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Molecular mapping of leaf rust resistance gene *Rph14* in *Hordeum vulgare*

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Abstract An incompletely dominant gene conferring resistance to *Puccinia hordei*, *Rph14*, identified previously in an accession of *Hordeum vulgare*, confers resistance to all known pathotypes of *P. hordei* in Australia. Knowledge of the chromosomal location of *Rph14* and the identification of DNA markers closely linked to it will facilitate combining it with other important leaf rust resistance genes to achieve long lasting resistance. The inheritance of *Rph14* was confirmed using 146 and 106 F_3 lines derived from the crosses 'Baudin'/'PI 584760' (*Rph14*) and 'Ricardo'/'PI 584760' (*Rph14*), respectively. Bulk segregant analysis on DNA from the parental genotypes and resistant and susceptible DNA bulks using DArT markers located *Rph14* to the short arm of chromosome 2H. DArT marker bPb-1664 was identified as having the closest genetic association with *Rph14*. PCR based marker analysis identified a single SSR marker, Bmag692, linked closely to *Rph14* at a map distance of 2.1 and 3.8 cm in the 'Baudin'/'PI 584760'and 'Ricardo'/'PI 584760' populations, respectively.

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Introduction

Leaf rust of barley, caused by *Puccinia hordei*, is one of the most destructive diseases in the major barley growing regions of the world (Park 2003). The deployment of monogenic seedling resistance has been practised as an economical and ecologically balanced option to control this disease. Several seedling resistance genes have been identified from cultivated barley and wild barley, of which 19 were designated *Rph1* to *Rph19* (Weerasena et al. 2004). The resistance provided by single *Rph* genes has often been overcome by new pathotypes, believed to have arisen via introduction or mutation (Park 2003). As a direct consequence, the number of effective *Rph* genes available to breeders is decreasing rapidly, suggesting the need for a new gene deployment strategy (Fetch et al. 1998). In this context, incorporating multiple seedling resistance genes in a single genotype was proposed as a way of increasing the life of the resistance of a cultivar and also to minimise the chance of resistance genes being rendered ineffective (Park 2003).

Resistance gene *Rph14* was identified in *H. vulgare* accession 'PI 584760' and was shown to be genetically independent of *Rph1* to *Rph13* (Jin et al. 1996) and *Rph15* (Chicaiza et al. 1996). Virulence for *Rph14* has not been detected in Australia (Park 2003) but has been reported, albeit rare (3%), in isolates collected from Europe, North America, South America and Africa (Fetch et al. 1998). Deploying this gene in combination with other effective *Rph* genes was therefore proposed as a strategy for long term management of the disease (Park 2003).

Genotypes carrying multiple genes may show the same phenotypic response to rust as those carrying a single gene due to the masking effect of one gene over another. This

can be overcome if pathotypes virulent on individual genes are available. In Australia, virulence for genes *Rph3*, *Rph7*, *Rph11*, *Rph14*, *Rph15* and *Rph18* has not been detected, making the selection of combinations based on these genes difficult (Park 2003). These drawbacks can be overcome by finding markers closely linked to the genes. In the last two decades, a large number of different marker technologies have been developed, of which polymerase chain reaction (PCR)-based markers such as simple sequence repeats (SSR), amplified fragment length polymorphisms (AFLP) and sequence tagged sites (STS) are well suited for marker-assisted-selection (MAS) (Mohler and Singrun 2004). These markers need very low amounts of DNA for genetic assays and results are highly reproducible. Recently, several mapping studies were conducted on *Rph* genes and DNA markers identified closely linked to *Rph2* (Borovkova et al. 1997), *Rph5* (Mammadov et al. 2003), *Rph6* (Zhong et al. 2003), *Rph7* (Brunner et al. 2000; Graner et al. 2000), *Rph15* (Weerasena et al. 2004), *Rph16* (Ivandic et al. 1998), *Rph17* (Pickering et al. 1998) and *Rph19* (Park and Karakousis 2002). In contrast, the chromosomal location of *Rph14* remains unknown. The present study was therefore conducted to map *Rph14* and to identify PCR based SSR or STS markers that could be used in MAS of the gene in barley breeding programmes.

