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Cloning and characterisation of a family of disease resistance gene analogs from wheat and barley

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Abstract The most common class of plant disease resistance (R) genes cloned so far belong to the NBS-LRR group which contain nucleotide-binding sites (NBS) and a leucine-rich repeat (LRR). Specific primer sequences derived from a previously isolated NBS-LRR sequence at the *Cre3* locus, which confers resistance to cereal cyst nematode (CCN) in wheat (*Triticum aestivum* L.) were used in isolating a family of resistance gene analogs (RGA) through a polymerase chain reaction (PCR) cloning approach. The cloning, analysis and genetic mapping of a family of RGAs from wheat (cv 'Chinese Spring') and barley (*Hordeum vulgare* L. cvs 'Chebec' and 'Harrington') are presented. The wheat and barley RGAs contain other conserved motifs present in known R genes from other plants and share between 55–99% amino acid sequence identity to the NBS-LRR sequence at the *Cre3* locus. Phylogenetic analysis of the RGAs with other cloned R genes and RGAs from various plant species indicate that they belong to a superfamily of NBS-containing genes. Two of the barley derived RGAs were mapped onto loci on chromosomes 2H (2), 5H (7) and 7H (1) using barley doubled haploid (DH) mapping populations. Some of these loci identified are associated with regions carrying resistance to CCN and corn leaf aphid.

Key words Barley (*Hordeum vulgare* L.) · Wheat (*Triticum aestivum* L.) · Plant disease resistance genes · Nucleotide binding site-leucine rich repeat (NBS-LRR) · Genetic mapping

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Introduction

The majority of plant disease resistance (R) genes cloned so far contain nucleotide-binding sites (NBS) and a leucine-rich repeat (LRR) domain. An interesting feature of this class of R genes is that they are involved in gene-for-gene resistance towards either fungal, viral, bacterial or nematode disease resistance (see review of Baker et al. 1997; Bent et al. 1994; Grant et al. 1995; Lawrence et al. 1995; Whitham et al. 1994; Lagudah et al. 1997; V. Williamson, personal communication). This class of R genes belongs to a superfamily, that is present in both dicotyledons and monocotyledons as suggested from sequence comparisons made between these isolated genes. Such analyses have revealed several highly conserved functional amino acid motifs, notably the NBS sequences (P-loop and Kinase-2a) and others of unknown function between the NBS and LRR region of this family of R genes.

The conservation between different NBS-LRR resistance genes opens the avenue for the use of polymerase chain reaction (PCR)-based strategies in isolating and cloning other R gene family members or analogs using degenerate or specific primers for these conserved regions. Strategies based on degenerate primers have been successfully utilised in the cloning of other putative NBS-LRR resistance gene analogs (RGA) from potato (*Solanum tuberosum* L.) (Leister et al. 1996) and soybean (*Glycine max* (L.) Merr.) (Kanazin et al. 1996; Yu et al. 1996). Some of these RGAs have been shown

to be associated with known R loci occurring in these plants, thus demonstrating the potential of the PCR-based strategy as an entry point into cloning other NBS-LRR-type R genes. This approach thus provides an alternative strategy to the classical methods of transposon tagging and map-based cloning strategies used so far.

Wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) are two economically important crops around the world which have been well studied in relation to the mapping of their genome for various R genes and other agronomic traits. The cloning of R genes from these two crops has taken a longer time due to its more complex and larger genome compared to other crops studied. Nevertheless, the benefits to be gained from the cloning of specific R genes, which are then used as cosegregating or 'perfect' markers in selection and as intergeneric sources of transgenes in transformation breeding, justifies continued effort.

In the study presented here, a PCR approach based on specific primers from the previously isolated NBS-LRR gene sequence at the *Cre3* cereal cyst nematode (CCN) resistance locus in *Triticum tauschii* L. (Lagudah et al. 1997) was used in the isolation of a family of RGAs from wheat (cv 'Chinese Spring') and barley (cvs 'Chebec' and 'Harrington'). The specific primers were derived from regions of conservation between the sequence at the *Cre3* locus and other known R genes (Grant et al. 1995). The cloning, sequence characterisation and chromosomal and genetic map location of the family of the RGAs from wheat and barley as well as their relationship to a wider family of cloned R genes and analogs from other plant species are reported in this paper.

Materials and methods

Genetic materials

Plant materials used for isolating RGAs were from wheat cv 'Chinese Spring' and barley cvs 'Chebec' and 'Harrington'. Barley doubled haploid (DH) populations of 'Steptoe' × 'Morex' (S × M), 'Chebec' × 'Harrington' (CH × H) used in the mapping studies were kindly supplied by R. Boyd (The University of Western Australia). Samples of genomic DNA from the 'Galleon' × 'Haruna Nijo' (G × Hn) DH population used in this study were based on those described in Langridge et al. (1995). All genomic DNA was extracted from leaf material as described in Lagudah et al. (1991b).

