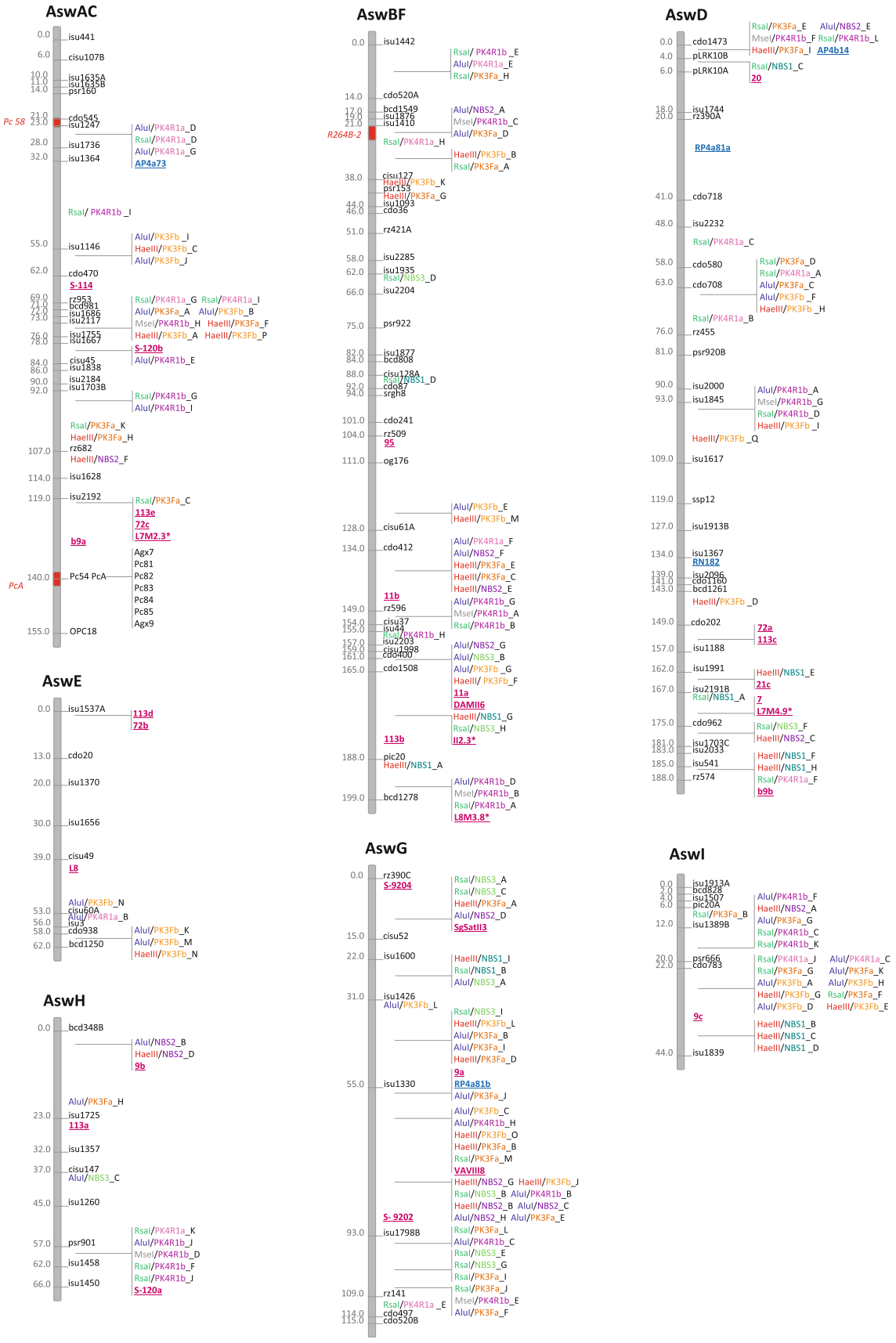
For each individual map, 240 markers for KO, 184 for Asw and 99 for MN were placed into bins. Several of the RGA-RFLP markers revealed by a single probe were assigned to regions on linkage groups described as homoeologous in the KO map (Wight et al. 2003). For example, two markers detected by probes RGA97 and L7M4.9 were found in KO22_44+18 and KO24_26_34. Another two detected by probes S-9202 and RGA9 were shared by KO6, KO16_23 and KO19+27. The low number of common markers found in the different MN linkage groups map prevented the detection of consistent homoeologous regions, although markers RGA-11, VAVIII8 and AP4b14b suggest the existence of relationships among the MN9, MN16 and MN22 groups.

The largest class of markers in each map was generated by NBS/PK profiling. Most of the polymorphic fragments obtained with the different enzyme/primer combinations used were located in the Asw and MN maps (91 and 97 %, respectively, compared to 70 % in the KO map). Thus, the advantage of using a more powerful fragment analyser that allowed the visualization of a larger number of fragments in Kanota and Ogle than in the other two pairs of parental lines did not translate into a higher proportion of polymorphic fragments (Supplementary Table S3). This was likely due to the increased probability of detecting co-migrating fragments in the Kanota and Ogle gels. Markers were found in nearly all linkage groups of the *Avena* maps. Seven out of 8 linkage groups in the Asw map, 23 out of 31 in the MN map, and 25 out of 29 in the KO map presented RGA markers. The exceptions were the small linkage groups of each map that contained few framework markers.