Materials and methods

Plant material

Cultivars 'Baudin' and 'Ricardo' were crossed with the stock possessing *Rph14* ('PI 584760'), and the resulting F_2 and F_3 populations were used to map *Rph14*. Although 'Baudin' carries *Rph12* and 'Ricardo' carries *Rph2* plus an uncharacterised seedling resistance to *P. hordei* (Park, unpublished), a pathotype virulent for all of these genes and avirulent for *Rph14* was used in the genetic analyses. The original stock carrying *Rph14* ('PI 584760') (Jin et al. 1996) was kindly provided by B. J. Steffenson (North Dakota State University, Fargo, ND). For greenhouse tests, seedlings were raised in 9-cm diameter pots containing a soil mix of pine bark fines and coarse sand. Pots were watered with a soluble fertiliser (Aquasol®, Hortico Pty Ltd, Revesby, NSW, Australia) at the rate of 35 g in 3 l of water per 100 pots, prior to sowing. F_2 and F_3 plants were raised by planting 25–30 seeds/pot. Seedlings of Australian series of differential genotypes (Park 2003) and parents were raised by sowing clumps (two per pot) of 5–7 seeds of each. The pots were transferred to temperature controlled greenhouse chambers (18 ± 2°C) under natural light and maintained until seedlings were ready for inoculation.

Seedling inoculations and disease assessment

Greenhouse inoculations were carried out on 9-day-old seedlings with fully expanded first leaves using urediniospores of *P. hordei* pathotype 5453P− (University of Sydney, Plant Breeding Institute Cobbitty Accession Number 010037) as described by Park and Karakousis (2002). Inoculated seedlings were incubated for 14–16 h at ambient temperatures in a misted dark room. The mist was generated by an ultrasonic humidifier. The seedlings were then moved to naturally lit greenhouse chambers at 20 ± 2°C and disease responses were recorded after 10–12 days, using a 0–4 scale infection type (IT) scale (Park and Karakousis 2002). Infection types of three or higher were regarded as indicative of susceptibility.

DNA extraction and bulk preparation

To extract genomic DNA, disease free leaves from 20–25 plants of each F_3 line were harvested as a bulk 5 days after disease screening. Genomic DNA was extracted by the Cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987) from 20 non-segregating resistant and 20 non-segregating susceptible F_3 lines derived from 'Baudin'/'PI 584760' and the parents. The DNA from all other F_3 lines was extracted using the Mixer Mill Method as outlined by Kota et al. (2006) for wheat seed. The concentration of DNA was determined using a spectrophotometer (Nanodrop™, Biolab, Australia), and all samples were adjusted to a final concentration of 50 or 100 ng/µl depending on the requirements of the individual experiment for further molecular analysis. Equal aliquots (5 µl) of DNA from 20 non-segregating resistant and 20 non-segregating susceptible F_3 lines were pooled to produce two DNA bulks for bulk segregant analysis (BSA) (Michelmore et al. 1991). BSA was conducted by Triticarte™ (Yarralumla, Australia) using Diversity Array Technology (DArT) markers as described by Wenzl et al. (2007).

SSR and STS analysis

A total of 16 SSR and 4 STS markers mapped previously to the short arm of barley chromosome 2H were evaluated for polymorphism between the parents as well as between DNA bulks (Table 1). Primers generating PCR products polymorphic between the parental lines and bulks were subsequently used to evaluate all F_3 lines of both populations. The relevant details of primers used in the present experiment are given in Table 1.

