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Mapping of the root lesion nematode (*Pratylenchus neglectus*) resistance gene *Rlnn1* in wheat

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Abstract The root lesion nematode, *Pratylenchus neglectus*, is an economically damaging pathogen of wheat and other crops. The development of *P. neglectus*-resistant wheat cultivars would be greatly accelerated through the use of molecular markers, as resistance phenotyping is extremely time-consuming. A greenhouse bioassay was developed to identify resistance phenotypes of doubled-haploid populations. Bulked-segregant analysis was used to identify AFLP markers linked to *P. neglectus* resistance in the wheat cultivar Excalibur. One resistance-linked AFLP marker was mapped close to chromosome 7A RFLP markers in a densely-mapped Cranbrook/Halberd population. One of the chromosome 7A RFLP probes, cdo347, was genotyped in the Tammin/Excalibur population segregating for response to root lesion nematode and showed 8% recombination with the *P. neglectus* resistance gene *Rlnn1*. The marker *Xcdo347-7A* was validated on Excalibur-and Krichauff-derived DH populations segregating for *Rlnn1* and showed 14% and 10% recombination, respectively, with *Rlnn1* in these populations.

Keywords *Triticum aestivum* · Nematode resistance · Rust resistance · Four colour · Genetic mapping · Marker-assisted selection

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Introduction

The root lesion nematode, *Pratylenchus neglectus*, is a migratory endoparasite that is common to many temperate regions of the world. This pathogen has been identified from broad-acre croping regions of North America, where it infects barley (Umesh and Ferris 1992), alfalfa (Griffin 1991), rangeland grasses (Griffin 1996) and potato (Ferris et al. 1994). In south-eastern Australia, the primary crop is winter-sown wheat, and *P. neglectus* has been shown to cause up to 27% yield loss in intolerant cultivars (Taylor SP et al. 1999; Vanstone et al. 1998). In addition to wheat, *P. neglectus* also infects chickpea, canola, barley, vetch and oat cultivars (Taylor et al. 2001) and has therefore proved difficult to control by crop rotation.

The development of resistant cultivars is the most efficient and cost-effective option for protecting crops against *P. neglectus* in low-value cropping systems as nematicides are prohibitive in cost and can have deleterious environmental effects. Wheat and barley cultivars grown under field conditions in south-eastern Australia have exhibited a range of responses to root-lesion nematode (Taylor et al. 2001; Vanstone et al. 1998). Wheat cultivars Excalibur and Krichauff expressed high levels of resistance.

In addition to *Pratylenchus neglectus*, the related species, *P. thornei*, and cereal cyst nematode (*Heterodera avenae*) are also common in south-eastern Australia, and all may be found within the same field (A. McKay, SARDI, personal communication). For this reason, cereal cultivars with an acceptable level of resistance to all three parasites are desirable. Several genes for resistance to *H. avenae* have been identified and mapped in wheat (Williams et al. 1996), *Aegilops tauschii* (Eastwood et al. 1994), rye (Taylor C et al. 1999) and barley (Barr et al. 1998; Kretschmer et al. 1997). None of the genes for resistance to *P. neglectus* or *P. thornei* have been located onto specific chromosomes in cereals.

Marker-assisted selection for traits like root lesion nematode resistance is an attractive option for wheat

breeders in south-eastern Australia because of the laborious nature of bioassays. This paper describes the inheritance of root lesion resistance in wheat cultivar Excalibur, the identification and validation of linked molecular markers and genetic association with leaf rust resistance gene *Lr20*.

Materials and methods

Plant materials

A Tammin (susceptible)/Excalibur (resistant) doubled-haploid (DH) population was used for marker identification. RAC860 (susceptible)/Excalibur and Trident (susceptible)/Krichauff (resistant) DH populations were used for marker validation. Parental lines were obtained from Prof. G. Hollamby (University of Adelaide) and DHs were produced by Dr. Neil Howes, (South Australian Research and Development Institute). DNA from the mapping population Cranbrook/Halberd was provided by the Australian National Wheat Molecular Marker Program.

