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The use of bulk segregant analysis to identify a RAPD marker linked to leaf rust resistance in barley

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Abstract An F_2 population from a cross between barley accession Q21861 and the Australian barley variety 'Galleon' was used to develop RAPD markers for resistance to barley leaf rust (*Puccinia hordei*). Resistant and susceptible DNA bulks were constructed following the classification of F_2 plants by leaf rust infection type. Bulked segregant analysis was then used to identify a 2.7-kb marker, designated $OU02_{2700}$ and located approximately 12cM from *RphQ*, a leaf rust resistance gene in Q21861. The marker was generated by PCR with the oligonucleotide primer OPU-02 (Operon). Infection types of F_3 progeny were used to confirm assignment of F_2 genotypes. $OU02_{2700}$ was shown, retrospectively, to be useful in the identification of individual F_2 plants that had been originally misclassified as having susceptible infection types. Both the RAPD marker and *RphQ* will be potentially useful in the development of new barley cultivars.

Key words Barley · *Puccinia hordei* · Resistance genes · Bulk segregant analysis · Polymerase chain reaction

Introduction

Barley leaf rust is caused by the fungus *Puccinia hordei* Otth. and has been reported worldwide (Clifford 1985). Twelve genes for resistance to the disease have been characterised in barley (*Rph1-12*), and additional sources of resistance are known to exist (Cotterill et al. 1995). Many of these genes have been used in breeding programmes, however, the detection of virulence to most of them in the *P. hordei* gene pool highlights the need for barley breeders to use long-term control strategies such as gene pyramiding (Nelson 1973) or the use of "slow rusting" resistances (Parlevliet 1983).

Q21861, a barley accession of unknown pedigree from CYMMIT, Mexico (Dill-Macky et al. 1992), is known to possess resistance to both barley leaf rust and stem rust (*P. graminis*). A single dominant gene, designated *RphQ*, is thought to control the leaf rust resistance (BJ Steffenson, personal communication) and would be useful in its own right. However, it would be highly desirable to use *RphQ* in combination with other sources of resistance.

Conventional gene pyramiding techniques rely on field and glasshouse screening with differential rust races. They may also require the use of controlled environment facilities if the resistance genes are temperature-sensitive. Consequently, the process is complex and time-consuming. However, the development of molecular marker technology, particularly the polymerase chain reaction (PCR) (Saiki et al. 1988) has considerably expanded the potential for pyramiding disease resistance genes. This is brought about by the ability to select for the target genes without the complications of working with living pathogens. Molecular markers have been identified for a number of disease resistance genes in cereal crops, which include barley (Heun 1992; Schuller et al. 1992; Graner and Bauer 1993; Jahoor et al. 1993; Chen et al. 1994), wheat (Hartl et al. 1993), oats (Penner et al. 1993) and rice (Yu et al. 1991).

One of the most time-consuming requirements of DNA marker development, the need to screen entire mapping populations with every probe or primer, has been removed by the development of bulked segregant analysis by Michelmore et al. (1991) in their work with downy mildew resistance in lettuce. The screening of contrasting DNA bulks made from individuals of the same phenotype in a

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segregating population means that testing the entire population is only required when polymorphisms between the bulks are detected. This results in a considerable time saving, particularly when used with PCR-based techniques such as random amplified polymorphic DNA (RAPD) (Williams et al. 1990). Bulked segregant analysis has now been used to detect markers linked to many traits, including *Uromyces appendiculatis* resistance in common bean (Miklas et al. 1993; Haley et al. 1993), factors controlling milling energy in barley (Chalmers et al. 1993), and fertility restoration in rapeseed (Delourme et al. 1994).

In this paper, we report the identification of a RAPD marker linked to the barley leaf rust resistance gene, *RphQ*.

