Theor Appl Genet (1997) 94:1060–1064

J. M. Kretschmer · K. J. Chalmers · S. Manning
A. Karakousis · A. R. Barr · A. K. M. R. Islam
S. J. Logue · Y. W. Choe · S. J. Barker
R. C. M. Lance · P. Langridge

RFLP mapping of the *Ha 2* cereal cyst nematode resistance gene in barley

Received: 5 December 1996 / Accepted: 20 December 1996

Abstract The cereal cyst nematode (CCN), *Heterodera avenae* Woll., is an economically damaging pest of barley in many of the world's cereal-growing areas. The development of CCN-resistant cultivars may be accelerated through the use of molecular markers. A number of resistance genes against the pest are well known; one of them, the single dominant *Ha 2* resistance gene, has been shown to be effective against the Australian pathotype and maps to chromosome 2 of barley. Segregation analysis identified two restriction fragment length polymorphism (RFLP) markers flanking the resistance gene in two doubled-haploid populations of barley. AWBMA 21 and MWG 694 mapped 4.1 and 6.1 cM respectively from the *Ha 2* locus in the Chebec × Harrington cross and 4.0 and 9.2 cM respectively in the Clipper × Sahara cross. Analysis of a further seven sources of CCN resistance in the form of near-isogenic lines (NILs) indicates that all available sources of resistance to the Australian pathotype of CCN in barley represent the *Ha 2* locus.

Key words CCN · RFLP · *Hordeum vulgare* · *Heterodera avenae* · Genetic mapping

Communicated by G. Wenzel

J. M. Kretschmer · K. J. Chalmers (✉) · S. Manning
A. Karakousis · P. Langridge
ARC Special Research Centre for Basic and Applied Plant Molecular Biology, Department of Plant Science, Waite Campus, University of Adelaide, South Australia 5064

Y. W. Choe · A. R. Barr · A. K. M. R. Islam
S. J. Logue · S. J. Barker
Department of Plant Science, Waite Campus, University of Adelaide, South Australia 5064

R. C. M. Lance
Agriculture Western Australia, South Perth, Western Australia 6151

Introduction

The cereal cyst nematode (CCN), *Heterodera avenae* Woll., is endemic in the major cereal-growing regions of the world (Meagher 1977). In Australia, where large areas are infested, yield losses in an intolerant barley cultivar may be up to 30%. Since chemical control in field crops is expensive, and employs very toxic chemicals, the development of disease-resistant cultivars is highly desirable for the cultivation of barley in infested areas.

Studies on the inheritance of resistance to *H. avenae* in barley identified two major resistance genes (*Ha 1* and *Ha 2*). These genes segregate independently and have been used to breed resistant varieties (Andersen and Andersen 1968; Cotton and Hayes 1969). *Ha 1* confers a limited resistance against many of the European pathotypes. However, it is ineffective against the single pathotype found in Australia (Andersen and Andersen 1982).

Ha 2 has been shown to be effective against the Australian pathotype. The resistance gene derived from *Hordeum pallidum* var. 191 (Cotton and Hayes 1969) has been incorporated into commercial varieties and has been located on the long arm of chromosome 2H (Andersen and Andersen 1973). The *Ha 2* gene confers resistance to *H. avenae*, arresting female development at about 15 days by causing the degradation of feeding sites in the root steele (Williams and Fisher 1993).

The selection of resistant lines in breeding programmes has been constrained by the test for resistance, a bio-assay which is time-consuming and expensive. Recent mechanisation and computerisation of the bioassay have reduced the cost but the test still takes 14 weeks and is confined to the winter months. The identification of markers linked to the gene conferring a resistant phenotype would therefore represent an important tool for barley breeders. The availability of detailed restriction fragment length polymorphism

(RFLP) maps of the barley genome (Langridge et al. 1995) now provides a means for the genetic location of closely linked, selectable markers.

