Theor Appl Genet (2000) 100:27–31

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Locating introgressions of *Hordeum bulbosum* chromatin within the *H. vulgare* genome

Received: 16 December 1998 / Accepted: 12 April 1999

Abstract Several disease-resistant recombinants between barley (*Hordeum vulgare*) and bulbous barley grass (*H. bulbosum*) have been obtained in recent years, but the process of characterization is often laborious and time-consuming. In order to improve the identification and chromosomal location of introgressed chromatin from *H. bulbosum* into the barley genome, we employed sequential genomic in situ hybridization (GISH) and fluorescence in situ hybridization (FISH). GISH enabled us to establish that an introgression was present in the disease-resistant recombinant line, and the subsequent use of FISH, with a short oligonucleotide sequence as probe, allowed us to locate the introgression on the long arm of barley chromosome 2H. These data were confirmed using RFLP probes that hybridize to barley chromosome 2HL.

Key words *Hordeum vulgare · Hordeum bulbosum* · Leaf rust resistance · Gene introgression · In situ hybridization

Introduction

Until recently the sexual transfer of genetic material from wild barley species into cultivated barley has been restricted to *H. vulgare* ssp. *spontaneum* (C. Koch) Thell. (Lehmann 1991). The main problem preventing successful alien gene transfer into cultivated barley (*Hordeum vulgare*) is its diploid constitution (2n=2x=14), which cannot tolerate as much genetic manipulation as polyploid cereal species such as bread wheat (*Triticum aestivum*; 2n=6x=42) and oats (*Avena sativa*; 2n=6x=42). Gene and chromatin transfers have been achieved regularly with these latter species (Jiang et al. 1994; Leggett 1996). Even with *H. bulbosum*, a species closely related to cultivated barley, several barriers such as incompatibility, hybrid instability, infertility and low levels of intergenomic crossing-over need to be overcome before the transfer of genetic material can be achieved (Pickering 1991). Despite these limitations, recombinant plants have been produced, some of which contain disease–resistance genes transferred from *H. bulbosum* into an *H. vulgare* background (Xu and Kasha 1992; Pickering et al. 1995). Recombinants were selected among progenies from interspecific hybrids by comparing plant phenotype and the response to pathogens. These recombinants were characterized using genomic in situ hybridization (GISH) and molecular analyses such as restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP) and random amplified polymorphic DNA (RAPD) (Xu and Kasha 1992; Pickering et al. 1995, 1997). However, the whole process of recombinant characterization is lengthy and labour-intensive. The aim of the present research presented here was, therefore, to establish a fast and efficient approach for detecting and localizing introgressed *H. bulbosum* chromatin in barley. To detect an introgression mediating disease resistance in the chromosomes of a recombinant plant we used GISH with labelled *H. bulbosum* genomic DNA and subsequently performed fluorescence in situ hybridization (FISH) with a $(CTT)_{10}$ oligonucleotide (Pedersen and Linde-Laursen 1994) to identify the particular chromosome arm(s) that carry the *H. bulbosum* DNA. The chromosomal location of the introgression was confirmed by RFLP analyses.

Communicated by P. Langridge

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Materials and methods

A partially fertile triploid hybrid was produced from a cross between *H. vulgare* cv 'Emir' (2n=2x=14) and a colchicine-induced autotetraploid of *H. bulbosum* HB2032 (2n=4x=28) (also known as PB8, Xu and Snape 1988). The triploid hybrid was backcrossed as male parent to 'Emir'. Selfed progenies were screened on the