PCR analysis

Specific PCR primers from the Kinase-2a (Kin2: 5′-TGATACTGG ATGATGTCTGG-3′) and another downstream conserved amino acid motif, EGF (rpEGF: 5′-GTGCTTCTTATGAACCCTTC-3′) derived from the NBS-LRR sequence at the *Cre3* locus (Lagudah et al. 1997) were used in isolating other NBS-LRR gene analogs from wheat and barley. Each 50-μl PCR reaction consisted of the following reagents: 2.5 μl 100 ng/μl total genomic DNA, 5 μl 10 × PCR buffer (Promega), 4 μl 25 m*M* $MgCl_2$, 5 μl 2 m*M* dNTPs, 5 μl each of 10 pmol/μl primer, 22.5 μl MQ H_2O and 1 μl *Taq* polymerase (Promega). Cycle conditions were: 5 cycles each of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 2 min; followed by 30 cycles each of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 50 s; and finally 1 cycle of denaturation at 94°C for 30s, annealing at 55°C for 30 s and elongation at 72°C for 5 min.

Cloning and analysis of PCR products

Five-microliter aliquots of PCR products were separated on a 1% agarose gel and visualised under UV light. The remaining products were purified and concentrated using 'Wizard PCR Prep' (Promega) and cloned into the pGemT vector (Promega) according to supplier's instructions.

Each PCR clone was selected for sequencing based on its hybridisation intensity on nylon membrane to [P^{32}]-labelled probe made from a pooled sample of individually eluted PCR bands and the *Cre3* NBS-LRR sequence. DNA hybridisation conditions were as described in Lagudah et al. (1991a). Selected clones were prepared and sequenced according to the supplier's instructions using 'ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase' (Perkin Elmer). Sequences were compared and analysed using BLAST (Altschul et al. 1990) and the Genetics Computer Group (GCG) packages from the University of Wisconsin.

Restriction fragment length polymorphism (RFLP) analysis and mapping

A range of restriction enzymes was used to digest genomic DNA and to analyse for restriction fragment length polymorphisms (RFLP) between the parental lines of the three sets of barley mapping populations. Restriction enzymes and RGA probe combinations resulting in clear RFLPs between the barley parents were used in subsequent mapping studies involving two of the barley RGA clones designated, ssCH4 and ssCH5. DNA hybridisation and washing were performed as described previously. Genetic mapping in the SxM population was based on 150 *Nco*I-digested DH progeny probed with the ssCH4 clone; 50 *Xho*I-digested DH progeny were used with the ssCH5 clone. In the CH × H population, 60 *Nco*I-digested DH progeny were used in mapping the ssCH4 clone. With the G × Hn DH population, the ssCH4 and ssCH5 clones were mapped with the aid of 120 *Bam*HI-digested, 60 *Hin*dIII digested and 60 *Nco*I-digested DH progeny. Mapping of RGA markers was done using 'MAPMAKER V 2.0' (Lander et al. 1987). The RGA loci identified bear the prefix 'Rl' (for RGA loci) followed by the name of the RGA clone used as the probe. In parenthesis, in capitals, is the first letter of the restriction enzyme used in obtaining the RFLP and in lowcase, the identification of the scored polymorphic band.

In the case of the wheat clones (ssCS3 and ssCS4), only their chromosomal locations in wheat and barley were determined using the wheat cv 'Chinese Spring' nullitetrasomic series and the barley cv 'Betzes' addition line series (Sears 1966; Islam et al. 1981). Genomic DNA of these genetic stocks was digested with the *Dra*I restriction enzyme and analysed for RFLP patterns using the RGAs as RFLP probes.

Results

PCR amplification of RGAs

Major PCR products in the 530-bp size range were obtained from wheat cv 'Chinese Spring' (CS) and

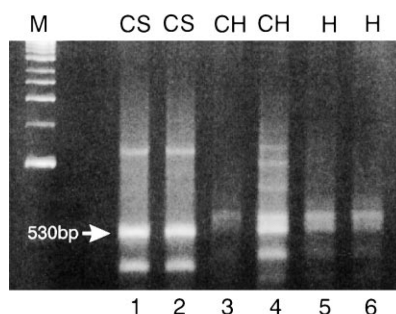

Fig. 1 PCR products amplified from genomic DNA of wheat cv 'Chinese spring' (*CS – lanes 1 and 2*) and barley cvs 'Chebec' (*CH – lanes 3* and *4*) and 'Harrington' (*H – lanes 5* and *6*) using the Kin2- and rpEGF specific PCR primers

barley cvs 'Chebec' (CH) and cv 'Harrington' (H) (Fig. 1). This was as expected since the Kin2 and rpEGF primers from the NBS-LRR sequence at the *Cre3* locus were 532 bp apart (Lagudah et al. 1997) (Fig. 2). In addition to this, several other PCR products of varying sizes were also observed from the wheat and barley, cultivars, but these were not reproducible (Fig. 1). This was also observed when the same set of PCR primers were used with sugarcane (*Saccharum spontaneum* L.) and sorghum (*Sorghum bicolor* L. (Moench)) genomic DNA (L. McIntyre, personal communication). None of the larger sized fragments were successfully cloned; however, cloned smaller fragments (< 530 bp), when sequenced, revealed no resemblance to resistance-like genes or contained no open reading frames. Some of these, on the other hand, showed close identity to retrotransposon-like elements as determined through BLAST searches of GenBank.