PCR was performed in a volume of 25 µl, containing 1 µl of DNA (~50 ng), 2.5 µl of 10× PCR buffer (Applied Biosystems, Australia), 2.5 µl of 2 mM dNTPs (dATP, dCTP, dGTP, dTTP) (Roche Dignostics, Australia), 1.5 µl

Table 1 Description of 20 DNA markers located on barley chromosome 2HS assessed for potential mapping in two barley populations

Marker name	Marker type	Primer sequence (5′–3′)	Reference
GMS003	SSR	TTTCAGCATCACACGAAAGC TTGCATGCATGCATACCC	Struss and Plieske (1998)
EBmac715	SSR	GCGAACATTGTCATGTTAGTA TGTCATGCCAGACCTATG	Ramsay et al. (2000)
EBmac607	SSR	GCGAACATTGTCATGTTAGTA AACCTTATGGATTTGGAGG	Ramsay et al. (2000)
Bmac518	SSR	ATATGGGTCACACTGAAAATC AGTTTGTTTTTACCAATAAGAGTG	Ramsay et al. (2000)
Bmag381	SSR	TTTTATTATTGCATCTAGGGC TATCAAGATCATGACGTCTCA	Ramsay et al. (2000)
Bmag341a	SSR	TCATGGAGACCGTTGTAGT CCACAAGCCTCTGTTCTC	Ramsay et al. (2000)
Bmac0093	SSR	CGTTTGGGACGTATCAAT GGGAGTCTTGAGCCTACTG	Ramsay et al. (2000)
Bmac134	SSR	CCAACTGAGTCGATCTCG CTTCGTTGCTTCTCTACCTT	Ramsay et al. (2000)
HVM23	SSR	TCGGTGAAGAAATACGAGGC TCTTTGTAGACCTACCGGTCC	Liu et al. (1996)
Bmac132	SSR	AACCTCCATAGTGTAGGGG GTTTGTTCTTTTGATTTTGTTG	Ramsay et al. (2000)
Bmac218	SSR	ATTGCATTGATTAACTCCTACA GGGGGAATCTTTGTGTAAG	Ramsay et al. (2000)
ABG358	STS	ATTCCAGAACCTCCTCGAC AAGCCACATCAACATAATGC	Kuenzel et al. (2000)
Bmag125	SSR	AATTAGCGAGAACAAAATCAC AGATAACGATGCACCACC	Ramsay et al. (2000)
MWG2133	STS	CTTTACCACGGTCTATGTCA GGTAAGACATGGAGGACCAT	Kuenzel et al. (2000)
ABG459	STS	GCCACCACGCTCTCCATTGT CCACGCTCGCTTGCTGACTC	Rodriguez et al. (2006)
HVM36	SSR	TCCAGCCGAACAATTTCTTG AGTACTCCGACACCACGTCC	Liu et al. (1996)
Bmag692	SSR	GCAAGGTATCTCTTGTATTTTG TGGCATCTACAATCTAAAACA	Ramsay et al. (2000)
GBM1251[a]	SSR	CCAGCAATAACAACGTGTGG TGTCTTTTATTTCCGGAGCG	Varshney et al. (2006)
GBM1115[a]	SSR	GTGCCGGTCCTTCATGTC GCCTTCACGTAGTCCCAGAC	Varshney et al. (2006)
ABC454	STS	TTCACAGCCGAAACACTTGT GCGTGCGAGGGGAAGGAGAA	Rodriguez et al. (2006)

[a] Expressed sequence tag derived SSR

of 2.0 mM $MgCl_2$, 1.5 µl (10 ng/µl) of forward and reverse primers (Sigma), 2.5 unit of Taq DNA polymerase (Applied Biosystems, Australia) and 13 µl of ddH_2O. PCR amplification was performed in a DNA theromocycler (Eppendorf, Germany) programmed for 5 min at 94°C for initial denaturation; followed by 35 cycles each consisting of denaturation at 94°C for 45 s, annealing at 53–60°C (depending on each primer pair) at 45 s, and extension at 72°C for 45 s; and a final extension at 72°C for 10 min. The annealing temperature for each primer pair was essentially the same as published in the Grain Genes database (http://wheat.pw.usda.gov/GG2/index.shtml), with the exception of 53°C used for Bmag692.