Table 1 Summary of the bin mapping of RGA markers in the three mapping populations Asw (*A. strigosa* × *A. wiestii*), MN (MN841801-1 × Noble-2) and KO (Kanota × Ogle)

	Asw	MN	KO
Total no. of mapped markers	184	99	240
RGA-RFLP NBS	33	30	46
RGA-RFLP PK	3	5	7
NBS profiling	38	29	64
PK profiling	110	35	123
Total no. of linkage groups	8	31	29
With RGAs	7	23	25
Total no. of bins	118	157	227
With RGAs	54	51	110
Total no. of RGA clusters	40	23	55
Total no. of RGA singletons	34	32	62

A notable proportion of bins contained RGA markers. In the KO and Asw maps, the proportion reached 48 and 45 %, respectively; in the MN map, this fell to 32 % in agreement with the low level of polymorphism detected between MN841801-1 and Noble-2. Markers assigned to a bin were of two types: (1) those with the same genotype in all the analysed lines; and (2) those with slight variations in their segregations. The latter were frequently seen in bins covering large genetic distances (larger than 15 cM). In this case, when a marker or a group of markers shared the same segregation, and this was different from that observed for a second marker or group of markers, the two (groups of) markers were maintained separately within a bin. RGAs were not equally distributed throughout the chromosomes. Indeed, all three maps showed a large number of clusters formed by two or more RGAs markers that shared a common segregation pattern. This clustering was especially notorious for markers obtained by both NBS and PK profiling. The largest number of clusters was observed in linkage groups AswBF and AswD with nine, and in KO7_10_28 with six. The MN groups had the smallest number of clusters, probably due to the smaller number of markers in this map. Several clusters were composed of either PK or NBS fragments generated by the same primer, e.g. in linkage group AswAC at 55 cM, in MN1 at 8 cM, and in KO5 at 18 cM. These fragments could have been derived from a single genomic sequence with sites for different restriction enzymes. However, the majority of clusters were composed of fragments generated from different primers, indicating that different genomic sequences had been targeted. These homogeneous clusters contained only PK or NBS primer-derived fragments (mapping, for example, to AswAC at 76 cM, to MN9 at 63.4 cM or to KO4_12 at 18 cM). However, mixed clusters containing both PK and NBS markers were more frequently observed (mapping, for example, to AswBF at 161 cM, to MN3 at 54 cM or to KO17 at 17 cM). Several clusters, mainly in the KO map, were located in areas of known reduced recombination attributed to the putative existence of centromeric regions or translocation breakage points (O'Donoughue et al. 1995; Wight et al. 2003). In such cases, the observed genetic clustering of RGA markers might not be attributable to any distinct feature of the RGA and *R* gene families. Examples of this clustering was observed at 75–100 cM in KO3+38, at 12–16 cM in KO2,


AswAC
AswBF
AswD
AswE
AswG
AswH
AswI

◀ **Fig. 1** Location of RGA-RFLP and NBS/PK profiling markers in the *A strigosa* × *A. wiestii* map (Portyanko et al. 2001).To the *left* of each linkage group, genetic distances are indicated in cM. To the *right* of each linkage group, framework markers are indicated in *black*; RGA-RFLP markers are indicated in *magenta* and *underlined*; RFLP-RGA markers previously mapped (Loarce et al. 2009) are indicated in *magenta* and with an *asterisk*; RFLP markers derived from NBS/PK profiling bands are indicated in *blue* and *underlined*. NBS/PK profiling markers are indicated following a colour code to identify the restriction enzyme and the primer that generated the marker. *Red* boxed regions on the linkage groups indicate the positions of previously mapped loci for resistance to *P. coronata* (Yu and Wise 2000; Kremer et al. 2001). The names of these resistance loci are indicated in *red* on the *left* of each linkage group (color figure online)

at 55–83 cM in KO7_10_28, at 43–56 cM in KO15, at 5–17 cM in KO17 and at 9–15 cM in KO30.