Greenhouse bioassay (EbbFlow test)

Wheat DH populations were assessed for response to *Pratylenchus neglectus* in a greenhouse maintained at 20°±3 °C. Square seedling tubes (5.5 cm wide × 12 cm long) were filled with steam-sterilised sand and held in purpose-built galvanised steel square mesh crates (5 × 5 arrangement). Crates were placed in EbbFow trays that were flooded for 4 min every 3 days to a depth of 10 cm using water from a reservoir below each tray. Water immediately drained into the reservoir and was monitored weekly for changes in pH. Each experiment comprised eight replicates of 50 lines (including 3 controls), and each EbbFlow tray held one experiment.

One pre-germinated DH seed was sown per seedling tube and, after emergence, each seedling was inoculated with 1,500 *P. neglectus* individuals per plant, pipetted into two 5-cm-deep holes on either side of the seedling. *P. neglectus* inoculum was obtained from carrot callus (Moody et al. 1973). In each experiment, controls comprised a susceptible wheat (cv. Machete) and a resistant triticale (cv. Abacus). Two days after inoculation, slow-release fertiliser (Osmocote Plus) was added (rate 4 g/kg of soil) and covered with 1 cm of organic material-free sand.

At 8 weeks after inoculation, the maturity of plants was recorded and shoots were removed. Soil was washed from the roots before the whole root system was cut into 1-cm sections. *P. neglectus* was extracted in a misting chamber for 96 h, and the resulting nematode suspension was collected and stored at 4 °C prior to counting. Two 0.5-ml aliquots were removed from each sample, and the number of nematodes present was counted using a dissection microscope (20×). The number of nematodes was $\log_e$-transformed and lines ranked in comparison to resistant and susceptible controls using Statistix for Windows (version 2.0) general analysis of variance (ANOVA).

Leaf rust response tests

Puccinia triticina pathotype [PBI accession no. 104-2,3,6,(7) (231)] was used to screen DHs derived from the Tammin/Excalibur cross. Four clumps of five to ten seeds of each line were planted in 9-cm pots. Excalibur, Tammin, Thew (*Lr20* differential) and Morocco (susceptible) were included as controls. Inoculation, incubation and scoring details are described in Bariana and McIntosh (1993). Due to the temperature sensitivity of *Lr20*, seedlings were incubated at 18–20 °C for 2 weeks.

DNA extraction

DNA was extracted using a DNA mini-prep method adapted from Rogowsky et al. (1991). Variations to the method were as described below. For the initial extraction, 750 µl of extraction buffer and 750 µl phenol-chloroform-isoamyl alcohol (25:24:1) were used. The extraction buffer was 0.1 *M* Tris-HCl, pH 8, 10 m*M* EDTA, 0.1 *M* NaCl, 1% sarkosyl, 2% insoluble polyvinylpoly-pyrrolidone. DNA was precipitated by the addition of 0.1 vol. of 3 *M* sodium acetate (pH 4.8) and 1 vol. of propan-2-ol.

Bulked segregant analysis

The amplified fragment length polymorphisin (AFLP) technique was used in bulked segregant analysis (BSA) (Michelmore et al. 1991) to identify putative markers for *P. neglectus* resistance in the Tammin/Excalibur DH population. The resistant bulk was formed by pooling 1 µg of DNA from each of the five most resistant lines, and the susceptible bulk was formed by pooling 1 µg of DNA from each of the five most susceptible lines. AFLP analysis was performed according to Vos et al. (1995) with some modifications. The restriction digestion of DNA samples and ligation of adaptors was performed in a single 60 µl reaction containing 10 m*M* Tris-HCl, pH 7.5, 10 m*M* Mg-acetate, 50 m*M* K-acetate, 0.5 m*M* DTT, 0.2 m*M* ATP, 5 U *Mse*I and 10 U *Pst*I, 1 U T4 DNA ligase, 50 µ*M* *Mse*I adapter and 5 µ*M* *Pst*I adapter. The mixture was incubated at 37 °C for 3 h, then placed at 4 °C overnight to ensure efficient ligation of the adaptors.

