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ORIGINAL PAPER

Resistance gene analogues of wheat: molecular genetic analysis of ESTs

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Abstract Using two divergent nucleotide binding site (NBS) regions from wheat sequences of the NBS-LRR (leucine rich repeat) class, we retrieved 211 wheat and barley NBS-containing resistance gene analogue (RGA) expressed sequence tags (ESTs). These ESTs were grouped into 129 gene sequence groups that contained ESTs that were at least 70% identical at the DNA level over at least 200 bp. Probes were obtained for 89 of these RGA families and chromosome locations were determined for 72 of these probes using nullitetrasomic Chinese Spring wheat lines. RFLP analysis of 49 of these RGA probes revealed 65 mappable polymorphic bands in the doubled haploid Cranbrook × Halberd wheat population (C × H). These bands mapped to 49 loci in C × H. RGA loci were detected on all 21 chromosomes using the nullitetrasomic lines and on 18 chromosomes (linkage groups) in the C × H map. This identified a set of potential markers that could be developed further for use in mapping and ultimately cloning NBS-LRR-type disease resistance genes in wheat.

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Introduction

In modern agriculture, manipulation of crop plant resistance to disease is a major tool for the minimisation of the potentially devastating effects of plant disease epidemics. Although at least 397 genes conferring resistance to a disease or pest have been identified in wheat (McIntosh et al. 2005), only a few, such as *Lr21* (Huang et al. 2003), *Lr10* (Feuillet et al. 2003) and *Pm3*(Yahiaoui et al. 2004 have been confirmed as cloned resistance genes. Information about the chromosomal location and genetic map position of disease resistance genes is a prerequisite for cloning and is also important in the generation of disease resistance-linked markers for deployment in the marker-assisted selection of breeding material. Therefore, our aim in this study was to generate a framework of genetic map positions for potential disease resistance genes for use in the mapping and cloning of new disease resistance genes (R genes) in the *Triticeae tribe*.

Many of the R genes cloned from a variety of plants code for proteins that contain a characteristic arrangement of conserved domains. There is a central nucleotide binding site (NBS) domain and a carboxy-terminal leucine rich repeat (LRR) region (Cannon and Young 2003). One class of NBS-LRR proteins also contains a domain related to the *Toll* and interleukin 1 receptor proteins (TIR domain). However, this class of proteins has only been observed in dicotyledonous plants, so cereals probably only contain NBS-LRR genes of the non-TIR class. Within the NBS domain of resistance proteins there are several, small, presumably functional, conserved domains, such as the P-loop, kinase domains, MHD, and GLPL motifs (Meyers et al. 2003; Huettel et al. 2002). These conserved motifs have been

utilised in the design of PCR-based cloning and mapping strategies to characterise R genes from members of the *T. tribe* such as barley (Collins et al. 2001; Madsen et al. 2003; Mammadov et al. 2005) and wheat (Seah et al. 1998; Spielmeyer et al. 1998; Maleki et al. 2003; Dilbirligi and Gill 2003). The approach has also been applied in a range of crops (Cheng et al. 2005), and to sugarcane (McIntyre et al. 2005), apricot (Soriano et al. 2005), apple (Calenge et al. 2005), cotton (He et al. 2004) and cocoa (Lanaud et al. 2004), demonstrating the widespread utility of this approach.

In the rice genome, a study of databases containing sequences from japonica and indica rice identified about 560 putative NBS-containing genes. These were grouped into 354 families that were at least 75% identical at the DNA level and were found by analysis of the degree of sequence identity to fall into 117 groups (Bai et al. 2002). A more recent study (Zhou et al. 2004) of the 441 alignable NBS-LRR gene sequences derived from the complete genome sequence of *Oryza sativa* var. Nipponbare identified 230 groups of genes that showed less than 30% sequence divergence. Examination of 28,000 rice expressed sequence tags (ESTs) for resistance gene analogue (RGA) gene families identified only about 153 expressed NBS-encoding gene families, of which 130 represented NBS-LRR genes (Monosi et al. 2004). Thus only a proportion of the RGAs present in the rice genome is expressed; presumably, the functional RGA genes are among this group. Strategies designed to detect R genes in genomic DNA, particularly in wheat with a much larger haploid genome size (1.6×10^4 Mb) than rice (430 Mb) (Arumuganathan and Earle 1991), are therefore likely to be hampered by non-functional R gene analogues. In addition, while full genome sequence information is available for rice, the size and complexity of the wheat genome means that only portions of the genome have been sequenced. However, a valuable resource for wheat genomics has been made available with the development of a comprehensive EST database that includes ESTs from a wide range of tissues and developmental stages (Lazo et al. 2004; Zhang et al. 2004; Qi et al. 2004). In order to target functional R gene analogues, we focussed on expressed R genes represented in the extensive public wheat EST databases.

R genes are often expressed at low levels and so their transcripts are likely to be rare in EST libraries. Therefore, we selected both wheat and barley ESTs to generate a good representative set of RGAs for the *T. tribe*. Once a representative set of non-cross-hybridising EST sequences had been assembled, RGA probes were generated for RFLP analysis. A set of nullitetrasomic wheat lines was used to determine the chromosome locations of the RGAs. A well-characterised doubled haploid (DH) Cranbrook × Halberd (C × H) mapping family (Chalmers et al. 2001; Lehmensiek et al. 2005), (http://rye.pw.usda.gov/cmap) was used to map the RGAs. This work complements previous studies that concentrated on the determination of chromosome location for expressed RGAs using wheat deletion lines (Dilbirligi et al. 2004) and represents an attempt at the generation of a comprehensive genetic map for expressed Triticeae RGAs in wheat. The mapped RGAs provide potentially powerful tools for the development of markers for resistance traits, and for the cloning of NBS-LRR-type resistance genes. This could include genes for broad spectrum, qualitative disease resistance, because recent studies in rice (Wisser et al. 2005) and sugarcane (McIntyre et al. 2005) found that in some instances there was significant association between RGA genes and quantitative resistance traits.

Materials and methods

Plant material

Chromosome locations of polymorphic RGA markers were determined using a series of Chinese Spring nullisomic-tetrasomic and D-genome ditelosomic lines (Sears 1966). For amplification of RGA sequences for use as probes, DNA or RNA from wheat (Chinese Spring) or barley (Morex or Golden Promise) was used. For linkage mapping analysis, DNA from DH progeny seedlings from Cranbrook × Halberd (C × H) (Chalmers et al. 2001; Lehmensiek et al. 2005) (DH lines) was used. Extraction of total genomic DNA, restriction digestion of DNA and preparation, processing and autoradiography of DNA blots was performed using standard protocols as described previously (Lagudah et al. 1991). RNA was extracted using the Qiagen RNeasy® Plant Mini kit (Qiagen Pty Ltd, Clifton Hill, Australia) according to the manufacturer's instructions.

Selection of RGA-candidate wheat and barley ESTs

Wheat and barley ESTs were retrieved from Genbank non-mouse and non-human EST entries using TBLASTN searches with the NBS regions, from the P-loop to the MHD motif [motifs as described in (Meyers et al. 2003)] of the two divergent NBS-LRR-gene sequences, *Go35* (AY124651) and *Yr10RGA* (AF149114). The results of these searches were filtered using the terms "Triticum" or "Hordeum" to retrieve

only wheat or barley sequences. Further searches were performed with other R genes, but no additional sequences were retrieved. Sequences were included where the *E* value obtained was < 1.0, unless two or more NBS-region motifs were observed. Where *E* values were high and several very similar ESTs were obtained, only one was selected.