Analysis of RGAs

A total of 14 clones from CS, 44 from CH and 34 from H were obtained after cloning the amplified PCR products (Fig. 1). Of these, 7 clones from CS (ssCS1-7), 8 from CH (ssCH1-8) and 9 from H (ssH1-9) showed strong hybridisation when probed with the eluted PCR bands including the NBS-LRR sequence at the *Cre3* locus. These clones were thus selected for sequencing which yielded 5 distinct clones with open reading frames, 2 from wheat (ssCS3 and ssCS4) and 3 from barley (ssCH4, ssCH5 and ssH3). The deduced amino acid sequence of these clones showed a varying level of relatedness to the NBS-LRR sequence at the *Cre3* locus. The most closely related sequence was ssCH4 (98.9% identity and similarity), followed by ssCS3 (92% similarity, 85.2% identity), and ssH3 (75% similarity, 60.2% identity) and ssCS4 (73.9% similarity, 60.8% identity), and the most divergent one was ssCH5 (71.6% similarity, 55.1% identity). Each of these clones resembled parts of R genes in that they contain internal signature motifs present in the other NBS-LRR class of R genes (Fig. 2). Furthermore, a BLAST search of the GenBank database with each of these sequences has revealed strong associations with several R genes, some of which include *I2* from tomato, *RPS2* and *RPM1* from arabidopsis. Consequently, these clones were tentatively classified as RGAs.

Phylogenetic analysis of the wheat and barley RGA clones isolated in this study and other PCR-derived RGAs and known R genes indicate that these RGAs and NBS-LRR resistance genes belong to a superfamily of multigenes present within and across a wide range of plant species (Fig. 3). This analysis was based on the amino acid sequences spanning the region between the Kinase-2a conserved amino acid motif the EGF or RCF(A/L)Y conserved motifs since the EGF motifs was not present in all RGAs and R genes characterised (Fig. 2). Several subclasses or clusters were observed; the RGAs isolated from this study, including a maize RGA (Mrga1, sequence kindly provided by N. Collins), was placed in the same cluster as the NBS-LRR sequence at the *Cre3* locus. This perhaps is an indication of the selection for a particular subclass of R genes isolated based on the specific primers used and the pre-selection of clones by DNA hybridisation when isolating these RGAs for analysis. Most of the other RGAs in Fig. 3 were isolated using degenerate primers from other conserved regions of known NBS-LRR containing R genes, namely the P-Loop, Kinase-2 and GLPLAL motifs.

Genetic mapping of RGAs

Only the two most divergent RGAs (ssCH4 and ssCH5) isolated from this study were selected for further RFLP mapping analysis using the S × M, CH × H and G × Hn DH mapping populations since the RGAs were expected to cross-hybridise. The RFLP profiles showing multiple banding patterns using these RGAs as probes identified these RGAs as belonging to a multigene family (Fig. 4). These clones were found to map mainly to the long arms of chromosomes 2H (2), 5H (7) and 7H (1) of barley, except in one case where clone ssCH4 detected one RGA locus on the short arm of 7H in the S × M population (Figs. 4 and 5). At least seven non-homologous loci associated with either the ssCH4 or ssCH5 RGA were identified across the three mapping populations (Fig. 5).

Two of these loci were located on the long arm of chromosome 2H which hybridised with ssCH4. In the CH × H population, the ssCH4 RGA identified a locus (Rlch4(Ne)) closely linked to the previously mapped phenotypic *Ha2* (barley CCN) resistance locus and mapped proximal to the flanking BCD453(B) RFLP marker (Fig. 5). The precise location of this RGA locus between these two markers is still being resolved. This

Fig. 2 Alignment of deduced amino acid sequences of putative RGAs from wheat and barley derived from this study (ssCH4, ssCH5, ssH3, ssCS3 and ssCS4; Genbank accession numbers: AF052395-99) and other cloned NBS-LRR type R genes (*Cre3*, *RPS2*, *RPM1*, *N* and *L6*.). *Arrows* show primer sites in *bold*. *Underlined* sequences in *bold* indicate internal conserved motifs