Methods for targeting markers to important plant genes have largely been based on the use of near-isogenic lines (NILs). A comparison of NILs with the recurrent and donor parent provides a means for the identification of DNA sequences arising from the introgressed region of the genome. This approach has identified tightly linked molecular markers for several important disease resistance genes, as reported by Williams et al. (1994) for the *Cre* locus in wheat, Barua et al. (1993) for the *Rh* locus in barley, and Schüller et al. (1992) for the *Ml-a* locus in barley. Importantly, loci conferring resistance to *H. avenae* have been mapped in bread wheat on the long arm of chromosome 2B (Williams et al. 1994), in *Triticum tauschii* on 2D (Eastwood et al. 1991), and in rye to the long arm of chromosome 6 (Asiedu 1986). Given the high level of synteny between wheat, rye and barley, it is probable that three loci can confer resistance to CCN: two on the long arm of group-2 chromosomes, and one on the long arm of group 6.

The objective of the present study was to identify molecular markers linked to the *Ha 2* gene for resistance to *H. avenae* which can be used for marker-assisted selection in barley. A further objective was to determine if additional loci could be identified from alternative sources. Such loci would provide an opportunity to pyramid genes for resistance to CCN.

Materials and methods

Plant material

The donor and recurrent parents used to create the near-isogenic lines are given in Table 1. Doubled-haploid (DH) populations were produced from crosses between the barley cultivars Chebec (CCN-resistant) and Harrington (CCN-susceptible) and between the barley cultivar Clipper (CCN-susceptible) and the Algerian land-race Sahara 3771 (CCN-resistant). The Chebec × Harrington population was generated by anther-culture while the Clipper × Sahara population was produced by the *Hordeum bulbosum* method (Finnie et al. 1989). Near-isogenic lines (NILs) were obtained from Dr. James M^{c}Key (Swedish University of Agricultural Science).

Nematode assay

A total of 88 doubled-haploid lines of the Chebec × Harrington and 148 lines of the Clipper × Sahara crosses, together with their parents, were scored for their resistance to *H. avenae* using the bioassay described by Fisher (1992). Seedlings were planted in 3-cm tubes filled with sterile soil and inoculated five times at 3-day intervals with 100 second-stage juveniles. After 12 weeks at 15°C the number of cysts formed on roots of each plant were counted.

DNA clones

Clones were obtained through the Australian Triticeae Mapping Initiative and from a barley root cDNA library (Murphy et al. 1995).

RFLPs

DNA extraction, restriction digestion and Southern blotting and hybridisation were carried out as described by Guidet et al. (1991). NIL leaf DNA was digested with *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV and *Hin*dIII. DNA membranes of the DH lines were screened with RFLP probes and the results analysed with Map Manager QT (version 3b3) software using the Kosambi map unit function (Kosambi 1944).

Results

Segregation of the nematode resistance genes

A total of 148 DH lines of the Clipper × Sahara, and 88 DH lines of the Chebec × Harrington populations were tested for resistance to *H. avenae*. The segregation of individuals from the cross Clipper × Sahara was 78 resistant : 70 susceptible ($\chi^2 = 0.41$) and that of Chebec × Harrington was 66 resistant : 22 susceptible ($\chi^2 = 22$). The χ^2 values would indicate that the segregation of the Clipper × Sahara population fits the expected 1 : 1 ratio for a single dominant gene for resistance to CCN. However, the observed ratio for the Chebec × Harrington population shows a significant deviation from the expected ratio.

RFLP analysis

Since it was known that *Ha 2* was located on 2HL, RFLP probes previously mapped to this arm were used to screen for linkage in the two crosses. For this purpose, 48 RFLP clones were surveyed for polymorphism between the parents of the crosses using five restriction enzymes (see Materials and methods). Polymorphism with at least one enzyme was found for 20 clones (42%) in the Chebec × Harrington cross and 23 clones (48%) in the Clipper × Sahara cross. The polymorphic clones were tested for linkage with the *Ha 2* resistance gene in the DH populations and linkage groups containing the *Ha 2* locus were identified (Fig. 1). In the Chebec × Harrington cross, *Ha 2* was located 4.4 cM and 6.1 cM from PSR 901 and AWBMA 21 respectively while in the Clipper × Sahara cross, *Ha 2* was located 4.0 cM from AWBMA 21.