Generation of a representative set of wheat and barley RGA ESTs

Wheat and barley EST sequences were analysed separately to identify a set of non-cross-hybridising RGA families. Initially, the TBLASTN results were inspected manually to identify conserved NBS-region motifs and the sequences were grouped according to the motifs they contained. Those with motifs at the 5′-end of the NBS domain, i.e. P-loop, kinase 2, kinase 3, or GSK motifs were grouped together in one group; those with motifs at the 3′-end of the NBS domain, i.e. CF, EGF or MHD motifs were grouped together as a second group. Sequences from either group and additional sequences that contained the GLPL motif in the central region of the NBS domain were placed in a third group. Some sequences fell into more than one group. The sequences within each of these three groups were analysed for sequence similarity using the CLUSTAL function of GCG and the resulting multiple sequence alignments were displayed using CLUSTREE. Neighbouring and, where appropriate, near neighbour DNA sequences within the multiple sequence alignments were compared using pair-wise BESTFIT analysis. Sequences that showed greater than 70% identity at the DNA level over at least 200 bp were considered to be members of the same family. A representative sequence (usually the longest) was chosen from each family. The results from each of the three groups were compared and a representative set of unique RGA families was selected. This analysis was performed separately for wheat and barley. The representative set of wheat RGA families was selected first. The set of RGA gene families generated from the barley ESTs was then compared with the wheat set and any RGA families not already represented were added to the final set of wheat and barley RGA EST families. Examination of the Genbank entry for each selected RGA and searching of the Graingenes database (http://wheat.pw.usda.gov) was undertaken to see if genetic map locations had already been determined for the RGA families. In general, sequences for which there was no existing map information were selected for analysis in this study.

Generation of DNA inserts and probes for genetic analysis of RGA families

Where possible, clones of ESTs corresponding to the representative set of RGA ESTs were obtained from the laboratory that lodged the sequence with Genbank. Inserts were PCR-amplified from the clones provided using sequence-specific primers. These primers were designed using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Clones were considered correct if a PCR product of the expected size was obtained using the specific primers. These inserts were used as templates for Southern analysis of DNA blots.

Where clones of selected ESTs were not obtained from external sources, an attempt was made to obtain the desired sequence by PCR of DNA or RT-PCR of RNA from wheat or barley tissue. All RT-PCR reactions with wheat RNA were used as templates for an additional PCR reaction. For barley, DNA from cultivar Golden Promise was used for PCR or RNA from cultivar Morex used for RT-PCR. PCR reactions were performed in a total volume of 25 µl containing 2.5 µl Qiagen 10 × PCR buffer, 0.2 mM dNTPs, 1.25 U *Taq* polymerase, 12 pmol each primer and 250 ng template. Cycling conditions were five cycles with 1 min at 94°C, 1 min at 55°C, 2 min at 72°C; 30 cycles with 30 s at 94°C, 30 s at 55°C and 50 s at 72°C; one cycle with 30 s at 94°C, 30 s at 55°C and 5 min at 72°C. RT-PCR was performed using the Qiagen OneStep RT-PCR kit with cycling conditions as recommended by the manufacturer, with an annealing temperature of 55°C for Chinese Spring and 54°C for Golden Promise. An aliquot of the amplifications was visualised on a 1% (w/v) agarose gel and if a fragment of the expected size was observed, the reaction was purified using the QIAquick PCR purification kit (Qiagen) and the manufacturer's protocol. Amplified products were ligated into pGEM®-T Easy vector (Promega, Madison, USA) and electroporated into DH10B cells. Single colonies were amplified by PCR using SP6 and T7 primers to identify those containing an insert of the anticipated size. DNA was prepared from cultures of selected colonies using the Qiagen QIAprep spin miniprep kit and sequenced using either T7 or SP6 primers and the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems). PCR-generated inserts were considered correct if the observed sequence was at least 80% identical to the published sequence. Inserts for use as templates for the generation of probes for Southern analysis of DNA blots were generated by amplification of clones with the correct sequence using sequence-specific primers.

DNA blots and RFLP analysis

*Eco*RI or *Hind*III was used for restriction digestion of 15 μg genomic DNA from the nullitetrasomic and ditelosomic lines. Parental blots of Cranbrook and Halberd genomic DNA were prepared from genomic DNA restricted with six enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Sac*I and *Xba*I). For some RGAs, an additional set of six enzymes (*Bgl*II, *Nco*I, *Nde*I, *Nsi*I, *Pst*I and *Xho*I) was used to look for polymorphisms between the parents. For linkage analysis, blots were prepared from restricted genomic DNA from 56 to 79 DH lines. Hybridisation of blots was carried out overnight at 65°C in aqueous hybridisation buffer (10% dextran sulphate, 6 × SSC, 0.05 M Tris–HCl, pH 8.0, 1 mM EDTA, 5 × Denhardt's, 0.2% SDS and 0.1 mg/ml salmon sperm DNA). Blots were washed in 2 × SSC, 0.1% SDS at 60–65°C. As each blot was used up to six times, the stringency of washing varied with the age of the blot.

Linkage analysis

Data obtained from scoring the segregation patterns of RGA markers in the DH lines was analysed using Map Manager QTXb20 (Manly et al. 2001) (http://mapmanager.org/mmQTX.html). The position of new RGA markers was determined relative to existing markers in the C × H linkage map (Chalmers et al. 2001; Lehmensiek et al. 2005), (http://rye.pw.usda.gov/cmap) using the "generate links report" function with a threshold value of $P = 0.05$. The final position of the new marker was optimised manually selecting the position that maximised the LOD score and minimised the number of crossovers. The number of double crossovers was determined, and the scoring of these individuals checked. Only RGA markers that gave three or fewer double-crossovers were assigned to a linkage group. Distances between markers were determined using the Kosambi function. Maps were visualised using MapChart (Voorrips 2002) (http://www.plant.wageningen-ur.nl/products/mapping/MapChart/mcdownload.htm).