The amplified PCR product (8 µl) and loading buffer (2 µl; 98% formamide, 10 mM EDTA [pH 8.0], 0.05% [wt/vol] Bromophenol blue and 0.05% xylene cyanol) were

Table 2 Observed frequencies of phenotypic classes in F_2 and F_3 populations derived from the crosses 'Baudin'/'PI 584760' and 'Ricardo'/'PI 584760'

Cross	Generation	Observed frequencies F_2—R:S F_3—NSR:SG:NSS	Tested ratio	χ^2
'Baudin'/'PI 584760'	F_2	99:53	3:1	7.90**
'Baudin'/'PI 584760'	F_3	36:81:29	1:2:1	2.43
'Ricardo'/'PI 584760'	F_3	22:47:37	1:2:1	5.60

R Resistant, *S* Susceptible, *NSR* non-segregating resistant, *SG* segregating, and *NSS* non-segregating susceptible

χ^2 (1 df) at $P = 0.01$ is 6.64 and χ^2 (2 df) at $P = 0.05$ is 5.99

Values followed by ** deviate from the expected ratio significanlty at $P < 0.01$

loaded in 2% agarose gel prepared in 1× Tris–borate EDTA (TBE) buffer (90 mM Tris–borate +2 mM EDTA [pH 8.0]) and subjected to electrophoresis at 100 V for approximately 1 h. The separated products were stained with ethidium bromide and visualised under ultra violet light.

The amplified DNA fragments derived from all primer pairs were also separated in 6% polyacrylamide gel following the procedure outlined by Sambrook et al. (1989). The gel was pre-run in 1× TBE buffer for approximately 40 min at 1,600 V, until the gel temperature reached to ~50°C. An equal volume of loading buffer (4 µl) was added to each PCR sample (4 µl) and denatured at 94°C for 4 min. The denatured PCR products were chilled on ice and 3 µl of each sample was loaded in each well of polyacrylamide gel. The loaded gel was subjected to electrophoresis at 1500 V for 1.5–2 h, depending on the approximate size of the amplified products. The separated DNA fragments were visualised by silver staining.

Chi-squared and linkage analysis

The phenotypic data obtained from rust testing the F_2 and F_3 populations were subjected to Chi-squared (χ^2) analysis to confirm the goodness-of-fit of observed ratios to theoretical expectations. The analysis of linkage between *Rph14* and the molecular markers was performed using Map Manager QTXb20—version 3 (Manly et al. 2001). The Kosambi mapping function was used to convert recombination frequencies to map distances in centi-Morgans (cM).

Results

Genetic analysis

Pathotype 5453P- produced a low IT (1 + CN) on the stock carrying *Rph14*, and a susceptible IT (3+) on seedlings of 'Baudin' and 'Ricardo'. F_2 seedlings derived from the cross 'Baudin'/'PI 584760', along with the parents and differential genotypes, were tested in the greenhouse against *P. hordei* pt. 5453P−. The infection types of F_2 seedlings were compared with the infection types of parents and those of differential genotypes and each F_2 seedling was categorised as susceptible or resistant. The ratio of resistant to susceptible F_2 individuals was 99:53, which showed a significant deviation from a 3:1 ratio (χ^2 3:1 = 7.90, $P < 0.01$, 1 df), expected for the segregation of a single gene (Table 2). Each susceptible and resistant F_2 plant was marked, transplanted to the field, harvested, and the F_3 progeny were tested against *P. hordei* pt. 5453P−. F_3 lines were scored as either non-segregating resistant, segregating or non-segregating susceptible. The number of F_3 lines included in these three classes confirmed to a 1:2:1 ratio (1 non-segregating resistant:2 segregating for susceptible:1 non-segregating susceptible), expected for segregation at a single locus (Table 2). Assuming single dominant gene segregation in a population, the progeny of the resistant F_2 plants should have been either non-segregating resistant or segregating and the progeny of susceptible F_2 plants should have been non-segregating susceptible. However, 20 plants scored as susceptible in the F_2 were scored segregating in the F_3, indicating that they were heterozygous for *Rph14* and that the parental F_2 plants had been misclassified (Fig. 1). This suggested that the inheritance of *Rph14* tended to be incompletely dominant, which would account for the excess susceptible F_2 plants that resulted in deviation from single gene model.