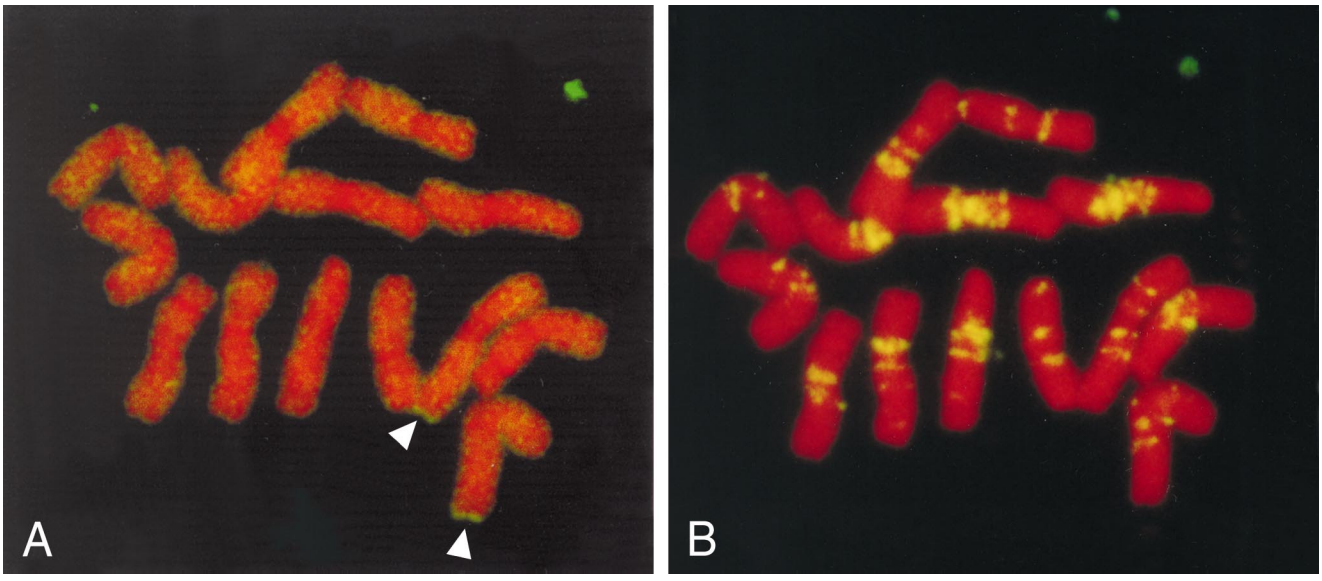

Fig. 1A, B GISH and FISH on a mitotic chromosome preparation from a leaf rust-resistant *H. vulgare* – *H. bulbosum* recombinant, 38P18. **A** GISH using total genomic DNA from *H. bulbosum* showing two signals (*arrowheads*). **B** FISH using the oligonucleotide sequence $(CTT)_{10}$ as probe on the same chromosome preparation. The hybridization patterns of the two barley chromosomes containing the introgressions establish the location on 2HL. *Magnification.* ×2300

basis of morphology and reaction to endemic fungal pathogens in glasshouse tests and in two sowing cycles in the field. Several plant selections were retained for further analyses including one (38P18) that was more resistant in the field to leaf rust (*Puccinia hordei* Otth, infection type 1) than 'Emir' (infection type 3+) on the scale of Levine and Cherewick (1952). There were no visible signs of infection on *H. bulbosum* genotype HB2032 in a glasshouse test. Homozygous leaf rust-resistant plants were subjected to GISH and FISH and were also backcrossed to 'Emir' to produce segregating BC_2F_2 populations for inheritance studies and further molecular analyses.

GISH and FISH were performed sequentially on the same root-tip squashes. GISH was performed as described by Pickering et al. (1997) except that 20 µl of hybridization mix was used for each preparation, which was then denatured at 80°C for 6–7 mins prior to hybridization. For rehybridization, the genomic probe was removed using the method of Heslop-Harrison et al. (1992).

For FISH, a $(CTT)_{10}$ oligonucleotide obtained from Research Genetics, (USA), was end-labelled with biotin-16-dUTP by terminal transferase (Boehringer Mannheim) according to the manufacturer's instructions. The procedure for FISH followed that published by Pedersen and Linde-Laursen (1994) except that preparations were denatured for 6 min at 80°C prior to hybridization with the denatured probe. The hybridized probe was detected using fluorescein isothiocyanate (FITC)-streptavidin (8 µg/ml) and one amplification with biotinylated anti-streptavidin (16 µg/ml). For GISH and FISH, propidium iodide (PI, 1 µg/ml Vectashield™) was used for counterstaining.

For GISH analysis, PI and fluorescein images of the chromosomes were captured with a cooled CCD camera system (Photometrics) on a Zeiss Axioplan 2 epi-fluorescence microscope. The images were digitized with IPLab Spectrum (Signal Analytics, Vienna, Va.), false-coloured and merged in Adobe Photoshop (Mountain View, Calif.). The FISH preparations were viewed and photographed directly on the Zeiss Axioplan 2 epi-fluorescence microscope using Ektachrome Elite II colour transparency film (400ASA). The photographic slides were subsequently digitized to obtain greater clarity for the final prints.