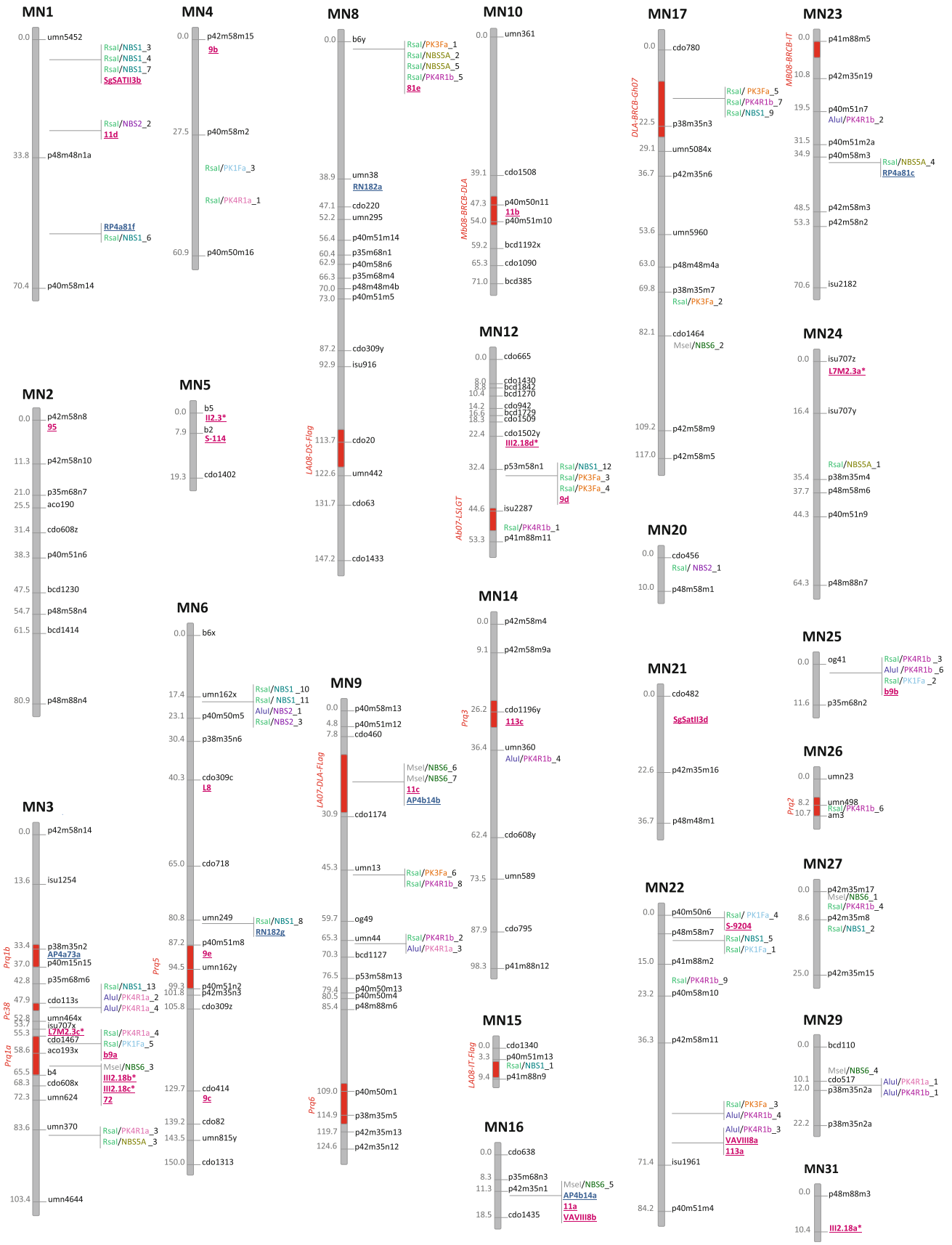
In the three maps, RGA-RFLPs were located either singly—roughly 50 %—or in the same cluster as the NBS/PK profiling markers. Within these, a number of RGA-RFLP markers based on the NBS domain were located in the same bins as in NBS profiling (10, 10 and 12 for the Asw, MN and KO maps, respectively, i.e., 26–33 % of the RGA-NBS probes mapped). These data indirectly showed that the NBS profiling markers identified regions harbouring NBS sequences, confirming the sequence analysis results of the profiling markers. A parallel conclusion was reached from the comparison of bins co-occupied by RGAs obtained from PK-based probes and PK profiling markers in the KO map. One of the three RFLPs mapped with PK probes was located in the same bin as a PK profiling marker. If most of the degenerated primers used in NBS/PK profiling were to detect sequences containing an NBS or a PK domain, as suggested by the cDNA profiling results, the two kind of motif-directed profiling employed here might be considered complementary since they reveal partially overlapping RGA sets.

Probes from excised NBS/PK profiling fragments with similarity to NBS or PK genomic sequences were used in RFLP experiments with two purposes: (1) to validate the map positions of the corresponding NBS/PK profiling fragments and (2) to determine their homologous locations in the studied oat maps. Supplementary Table S4 summarizes the mapping data of these markers. The map positions of the RFLPs produced by probes RN182 and AP4a73 were the same as those obtained for their corresponding profiling markers *Rsa*I/NBS1_8 and *Alu*I/PK4R1a_G, respectively. The first was close to the partial resistance QTL *Prq5* on MN6 (Portyanko et al. 2005) and the second was located on a homologous region to *Pc58* (Jackson et al. 2008) in the AswAC linkage group (Figs. 1, 2). The two other clones that generated polymorphic hybridization patterns (AP4b14 and RP4a81) identified loci in linkage groups other than those where the original fragments were mapped. However, some of these RFLPs clustered with other PK profiling markers, e.g. in linkage groups AswD, AswG or KO3_38.