A 4-µl aliquot of the restriction/ligation mixture was used as template DNA in a polymerase chain reaction (PCR) to generate pre-amplified material. The 25-µl 'preamplification' reaction contained 20 m*M* Tris-HCl, pH 8.4, 50 m*M* KCl, 0.2 m*M* dNTP, 1.5 m*M* $MgCl_2$, 75 ng each of primers *Pst*I+A (5′-GACTGCGTACATGCAG+A-3′) and *Mse*I+C (5′-GATGAGTCCTGAGTAA+C) and 1 U *Taq* DNA polymerase (Life Technologies, Gaithersburg, Md.). PCR amplification was performed for 20 cycles: 30 s at 94 °C, 1 min at 56 °C, and 1 min at 72 °C. The PCR products were diluted with 7 vol. water and stored at –20 °C as 'preamplified stock'. Selective amplification was performed using combinations of 10 *Mse*I+CNN and 12 *Pst*I+ANN primers. The *Pst*I selective primers were labelled with one of three fluorescent tags, HEX, TET or FAM. The volume of the selective PCR was 20 µl and consisted of 20 m*M* Tris-HCl, pH 8.4, 50 m*M* KCl, 0.2 m*M* dNTP, 1.5 m*M* $MgCl_2$, 25 ng *Mse*I+CNN, 12.5 ng *Pst*I+ANN, 0.5 U *Taq* DNA polymerase (Life Technologies) and 5 µl 'preamplified stock' as template DNA. PCR amplification was performed for 34 cycles using a 'touchdown' profile: 30 s at 94 °C, 30 s annealing (see below), 1 min at 72 °C. The annealing temperature for the first cycle was 65 °C, with a by 1 °C reduction per cycle for the next ten cycles. The remaining 24 cycles were with annealing at 56 °C. The products were diluted with 10 vol. water. PCR reactions were performed in a PTC-225 (MJ Research Carlsbad, Calif.) thermocycler.

Gel electrophoresis/analysis

Prior to gel electrophoresis, 3.5 µl of diluted selective PCR product was dried, and a 2-µl aliquot of loading buffer (75% formamide, 25% GeneScan 500 standard) was added. Samples were denatured and electrophoresed on 6% sequencing gels at 30 W on an Applied Biosystem 373 DNA Sequencer according to the manufacturer's instructions. The AFLP data generated from gel electrophoresis was analysed using GENESCAN software. AFLP designations were based on the primer combination used (*Pst*I+CNN/*Mse*I+ANN) and the amplified fragment size.

Genetic mapping and marker validation

The Cranbrook/Halberd map, based on 161 DHs, has a length of 4,020 cM, includes 813 markers and has an average distance between markers of 4.9 cM (Australian National Wheat Molecular Marker Program, unpublished). The position of the AGC/CCT 179 AFLP marker on this map was determined using MAP MANAGERQTB version 28ppc (Manly and Olsen 1999). For restriction fragment