Analysis of near-isogenics with RFLPs

A further seven sources of CCN resistance were available for study in a series of near-isogenic lines with a common background. To investigate whether the markers obtained for the Chebec and Sahara genes are also indicative of the presence of *Ha 2* or some other locus in other genetic backgrounds, seven near-isogenic lines (Table 1) were screened with those markers showing closest linkage. With the exception of Siri, all of the

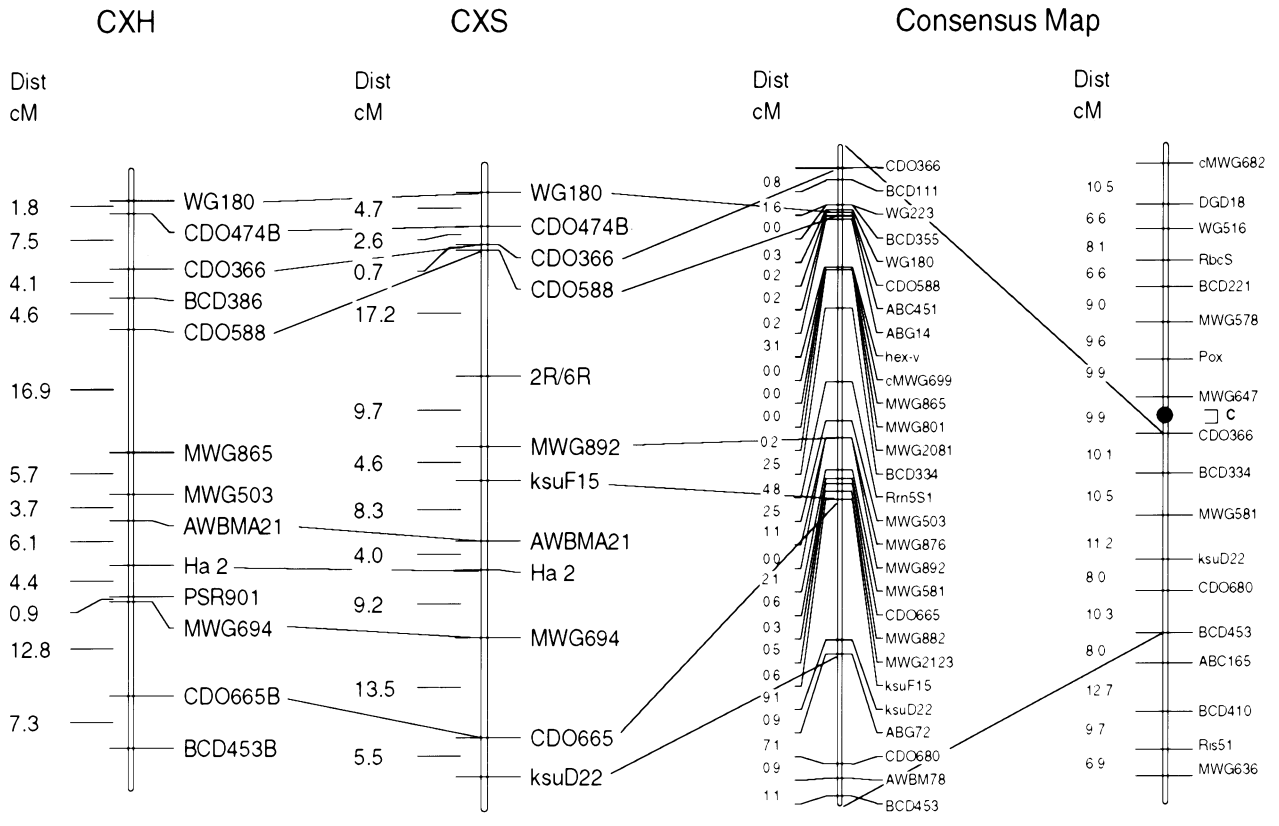

Fig. 1 The map location of the *Ha 2* locus in the Chebec × Harrigton (C × H) and the Clipper × Sahara (C × S) crosses. The location of the RFLP probes mapped in the two crosses are shown relative to a portion of the full barley consensus map and relative to the skeletal map of chromosome 2H (Langridge et al. 1995)