RFLP analyses were performed on DNA extracted from young leaves of four leaf rust-resistant selections of 38P18 and its parents, 'Emir' and HB2032, according to the method of McCouch et al. (1988). The DNA was digested with the restriction enzymes *Eco*RI, *Eco*RV, *Hin*dIII and *Dra*I. Since the results from GISH and FISH indicated an introgression on the long arm of chromosome 2 (2HL) (see Results), the following RFLP probes that had previously been mapped on 2HL (starting from the outermost position) were used: MWG2225, MWG949, MWG829, MWG2200, BG123, MWG866 and cMWG660 (Graner et al. 1991; Langridge et al. 1995; A. Graner, personal communication). Southern blots, probe labelling and filter hybridization followed the methods described by Devos et al. (1992). RFLP analyses using MWG949 were also performed on individual and pooled samples of resistant and susceptible plants derived from the BC_2F_2 population using the method of Pickering et al. (1995).

Sequence-tagged-site (STS) analyses were carried out in parallel to RFLP analyses on DNA extracted from 1 leaf rust-resistant plant of 38P18 and the parents ('Emir' and HB2032) by polymerase chain reaction (PCR) with primers that amplify 2HL marker sequences from barley DNA, as described by Korzun and Künzel (1996). The following STS markers (which yielded polymorphisms among the PCR products obtained from DNA of *H. vulgare* and *H. bulbosum*) were used in an attempt to confirm the chromosomal site of the *H. bulbosum* chromatin: MWG949, MWG080, MWG2076, MWG2303 and MWG2068 (from most distal to proximal location, respectively; see consensus map of Langridge et al. 1995; A. Graner personal communication).

Results

Inheritance of leaf rust resistance and chlorotic leaves

Selfed progeny of plant 38P18 segregated for leaf rust resistance (13 plants) or susceptibility (4 plants), and for green (23 plants) or chlorotic leaves (9 plants). From the BC_2F_2 population of 287 plants, 208 resistant and 79 susceptible plants were recorded, a ratio not significantly different from the 3 resistant: 1 susceptible expected when resistance is conferred by a single dominant gene (χ^2=0.85; 1 *df*). From another BC_2F_2 population involving a cross between a chlorotic leaf rust-resistant plant of 38P18, 148 green and 43 chlorotic plants were