Co-localization of phenotypic resistance loci and RGA markers

RGA markers were found close to the two *P. coronata* resistance loci in the *Asw* map. The RGA-RFLP marker b9-1 assigned to the *Asw*AC linkage group lay close to the *PcA* region. The latter consists of a least five recombinationally separable loci with different specificities within a 5 cM region (Yu et al. 2001). Six NBS/PK profiling markers mapped to the region containing the *R246B-2* locus in linkage group AswBF (Yu and Wise 2000; Portyanko et al. 2001) (Fig. 1). Moreover, a group of four PK markers targeted the region where *Pc58* was positioned by comparative mapping in the AswAC linkage group (Jackson et al. 2008). In the hexaploid MN map, five out of eight QTLs for partial resistance to *P. coronata* were associated with RGA markers, namely *Prq1a*, *Prq1b*, *Prq2*, *Prq3* and *Prq5* (Fig. 2). These QTLs, originated from MN841801-1, have been evaluated in different field environments and greenhouse assays using different pathogen isolates (Portyanko et al. 2005; Acevedo et al. 2010). Thus, associations were found between the RGA markers and two consistently detected QTLs, *Prq1a* and *Prq2* (in linkage groups MN3 and MN26, respectively). RGA markers of all the types assayed in this work were located in the region covered by *Prq1a*, whereas a PK marker located to a position coincident with *Prq2*. NBS-RGAs were located within the interval of the other QTLs, *Prq1b*, *Prq3* and *Prq5*. Moreover, other minor QTLs detected in that map, such as those located in MN9, MN10, MN12 and MN17, co-localized with both NBS- and PK-based RGA markers. Interestingly, some RGA-RFLP markers detected by the same probe appeared linked to resistance loci located in different maps. Thus, markers b9-1 and 72 were located at the interval of the QTL *Prq1a* in MN3. These markers were at homologous positions on the AswAC group linked to the *PcA* complex. Further, RGA-RFLP 113 located in the *PcA* region was found in the *Prq3* region in group MN14, linked to a QTL for resistance to Bydv in KO22_44+18 (*Bydq Pav-NY*). Analogously, markers revealed by RN182 were linked to *Prq5* in MN6, in KO4_12 and KO13 in regions where resistance to two crown rust isolates (PC54 and PC59) have been mapped (Bush and Wise 1996) and to KO6 in the interval covering a second QTL for *Bydq Pav-NY* (Barbosa-Neto et al. 2000).

To search for a possible association between resistance loci and RGA markers in the reference KO map, markers closely linked to the resistance loci in the original maps were identified in the KO map by comparative mapping (Supplementary Fig. S3). Two traits were selected for this study: resistance to *P. coronata* and tolerance to Bydv (because of the significant number of loci for these traits identified in different oat maps). The reference KO map