lines showed a polymorphism with respect to the recurrent CCN-susceptible parent Ingrid, when probed with AWBMA 21 (Fig. 2). Three allelic variants were observed with this most-closely linked probe suggesting allelic variation at the *Ha 2* locus. The NILs were polymorphic for the closely linked RFLP markers MWG 503 and MWG 693. However, BCD 453 and ksuF15 detected polymorphism between the parental lines but not within the NILs themselves, thus defining the limits of the introgressed segments (Table 2). Linkage analysis would therefore indicate an introgressed region of between 10 and 20 cM in each NIL.

Discussion

The RFLP mapping in this study confirms the previous assignment of the CCN resistance locus to the long arm of chromosome 2H established by means of linkage to morphological markers (Cotton and Hayes 1969; Andersen and Andersen 1973). The results also indicate that the resistance loci in Sahara 3771 and Chebec represent the same locus, *Ha 2*. Indeed it appears that all available sources of resistance to CCN in barley involve the *Ha 2* locus. This result was disappointing since it had been hoped that several loci would be identified and that these could be use to pyramid resistance to this nematode. Pyramiding of several resistance genes to a single pathogen is expected to provide particularly durable resistance. Previous work had indicated that there are at least three potential loci conferring resistance to CCN; two on the long arm of group-2 chromosomes, including *Ha 2*, and one on the long arm of group 6 (Andersen and Andersen 1973; Asiedu 1986). Based on the results presented here, it seems unlikely that useful resistance is present in barley at alternative sites. It may be that alleles at the alternative loci only confer tolerance to the nematode and were not included in the original survey for sources of CCN resistance. The bioassay for resistance is a recent development and no reliable assay for tolerance has been implemented. However, tolerance genes may provide a useful addition to the plant's defences and efforts are underway to develop a bioassay for tolerance.

This study has used NILs to identify RFLP markers linked to the cereal cyst nematode resistance gene *Ha 2*. As the NILs should be identical, apart from a segment of DNA containing the gene selected for, any RFLPs between the NILs should be closely linked to *Ha 2*. Examination of the NILs has shown that the RFLP markers used in the present study provide a new tool

Table 1 Parents used to create near-isogenic lines together with the number of backcrosses (BC)

Donor parent	Recurent parent	Gene	Reference
Martin 403-2	Ingrid (BC7)	*Ha 2*	O'Brien and Fisher (1977, 1979)
Morocco	Ingrid (BC7)	*Ha 2*	O'Brien and Fisher (1977, 1979)
Marocaine	Ingrid (BC7)	*Ha 2*	O'Brien and Fisher (1977, 1979)
Siri	Ingrid (BC7)	*Ha 2*	Cotten and Hayes (1969)
No. 191	Ingrid (BC7)	*Ha 2*	Andersen and Andersen (1968)
CI-4226	Ingrid (BC7)	*Ha 2*	Cook and York (1982)
Bajo Aragon-1-1	Ingrid (BC7)	*Ha 2*	Neilsen (1982)