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              Kin2 -->
              1                                                50
ssCS3      ..ILDDVWCN ADVGNQ.ELP KLLSPLKKGK KESKILVTTR SKYALPDLCP
ssCS4      ..ILDDVWCN KDVGEQ.NLP QLLSSFKVGK RGSKILATSR NVDAFSDLGP
ssH3       ..ILDDVWCN KDVGEQ.NLP QLLSPFKIGK RGSKILATSR NVDAFFDLGP
ssCH4      ..ILDDVWCN KDVSDQ.NLP ELLSPLKVGK RGSKILVTTR SKYALPDLGP
ssCH5      ..ILDDVWCS KNVNEQKKLP ELLPPLIAAH NGSKILVTSR HHDALLELGP
Cre3       ..ILDDVWCN KDVSDQ.NLP ELLSPLKVGK RGSKILVTTR SKYALPVLGP
Cre3 CD2   .LVLDDVWCN ADVGNQ.ELP KLLSPLKKGK KGSKILVTTR SKYALPDLCP
RPS2       .LLLDDVW.E EI.DLEKTGV P.R.PD..RE NKCKVMFTTR SIALCNNM..
RPM1       IVVLDDVWTT GL..WREISI AL..PD..GI YGSRVMMTTR DMNVASFP..
N          LIVLDDI..D NKDHYLEYLA GDLDWF..GN .GSRIIITTR DKHLIEKN..
L6         LVVLDDV..D EKFKFEDMLG SPKDFI..SQ ..SRFIITSR SMRVLGTL..

           51                                               100
ssCS3      GVR.YTAMPI TEVDDTAFFE LFMHYALEDG .QDQ....SM FQNIGVEIAK
ssCS4      DVR.CANFPI SDLDDSMFLE LFMYYALGDW .NXNNGAGST LWNIGAHIAE
ssH3       DVR.CATFPI SDLDDSMFLE LFMYYALGDG .NANDGVGST LWNIGAHIAE
ssCH4      GVR.CTAIPV PEFDDTAFFE LFMHYALEEG .QDQ....SL FCIIGEEIAK
ssCH5      QVRVIDNYAI PELEDEVFLE LLMHNAFGQR IRDDDASYRQ LLKFGSKIAQ
Cre3       GVR.CTAIPV PEFDDTAFFE LFMHYALEEG .QDQ....SL FCIIGEEIAK
Cre3 CD2   GVR.YTAMPI TEVDDTAFFE LFMHYALEDG .QDQ....SM FQNIGVEIAK
RPS2       GA..EYKLRV EFLEKKHAWE LFCSKVWRKD LLES....SS IRRLAEIIVS
RPM1       YGIGSTKHEI ELLKEDEAWV LFSNKAFPAS LEQCRT..QN LEPIARKLVE
N          DI....IYEV TALPDHESIQ LFKQHAFGKE VPNE....N. FEKLSLEVVN
L6         NENQCKLYEV GSMSKPRSLE LFSKHAFKKN TP.P....SY YETLANDVVD

           101                                              150
ssCS3      KLKGSPLAAR TVGGNLRRQQ DVDHWRRVGD QDL....... .FKVWTGPLW
ssCS4      KLKRSPLAAS TVGGQLRTRK NVELWRRARD QDL....... .LNETMGALS
ssH3       KLKRSPLAAS TVGGQLRKRK NAEFWRRARN QDL....... .LNETMGALS
ssCH4      KLKGSPLAAR TVGGNLRRQP DVDHWRRVRD QDL....... .FKVWGGPLW
ssCH5      KLKKSPLLAS VVGQMLNKNH DVDHWGSIAE QKI....... .LDEYMGPLW
Cre3       KLKGSPLAAR TVGGNLRRQP DVDHWRRVRD QDL....... .FKVWGGPLW
Cre3 CD2   KLKGSPLAAR TVGGNLRRQQ DVDHWRRVGD QDL....... .FKVWTGPLW
RPS2       KCGGLPLALI TLGGAMAHRE TEEEWIHASE VLTRFPAEMK GMNYVFALLK
RPM1       RCQGLPLAIA SLGSMMSTKK FESEWKKVYS TLNWELNNNH ELKIVRSIMF
N          YAKGLPLALK VWGSLLHNLR LTEWKSAIEH MKNN.SYSGI IDKLKISYDG
L6         TTAGLPLTLK VIGSLLFKQE IAVWEDTLEQ LRRTLNLDEV YDRLKISYDA

                                                     rpEGF
           151                                      <--   194
ssCS3      WSYYQLGEQA RR.CFAYCSI FPRRHRLYRD ELVRLWMAEG FIRS
ssCS4      WSYQHLNAQI RQ.CFAYCSM FPRGHHLRRN VLVKLWVAEG FIRS
ssH3       WSYQHLNEQI RQ.CFAYCSM FPRGHHFRLH VLVKLWVAEG FIRS
ssCH4      QWSYYQLGEA RR.CFAYCSI FPRRHRLYRD DLVRLWVAEG FIRS
ssCH5      WCYQHLDEQV KR.CFAYCSV FPRRRHLYRD KLVNLWVAEG FIRS
Cre3       WSYYQLGEQA RR.CFAYCSI FPRRHRLYXD DLVRLWVAEG FIRS
Cre3 CD2   WSYYQLGEQA RR.CFAYCSI FPRRHRLYRD ELVRLWMAEG FIRN
RPS2       FSYDNLESDL LRSCFLYCAL FPEEHSIEIE QLVEYWVGEG FL..
RPM1       LSFNDLPYPL KR.CFLYCSL .......... .......... ....
N          LEPKQQEMFL DIACFLRG.. .......... .......... ....
L6         LNPEAKEIFL DIACFFIG.. .......... .......... ....
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will be discussed later. Using the G × Hn DH, we identified a relatively more distal RGA locus (Rlch4(BaNc)) after having probed with the ssCH4 RGA. None of the barley RGAs mapped onto chromosome 2H of the S × M population based on the restriction enzymes used to detect polymorphisms.