Results

Selection and generation of a representative set of wheat and barley RGA ESTs

A flow chart of the sequence analysis procedure and the numbers of EST sequences obtained at each step is given in Fig. 1. TBLASTN searches of Genbank non-mouse and non-human EST entries with the NBS regions (from the P-loop to the MHD motif) of the two divergent R gene-like sequences, *Go35*(AY124651) and *Yr10RGA* (AF149114) were performed and the crude search results filtered using the terms "Triticum" or "Hordeum" to retrieve only wheat or barley sequences. This retrieved 115 RGA EST sequences for wheat and 96 RGA EST sequences for barley. Further searches were performed using other known NBS-LRR resistance gene sequences, but no additional sequences were identified. A complete alpha-numerically ordered list of Genbank accession numbers of retrieved sequences is given in Supplementary Table A. Wheat and barley EST sequences were analysed separately to identify a representative set of RGA families. Sequences that showed greater than 70% DNA sequence identity over at least 200 bp were considered to be members of the same family. The 115 wheat ESTs were grouped into 77 RGA families while the 96 barley ESTs fell into 65 families. Thirteen of these barley families were already represented in the wheat RGA family set, resulting in a combined set of 129 RGA families. Table 1 shows the RGA family number assigned to each retrieved EST sequence. Examination of Genbank and Graingenes databases and literature sources showed that chromosome location information was already available for 39 of these families; 13 in barley and 26 in wheat (See Table 1). This included RGA3, which showed significant nucleotide sequence similarity to the cloned wheat disease resistance gene *Lr*21, and RGA115, which showed significant sequence similarity to *Lr*10. No EST was identified that showed significant sequence homology to cloned wheat resistance gene *Pm*3. Fifteen of the RGA families for which sequence information was available were not analysed further; thus 114 RGA families (including RGA3/*Lr*21, but not RGA115/*Lr*10) were selected as targets for analysis in this study (Fig. 1).

Generation of DNA inserts, chromosomal location and genetic analysis of RGA families

Clones of 40 of the 114 ESTs selected for analysis were obtained from external sources and were found to give a band of anticipated size on PCR-amplification with sequence-specific primers. Clones of a further 49 ESTs were generated by PCR or RT-PCR from wheat or barley DNA or RNA and found by DNA sequencing to have the correct sequence giving a total of 89 probes.

Genomic gel blot analysis using the RGA probes gave a range of hybridisation patterns. Analysis of restricted genomic DNA from the Cranbrook and Halberd parents gave both simple and complex hybridisa-

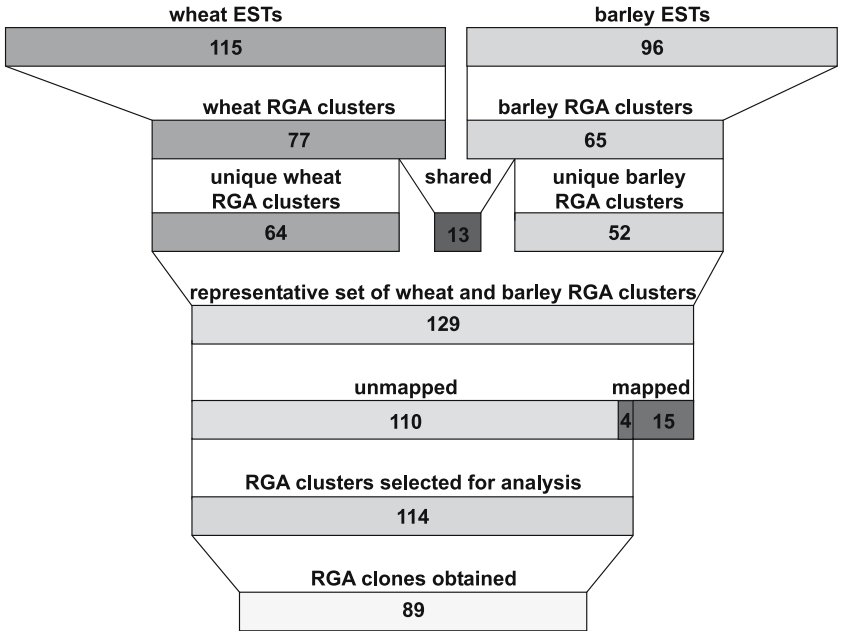


Fig. 1 Flow sheet of sequence analysis of EST RGAs in the generation of a representative set of wheat and barley RGAs for analysis of chromosome locations of RGA genes in wheat

tion patterns. For example, RGA62 gave only one major non-polymorphic hybridising band, with a few minor bands (Fig. 2a), while RGA87 gave many polymorphic hybridising bands (Fig. 2c). An example of the complete gel blot analysis for RGA71 is also given in Fig. 2. The hybridisation of the restricted genomic DNA from the Cranbrook and Halberd parents (Fig. 2b) shows three main hybridising bands, presumably corresponding to the A, B and D wheat genomes, with polymorphism between the parents visible in DNA digested with *Dra*I and *Eco*RV. The segregation of the polymorphic band obtained by *Dra*I digestion of genomic DNA from a selection of C × H DH lines is clearly visible (Fig. 2d). The hybridisation of the probe to genomic DNA from the nullitetrasomic and ditelosomic lines digested with *Hind*III shows the absence of a band hybridising to RGA71 in CS N5A-T5D, CS N5B-T5D and CS N5D-T5B, showing the presence of an RGA71 homologue in 5A, 5B and 5D, respectively. The band missing in CS N5D-T5B is also missing in CS Dt5DL, demonstrating that the band is on the short arm of 5D (Fig. 2e).

Genomic gel blot analysis of 21 nullitetrasomic and seven ditelosomic lines revealed a chromosome location for 73 of these probes, as shown in Table 1. No result was obtained for the remaining 16 probes, either because of lack of polymorphism or because poor hybridisation gave unreadable autoradiographs. Of the 73 probes that gave a result, 19 probes identified a single locus, 41 probes identified two or three loci on homoeologous chromosomes, eight probes identified one or more homoeologous loci and one or more loci on a chromosome of a different group, and five probes identified loci on chromosomes from different groups only. All homoeologous groups of chromosomes were represented, with the most loci (loci on homoleogous chromosomes were counted only once) identified on group 7 chromosomes (18) and the fewest loci identified on group 4 chromosomes (5).

The results of the linkage group analysis for the RGA probes in the C × H mapping population are given in Table 1 and Fig. 3a–g. In this analysis, 49 RGA probes gave 65 mappable bands that revealed 49 distinct loci. Of the 49 probes that gave mappable bands, 37 probes mapped to a single position in the C × H map. Six probes mapped to approximately equivalent positions on two or more homoeologous chromosomes. Of these six probes, one (RGA 40) mapped to two adjacent loci on 6D and another (RGA 20, mapping to 2B and 2D) also mapped to an additional position on an unrelated chromosome (7B). A further six probes mapped to more than one locus on unrelated chromosomes. In the 45 cases where there was information from both the nullitetrasomic analysis and the C × H mapping population, equivalent chromosome location and map position data were obtained for 40 probes. There were nine clusters where RGA probes were found to have the same map position, or where the map positions differed by less than 1 cM. Analysis of the hybridisation patterns of the parent blots for probes localising in similar map positions revealed similar banding patterns for probe pairs RGA46/95, RGA81/98 and RGA61/119. Thus, these probe pairs are equivalent and are hybridising to the same RGA locus. However, seven clusters were identified by probes with distinct hybridisation patterns, making them genuine clusters. The 40 remaining probes that did not yield a linkage group in the C × H mapping