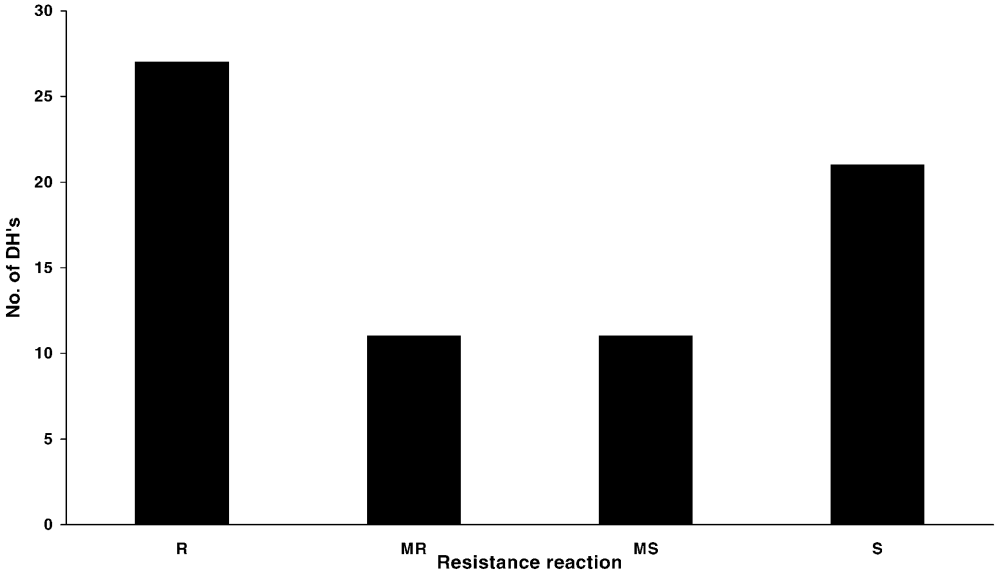

Fig. 1 Reaction of Tammin/Excalibur doubled haploids to inoculation with *Pratylenchus neglectus* (*S* Tammin, *R* Excalibur). *R* resistant, *S* susceptible, *M* moderately

length polymorphisin (RFLP) marker validation, restriction endonuclease digestion and Southern hybridisation experiments, standard methods were followed (Rogowsky et al. 1991).

Microsatellite amplification was performed as described by Röder et al. (1998), with the following minor modifications. Total reaction volume was 12 µl and consisted of 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 1.50 m*M* $MgCl_2$, 0.8 m*M* of each dNTP, 75 ng of each primer and 0.5 U *Taq* DNA polymerase, with 10 ng DNA template. PCR amplification was performed in 34 cycles using a touchdown protocol. The denaturation was performed at 94 °C for 30 s and extension at 72 °C for 30 s. The annealing temperature in the first cycle was 59 °C for 30 s, and this was reduced each cycle by 0.5 °C for the next 19 cycles until 50 °C was reached; the remaining 29 cycles all had an annealing temperature of 50 °C. Amplification products were separated on 6% acrylamide gels and visualised after staining with ethidium bromide.

The flour colour PCR assay was derived from that reported by Parker and Langridge (2000), although primer sequences were altered to improve amplification. Primers were originally designed to specifically amplify the Schomburgk allele associated with yellow flour colour on chromosome 7A. Primer sequences were: 5′TAACAGCGGATCTGATGC3′, 5′TAACAGCGGATATGATGG3′. PCR product amplification and separation details were described under microsatellite analysis.

Results

Inheritance of root lesion nematode resistance

Seedling bioassays were used to determine the *P. neglectus* responses of 70 Tammin/Excalibur DH lines. Classes for reaction to *P. neglectus* were determined by comparing DH lines to controls using ANOVA. The number of nematodes collected from roots varied between tests, so DH lines were ranked with respect to the resistant triticale control, ev. Abacus, and the susceptible wheat control, cv. Machete. Lines with means that were not significantly different (P=0.05) from the resistant and susceptible controls in more than one experiment were classified as resistant or susceptible and used for linkage analysis. Comparison of classes with either a resistant or moderately resistant response with those showing a susceptible or moderately susceptible response showed segregation at a single locus (resistant/moderately resistant=38, susceptible/moderately susceptible=32, for a 1:1 ratio, $\chi^2 = 0.22$, P>99.9) (Fig. 1). Twenty-five lines with consistent phenotypes for reaction to *P. neglectus* in three to five separate experiments over 2 years were used for marker development. Four of these lines showed moderate levels of resistance or susceptibility in at least one experiment. Lines with consistent resistance phenotypes from RAC860/Excalibur (30) and Trident/Krichauff (31) crosses were used for marker validation.

Bulked segregant analysis

One hundred and twenty *Pst*I-*Mse*I primer combinations were used to screen resistant (R) and susceptible (S) bulks together with the parents Tammin and Excalibur. An average of five AFLPs were observed between the parents for each primer set. From 600 loci, 65 AFLPs differentiated the R and S bulks and the parents. The reproducible between-bulk AFLPs were used to screen 25 Tammin/Excalibur DH lines which had consistent phenotypes for reaction to *P. neglectus*. These AFLPs showed 8–14% recombination with the resistance locus.