On chromosome 5H, two or possibly three independent RGA loci were identified. The ssCH5 clone

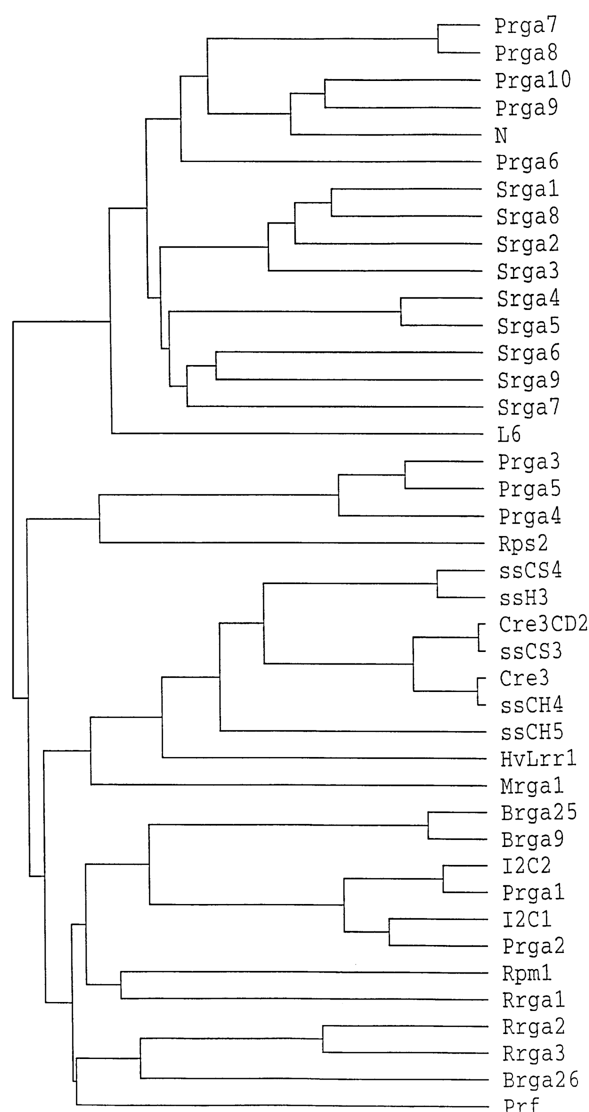


Fig. 3 Phylogenetic analysis of deduced amino acid sequences of RGAs isolated from wheat (ssCS3 and ssCS4), barley [ssH3, ssCH4, ssCH5 and HvLrr1 (courtesy of M. Robertson, CSIRO], Brga 9, 25 and 26 (courtesy of N. Collins, CSIRO), potato (Prga) (Leister et al. 1996), soybean (Srga) (Kanazin et al. 1996), rice (Rrga) (Xue et al. unpublished, GenBank accession nos.: y09807, y09810 and y09812), maize (Mrga) (courtesy of N. Collins, CSIRO) and other NBS-LRR-type R genes – *Cre3* and *Cre3*CD2 (Lagudah et al. 1997), *N* (Whitham et al. 1994), *RPS2* (Bent et al. 1994), *RPM1* (Grant et al. 1995), *PRF* (Salmeron et al. 1996), *L6* (Lawrence et al. 1995) and *I2C1* and *C2* (Ori et al. 1997)

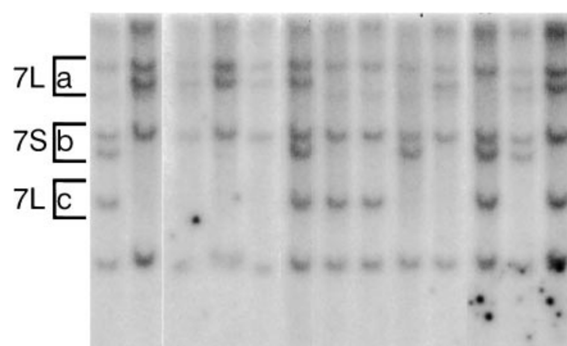


Fig. 4 DNA hybridisation patterns of *NcoI*-digested segregating progeny from the 'Steptoe' × 'Morex' DH population probed with the ssCH4 RGA. The *first two lanes* show the RFLP profile of the 'Steptoe' and 'Morex' parents and three independently segregating polymorphic regions indicated (a, b and c), two of which map onto the long (*L*) arm of chromosome 7H and one on the short (*S*) arm of 7H