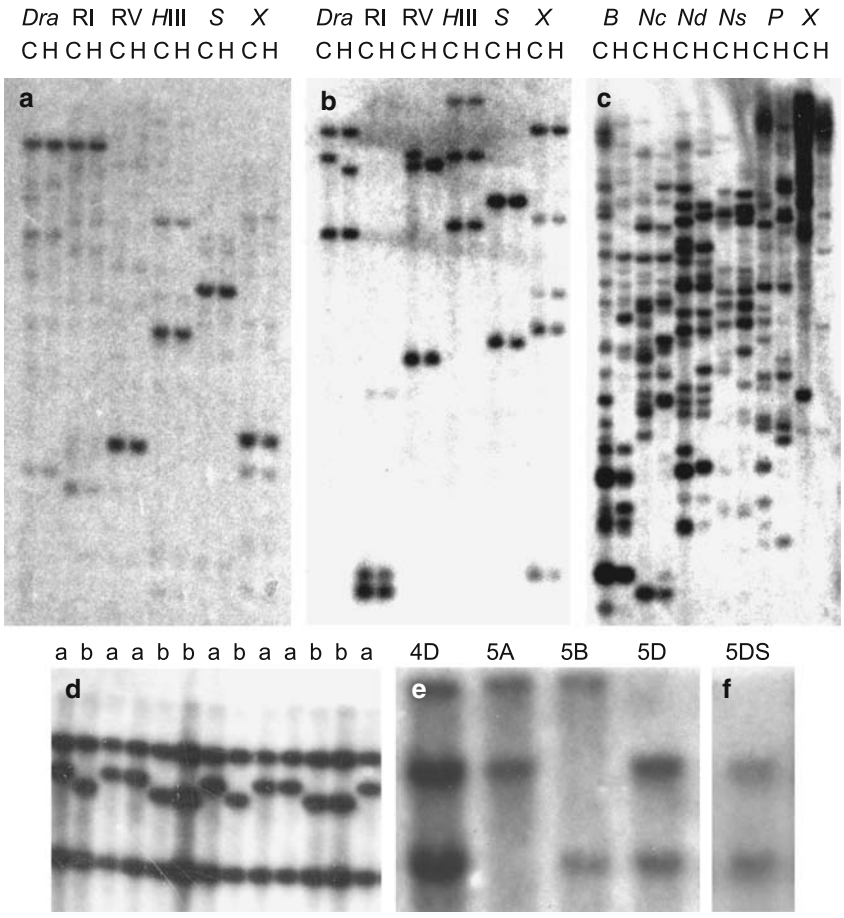


Fig. 2 DNA gel blots of Cranbrook and Halberd genomic DNA probed with **a** RGA62, **b** RGA71, and **c** RGA87. For each blot, *lanes C* contain Cranbrook DNA; *lanes H* contain Halberd DNA. For blots (**a**, **b**), DNA was restricted with *Dra*I (*Dra*),*Eco*RI (RI), *Eco*RV (RV), *Hind*III (*H*III),*Sac*I (*S*) or *Xba*I (*X*). For blot (**c**), DNA was restricted with *Bgl*II (*B*), *Nco*I (*Nc*), *Nde*I (*Nd*), *Nsi*I (*Ns*), *Pst*I (*P*) and *Xho*I (*X*). Blot **d** contains genomic DNA from selected doubled haploid lines of Cranbrook × Halberd probed with RGA71. Lanes labelled *a* show the Cranbrook hybridisation pattern while lanes labelled *b* show the Halberd hybridisation pattern. Blot **e** shows genomic DNA restricted with *Hind*III. The blot contained DNA from CS nullitetrasomic lines CS N4B-T4D, CS N5A-T5D, CS N5B-T5D and CS N5D-T5B, labelled with the missing chromosomes *4D*, *5A*, *5B*, *5D*, respectively. Blot **f** shows a lane containing DNA from ditelosomic line CS Dt5DL (missing *5DS*)

population either gave segregating bands that were unlinked, or the hybridisation pattern was not clear, or polymorphisms were not observed (Table 1).

Preliminary examination of the results suggests that there might be a preferential distribution of RGAs within the wheat genome. Polymorphic RGA-hybridising bands mapped to each homoeologous group of chromosomes, with the most bands mapped to group 1 chromosomes (12) and the fewest (4) mapped to chromosomes in groups 3, 4 and 6. More RGA-hybridising bands mapped to the A genome (34) than the B (17) or D (11) genomes. However, we postulated that this apparent distribution might be as a result of the distribution of markers in the C × H map, rather than a preferential distribution in the genome. We therefore tested if the distribution of RGA-hybridising bands between homoeologous chromosome groups or among genomes was significantly different from the distribution of all markers in the C × H population using the chi-squared test. For the distribution of RGA markers between chromosome groups ($\chi^2 = 0.773$, six degrees of freedom, for significance $\chi^2 > 12.59$) and among genomes ($\chi^2 = 2.75$, two degrees of freedom, for significance $\chi^2 > 5.99$), we found there was no significant difference between the distribution of RGA markers and all markers in the C × H map.

Discussion

Generation of a representative set of wheat and barley RGA ESTs

It has been suggested that only a small fraction of NBS-LRR-encoding RGAs in plants code for functional

Table 1 Chromosome location of wheat and barley RGA ESTs in wheat as determined by the analysis of polymorphic bands in nullitetrasomic and ditelosomic lines of Chinese Spring and by linkage analysis of Cranbrook × Halberd doubled haploid lines

RGA family[a]	Genbank accession[b]	Existing map data	Chromosome location (nullitetrasomic lines)	Chromosome location (C × H DH lines)
1	**BE400250**	*Hv Mla1* 5S(1H)[c]		
2	**BE405711**	3BL[d]	Not polymorphic	Not polymorphic
3	**BE498831**	*A tauschii* putative RGA: 1DS[c], *Lr21*[c], 1BS[d]	1B,2A	1A,1B,1D
4	**BE499177**	3AS,3DS[e], 3AS[d]		
4	BE638124	6AL[e], 6AL[d]		
5	**BE500158**	6AL[d]	6A,6B	6A
5	BQ281343			
5	CA657592			
6	**BF482358**	4BS[d]	4A,4D,5B	Unlinked
7	**BJ255861**		2B, 2D, 6A,6B,6DS	6A
8	**BJ258770**		1A,1B,1DL	1A
9	**BJ269157**	Hv1LRR2: 1HS[c]	7A, 7D	2B,4A
10	**BJ270203**		1A,1B,1DS, 2A	1A,1D
10	BJ270209			
10	BJ270399			
10	BJ275205			
11	**BJ272146**			
11	BJ274706			
12	**BJ286558**		3A	Unlinked
12	BM259093			
12	CA686262			
13	**BJ295708**		4A, 4B	1A, 4B
14	**BJ300496**		3A, 3B	3B
15	**BQ239089**		5B	Not polymorphic
16	AV834770	*T. ventricosum* V6: 2S[c]		
16	**BQ241493**		5A, 5B	Unlinked
17	**BQ246913**		5A, 5DS	Not polymorphic
18	AU252328			
18	**BQ579469**	*T. aestivum* T10 rga 2-1A: 1AS[c]	1A,1DS	Not polymorphic
19	**BQ753146**		2B,2DS	2D
19	CA654826			
20	**BQ802253**		7A,7B,7D	2B,2D,7B
21	BI479697			
21	**BQ802688**		4A,7A	4A, 7A
21	BU968942			
21	CA600125			
22	**BQ803195**		7A,7B,7DL	Not polymorphic
23	**BQ901277**		Not clear	Unlinked
23	BQ901540			
23	BQ902435			
24	**BU100242**		7B,7D	1A
25	**CA500988**		3A,3B,3DL	2A
25	CA691224			
26	**CA600403**			
27	**CA606728**			
28	**CA610289**	HvRGA1R: 1(7H)[c]		
29	**CA652512**		5B,6A	Not polymorphic
30	**CA662188**			
31	**CA662651**			
32	**CA676926**	*Hv b7*[c]: 1H[f]		
33	**CA679534**			
34	**CA681703**			
35	**CA725884**	HvRGA1R: 1(7H)[c]	1A, 7A, 7D	7A
35	CA726483			
36	**CA726158**	*Hv Mla1-2*: 5S[c]	1B,1DL	1A
37	**CA733486**			
38	**CA741642**			
39	**CA742788**		4A	4A
40	**CA742849**	Hv rga S-9204[c]: 2H[g]	6D,6DL	6A, 6D(x2)