Mapping of AFLP markers

The AFLP marker AGC/CCT 179, which showed good linkage to resistance in the Tammin/Excalibur debulks, was assayed on the densely mapped Cranbrook/Halberd population, and it was found to be linked with the RFLP loci *XksuH9C*, *Xpsr121*, *Xpsr680A* and *Xcdo347* (Fig. 2A). The RFLP probes cdo347, psr121 and psr680 revealed polymorphisms between Tammin and Excalibur. *Dra*I-digested DNA samples from 25 Tammin/Excalibur DHs were probed with cdo347, psr121 and psr680. These RFLP markers showed close genetic asso-

Table 1 Summary of recombination of marker and gene loci with the root lesion nematode resistance gene *Rlnn1*

Population	Number of progeny (*Rlnn1:rlnn1*)	Locus[a]	Recombination fraction (%) with *Rlnn1*	Map distance (Kosambi)(cM) with *Rlnn1*	LOD score
Tammin/Excalibur	25 (12:13)	*XAGC/CCT179-Xcdo347-Xpsr121-Xpsr680-Xschfc3*	8	8	4.5
RAC860/Excalibur	30 (11:19)	*Xcdo347-Xpsr121-Xpsr680*	13	14	3.4
Trident/Krichauff	31 (18:13)	*Xcdo347-Xpsr680-Xfba349*	10	10	5.1
		Xgwm344	19	21	2.5

[a] Loci joined by dashes co-segregate

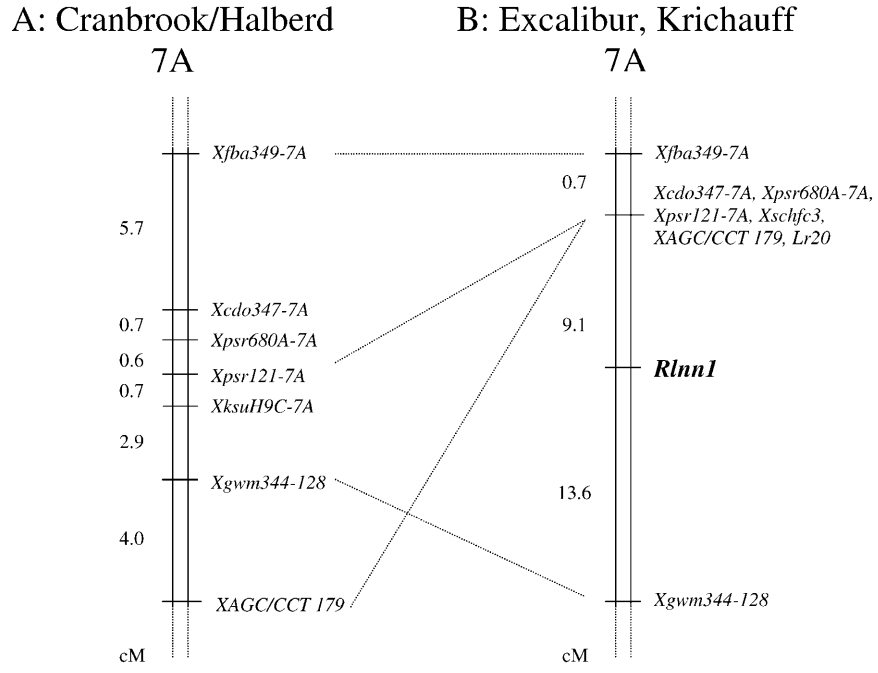

Fig. 2 **A** Partial map of chromosome 7A from a Cranbrook/Halberd cross showing the mapping of the *Rlnn1*-linked XAGC/CCT 179 AFLP marker identified by bulked-segregant analysis in order to identify adjacent RFLP and SSR markers for *Rlnn1* linkage validation. **B** Partial consensus map of chromosome 7A from Tammin/Excalibur, RAC860/Excalibur and Trident/Krichauff crosses, showing linkage of STS, SSR and RFLP markers with *Rlnn1*. Dashed lines show relative positions of loci in the two maps