MN1
0.0 umn5452
RsaI/NBS1_3
RsaI/NBS1_4
RsaI/NBS1_7
SgSATII3b
RsaI/NBS2_2
11d
33.8 p48m48n1a
RP4a81f
RsaI/NBS1_6
70.4 p40m58m14
MN4
0.0 p42m58m15
9b
27.5 p40m58m2
RsaI/PK1Fa_3
RsaI/PK4R1a_1
60.9 p40m50m16
MN8
0.0 b6y
RsaI/PK3Fa_1
RsaI/NBS5A_2
RsaI/NBS5A_5
RsaI/PK4R1b_5
81e
38.9 umn38
RN182a
47.1 cdo220
52.2 umn295
56.4 p40m51m14
60.4 p35m68n1
62.9 p40m58n6
66.3 p35m68m4
70.0 p48m48m4b
73.0 p40m51m5
LA08-DS-Flag
87.2 cdo309y
92.9 isu916
113.7 cdo20
122.6 umn442
131.7 cdo63
147.2 cdo1433
MN10
0.0 umn361
39.1 cdo1508
Mb08-BRCB-DLA
47.3 p40m50n11
11b
54.0 p40m51m10
59.2 bcd1192x
65.3 cdo1090
71.0 bcd385
MN17
0.0 cdo780
DL4-BRCB-Gh07
RsaI/PK3Fa_5
RsaI/PK4R1b_7
RsaI/NBS1_9
22.5 p38m35n3
29.1 umn5084x
36.7 p42m35n6
53.6 umn5960
63.0 p48m48m4a
69.8 p38m35m7
RsaI/PK3Fa_2
82.1 cdo1464
MseI/NBS6_2
109.2 p42m58m9
117.0 p42m58m5
MN23
MB08-BRCB-IT
0.0 p41m88m5
10.8 p42m35n19
19.5 p40m51n7
AluI/PK4R1b_2
31.5 p40m51m2a
34.9 p40m58m3
RsaI/NBS5A_4
RP4a81c
48.5 p42m58m3
53.3 p42m58n2
70.6 isu2182
MN2
0.0 p42m58n8
95
11.3 p42m58n10
21.0 p35m68n7
25.5 aco190
31.4 cdo608z
38.3 p40m51n6
47.5 bcd1230
54.7 p48m58n4
61.5 bcd1414
80.9 p48m88n4
MN5
0.0 b5
II2.3*
7.9 b2
S-114
19.3 cdo1402
MN12
0.0 cdo665
8.0 cdo1430
8.8 bcd1842
10.4 bcd1270
14.2 cdo942
16.6 bcd1729
18.3 cdo1509
22.4 cdo1502y
III2.18d*
32.4 p53m58n1
RsaI/NBS1_12
RsaI/PK3Fa_3
RsaI/PK3Fa_4
9d
Ab07-LSLGT
44.6 isu2287
RsaI/PK4R1b_1
53.3 p41m88m11
MN24
0.0 isu707z
L7M2.3a*
16.4 isu707y
RsaI/NBS5A_1
35.4 p38m35m4
37.7 p48m58m6
44.3 p40m51n9
64.3 p48m88n7
MN20
0.0 cdo456
RsaI/ NBS2_1
10.0 p48m58m1
MN6
0.0 b6x
17.4 umn162x
RsaI/NBS1_10
RsaI/ NBS1_11
AluI/NBS2_1
RsaI/NBS2_3
23.1 p40m50m5
30.4 p38m35n6
40.3 cdo309c
L8
65.0 cdo718
80.8 umn249
RsaI/NBS1_8
RN182g
87.2 p40m51m8
9e
94.5 umn162y
99.3 p40m51n2
101.8 p42m35n3
105.8 cdo309z
129.7 cdo414
9c
139.2 cdo82
143.5 umn815y
150.0 cdo1313
MN9
0.0 p40m58m13
4.8 p40m51m12
7.8 cdo460
LA07-DLA-Flag
MseI/NBS6_6
MseI/NBS6_7
11c
AP4b14b
30.9 cdo1174
45.3 umn13
RsaI/PK3Fa_6
RsaI/PK4R1b_8
59.7 og49
65.3 umn44
RsaI/PK4R1b_2
AluI/PK4R1a_3
70.3 bcd1127
76.5 p53m58m13
79.4 p40m50m13
80.5 p40m50m4
85.4 p48m88m6
Prq6
109.0 p40m50m1
114.9 p38m35m5
119.7 p42m35m13
124.6 p42m35n12
MN14
0.0 p42m58m4
9.1 p42m58m9a
Prq3
26.2 cdo1196y
113c
36.4 umn360
AluI/PK4R1b_4
62.4 cdo608y
73.5 umn589
87.9 cdo795
98.3 p41m88n12
MN15
LA08-IT-Flag
0.0 cdo1340
3.3 p40m51m13
RsaI/NBS1_1
9.4 p41m88n9
MN16
0.0 cdo638
8.3 p35m68n3
11.3 p42m35n1
MseI/NBS6_5
AP4b14a
11a
VAVIII8b
18.5 cdo1435
MN21
0.0 cdo482
SgSatII3d
22.6 p42m35n16
36.7 p48m48m1
MN25
0.0 og41
RsaI/PK4R1b_3
AluI/PK4R1b_6
RsaI/PK1Fa_2
b9b
11.6 p35m68n2
MN26
0.0 umn23
Prq2
8.2 umn498
RsaI/PK4R1b_6
10.7 am3
MN22
0.0 p40m50n6
RsaI/ PK1Fa_4
S-9204
p48m58m7
RsaI/NBS1_5
RsaI/PK1Fa_1
15.0 p41m88m2
RsaI/PK4R1b_9
23.2 p40m58m10
36.3 p42m58m11
RsaI/PK3Fa_3
AluI/PK4R1b_4
AluI/PK4R1b_3
VAVIII8a
113a
71.4 isu1961
84.2 p40m51m4
MN27
0.0 p42m35m17
MseI/NBS6_1
RsaI/PK4R1b_4
8.6 p42m35m8
RsaI/NBS1_2
25.0 p42m35m15
MN29
0.0 bcd110
MseI/NBS6_4
10.1 cdo517
AluI/PK4R1a_1
12.0 p38m35n2a
AluI/PK4R1b_1
22.2 p38m35n2a
MN31
0.0 p48m88m3
10.4 III2.18a*
MN3
0.0 p42m58n14
13.6 isu1254
Prq1b
33.4 p38m35n2
AP4a73a
37.0 p40m15n15
42.8 p35m68m6
RsaI/NBS1_13
AluI/PK4R1a_2
Pc38
47.9 cdo113s
AluI/PK4R1a_4
52.8 umn464x
53.7 isu707x
L7M2.3c*
55.3 cdo1467
RsaI/PK4R1a_4
RsaI/PK1Fa_5
Prq1a
58.6 aco193x
b9a
MseI/NBS6_3
65.5 b4
III2.18b*
III2.18c*
68.3 cdo608x
72.3 umn624
72
83.6 umn370
RsaI/PK4R1a_3
RsaI/NBS5A _3
103.4 umn4644
Prq5

◀ **Fig. 2** Location of RGA-RFLP and NBS/PK profiling markers in the MN841801-1 × Noble-2 map (Portyanko et al. 2005; Acevedo et al. 2010).To the *left* of each linkage group, genetic distances are indicated in cM. To the *right* of each linkage group, framework markers are indicated in *black*; RGA-RFLP markers are indicated in *magenta* and *underlined*. RFLP-RGA markers previously mapped (Loarce et al. 2009) are indicated in *magenta* and with an *asterisk*. RFLP markers derived from NBS/PK profiling bands are indicated in *blue* and underlined. NBS/PK profiling markers are indicated following a colour code to identify the restriction enzyme and the primer that generated the marker. *Red* boxed regions on the linkage groups indicate the positions of previously mapped QTLs affording partial resistance to *P. coronata* (Portyanko et al. 2005; Acevedo et al. 2010). The names of these resistance QTLs are indicated in *red* on the *left* of each linkage group (color figure online)

was divided into 227 bins limited by the framework markers (Wight et al. 2003). The frequencies of bins harbouring *P. coronata* resistance markers and RGA markers was counted and Chi-squared tests performed, one taking into account NBS-based markers (both NBS-RFLPs and NBS/profiling markers) and the other focusing on PK-based markers (both PK-RFLPs and PK/profiling markers). The null hypothesis assumed an independent distribution of bins occupied by resistance loci and bins occupied by RGA(s), as described by González et al. (2010) (Table 2). The markers for resistance to *P. coronata* occupied 16 bins, while the NBS markers occupied 70. Eleven bins were co-occupied by a resistance marker and by one or more NBS markers. The PK markers occupied 71 bins and 9 were co-occupied by a resistance marker and PK marker(s). The null hypothesis was rejected in both Chi-squared tests, which suggests the existence of an association between the distribution of the resistance loci to *P. coronata* and NBS- and PK-based markers in the KO map. Analogously, a similar, strong association was observed between the distribution of 28 QTLs for tolerance to Bydv and the distribution of NBS and PK markers.