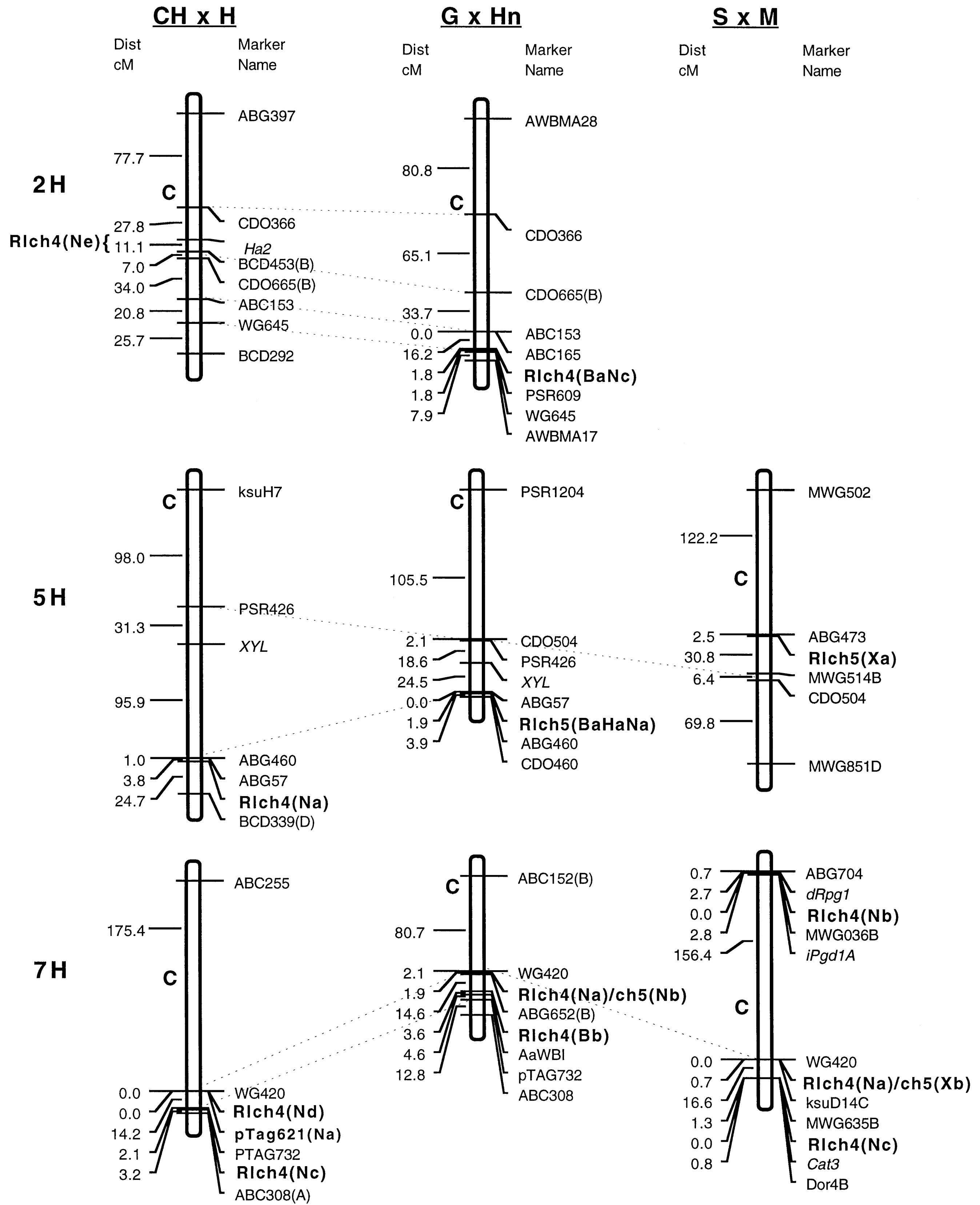
identified a locus Rlch5(Ba/Ha/Na) in the G × Hn population which cosegregated with the ABG57 RFLP marker on the distal end of chromosome 5H. Similarly, the ssCH4 RGA was also linked by 3.8 cM to the ABG57 RFLP marker in the CHxH DH population. However in the S × M DH population, a more proximal RGA locus Rlch5(Xa), was detected (Fig. 5).

Three independent RGA loci were identified on chromosome 7H, one on the short arm which mapped 2.7 cM proximal to the phenotypic stem rust R locus *Rpg1* and the other two on the distal end of the long arm (Fig. 4). The Rlch4(Nb) locus on the short arm was detected only on the S × M DH and not on the other two mapping populations. In contrast, the other two RGA loci on the long arm were detected across the three mapping populations and may represent homologous locus The two RGA loci were separated by a mean distance of 15.1 cM from one another. The more proximal locus was consistently linked to the WG420 RFLP marker, which was identified by both the ssCH4 and ssCH5 RGAs in the G × Hn and S × M populations. No mapping of the ssCH5 RGA on the CH × H DH population was done. Whether this is the same locus that the two RGAs have identified as evidence of cross-hybridisation or a cluster of NBS-LRR sequences around the RFLP WG420 marker remains to be resolved since the two RGAs share only 69.5% DNA sequence identity.