The inheritance of *Rph14* was further confirmed by screening F_3 progeny from the cross 'Ricardo'/'PI 584760' with *P. hordei* pt. 5453P−. The ratio of 22 non-segregating resistant:47 segregating:37 non-segregating susceptible observed in F_3 lines conformed to a 1:2:1 ratio, expected for segregation at a single resistance locus (Table 2). The mismatching of F_2 plants with F_3 lines was due to the loss of 6 F_2 plants during generation advancement in the field. The ratio of resistant to susceptible plants within each segregating F_3 line from both the populations was determined and homogeneity χ^2 was also calculated before pooling the F_3 lines for molecular analysis. The results suggested that

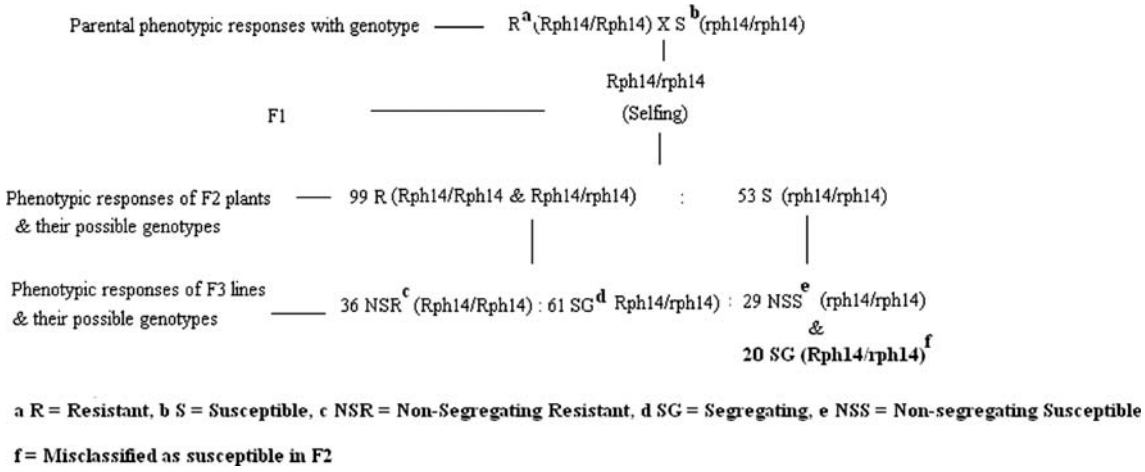

Fig. 1 Observed phenotypic responses in F_2 and F_3 generation to explain the possible genotypes of F_2 and F_3 plants

the F_3 lines segregated for a single gene and that the data were homogeneous (data not presented).

Molecular mapping

BSA using DArT markers was performed on DNA from the parental genotypes and resistant and susceptible DNA bulks. A total of 2,085 markers were initially screened on parental lines and each was scored as either present or absent based on hybridisation intensity. Markers showing variation in hybridisation intensity between parents were then screened on resistant and susceptible bulks. Amongst 386 informative DArT markers, only eight (bPb-7229, bPb-6755, bPb-2501, bPb-7299, bPb-7906, bPb-3190, bPb-1664 and bPb-9925), positioned previously on chromosome 2HS, showed the maximum contrasting variation in hybridisation intensity with DNA from resistant and susceptible bulks (data not presented). The highest variation was observed with marker bPb-1664, indicating that it had the closest genetic association with *Rph14*. The consensus map developed by linking DArT markers with previously mapped SSR, STS and RFLP markers (Wenzl et al. 2006) suggested that the markers bPb-2501, bPb-7906 and bPb-3190 were located 1 cM proximal to the marker bPb-1664, whereas the marker bPb-7299 was located 3.7 cM distal to bPb-1664. Based on these results, *Rph14* was located on chromosome 2HS (Fig. 2).

To identify PCR based markers closely linked to *Rph14*, 16 SSR and four STS markers previously positioned on chromosome 2HS were analysed initially to demonstrate polymorphism between the parents and between the two DNA bulks. Out of the 20 markers, only one SSR marker, Bmag692, generated PCR products that were polymorphic between parents and between DNA bulks. The association between Bmag692 and *Rph14* was determined by screening the marker on 146 F_3 lines derived from the crosses 'Baudin'/'PI 584760' and 106 F_3 lines derived from 'Ricardo'/'PI 584760'. The segregation of marker Bmag692 on a set of F_3 lines and the parental genotypes is shown in Fig. 3. Linkage analysis suggested that Bmag692 was linked to *Rph14* with map distances of 2.1 cM in the 'Baudin'/'PI 584760' population and 3.8 cM in the 'Ricardo'/'PI 584760' population. The consensus map developed using BSA data located the SSR marker Bmag692 at a map distance of 7.5 cM distal to the DArT marker bPb-1664, which was completely associated with *Rph14*.