Table 1 continued

RGA family[a]	Genbank accession[b]	Existing map data	Chromosome location (nullitetrasomic lines)	Chromosome location (C × H DH lines)
41	**CA499328**	Hv rga S-18[c]: 2H[g]	2B	Unlinked
42	**AJ480573**			
44	**AV835532**		Smear	2B
45	AV931700			
45	**AV931701**			
46	**AV939166**		7A,7DS	7A
47	**BF259304**		No hybridisation	No hybridisation
48	AV832695			
48	**BJ473038**		7B	7A
48	CB879462			
48	CB879858			
49	**BQ465430**		6A,6B,6DL	6A
50	**BQ469814**		Smear	Not polymorphic
51	**BQ740040**		Not clear	Not polymorphic
52	**BU998087**		No hybridisation	No hybridisation
53	**CA692539**		6B	Not polymorphic
54	**AJ466357**		6A,6B	Not polymorphic
55	**AU252332**	HV1LRR1: 1HS[c]		
56	**AU252333**	HV1LRR1: 1HS[c]		
57	**AV832768**		6A	6A
57	BJ470031			
58	BJ226439			
58	**BJ246024**		7A	7A,7D
58	BJ301559			
59	AJ465556			
59	**BJ245188**		7A,7B,7DL	Not polymorphic
60	**CA501245**		5B, 5DS	1A
61	BF065859			
61	BF474327			
61	BF482366	3AL[d]		
61	**BM134773**		3A	3A
61	CA723707			
62	BQ743901			
62	**BQ752781**		3B	Unlinked
62	CA604604			
63	**BE591190**	7BS, 7DS[e], 7AS[d]	7A,7B,7D	7B
63	BJ465016			
63	BJ482257			
63	BU970049			
64	**CD910548**			
65	**BE517538**		5DL, 6A	5D
66	**CA497657**			
67	AV915815			
67	AV926502			
67	AV926503			
67	AV926862			
67	BG343364			
67	**BQ744464**		1B,1DL	Unlinked
67	CA032758			
68	**CA617198**			
69	**AV834807**		1B	1B
70	**BM134978**			
70	BM134985			
71	AV833466			
71	BE406845	5BS[e], 5BL[d]		
71	BE429766			
71	BJ472446			
71	BQ763973			
71	**BU099584**		5A,5B,5DS	5A
71	BU672244			
71	CA007089			

Table 1 continued

RGA family[a]	Genbank accession[b]	Existing map data	Chromosome location (nullitetrasomic lines)	Chromosome location (C × H DH lines)
71	CA019191			
71	CA730176			
71	CA744190			
71	CD874554			
72	**CA736742**		7B, 7D	7B
73	**CA740494**			
74	CA723870			
74	**CA724373**		5A,5DL,6A	5B
75	AV836263			
75	BJ278446			
75	**CA498317**		2B	2B
75	CD055513			
76	AV833874			
76	BE518223	1BS,1AS[e], 1BS[d]		
76	BE591154	3AL[e], 3BL[d]		
76	BF475048	1BS[d]		
76	BM375632	*Hv b6*[c]; 3H, 1H[f]		
76	BU968741			
76	CA655300			
76	**CA696482**			
77	**BJ213107**		5A, 5B, 5DL	Unlinked
78	**BJ316279**		2B, 2D	2D
79	**BJ277253**		3A	Unlinked
79	CD056619			
80	AL826387			
80	**BQ743300**			
81	**BF484437**	5BL[d]	5B	5B
82	AV915861			
82	**BJ207304**		Not clear	7A
83	CA614919			
83	**CA744411**			
84	**BJ276947**			
85	**BJ225910**	*T. turgidum* dw-RGA3[c]	5A	Not polymorphic
86	**CA727476**		7A,7B,7DS	Not polymorphic
87	**BE405507**	6BL, 6DL, 7AL, 7BL[e], 6BL, 7AL[d], Hv S-9204[c]: 2H[g]	6B,6DL	6A,7A
88	**AV835434**		7B	Not polymorphic
88	CA024583			
89	**AV835525**		Not clear	6A
90	**AV836013**			
90	BU994795			
91	**AV836112**		No hybridisation	5B
92	**AV836316**		2A,2B	Unlinked
93	AV923176			
93	**AV925982**		5A,5DL,6A	Not polymorphic
94	**AV938195**	Hv S-226, S9217[c]: 1H[g]		
95	**AV947188**		7A,7B,7DS	7A
96	AJ469133			
96	AL504844			
96	**BF254589**			
97	**BF266730**		7B	7B
98	**BF620979**		5A,5B,5DL	5A, 5B
99	**BF621118**			
100	**BF631540**		2B,2DS,5B	2B
101	**BI946756**		5DL	Not polymorphic
102	AV835430			
102	**BI952460**			
103	**BJ452831**		Not clear	Not polymorphic
104	**BJ463154**		No poly seen	Unlinked
105	**BJ464246**		2DL	2D
106	**BJ471122**		6A,6B,6DS	Not polymorphic

Table 1 continued

RGA family[a]	Genbank accession[b]	Existing map data	Chromosome location (nullitetrasomic lines)	Chromosome location (C × H DH lines)
107	**BJ477899**	*Hv* rga S-L8[c]: 3H[g]		
107	BU969301			
108	**BJ479696**		5B,2DS	Not polymorphic
109	**BJ484626**		3A,3B,3DL	2A
111	**BQ461468**		1B,1DL	1B
112	**BQ463162**		No hybridisation	No hybridisation
113	**BQ464722**		4B, 4DS	4A
114	**BQ465303**	*Hv* rga S-L8[c]: 3H[g]		
115	**BQ465725**	*T. turgidum* rga1*T. aestivum Lr10*: 1AS (7H)[c]		
116	**BQ468699**		7A,7B,7D	7A,5B,7B
117	**BQ756559**		2A, 3B, 3DL	3A,3B
118	AJ474842			
118	**BU987263**		Not polymorphic	Not polymorphic
119	AV909710			
119	AV911609			
119	**BU987965**		3A, 3B, 3DL	3A
119	BU996533			
119	BU996723			
119	CB862991			
120	**CA009070**	*Hv b1*[c]	No hybridisation	Not polymorphic
121	**CA011604**		6A, 6DS	Not polymorphic
122	**CA017389**		Not polymorphic	Not polymorphic
123	**CA020245**		7B,7DL	Not polymorphic
124	**CA033056**		5B,5D	Unlinked
125	**CB882202**		1A,1B,1DL	Unlinked
126	**BE418533**	2DL[e]		
127	**BF199788**	*Hv b7*[c]: 6H[f], 6DL, 6BL[d]		
128	**BF473313**	1AL, 1BL, 1DL[e], 1AL[d]		
129	**BE445244**	4AS, 5BS[e], 4AS, 5BL[d]		
130	**BE605005**	1AL[d]		
131[h]	**BE426789**	1AL, 4AL, 6BL[e], 4AL, 6BL, 7BL[d]		