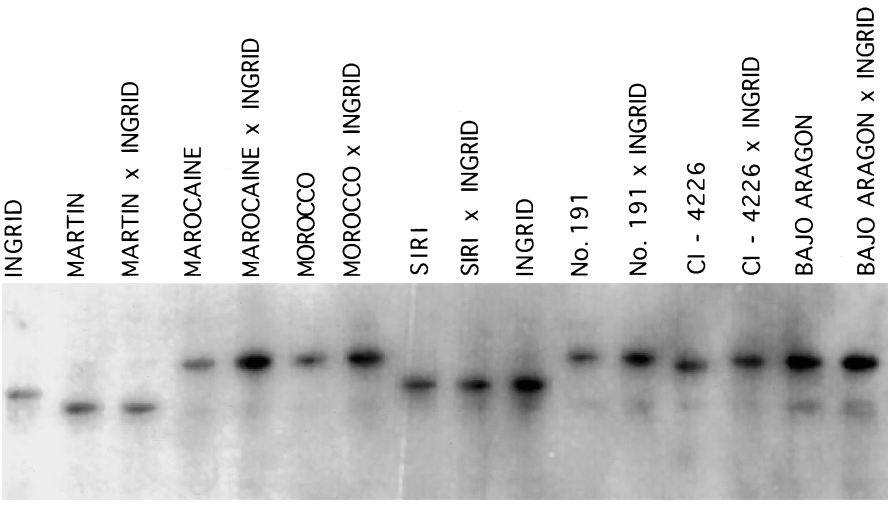

Fig. 2 Southern analysis of NILs. DNA was digested with *Hin*dIII and probed with AWBMA 21. With the exception of Siri, all of the lines show polymorphism with respect to the recurrent CCN-susceptible parent Ingrid

Table 2 Usefulness of RFLP markers in different genetic backgrounds, tested on the seven near-isogenic lines (+ denotes detection of RFLP polymorphism completely linked to the *Ha 2* resistance gene, − denotes absence of RFLP polymorphism completely linked to the *Ha 2* resistance gene)

Accession	Probe				
	ABWM21	cMWG 503	cMWG 694	BCD 453	KSUF15
Martin 403-2	+	−	+	−	−
Marocaine	+	−	+	−	−
Morocco	+	−	+	−	−
Siri	−	+	−	−	−
Var. 191	+	−	−	−	−
CI-4226	+	−	+	−	−
Bajo Aragon	+	−	−	−	−

for the successful identification of the *Ha 2* resistance gene, irrespective of the genetic background. The use of these probes will enable the specific selection of the *Ha 2* resistance gene in backcross programmes.

In the Clipper × Sahara cross, *Ha 2* resistance segregated in the 1:1 ratio expected for a single gene. The Chebec × Harrington anther-culture-derived cross showed a skewed segregation around the *Ha 2* resistance locus. On the long arm of chromosome 2, significantly more "Chebec" than "Harrington" alleles were detected. Distorted segregation ratios have been observed in several DH populations. One possible explanation for the skewing in this region is the indirect selection of this region during the production of the DH lines (Powell et al. 1986) as has been previously reported in this population (Logue et al. 1995).

RFLP markers can provide powerful tools for the indirect selection of agronomically important genes in a breeding program (Beckman and Soller 1983; Tanksley 1983). The objective of the present study was to identify RFLP markers that could be used to select for CCN resistance in barley breeding programs. Marker-based selection allows screening for resistance at early stages of plant development. Molecular markers offer several important advantages over the bioassay for CCN resistance (Table 3).

Although a bioassay will be required in late-generation screening and during pure-seed production, the

Table 3 Relative advantages of RFLP and bioassy

Feature	RFLP	Bioassay
Time	4 days	3 months
Cost per test	$4	$4
Suitability of transplanted seedlings for crossing	Yes	No
Plants needed for reliable assay	1	6–10
Multiple-trait selection	Yes	No
Identification of heterozygotes	Yes	No

RFLP markers described here can effectively replace the bioassay in all early generation screens. New malting-quality barley germplasm carrying the CCN resistance gene from Sahara and Chebec will enter pre-release testing within the next 2 years. The markers described here will have played an important role in the development of these lines. Of particular agricultural significance will be the availability of CCN-resistant malting-quality varieties permitting an increase in the cultivated area of malt barley and leading to higher growth returns. Additionally, wheat and barley showing resistance to CCN will allow growers to effectively reduce the levels of nematodes in soils without the present heavy reliance on rotation with resistant crops, such as canola. This will provide growers with more flexibility in rotations by being able to grow CCN-susceptible, but otherwise excellent, varieties of wheat and oats.