Discussion

The inheritance of leaf rust resistance in barley accession 'PI 584760', a single leaf rust resistant plant selection from *H. vulgare* accession 'PI 531901', was previously investigated by Jin et al. (1996). The accession 'PI 531901' is a land race from Ethiopia (Jin and Steffenson 1994). The resistance in 'PI 531901' was shown to be governed by an incompletely dominant gene that was not allelic to previously designated leaf rust resistance genes *Rph1* to *Rph13*. The new allele symbol *Rph14* was therefore assigned to the single resistance gene identified in 'PI 584760'. In the present study, the inheritance of *Rph14* was confirmed using F_2 and F_3 populations derived from crosses between 'PI 584760' and the cultivars 'Baudin' and 'Ricardo'.

In the present study, BSA using DArT markers positioned *Rph14* on chromosome 2HS. Previous mapping studies of leaf rust resistance genes in barley have shown that *Rph15* (Weerasena et al. 2004), *Rph16* (Ivandic et al. 1998) and *Rph17* (Pickering et al. 1998) are also located on chromosome 2HS. These genes are also potentially important in barley breeding in Australia because they confer resistance to all known Australian pathotypes of *P. hordei* (Park, unpublished). Chicaiza et al. (1996) demonstrated

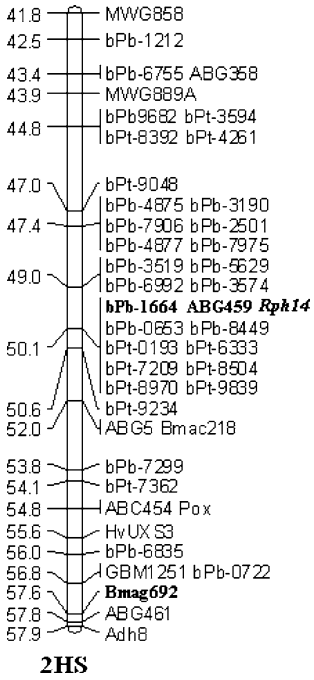

Fig. 2 Partial consensus map of barley chromosome 2HS developed by linking DArT markers to SSR, RFLP and STS loci (Wenzl et al. 2006) and showing the genomic location of *Rph14*. The complete linkage between DArT marker bPb-1664 with *Rph14* was based on the maximum contrasting hybridisation intensity observed between susceptible and resistant DNA bulks with the marker. The SSR marker Bmag692 positioned 57.6 cm from telomere on the consensus map, showed close linkage to *Rph14* when screened on 146 and 106 F3 lines derived from 'Baudin'/'PI 584760' and 'Ricardo'/'PI 584760', respectively

independent segregation of *Rph14* in a cross between *H. spontaneum* accessions 'PI 355447' (carrying *Rph15*) and 'PI 584760'. However, the relationships of *Rph14* with the genes *Rph16* and *Rh17*, also located on chromosome 2HS, are currently not known. Weerasena et al. (2004) studied the genetic relationship between *Rph15* and the gene *Rph16* and demonstrated that they are allelic. Therefore, *Rph16* should not be associated with *Rph14* because its allele (*Rph15*) was shown to be genetically independent from *Rph14*. The gene *Rph17* was characterised in a recombinant line derived from a cross between *H. vulgare* and *H. bulbosum*. This recombinant line also carries the powdery mildew resistance gene *Mlhb*, which is tightly linked to *Rph17* (Pickering et al. 1998). Therefore, knowledge of any potential linkage between *Rph17* and *Rph14* could be useful in barley breeding in attempts to construct combinations of *Rph14*, *Rph17* and *Mlhb*.