Table 2 Chi-squared values for the probability of the independent distribution of NBS and PK markers along with markers linked to resistance loci (major genes and QTLs affording resistance to *P. coronata* [Pcr] or QTLs for tolerance to barley yellow dwarf virus [Bydq]), in the KO framework map (which has 227 bins)

	NBS	PK	Pcr	Bydq
Bins[a]	70	71	16	28
Co-occupied bins[b]				
NBS			11	15
PK			9	14
$\chi^2_{df=1}$				
NBS			11.76[d]	7.8[c]
PK			4.9[c]	5.3[c]

[a] Number of bins occupied by each kind of marker: NBS, PK and markers for resistance loci

[b] Number of bins co-occupied by NBS or PK markers and markers for the respective class of resistance loci

[c] χ^2 at the 5 % level

[d] χ^2 significant at the 1 % level

It is well known that the recombination frequency is not evenly distributed across the genome of most plant species and that the density of genes can differ widely in different genome areas. The co-localization of RGA markers with resistance loci might therefore be due to the existence of regions of reduced recombination or gene-rich areas, rather than any true functional relationship. To test this, the association between RGA markers and QTLs for other morphological and agronomic traits, such as days to heading, plant height, yield, milling yield and test weight, were also examined. Since a similar distribution was observed for the NBS- and PK-based markers, the analyses shown in Table 3 refer to the total number of RGAs obtained. The analysed traits were not expected to be related with RGAs and, indeed, the Chi-squared test showed the RGAs not to be significantly associated with any of the QTLs for the agronomic traits considered.

Discussion

RGA marker development

RFLPs with genomic and cDNA probes consisting of RGA sequences carrying an NBS domain from closely related species have provided an effective mean of using a set of informative sequences to increase the number of RGA markers in three oat genetic maps. Between 21 and 28 RGA-RFLPs were positioned in the oat maps studied (Supplementary Table S2). The probability of mapping loci detected by polymorphic RGA probes in the maps of Asw and KO was roughly 50 % higher than for other kinds of random cDNA or genomic probes reported by other authors (Kremer et al. 2001; O'Donoughue et al. 1995). Different studies have shown a high level of diversity between different populations in some *R* genes, and consequently in related RGAs, which has been assumed to evolve rapidly in response to changes in pathogen populations (Kuang et al. 2004). In spite of this, enough conservation of NBS sequences was detected among genera of the *Poaceae* to allow the use of RGA clones from wheat and barley in *Avena* species.

The paucity of common markers in the oat maps and the frequent rearrangements that have occurred during the evolution of *Avena* species (Jellen et al. 1994; Sanz et al. 2010) have seriously hindered the establishment of clear homoeologous relationships among linkage groups. These have been reported by other authors on the basis of three or four markers in common (Wight et al. 2003; Oliver et al. 2011) and through the coalignment of the oat and

Table 3 Chi-squared values for the probability of the independent distribution of RGA markers and oat QTLs for days to heading (Dh), plant height (Ph), yield (Yi), milling yield (Myi), test weight (Tw) and straw yield (Syi) in the KO map (which has 227 bins)

	RGA	Dh	Ph	Yi	Myi	Tw	Syi
Bins[a]	110	58	49	46	30	26	23
Co-occupied bins[b]		29	20	25	13	14	11
$\chi^2_{df=1}$[c]		0.068	1.46	0.45	0.35	0.06	0.06

[a] Number of bins occupied by RGA markers (NBS + PK markers) or markers for each class of trait

[b] Number of bins co-occupied by RGA markers and markers for the respective class of trait

[c] χ^2 values underlined indicate that the null hypothesis is accepted $\chi^2_{df=1} = 3.84$, $P = 0.05$)

Brachypodium distachyon maps (Gutierrez-Gonzalez and Garvin 2011). The addition of RGA-RFLPs to the hexaploid maps strengthens the suggested homoeology between several linkage groups (Fig. 2; Supplementary Fig. 3). In addition, comparison between maps confirms the relationships between MN3 and KO17 and MN6 and KO6 reported by other authors (Portyanko et al. 2005; Loarce et al. 2009) and helps to establish new relationships, like those between AswBF and KO14 or AswG and MN22. Comparative analysis of the RGA-RFLP markers studied in this work in different species has produced little evidence of macrosyntenic relationships between oats, wheat and barley. However, the locations on groups KO4_12 and KO13 of the RN182 RFLP markers produced by a sequence with similarity to the *Lrk10* wheat gene (Supplementary Table S4) provide a good example of the genomic rearrangements that have occurred during oat evolution. Groups KO4_12 and KO13 have been related to homoeologous groups 1 and 3 of wheat, respectively (Cheng et al. 2002) Moreover, in the updated version of the KO map (Tinker et al. 2008), KO13 has been linked to KO4_12. Therefore, this new group, KO4_12_13, represents a translocation between the chromosomes of homoeologous groups 1 and 3 of wheat.