Materials and methods

Screening the F_2 mapping population for leaf rust infection type

Eighty-four F_2 plants from the cross Q21861/'Galleon' and the parent lines were grown in individual pots under glasshouse conditions. The cross was chosen because of the presence of resistances to both stem rust and leaf rust in Q21861. Six weeks after sowing, and following screening by inoculation with *P. graminis*, the seedlings were inoculated with *P. hordei* octal race 210 by an aerosol spray of spores suspended in light oil. Following incubation at 17°C for 16 h, the plants were transferred to a controlled environment chamber with a 15°/25°C night/day regime. Leaf rust infection types were assessed 10 days after inoculation using a 0–4 scale, where 0 means high resistance and 4 high susceptibility (Parlevliet 1976). The F_2 plants were subsequently transplanted to the field for production of F_3 seed.

F_3 disease resistance screening

F_3 progeny from all of the susceptible and some of the resistant F_2 plants were screened for leaf rust infection type in two sessions. Thirty seeds from each of the F_3 progenies tested were planted in pots. The germinating seedlings, which ranged in number per progeny from 13 to 30, were raised in a glasshouse for 13 days before aerosol inoculation with *P. hordei* octal race 210, and then transferred to a dew chamber for 16 h at 20°C. They were then grown in a growth cabinet for 12 days at a 13°/23°C, 12-h day/night regime before the individual plant infection types were scored.

DNA extraction

Leaf tissue samples were collected from the F_2 plants prior to inoculation with *P graminis*, and stored in liquid nitrogen. Nine of the samples were accidentally destroyed as they were removed from cold storage, and additional tissue could not be harvested due to the risk of contamination with fungal DNA. Extraction of the DNA from the remaining 75 samples was carried out using a modification of the method of Graham et al. (1994). The frozen samples were ground in sterile 1.5-ml Eppendorf tubes, and 800 µl of CTAB buffer was added to each tube before incubation at 55°C for 15 min. After centrifugation at 13000 rpm for 5 min, the supernatant was transferred to a 1.5-ml tube by wide-bore pipette. Approximately 400 µl of chloroform:iso-amyl alcohol (24:1) was added to the tubes, and the contents were mixed by gentle inversion. The samples were centrifuged at 12000 rpm for 1 min, the upper aqueous phase was transferred to new 1.5-ml tubes and 70 µl of 7.5 *M* ammonium acetate was added, followed by 750 ml of ice-cold absolute ethanol. The precipitated DNA was washed in 70% ethanol, air-dried and resuspended in TE buffer.

Bulk segregant analysis with RAPDs

Eight resistant and eight susceptible F_2 plants were selected for the respective bulk DNA samples. DNA concentration in the working solutions of approximately 25 ng/ml in ddH_2O was confirmed by spectrophotometric analysis at 260 nm.

PCR was carried out on the bulk and parental DNA samples using 10-bp oligonucleotide primers with arbitrary sequence (Operon) on a Perkin Elmer Cetus 9600 thermal cycler. The optimal mix for a 25-µl PCR reaction was determined by titration to be: 10×PCR buffer, 2.5 µl; dNTPs (dATP, dCTP, dGTP, dTTP), 200 m*M*; *Taq* DNA polymerase, 1 unit; template DNA, 50 ng; primer, 0.2 m*M*.

The thermal cycle for the PCR amplification followed a modified version of that used by Williams et al. (1990). The template DNA was initially denatured at 96°C for 2 min and then subjected to 10 cycles at 36°C for 30 s, 72°C for 1 min and 94°C for 20 s. This was followed by 30 cycles at 37°C for 30 s, 72°C for 1 min and 94°C for 15 s.

Scoring and analysing RAPD data

Following amplification, the samples were subjected to electrophoresis in 1.5% agarose gels which were subsequently stained with ethidium bromide and viewed under ultra-violet light. When a primer was found to give rise to a polymorphism between the bulks, it was used to amplify DNA from the individual F_2 plants in the population to verify if the PCR product was linked to the target gene.