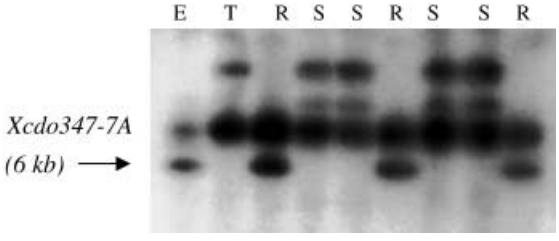

Fig. 3 Validation of linkage of RFLP marker *Xcdo347-7A* with *Rlnn1* by Southern analysis of segregating lines. DNA from individual DH plants with known scores for reaction to *P. neglectus* from the cross Tammin/Excalibur was digested with *Dra*I and probed with the cdo347 clone. *Lanes*: *1* Excalibur, *2* Tammin, *3*, *6*, *9* resistant DHs, *4*, *5*, *7*, *8* susceptible DHs. The 6-kb *Xcdo347-7A* fragment (*arrow*) showed 9% recombination with *Rlnn1* in 25 DHs from this cross

ciation with *P. neglectus* resistance (Fig. 2B, 3, Table 1). No recombination between the markers *Xcdo347-7A*, *Xpsr121-7A* and *Xpsr680-7A* was observed in 81 additional lines (data not shown). This experiment confirmed the Cranbrook/Halberd mapping results. The dominant flour colour marker *Xschfc3* was also assayed on 25 Tammin/Excalibur DHs with consistent phenotypes for reaction to *P. neglectus*. The *Xschfc3* marker showed the same association with *P. neglectus* resistance as the other three chromosome 7A RFLP probes (Fig. 2B, Table 1).

Mapping of *Lr20*

Cultivar Excalibur carries the resistance gene cluster *Lr20*/*Sr15*/*Pm1* and cultivar Tammin lacks it (Bariana, unpublished). Tammin/Excalibur-derived DHs segregated for leaf rust resistance gene *Lr20*. Leaf rust response tests on 81 lines showed co-segregation of *Lr20* with the RFLP markers *Xcdo347-7A*, *Xpsr121-7A* and *Xpsr680-7A*.

Genetic association of RFLP markers and *Lr20* with resistance to *P. neglectus*

Comparison of the *P. neglectus* bioassay, RFLP and rust response data showed a close genetic association (8–14% recombination) of *P. neglectus* resistance with markers *Xcdo347-7A*, *Xpsr121-7A*, *Xpsr680-7A* and *Lr20*. These results placed *P. neglectus* resistance in

chromosome arm 7AL. This is the first report on the chromosomal location of resistance to *P. neglectus*. We designated the root lesion nematode resistance in cv. Excalibur as *Rlnn1* (Root lesion nematode neglectus) (R.A. McIntosh, personal communication).

Validation of RFLP markers

The ability of molecular markers to predict *P. neglectus* resistance was validated using populations from the South Australian wheat breeding programs. The RFLP markers *Xcdo347-7A*, *Xpsr121-7A* and *Xpsr680-7A* predicted the presence of *Rlnn1* in 30 RAC860/Excalibur-derived DHs and 31 Trident/Krichauff-derived DHs with 86% and 90% accuracy, respectively (Table 1). The RFLP marker *Xfba349* also had 91% accuracy in the 31 phenotyped Trident/Krichauff-derived DHs, although one recombinant between *Xfba349* and *Xcdo347-7A* and *Xpsr680-7A* was observed when an additional 27 lines were screened with markers. The simple sequence repeat (SSR) marker *Xgwm344-128* showed 19% recombination with *Rlnn1* in the 31 Trident/Krichauff-derived DHs (Fig. 2). Mapping and phenotypic data from all three populations was used to construct a consensus map of the distal region of chromosome 7A using the program JOINMAP 2.0 (Stam 1993) (Fig. 2B).