[a] **RGA families** that are bold and underlined were selected (114 total) for analysis; ***RGA families*** in italics were selected but samples of clones were not obtained

[b] **ESTs** with genbank accessions in bold and underlined were selected as the representative sequence for one of the 129 representative RGA families

[c] Significant sequence similarity to a sequence with map information in Genbank entry determined by NBLASTN search

[d] Dilbirligi et al. (2004)

[e] EST map location given in Graingenes e database, http://wheat.pw.usda.gov/e/

[f] Leister et al. (1998)

[g] Madsen et al. (2003)

[h] RGA family numbers 43 and 110 were not assigned, hence total number of families = 129

resistance genes (Dilbirligi and Gill 2003). As it is unlikely that non-functional genes are expressed, one strategy for selectively targeting potentially functional RGAs is to focus on expressed genes. However, expressed RGAs are relatively rare. For example, in rice, 130 NBS-LRR gene-encoding cDNA gene families were identified from 28,000 full-length cDNA clones (Monosi et al. 2004) although 230 families of NBS-LRR sequences were identified in the rice genome (Zhou et al. 2004). In this study, we also found that expressed cereal RGAs are relatively rare. In January 2004, there were 577,538 wheat and 377,074 barley ESTs in Genbank (Lazo et al. 2004). Our searches of the Genbank wheat and barley ESTs (conducted primarily in July–October 2003) yielded 211 RGA sequences that were grouped into 129 sequence families.

In order to estimate our coverage of the wheat RGAs, we compared the number of RGA gene families identified in this study with the number predicted for rice. Estimates of the number of families of NBS-containing gene sequences in the rice genome vary from 117 (Bai et al. 2002) to 230 (Zhou et al. 2004). Analysis of rice cDNAs identified 153 sequence families