Using the wheat nullitetrasomic series and barley addition lines, we found sequences related to the wheat RGA ssCS3 to be located on chromosome 2B of wheat and 2H of barley, whereas the ssCS4 RGA was found on chromosome 2A of wheat and 2H of barley (data not shown).

Discussion

This paper describes the PCR-based isolation of five R gene-like sequences or RGAs from wheat and barley (Fig. 2) of which the two most diverse were more intensively characterised by genetic mapping (Fig. 5). Lines of evidence which allow these PCR-derived clones to be identified as RGAs or portions of R genes were based on amino acid sequence comparisons which indicated


CH x H
G x Hn
S x M
Dist cM
Marker Name
2H
5H
7H
Rlch4(Ne)
ABG397
CDO366
Ha2
BCD453(B)
CDO665(B)
ABC153
WG645
BCD292
AWBMA28
ABC165
Rlch4(BaNc)
PSR609
AWBMA17
ksuH7
PSR426
XYL
ABG460
ABG57
Rlch4(Na)
BCD339(D)
PSR1204
CDO504
Rlch5(BaHaNa)
CDO460
MWG502
ABG473
Rlch5(Xa)
MWG514B
MWG851D
ABC255
WG420
Rlch4(Nd)
pTag621(Na)
PTAG732
Rlch4(Nc)
ABC308(A)
ABC152(B)
Rlch4(Na)/ch5(Nb)
ABG652(B)
Rlch4(Bb)
AaWBI
pTAG732
ABC308
ABG704
dRpg1
Rlch4(Nb)
MWG036B
iPgd1A
Rlch4(Na)/ch5(Xb)
ksuD14C
MWG635B
Cat3
Dor4B

the presence of conserved motifs [e.g. T(T/S)R, G(L/S)PLA(A/I/L), CF(A/L)YC(S/A)] that are characteristic of other established NBS-LRR classes of R genes (see Fig. 2). Furthermore, some of the RFLPs detected by the ssCH4 and ssCH5 probes on the parental lines of the CH × H and Cl × S mapping populations also cross-hybridised to a genomic fragment which consists only of a LRR domain associated with the NBS-LRR sequence at the *Cre3* locus (data not shown). This therefore indicates that these PCR clones from wheat and barley also contain LRR domains like the other established R genes. Additionally, sequence homology of the five clones as compared to the NBS-LRR sequence at the *Cre3* locus (Lagudah et al. 1997) ranged from 55.1% to 98.9% identity at the amino acid sequence level, and their relatedness to other R genes and cloned RGAs described by the phylogenetic tree (Fig. 3) lends further support to their being RGAs. Michelmore (1996) alluded to the need for further genetic evidence in the characterisation of these PCR-isolated clones as RGAs. The questions therefore being raised here are: (1) are these sequences or clones from wheat and barley linked to or cosegregate with known phenotypic R loci in wheat and barley? (2) do these NBS containing sequences occur in clusters, which have been observed in many R loci (Kesseli et al. 1993; Pryor and Ellis 1993)?

The nucleotide sequence of the ssCH4 from barley bears 98.7% identity to that of the NBS-LRR sequence at the *Cre3* locus derived from *Triticum tauschii* (Lagudah et al. 1997) which confers resistance to cereal cyst nematode (CCN) in wheat as compared to 69.0% for ssCH5. One member of the NBS-LRR gene family locus was implicated as the candidate *Cre*3 CCN resistance gene. It was therefore of interest to examine whether ssCH4 maps in barley to regions potentially associated with CCN resistance. There are currently four known R genes in barley (*Ha1*, *Ha2*, *Ha3* and *Ha4*) which confer resistance to CCN, of which *Ha2* and *Ha4* are of interest to Australian breeders for the resistance they confer to the Australian CCN pathotype as compared to the European pathotype (Andersen and Andersen 1982; K.J. Chalmers, personal communication). The *Ha2* and *Ha3* loci are closely linked (Andersen and Andersen 1982) on the long arm of chromosomes 2H (Krestchmer et al. 1997) (see Fig. 5). The *Ha4* gene derived from barley cv ‘Galleon’ was recently mapped to the distal long arm of chromosome 5H (Barr et al. 1998) in the region carrying a xylanase (*XYL*) locus in the G × Hn population (see Fig. 5).