The NBS/PK profiling technique appears to be an efficient method for marker development and mapping in oats. The large number of loci assigned to the three studied genetic maps is in the upper range of other multilocus methods, such as AFLP, when used with oats (Jin et al. 2000; Groh et al. 2001). NBS profiling results in oats were similar to the reported in other species with respect to the level of polymorphism and mapping feasibility of the generated markers. In contrast, the use of primers targeted to PK sequence motifs is relatively new (Vossen et al., in preparation). In oats, polymorphic fingerprinting occurred with six out of seven PK primers tested in combination with at least one of the enzymes used. Although differences were observed among mapping populations, primers PK3Fa and PK3Fb derived from the conserved PK domain VIb, plus primers PK4R1a and PK4R1b derived from domain VII (underlined in Supplemental Fig. 2b), were the most productive in terms of the number of polymorphic bands. The Pk3Fb and PK4R1a also performed well in potato (Vossen et al., in preparation), but here also the PK1Fa and Pk1Fb primers performed well, suggesting species-specific sequence conservation

Molecular nature of NBS and PK markers

The percentage of sequences showing similarity to NBS or PK genes or RGAs was 25 %, similar to that obtained for NBS sequences in lettuce (Syed et al. 2006) and peanut (Leal-Bertioli et al. 2009), but lower than the 50–65 % reported by others in potato and apple (Van der Linden et al. 2004; Calenge et al. 2005). These discrepancies might be explained by the technical difficulties in these experiments. Genomic bands selected for cloning and sequencing were chosen after verifying that they genetically mapped close to resistance loci. Some, however, were likely too faint to be properly excised from the polyacrylamide gels. This problem has been reported by other authors (González et al. 2010; Jo et al. 2011). Evidence of this was found when cloned bands excised from the gels were used as probes in RFLP experiments. Two probes, AP4b14 and RP4a81, shared bins with NBS/PK profiling markers different from those initially imagined and likely derived from neighbouring bands in the gels (Figs. 1, 2; Supplementary Table S4). Alternatively, the above discrepancies might be caused by the evolutionary distance between the species studied. Primers NBS1, NBS2 and NBS3 were designed to amplify the less conserved DNA upstream of the P-loop and might have a bias for NBS sequences in GenBank that are absent in oat. The specificity of the primers designed to detect NBS and PK sequences in the oat genomes was, however, confirmed by the positive results obtained using cDNA. Our results indicate that the majority of the amplified fragments were truly derived RGAs and confirm the constitutive expression of the NBS sequences (Brugmans et al. 2008). Similarly, the large number of bands generated by cDNA PK profiling indicates that at least some of the PK genes are continuously transcribed in non-infected oat leaves. Most of the profiling bands revealed no

polymorphism for the parental genotypes (Supplementary Fig. S1). However, some polymorphisms (presence/absence or in band intensity) were found. Sequence information for these fragments is proving to be useful in the development of STS markers (work underway), and their genetic mapping is in progress to identify genomic regions harbouring differentially expressed RGA sequences. Differences in patterns of expression in plants subjected to pathogen infection, analysed by cDNA NBS/PK profiling, would be helpful in identifying sequences involved in the establishment of plant defence responses. This would be of particular interest for sequences related to PKs that participate either in the perception of pathogen effectors, like the *R* genes, tomato *Pto* (Martin et al. 1993), rice *Xa21* (Song et al. 1995) or barley *Rpg1* (Brueggeman et al. 2002), or in the signalling pathways required for the induction of defence mechanisms (Romeis 2001).

Genomic distribution of RGA markers

A large number of the markers obtained in this work, including the RGA-RFLP markers and mainly those obtained by NBS/PK profiling, were located in clusters in the different oat maps (Table 1). Consequently, only 18.4, 32.3 and 25.8 % of the markers were found as singletons in the Asw, MN and KO maps, respectively. A number of RGA-RFLP markers were found in the same clusters as NBS/PK profiling markers (Figs. 1, 2; Supplementary Fig. S3). This co-localizations of markers obtained with the two methods has also been reported in apple (Calenge et al. 2005), reinforcing the hypothesis that both techniques target genomic sequences bearing NBS or PK domains. Broad marker clustering is seen in published NBS profiling linkage maps for apple, lettuce and potato (Calenge et al. 2005; Syed et al. 2006; Brugmans et al. 2008). This marker organization is in agreement with the information derived from wide genetic mapping and genomic studies in various species. This shows that disease resistance genes, as well as NBS-based RGAs, are often found in clusters containing closely related genes or in mixed clusters containing members of different gene families (Meyers et al. 2003; McHale et al. 2009; Bakker et al. 2011). The present results therefore suggest that the genomic location pattern of NBS sequences in oats might be similar to that described in other plant species. Receptor-like proteins have also been found to cluster in the genomes of different species (Martin et al. 1993; Brueggeman et al. 2002). Interestingly, the oat PK profiling markers located both in homogeneous clusters and as singletons show a more even distribution within linkage groups than either of the NBS profiling markers. This was also observed for the mixed clusters containing markers derived from both NBS and PK primers. This was particularly obvious in the Asw map (Figs. 1, 2; Supplementary Fig. S3). Thus, PK profiling seems to be a good complement to NBS profiling for identifying additional genetic regions potentially involved in resistance.