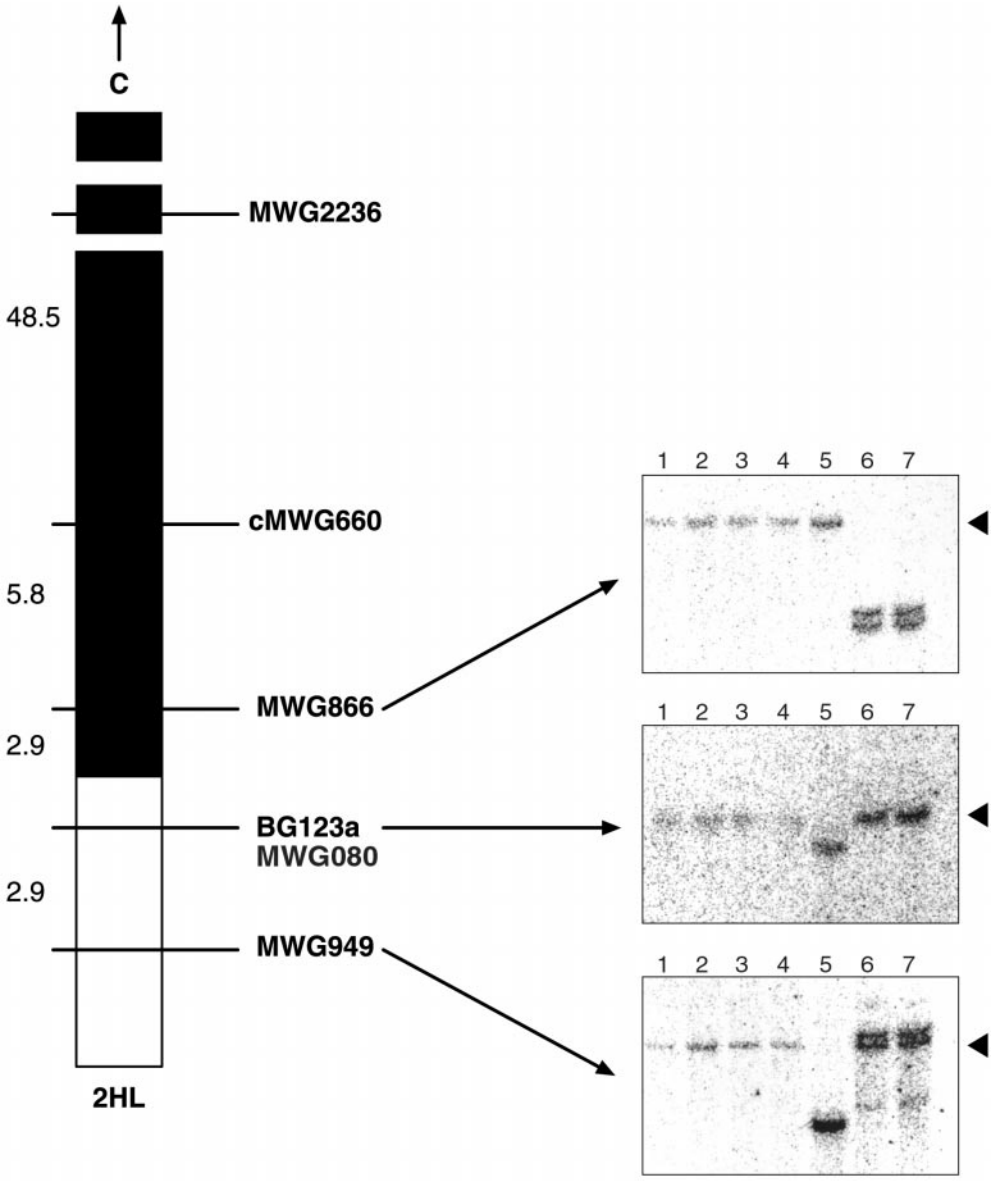

Fig. 2 Diagram of part of barley chromosome 2HL with the *H. bulbosum* introgression shown distally in *white*. The genetic distances (in centiMorgans) and the probe map order are according to Dr. A Graner (personal communication). The autoradiograms of RFLP analyses represent three probes and show the presence of *H. vulgare*- (MWG866) or *H. bulbosum*-specific (BG123a and MWG949) fragments (*arrowheads*) in DNA derived from plants of four disease-resistant recombinants (*lanes 1–4*), *H. vulgare* cv 'Emir' (*lane 5*), and *H. bulbosum* (HB2032, *lanes 6* and *7*). The restriction enzyme used for DNA digestion was *Dra*I

recorded, a ratio consistent with chlorosis being governed by a single recessive gene (χ^2=0.51; 1 *df*). The two genes for leaf rust resistance and chlorosis were unlinked, since in a subset of the population, the observed numbers of green resistant (87), green susceptible (25), chlorotic resistant (26) and chlorotic susceptible (9) plants did not differ significantly from a 9:3:3:1 ratio expected for independent inheritance in an F_2 population (χ^2=0.56; 3 *df*).

Detection and chromosomal location of *H. bulbosum* chromatin introgressed into the barley genome

Two chromosomes, each carrying a distal signal of increased intensity, were observed by GISH (Fig. 1A) using fluorescein-labelled genomic *H. bulbosum* DNA to chromosomes of a green leaf-rust resistant plant from 38P18. After removal of the genomic probe and subsequent re-probing with the labelled oligonucleotide sequence $(CTT)_{10}$, the chromosome arms showing terminal GISH signals were identified as 2HL (Fig. 1B).