BSA is the most efficient method to identify markers associated with a target locus, because it overcomes the need to construct a complete genetic map. BSA was used successfully in several previous studies to locate resistance genes in barley using PCR based markers (Poulsen et al. 1995; Mammadov et al. 2003; Weerasena et al. 2004; Zhong et al. 2003; Agrama et al. 2004; Genger et al. 2005). The suitability of hybridisation based DArT markers for BSA in barley was examined by Wenzl et al. (2007), who used BSA-DArT to map the gene *mPub*, controlling pubescent leaves, in a 'Steptoe'/'Morex' Doubled Haploid (DH) population, and the aluminium tolerance gene *Al* using bulks from a 'Dayton'/'Zhepi' DH population. BSA-DArT data revealed that these genes were positioned on the same chromosomes within a short distance (~5 cM) from previously mapped locations (Wenzl et al. 2007). In the present study, SSR marker Bmag692 was found to be closely linked to *Rph14*. The consensus map developed using BSA data located this marker at a map distance of 7.5 cM from the DArT marker bPb-1664, which was completely associated with *Rph14*. This result further demonstrates the efficiency of the method in identifying the precise genomic location of the target locus. Lee and Neate (2006) successfully used DArT markers to map genes R*sp*1, R*sp*2 and R*sp*3 conferring resistance to *Septoria* speckled leaf blotch in barley. Rheault et al. (2007) used DArT and SSR markers to identify genomic regions contributing resistance to Fusarium head blight in barley. In addition to mapping studies in barley, DArT has been established for many important crop species such as rice, wheat, cassava, barley, sugarcane, sorghum, banana and pigeonpea (Gupta et al. 2008). These studies opened new avenues to map important traits using BSA-DArT method for crop improvement.

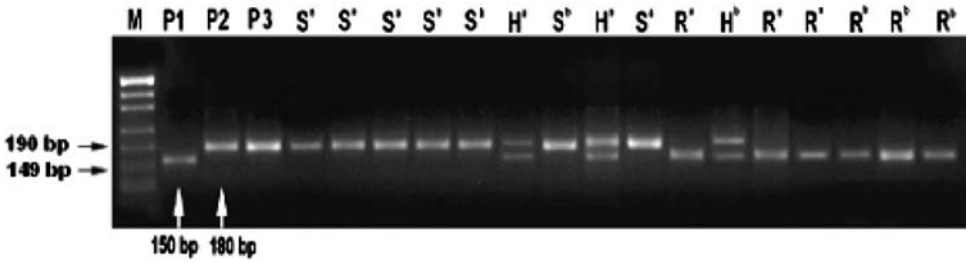

Fig. 3 Linkage analysis of *Rph14* with marker Bmag692 using F_3 lines from the population 'Baudin'/'PI 584760' (**a**) and 'Ricardo'/'PI 584760' (**b**); *M* pUC19/MspI DNA marker, *P1* resistant parent ('PI 584760'), *P2* susceptible parent ('Baudin'), *P3* susceptible parent ('Ricardo'), *S* non-segregating susceptible progeny, *H* segregating progeny, *R* non-segregating resistant progeny. The marker generated PCR products of approximately 150 and 180 bp from resistant and susceptible parents, respectively

The frequency of virulence for *Rph14* in Europe, North America, South America, and Africa was reported by Fetch et al. (1998) to be less than 3%. Virulence for this gene has not been detected in Australia (Park 2003). The widespread effectiveness of *Rph14* means that it could be an useful source of resistance, especially if it is combined with other seedling resistance genes to increase durability. The map information generated and the linked markers identified in the present study should improve efforts to combine *Rph14* with other resistance genes. The close linkage and co-dominance of Bmag692 mean that it will be useful in assisting selection for *Rph14*. The efficiency of using this marker in MAS could be improved by either identifying a second marker flanking *Rph14* or by further fine mapping studies. Given that the barley DArT markers were sequenced recently (Wenzl, personnel communication), it should be possible to use this sequence information for DArT markers closely linked to the target locus to develop PCR based STS markers for regular genetic assays and marker-assisted selection.

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