Co-localization of RGA markers and known loci for resistance

Comparison of the map positions of the NBS- and PK-based markers with the phenotypic resistance loci in the Asw and MN maps revealed co-localization. In the diploid Asw map, the RFLP-RGA marker b9-1 was the closest RFLP marker to the important region for resistance to *P. coronata*, *PcA* (Fig. 1). It has also been postulated that gene *Pc94* might be close to or part of *PcA* (Rines et al. 2007). Other resistance loci may also be linked to *Pca* (Yu et al. 2001). Interestingly, in the region between *PcA* and the first RFLP locus detected by the anonymous probe isu2192 (Kremer et al. 2001; Portyanko et al. 2001), several RGA-RFLP markers were located, e.g. the previously mentioned b9-1 along with 113e, 72c and L7M2.3. This suggests that the region close to *Pca* is rich in RGA sequences. This region mapped distally in AswAC, coincident with the end of chromosome arm 1Aswα (Sanz et al. 2012). It is known that the recombination frequency is high at terminal chromosomal regions. Further, it has been reported than gene clusters of RGAs and resistance loci can span large genetic distances from 10 to 20 cM (Meyers et al. 1998; McHale et al. 2009). Therefore, it might be postulated that the resistance genes mapping to the *PcA* region, and the RFLP-RGA markers located close to it, are members of one or several complex families of NBS-LRR resistance sequences. Homologous RGA members of these families are located in the intervals covered by QTLs for partial resistance to crown rust in MN3 and MN14 (Fig. 2), indicating that these resistance loci are likely related to the *PcA* loci. It has previously been shown that *R* loci in some genera of the *Solanaceae* are present at corresponding positions and that there is good conservation of the positions of resistance loci and co-localized RGA markers within related species and genera (Grube et al. 2000; Bakker et al. 2011). The present results regarding the relationships among *PcA*, and *Prq1a* and *Prq3*, and among *Prq5* (in MN6) and the resistance loci on KO4_12 and KO13 support the idea that the comparison of RGA-RFLP loci in different oat maps should allow regions of potential homology or homoeology with disease resistance loci to be defined, as proposed by Oliver et al. (2011).

PK profiling provided markers for the second resistance locus, *R246B-2*, that segregated in the Asw RIL population. In addition, PK markers co-localized with the QTLs *Prq1a* and *Prq2* in the MN map. Apart from the receptor-like kinase class of *R* genes that confer monogenic resistance (Martin et al. 1993; Song et al. 1995; Brueggeman et al.

2002), there was little evidence that QTLs for resistance also encode receptor-like kinases (Llorente et al. 2005). The present results therefore support the idea that genes underlying quantitative resistance share structural and functional similarities with *R* genes encoding PKs. This follows the same reasoning as that explaining the frequent relationships among resistance QTLs and NBS-based markers with similarity to the NBS-LRR class of *R* genes (Calenge et al. 2005; McHale et al. 2009; Rietman et al. 2012).

The usefulness of the RGA markers developed in the present work may be helpful in breeding for resistance, as suggested by comparative mapping with the KO map (which is widely used as a reference oat map). The mapping of RGAs on the KO map showed that both the NBS- and PK-based markers significantly co-localized with loci conferring resistance to *P. coronata* or tolerance to Bydv (Table 2). In contrast, in the regions occupied by loci involved in different agronomic traits, there was a paucity of RGA markers (Table 3). Some markers based on NBS domains, RFLPs or profiling markers co-localize with major genes for resistance to *P. coronata*, i.e., *Pc39* (KO16_23), *Pc54*/*Pc59* and *Pc68* (KO4_12) and *Pc58* (KO17), as well as with a locus conferring resistance to *P. graminis*, i.e., *Pg13* (KO3+38). The PK-RFLP and PK profiling markers co-localize with *Pc71* (KO11_41+20) and *Pc91* (KO3+38), whereas clusters of mixed markers based on NBS and PK domains co-localize with genes *Pc38* (KO17), *Pc94* (KO17) and *PcX* (KO4_12) (Supplementary Fig. S3). This latter kind of cluster was also the most frequently associated with the QTLs for partial resistance to *P. coronata* and QTLs for tolerance to Bydv. The localization of resistance phenotypes to map regions that contain both NBS-LRR genes and receptor-like kinase genes has been previously described (McHale et al. 2009). Although both classes of genes encode shared PFAM domains, like kinase 1, 2 and 3 domains, there is a clear distinction between the sequences within these domains from PK and NBS genes, and this is reflected in the sequences of the degenerate primers. Finer mapping would help clarify which of the two classes of genes present in the mixed clusters might be responsible for each resistance QTL.

Despite the large number of sequences and primer combinations successfully used, no RGA was found to be associated with resistance to *B. graminis* locus *Eg5* (KO22_44+18), neither were such associations seen with respect to some QTLs for tolerance to Bydv. As indicated by Calenge et al. (2005), this might be explained by taking into account that a number of fragments were non-polymorphic between the parents of the mapping populations (Supplementary Tables S2 and S3). Further, it is likely that some of the NBS and PK sequences in the genome were not targeted by the primers used. Moreover, it has been described that genes other than *R* genes involved in the upstream defence pathway might potentially be responsible for QTL-related resistance (Wang et al. 2007).

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