References

Andersen K, Andersen S (1968) Inheritance of resistance to *Heterodera avenae* in barley. Nematologica 14:128–130

Andersen K, Andersen S (1973) Linkage between marker genes on barley chromosome 2 and a gene for resistance to *Heterodera avenae*. Hereditas 73:271–276

Andersen K, Andersen S (1982) Suggestions for the determination and terminology of pathotypes and genes for resistance in cyst-forming nematodes especially *Heterodera avenae*. EPPO Bull 12:379–386

Asiedu R (1986) Resistance to *Heterodera avenae* Woll. located in the rye genome of triticale. PhD thesis, University of Adelaide

Barua UM, Chalmers KJ, Hackett CA, Thomas WTB, Powell W, Waugh R (1993) Identification of RAPD markers linked to a *Rhynchosporium secalis* resistance locus in barley using near-isogenic lines and bulked segregant analysis. Heredity 71:177–184

Beckman JS, Soller M (1983) Restriction fragment length polymorphism in genetic improvement: methodologies, mapping and costs. Theor Appl Genet 67:35–43

Cook R, York PA (1982) Resistance of cereals to *Heterodera avenae*: methods of investigation, sources and inheritance of resistance. EPPO Bull 12:423–434

Cotton J, Hayes JD (1969) Genetic resistance to the cereal cyst nematode (*Heterodera avenae*). Heredity 24:593–600

Eastwood RF, Lagudah ES, Appels R, Hannah M, Kollmorgen JF (1991) *Triticum tauschii*: a novel source of resistance to cereal cyst nematode (*Heterodera avenae*). Aust J Agric Res 42:69–77

Finnie SJ, Powell W, Dyer AF (1989) The effect of carbohydrate composition and concentration on anther-culture response in barley (*Hordeum vulgare* L.). Plant Breed 103:110–118

Fisher JM (1992) Towards a consistent laboratory assay for resistance to *Heterodera avenae*. EPPO Bull 12:445–449

Guidet F, Rogowsky R, Taylor C, Song W, Langridge P (1991) Cloning and characterisation of a new rye-specific repeat sequence. Genome 34:81–87

Kosambi DD (1944) The estimation of map distances from recombinational values. Ann Eugen 12:172–175

Langridge P, Karakousis A, Collins N, Kretschmer J, Manning S (1995) A consensus linkage map of barley. Mol Breed 1:389–395

Logue SJ, Oti-Boateng C, Karakousis A, Kretschmer JM, Manning S, Lance RCM, Langridge P (1995) Segregation analysis of DNA markers in anther culture-derived populations of barley (*Hordeum vulgare* L.). 7th Aust Barley Tech Symp, Perth, pp 210–216

Meagher JM (1977) World dissemination of the cereal nematode (*Heterodera avenae*) and its potential as a pathogen of wheat. J Nematol 9:9–15

Murphy PJ, Karakousis A, Smith SE, Langridge P (1995) Cloning functional endomycorrhizal genes. In Stocchi V, Bonfarte P, Nuti M (eds) Biotechnology of ectomycorrhizae. Plenum Press, New York, pp 77–83

Nielsen, CH (1982) Heredity of *Heterodera avenae* resistance originating from two barley cultivars and one spring wheat cultivar. EPPO Bull. 12:457–461

O'Brien PC, Fisher JM (1977) Development of *Heterodera avenae* on resistant wheat and barley cultivars. Nematologica 33:390–397

O'Brien PC, Sparrow DHD, Fisher JM (1979) Inheritance of resistance to *Heterodera avenae* in barley. Nematologica 25:348–352

Powell W, Borrino EM, Allison MJ, Griffiths DW, Asher MJC, Dunwell JM (1986) Genetical analysis of microspore derived plants of barley (*Hordeum vulgare*). Theor Appl Genet 72:619–626

Schüller C, Backes G, Fishbeck G, Jahoor A (1992) RFLP markers to identify the alleles on the *Mla* locus conferring powdery mildew resistance in barley. Theor Appl Genet 84:330–338

Tanksley SD (1983) Molecular markers in plant breeding. Plant Mol Biol Rep 1:3–8

Williams KJ, Fisher JM, Langridge P (1994) Identification of RFLP markers linked to the cereal cyst nematode resistance gene (*Cre*) in wheat. Theor Appl Genet 83:919–924