Linkage analysis

Linkage distance was calculated using MAPMAKER (Lander et al. 1987).

Results and discussion

Segregation analysis of the Q21861 leaf rust resistance gene, *RphQ*

At the commencement of this study, barley accession Q21861 was known to possess a gene for resistance to leaf rust (PJ Cotterill, personal communication) and 'Galleon' was thought to be wholly susceptible to the disease. A recent survey of Australian barley cultivars by Cotterill et al (1994) reported that 'Galleon' was slightly less susceptible to leaf rust than many other varieties, suggesting that it possesses a low level of resistance to leaf rust. In our experiment, a low level of resistance was again observed in 'Galleon'. However, this did not appear to affect the identification of Q21861-type plants in the F_2 mapping population.

Unfortunately, it was not possible to postulate the genotypes of all of the individual plants from the F_2 leaf rust infection-type data due to contamination with *P. graminis* from a preceding inoculation and screening. Therefore, the F_2 genotypes were checked by screening F_3 progeny from all of the leaf rust-susceptible and some resistant F_2 plants. This was successful and resulted in 60 resistant and 24 susceptible F_2 plants being identified. The distribution fitted a 3:1 segregation ratio (χ^2=0.571, p>0.25, 1 *df*), confirming the presence of a single, dominant leaf rust resistance gene (*RphQ*) in Q21861.

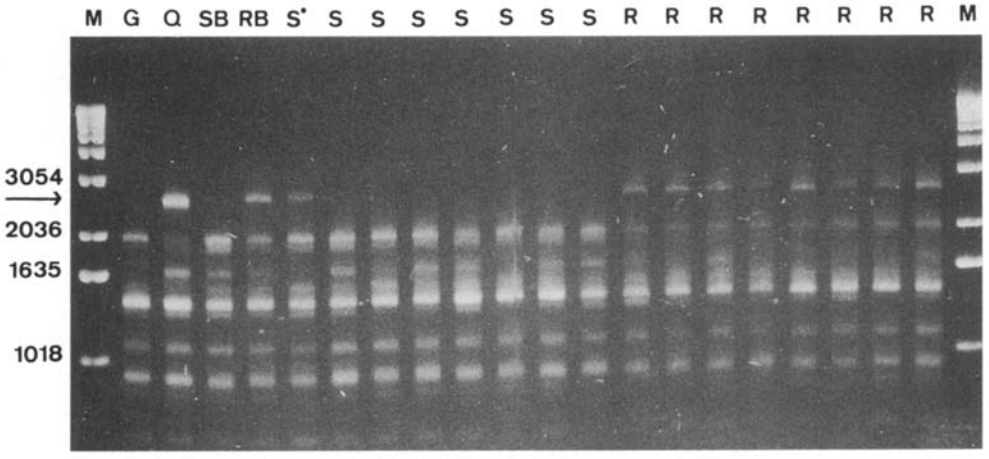

Fig. 1 A 1.5% agarose gel showing the results of PCR amplification with the random primer OPU-02 of *G* 'Galleon', *Q* Q21861, *SB* the leaf rust-susceptible bulk, *RB* the leaf rust-resistant bulk, *S* individual F_2 plants from the leaf rust-susceptible bulk, and *R* individual F_2 plants from the leaf rust-resistant bulk. *M* 1-kb ladder. The DNA sample marked *S** was found to have been extracted from a leaf rust resistant plant that had been originally misclassified as susceptible. Arrow indicates the marker, $OU02_{2700}$