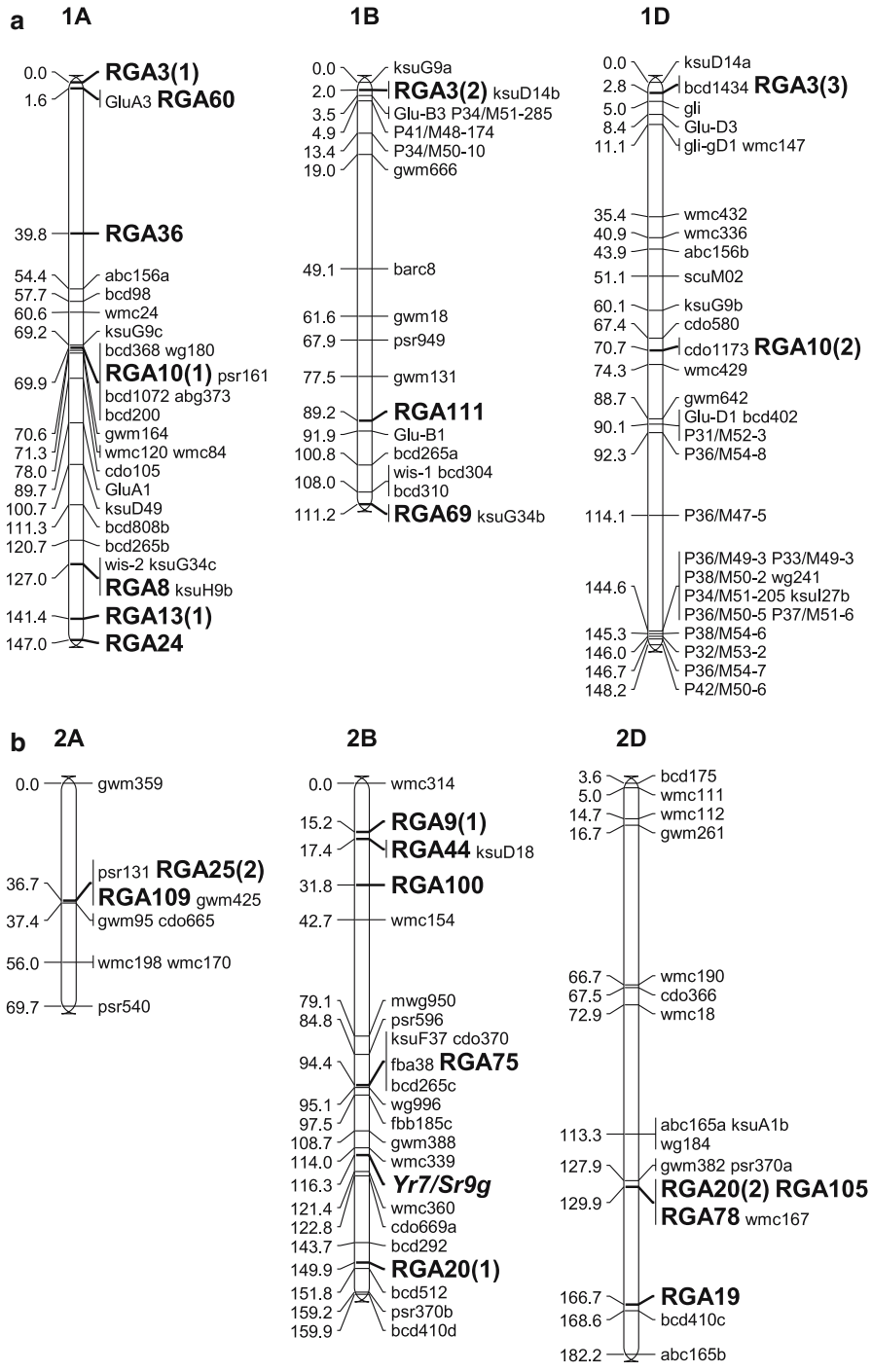


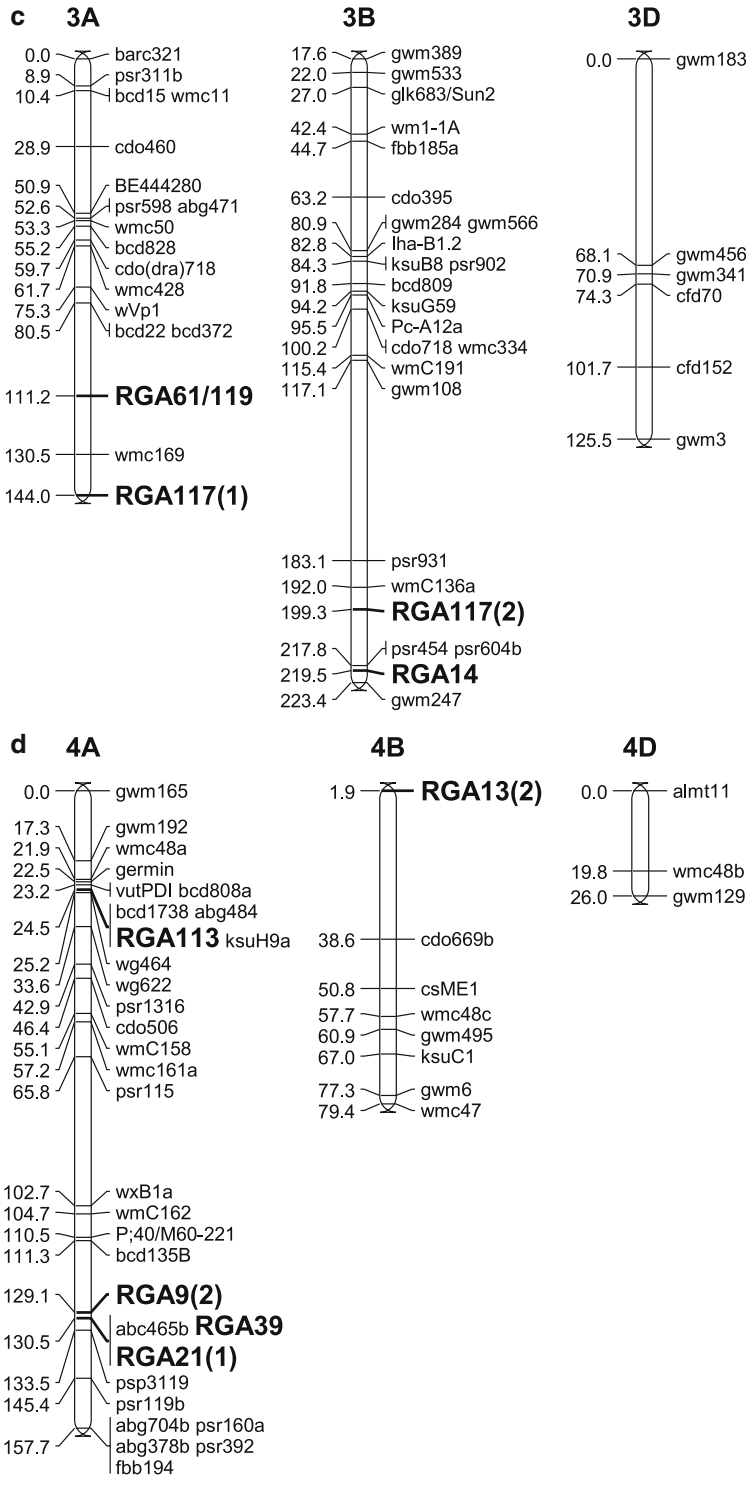
Fig. 3 **a–g** Genetic linkage groups for RGA families (*bold*) determined in Cranbrook × Halberd doubled haploid lines, relative to existing microsatellite markers (*normal*) for each chromosome group of wheat. Probes for RGA81 and RGA 98(2) (group 5) were shown to be duplicate probes on the basis of identical RFLP banding patterns for the digests of the Cranbrook and Halberd parent lines with six different restriction enzymes. Probes for RGA61/116 (group 3) and RGA 46/95 (group 7) were also duplicate probes

of expressed NBS-encoding genes (Monosi et al. 2004). The number of NBS-containing sequence groups that we identified using wheat and barley ESTs (129) is therefore similar to the number of expressed NBS-containing gene sequence groups identified in rice. Also, our approach identified a similar number of potential R-gene candidates to that identified in another recent study of expressed RGAs in wheat (Dilbirligi and Gill 2003) where RNA fingerprinting and data mining identified 125 R-gene candidates (not grouped into families) with sequence similarity to known R genes. Thus, if the number of expressed NBS-containing sequences in wheat is similar to that in rice (a currently untested assumption), then we have achieved a reasonable coverage of wheat NBS-containing RGAs.

Identification of RGA gene clusters

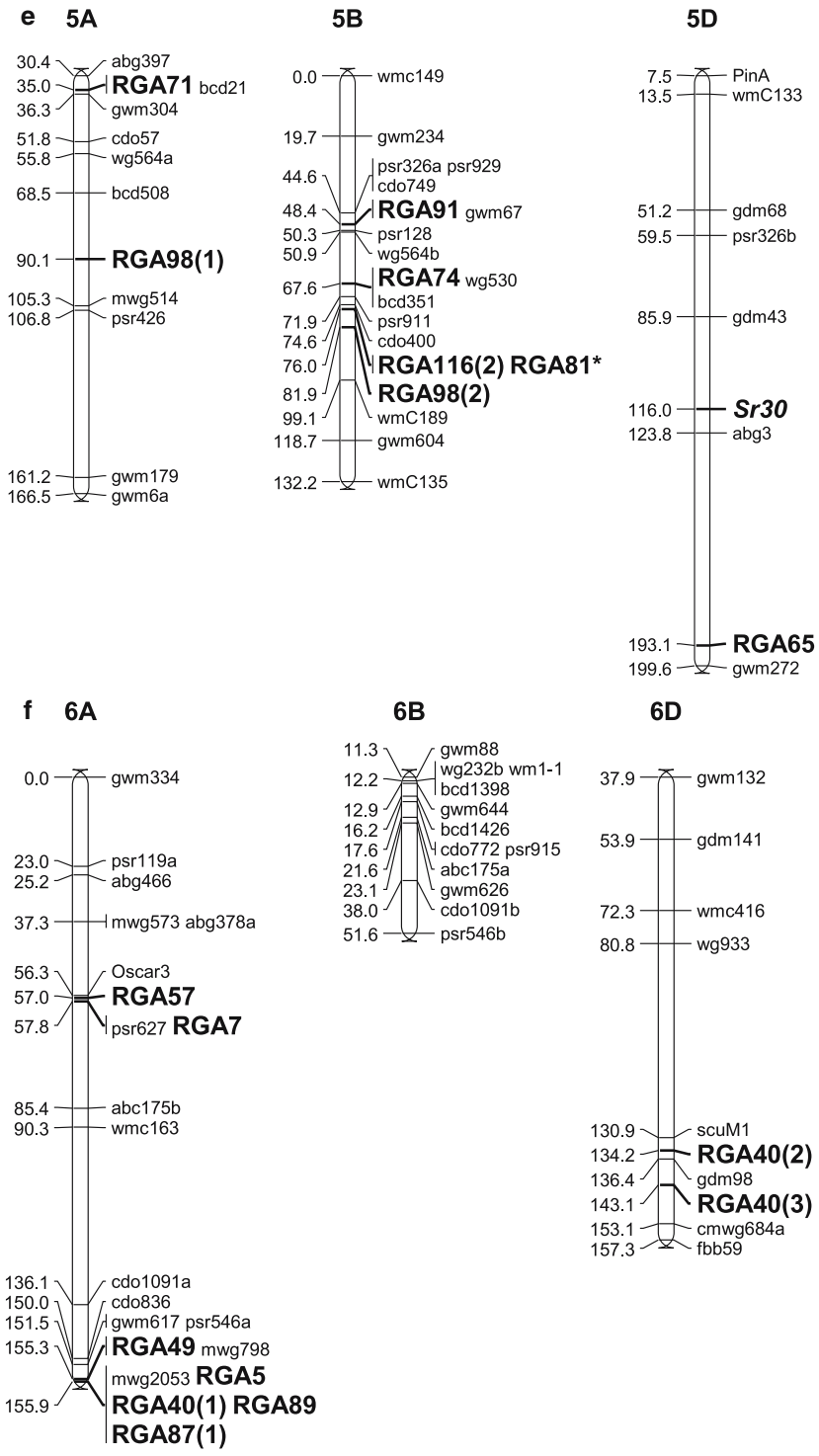
In this study, we used EST sequences to generate probes. Given that these sequences are not full