RFLP/STS analyses

The chromosomal location of the *H. bulbosum* introgression was confirmed and genetically defined by RFLP analyses (Fig. 2). These analyses revealed the presence in the recombinants of *H. vulgare*-specific fragments with probes cMWG660 and MWG866 and *H. bulbosum*-specific fragments with probes BG123, MWG2200, MWG829 and MWG949. One exchange of the barley chromosome arm 2HL with its *H. bulbosum* homoeologue had obviously occurred between markers BG123a and MWG866. A second crossover more distal-

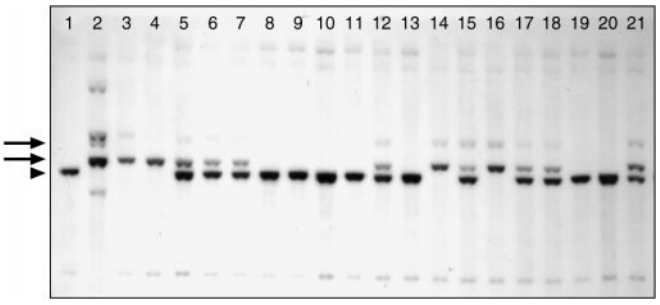

Fig. 3 RFLP analysis using probe MWG949 of individual leaf rust-resistant plants (*lanes 4–7* ex green plants, *15–18* ex chlorotic plants) and susceptible plants (*lanes 8–11* ex green plants, *19–20* ex chlorotic plants) and pooled samples of 5 resistant (*lane 12* ex green plants, *lane 21* ex chlorotic plants) and 5 green susceptible (*lane 13*) plants derived from a BC_2F_2 population. Lane 1 'Emir', *lane 2* HB2032 (the *H. bulbosum* parent). *Lanes 3, 14* selfed progeny from 38P18/20/2, which was the resistant recombinant plant used to make the BC_2F_2 population. *Lane 3* 38P18/20/2/7 (homozygous resistant green plant), *lane 14* 38P18/20/2/1 (homozygous resistant chlorotic plant). Note that 2 *H. bulbosum*-specific fragments (*arrows*) are always associated with resistant plants either with or without the 'Emir'-specific fragment (*arrowhead*), indicating heterozygosity or homozygosity, respectively, for the introgression. The *H. bulbosum*-specific fragments were absent in all the susceptible plants

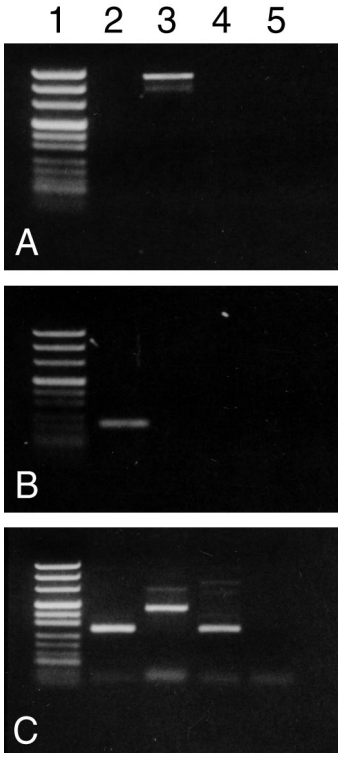

Fig. 4A–C PCR products obtained with sequence-specific primer sets for the RFLP probes MWG949 **(A)**, MWG080 **(B)** and MWG2303 **(C)** mapped on barley chromosome 2HL. *Lane 1* Molecular marker (pUC 'Fermentas'), *lane 2* 'Emir', *lane 3 H. bulbosum* HB2032, *lane 4* leaf rust-resistant recombinant from 38P18, *lane 5* without DNA (control). **A** Amplification of a fragment (approx. 900 bp) was only observed with HB2032 (lane 3). **B** Amplification of a fragment (200 bp) was only observed for 'Emir' (*lane 2*). **C** 'Emir'-specific fragments (284 bp) were observed for 'Emir' (lane 2) and the recombinant (lane 4), whereas an *H. bulbosum*-specific fragment (approx. 500 bp) was obtained for HB2032 (*lane 3*)

ly located than MWG949 is theoretically possible, since only 'Emir' fragments were observed with the most distally located probe, MWG2225 (not illustrated). However, with this same probe and restriction enzymes *Hin*dIII and *Eco*RV, 1 'Emir'-specific fragment was absent in homozygous resistant recombinants, whereas in heterozygotes and homozygous susceptible progenies, all the 'Emir'-specific fragments were present. MWG2225 detects two copies in *H. vulgare*, one of which was mapped as a null allele on 2HL and the other on chromosome 5H on the 'Igri'×'Franka' map (A. Graner, personal communication). If the *H. bulbosum* parent also carried the null allele on chromosome 2HL, then *H. bulbosum*-specific fragments and 1 or more 'Emir'-specific fragments would not be observed in the homozygous recombinants and a double crossover would, therefore, be unlikely. The recombinants showed only 'Emir'-specific fragments using the most proximal probe, cMWG660 (not illustrated).