Discussion

This study reports mapping of the *P. neglectus* resistance locus *Rlnn1* in Australian wheat cultivar Excalibur using a combination of BSA and genetic mapping. *Rlnn1* is the first root lesion nematode resistance gene mapped onto a specific chromosome in any species. We confirmed the usefulness of BSA (Michelmore et al. 1991) for identifying trait-linked markers in wheat. Because of the variable nature of the bioassay of *P. neglectus* resistance, the bulks used for BSA contained only five consistently resistant and susceptible DHs. Despite this, a *Rlnn1*-linked AFLP marker was identified. Chromosome location of the *Rlnn1*-linked AFLP marker was determined through its genetic association with chromosome arm 7AL-specific RFLP markers in a densely mapped Cranbrook/Halberd population. Resistance to the other important nematode parasite of wheat, *Heterodera avenae*, has been mapped in hexaploid wheat to chromosome 2B (Williams et al. 1996), in *Aegilops tauschii* to chromosome 2D (Eastwood et al. 1994), in *Aegilops ventricosa* to chromosome 5Nv (Ogbonnaya et al. 2001) and in rye to chromosome 6R (Taylor C et al. 1999). None of the nematode resistance genes have been mapped to chromosome 7A, whereas genes for resistance to other diseases located in the distal region of chromosome 7AL which carries *Rlnn1* include *Lr20*/*Sr15*/*Pm1* (Ma et al. 1994; Sears and Briggle 1969) and *Sr22* (The and McIntosh 1975). The *Rlnn1*-linked RFLP marker *Xcdo347* was previously mapped to chromosome 7A in the Synthetic/Opata population (Nelson et al. 1995) and in Yarralinka/Schomburgk population by Parker et al. (1998). Genetic associations of *Rlnn1* with the *Xcdo347-7A* locus and the leaf rust resistance gene *Lr20* further confirmed the chromosomal location. The mapping of AFLP markers in comparative populations of the same species, previously described by Waugh et al. (1997) obviates the need for cloning AFLP bands to produce RFLP or PCR markers. This mapping can identify RFLP or SSR markers and can also facilitate the identification of markers linked more closely to the trait of interest by marker walking but, most importantly, it provides information on chromosomal location without the expense of creating a full genetic map for each cross. Armed with the chromosomal locations of resistance genes, the plant breeder can make more informed decisions about combining genes which may be linked in coupling or repulsion.

The other important aspect of the study included validation of putatively linked markers on subsets of thoroughly phenotyped progeny from several populations. This strategy is recommended for BSA of resistance to diseases that are difficult to assay. Markers linked to such traits – for example, lower nematode multiplication rate – can increase the selection efficiency greatly. The usefulness of a molecular marker for *P. neglectus* resistance was underlined by the difficulties experienced in accurately assaying resistance phenotypes in this study. Three hundred and eight-three DH lines (5–8 replicates) were assessed in up to five separate experiments to find 86 lines with consistent reactions to *P. neglectus*. Because of the high cost of the bioassay, no high-throughput screening service is currently available to breeders in Australia.

Young (1999) emphasised that only a few of the reported molecular markers are linked with resistance to diseases that are difficult to assess. Using subsets of populations allows lines with intermediate or inconsistent phenotypes to be avoided in the BSA for marker development. While this may reduce the possibility of mapping loci with minor effects, it would greatly increase the probability of identifying genomic regions controlling large proportions of phenotypic variation. Once the closely linked markers are identified based on thoroughly phenotyped representatives of the population, markers can then be validated in breeding populations as described by Williams et al. (1999) and in this study.

In addition to the AFLP marker, one dominant PCR marker (*Xschfc3*) and one SSR and four RFLP markers with linkage to *Rlnn1* were identified. The relatively small size of populations tested and the lack of recombination between RFLP markers prevented the fine mapping of *Rlnn1* with respect to previously mapped markers and failed to clarify the gene order. The clustering of RFLP markers does, however, increase the number of probes available to reveal polymorphisms for use in marker-assisted breeding, which are often difficult to find among closely related wheat breeders' lines. Although the dominant flour colour marker locus *Xschfc3* was not

validated across genetic backgrounds, it showed close genetic association with *Rlnn1* in the Excalibur/Tammin DH population. Our study confirms the previously reported linkage of *Xschfc3* with *Xcdo347* (Parker et al. 1998).

The *Rlnn1*-linked markers developed for Excalibur also detected *P. neglectus* resistance in another cultivar, Krichauff. These two cultivars showed similar levels of resistance determined by multiplication rate of *P. neglectus* over five sites in two separate trials (Taylor SP et al. 1999; Vanstone et al. 1998). Krickhauff is derived from a cross involving cv. Aroona, which is known to carry *Lr20* (Bariana, unpublished). The future aim of this program is to convert the *Xcdo347* RFLP marker and/or AFLP marker *XAGC/CCT179* into a PCR-based assay. Such conversion would assist in high-throughput use of this marker in breeding programs.

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