Fig. 3 continued



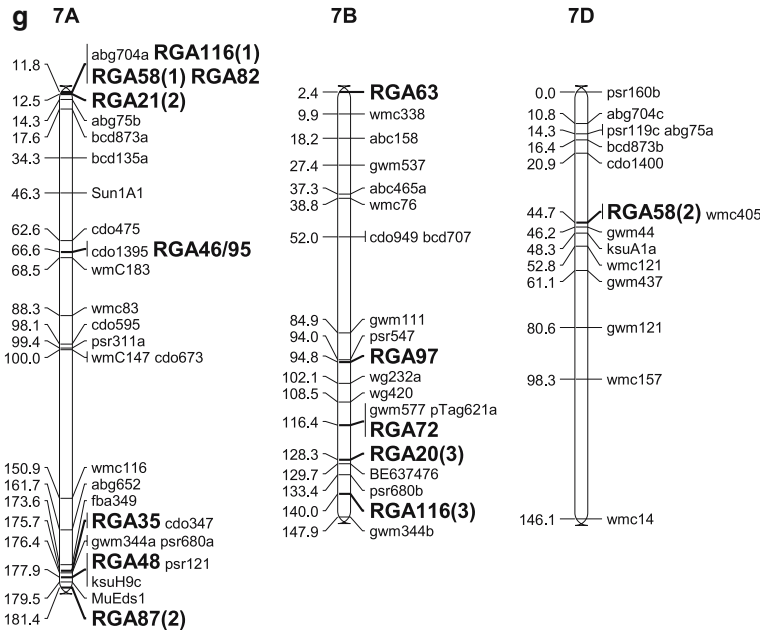
length, we were unable to assign the sequences to particular NBS-LRR gene classes (Monosi et al. 2004). There was also the potential for identifying several probes for the same RGA because different ESTs may have contained a partial sequence from a different portion of the same gene and hence generated sequences that did not overlap. These sequences would not have shown any sequence similarity, even though they were derived from the same gene. However, we could identify when this had occurred and found that it was not a major factor limiting the usefulness of the mapping exercise. We identified nine potential RGA clusters. These could be explained either by the presence of genuine RGA clusters or by

Fig. 3 continued



the use of multiple probes for the same RGA. By examining the hybridisation pattern of the RGA probes to the parent digests, we were able to determine that the use of multiple probes for the same RGA had only occurred in three instances, and that seven clusters (one contained both duplicate and different RGA probes) were genuine RGA clusters. In one instance, two RGAs that mapped to close but not identical loci [RGA 81/98(2)] were found to give similar hybridisation patterns with parent DNA blots. This discrepancy is probably due to a small number of phenotyping errors giving a small error in map position. Disease resistance genes are frequently observed in clusters (Dilbirligi et al. 2004), so our observation of clusters of RGAs in the C × H mapping population was not unexpected.

Fig. 3 continued



Comparison of physical chromosome location with map location

In most cases where comparison could be made (35/45 cases), we observed an agreement between the results for physical chromosome location obtained with the nullitetrasomic lines and the linkage group observed in the C × H mapping data. In a further five instances, the RGA location predicted from nullitetrasomic analysis and the map data was in the same homoeologous chromosome group. Given that most RGA probes hybridised to several bands (Table 1), it is likely that the different enzymes used for restriction of genomic DNA for the two types of analysis could have resulted in the identification of different bands corresponding to different loci in some instances. In addition, there may be differences between the location of RGAs in Chinese Spring and in the C × H mapping population.

There was good agreement between the results we obtained and those observed in other studies. In this work, the RGA family represented by probe RGA3 (BE498831) (which shows significant sequence homology to *Lr*21, see Table 1) mapped to linkage groups corresponding to the distal region of the short arms of the group 1 chromosomes. This corresponds with the physical location of this EST determined using wheat deletion lines (Dilbirligi et al. 2004) and the known location for *Lr*21 on 1DS (Huang et al. 2003). In addition, other RGAs and known disease resistance genes have been found in this region, such as *Yr*10 (1BS) and *Lr*40 (1DS) (Spielmeyer and Lagudah 2003; Spielmeyer et al. 2000). A correspondence between the physical chromosome location determined from wheat deletion lines (Dilbirligi et al. 2004) and the genetic location determined in our study by linkage analysis was also observed for RGA5, RGA63, RGA81 and RGA87. This correspondence between physical and genetic map information gives confidence in the validity of the C × H linkage group data. However, it is interesting to note that there were no RGAs identified that co-localised with the two resistance genes mapped in C × H, namely *Yr7/Sr9* on chromosome 2B and *Sr30* on 5D. If these R genes are members of the NBS-LRR class, then this is evidence that the expressed NBS-containing sequences described in this study do not represent a complete set of NBS-LRR resistance genes in the wheat genome.

Distribution of RGAs in the wheat genome

Although we observed most RGA markers on A genome chromosomes and on group 1 chromosomes, we found that there was no significant difference between the distribution of RGA markers and all markers on the C × H map. Thus, we could not draw any firm conclusions about the preferential location of RGAs within the wheat genome. This highlights one of the limitations of the mapping approach used, in that the mapping of RGA markers was dependent on the distribution of existing markers in the C × H map. We obtained 26 RGA markers that were unlinked in the C × H map. These may have mapped to chromosome

regions not well-represented in the C × H map, such as the RGA-rich subtelomeric regions (Dilbirligi et al. 2004).

A further limitation of the mapping approach used is that it relies on the detection of polymorphic bands on hybridisation of RGA probes to digested genomic DNA blots. Although we were able to obtain map positions for 49 probes, we found that 25 did not show any polymorphic bands in the two parents, and a further 13 probes gave polymorphic bands that were unlinked in the mapping population.

The RGA markers used in this study have been mapped using RFLP analysis with probes generated using primers for the NBS regions of the RGAs. The NBS region is a relatively highly conserved region within RGA sequences, so these primers are unlikely to be useful for PCR-based discrimination between lines where differences between resistance and susceptibility are due to the presence of functional and non-functional R-gene homologues. Further work would be required to generate and confirm a PCR-based marker derived from these RFLP markers. In this study, we observed RGAs in all 21 chromosomes using nullitetrasomic lines and mapped RGAs to linkage groups representing 18 chromosomes in the C × H map. No RGAs were found that mapped to the linkage groups for 3D, 4D, 6B. We have determined the chromosome location for 71 RGA families and a map location for 49 RGA loci. Therefore, we have described a set of RGA markers covering a significant portion of the wheat genome that could be developed further for use in the mapping and ultimate cloning of resistance genes in wheat.

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