Interestingly, the ssCH4 RGA was mapped into the region containing the *Ha2* locus on chromosome 2H in the CH × H population (see Fig. 5). The precise location of this RGA locus (Rlch4(Ne)) between the *Ha*2 and BCD453(B) RFLP marker is still being clarified, the reason being the identification of four apparent recombinants between the previously scored *Ha2* phenotypes and ssCH4 markers, which suggests linkage rather than cosegregation between the two markers. One of these apparent recombinants has been retested in a CCN bioassay, and the results confirmed that either there was a misclassification of the previous *Ha2* phenotype or that a different line from the original DH line was used in the current mapping studies. In either case, the RFLP pattern detected by the ssCH4 RGA clone correctly predicted the *Ha2* phenotype. Results for the remaining three recombinants have yet to be obtained and would ultimately determine the association of the ssCH4 marker with the *Ha2* locus. A second non-homologous locus Rlch4(Ba/Nc), was identified by the ssCH4 RGA in the G × Hn DH (see Fig. 5) as being distinct from Rlch4(Ne) on CH × H DH based on the relative positions of common markers. This marker was deduced to be associated with an orthologous locus in wheat detected by the *Cre3* candidate gene and the linked PSR609 RFLP marker, confirming the syntenic relationship between wheat and barley group 2 chromosomes (W. Spielmeyer and E. Lagudah unpublished) (see Fig. 5). The RGA clones ssCH4 and ssCH5 mapped to regions distal to the *XYL* quantitative trait locus (QTL) in the CH × H and G × Hn DH populations. In the G × Hn DH population, the *Ha4* locus was mapped into the interval containing the *XYL* QTL (K. J. Chalmers, personal communication; Langridge et al. 1995). A finer resolution of the region is further required to determine the linkage between the ssCH5 RGA and the *Ha4* locus. In the S × M DH population a relatively proximal location was also detected by ssCH5 (see Fig. 5).

Results from this paper and other studies (D. Leister, personal communication) suggests that a group of R-like genes or NBS-containing sequences are clustered in the region of the *Rpg1* stem rust R locus on the short arm of chromosome 7H in the S × M DH population. In addition to the ssCH4 RGA locus Rlch4(Nb) reported in this study (see Fig. 5), a further two different NBS-containing RGAs from maize and barley (N. Collins and D. Leister, personal communication) have been mapped in close linkage to *Rpg1*; one of these cosegregated with the *Rpg1* locus. A corn leaf aphid QTL has also been mapped between *Rpg1* and the iPgd1 A RFLP marker (Moharramipour et al. 1997) where the ssCH4 RGA locus Rlch4(Nb) was located in

Fig. 5 Genetic distribution of the PCR-derived RGAs from barley (ssCH4 and ssCH5) in the ‘Chebec’ × ‘Harrington’ (CH × H), ‘Galleon’ × ‘Haruna nijo’ (G × Hn) and ‘Steptoe’ × ‘Morex’ (S × M) doubled haploid mapping population. Previous marker location on the CH × H, G × Hn maps were from Langridge et al. (1995), and the S × M maps were from Kleinhofs et al. (1993). Only the most proximal and distal markers of each chromosome together with the flanking markers around the resistance-like (*Rl*) loci identified by the two RGAs (ch4 and ch5) and common markers are included. *Capital letters* in *parenthesis* identify the restriction enzyme used (*B BamHI*, *H*, *Hind*III, *N*, *Nco*I, *X*, *Xho*I), and *lowercase letters* identify the polymorphic loci associated with each digest

this study (see Fig. 5). Further studies are required to determine the level and nature of the association between the ssCH4 RGA derived from a candidate gene for CCN resistance (*Cre3*) and aphid R loci or QTLS. The significance of such a study is based on the fact that the feeding habits of both the nematode and aphid are quite similar, and speculations on possible similarities in the structure of their respective R genes may not be far-fetched. Similar observations have previously been reported in tomato, where a tight linkage was found between the root knot nematode (*Mi*) and potato aphid (*Meu*) resistance loci (Kaloshian et al. 1995). Now shown to be the same gene (Rossi et al. 1998). Two of the resistance genes against Russian wheat aphid (*Dn1* and *Dn5*) have also been reported to be on chromosome 7 in wheat (Schroeder-Teeter et al. 1994; Marais and Du Toit 1993). The *Dn5* gene has been mapped to the long arm of 7D (G. Marais, personal communication). It remains to be shown whether *Dn5* is actually linked to either of the RGA loci detected by ssCH4 and ssCH5 on the long arm of 7H. One of the two or three loci identified by either the ssCH5 or ssCH4 RGA on the long arm of chromosome 7H (Fig. 5) of barley could also be related to the *CreR* CCN-resistant loci found in rye (*Secale cereale* L.). Chromosome 6L of rye on which the *CreR* resistance locus resides (Asiedu et al. 1990), contains a multiple translocation chromatin, consisting of chromosome arms from 3L and 7L (Devos et al. 1993) .

The observations from this study indicate that the barley RGAs map into regions containing phenotypically characterised R loci and in some regions are associated with a cluster of other RGAs. Some of these phenotypes include resistance to CCN (*Ha2*) and corn leaf aphid in barley. There are other possible orthologous R gene loci which have been characterised in wheat and rye and these will require further study.

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Note added in proof:
The RGA locus Rlch4(Ne) is now shown to be different from the *Ha2* locus.