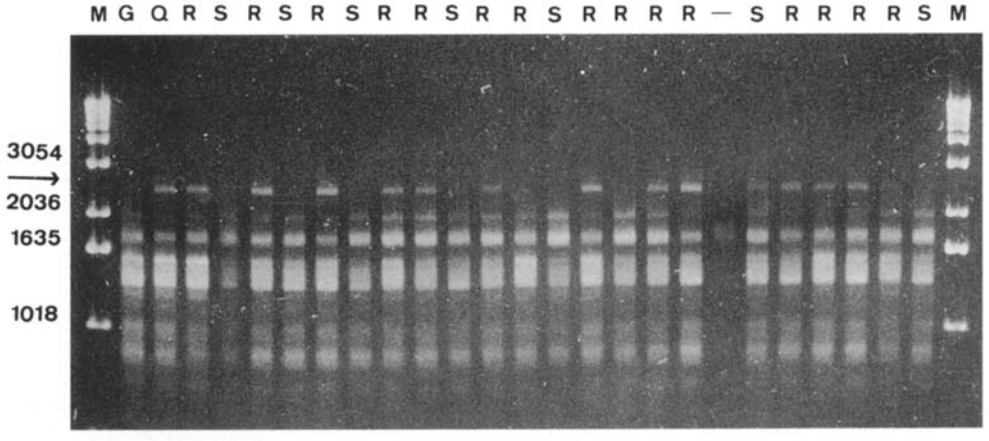

Fig. 2 A 1.5% agarose gel showing the results of PCR amplification with the random primer OPU-02 of *G* 'Galleon', *Q* Q21861, *R* resistant F_2 plants S susceptible F_2 plants. *M* 1-kb ladder. Arrow indicates the marker, $OU02_{2700}$

Detection of a RAPD marker linked to leaf rust resistance in Q21861

Resistant and susceptible bulk DNA samples were each constructed from 8 plants of the appropriate F_2 infection types and subjected to PCR using random oligonucleotide primers. One of the first eight primers tested produced a polymorphic band between the leaf rust resistant and susceptible samples (Fig. 1). The band, generated from primer OPU-02 (5′-CTGAGGTCTC), appeared to be approximately 2.7 kb in size and was designated $OU02_{2700}$, following the system used by Michelmore et al. (1991). Amplification of the individual DNA samples out of the bulks with OPU-02 revealed that all of the resistant plants and only 1 susceptible plant generated the polymorphic band, indicating strongly that the band was linked to *RphQ*.

When all of the 75 surviving individual DNA samples were screened with OPU-02 (Fig. 2), the RAPD band was observed to be segregating 53:22, which fit a 3:1 distribution (χ^2=0.751, P>0.25, 1 df). Analysis of the F_2 genotype and RAPD data with MAPMAKER gave a linkage distance between *RphQ* and $OU02_{2700}$ of 12.0 cM with a LOD score of 23.04. This distance is close enough for the RAPD to have practical value, particularly for selection within large breeding populations to obtain a manageable number of lines for multi-locational yield trials. The use of this marker would enable the selection of breeding lines for yield testing, of which the majority would be leaf rust resistant. $OU02_{2700}$ may also have some use in a backcrossing programme.

Retrospective analysis of the F_2 data using $OU02_{2700}$

When the F_2 population was inoculated with *P. hordei*, it was originally believed that sufficient time had passed after the *P. graminis* screening to avoid the results being confused by stem rust infection of the newly produced leaves. However, examination of the F_2 leaf rust infection type data revealed a number of inconsistencies with the expected results and it was concluded that some stem rust infection had actually occurred and that resistant stem rust pustules had been confused with those of leaf rust. This problem was resolved by screening F_3 populations derived from individual F_2 plants. Eleven F_2 plants that were originally thought to be susceptible were identified as possessing *RphQ*, and the RAPD data revealed that all 11 of those lines possessed $OU02_{2700}$. This data highlighted the potential usefulness of DNA markers in resolving phenotypic classification errors. It was also significant that the single

F_2 plant in the susceptible bulk which generated $OU02_{2700}$ was one of the misclassified individuals and that despite this, bulked segregant analysis was successfully used to identify the marker. This indicates that when the bulks are constructed from enough individuals, in this case 8, the bulked segregant method is sufficiently robust to cope with a low level of phenotypic misclassification.

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