RFLP analysis of restricted DNA from individual and pooled samples (resistant and susceptible to leaf rust) from the segregating BC_2F_2 population confirmed that the disease resistance gene co-segregated with MWG949, which is located on chromosome 2HL (Fig. 3).

In contrast to the data obtained from the RFLP marker analyses, no amplification product was obtained with primers of the most distally located STS marker (MWG949) from DNA of 'Emir' and the three recombinants, whereas a fragment of about 900 bp was amplified from DNA of the *H. bulbosum* parent HB2032 (Fig. 4A). Conversely, with MWG080, which maps approximately 2.7–2.9 cM more proximally than MWG949 (Langridge et al. 1995; A. Graner personal communication), there was no amplification product for HB2032 and the 3 recombinants, while with DNA of 'Emir' a fragment of 200 bp was obtained (Fig. 4B). STS analyses with the remaining three probes (MWG2076, MWG2303 and MWG2068), which are located still more proximally, showed polymorphisms of fragments between *H. vulgare* ('Emir') and *H. bulbosum*, but only 'Emir'-specific PCR products were obtained with DNA from the recombinants (MWG2303, Fig. 4C).

Discussion

A recombinant plant (38P18) derived from a cross between *H. vulgare* and *H. bulbosum* was shown to carry an introgression distally on chromosome 2HL, which transferred leaf rust resistance from *H. bulbosum* to barley. The location of a recessive gene conferring chlorotic leaves is unknown. This is the second reported transfer of a dominant leaf rust-resistance gene from *H. bulbosum* into *H. vulgare*; the first reported gene was localized

to the short arm of barley chromosome 2 (2HS; Pickering et al. 1998). In both cases, however, it is not possible at present to determine whether more than one resistance gene exists in the blocks of DNA transferred from *H. bulbosum*. The combination of GISH and FISH proved suitable for identifying the chromosomal position of an introgression which could be confirmed by subsequent RFLP analysis. The procedure took 2.5 days to accomplish and eliminated the need for extensive molecular analysis (e.g. bulked segregant analysis) to locate introgressions in progeny from this wide hybrid.

Recently, Schubert et al. (1998) used a similar combination of GISH, FISH and N-banding to detect and localize deletions and interspecific translocations involving barley chromosomes added to the hexaploid wheat genome in progeny from intergeneric crosses of wheat, *Aegilops cylindricum* and barley. The present paper provides a further example of an efficient, rapid, reliable and inexpensive technique for genome analysis of progeny from interspecific hybrids using FISH with oligonucleotides instead of N-banding for chromosome-arm identification. This approach can be used for routine testing as long as the size of the introgression does not fall below the limits of resolution obtainable with GISH.

The deviations in STS data from that of RFLP analyses using MWG949 might be due to problems arising when primers are used to amplify DNA from multi-copy targets of non-homologous DNA (Blake et al. 1996; Erpelding et al. 1996; Talbert et al. 1996). Whereas evidence for an *H. bulbosum* introgression was obtained from the RFLP analysis, there was no amplification of a fragment in 'Emir' or the recombinants compared with a fragment of 900 bp observed for HB2032 using the STS primers relating to MWG949. Since more than one fragment was observed in the RFLP analysis of the *H. bulbosum* parent, hybridization sites at more than one locus may exist. It is possible, therefore, that primers for particular STS markers may preferentially hybridize and amplify sequences from a site at a different location from the one under investigation (see also Erpelding et al. 1996). Talbert et al. (1996) have previously reported anomalies in the results following the use of STS primers among different species and Blake et al. (1996) presented data showing that some mismatching between RFLP probes and their respective STS primers can occur. Hence, care should be exercised when using STS markers in species other than those from which they were developed and when multi-locus sites are expected. In such cases RFLP analyses are obviously more reliable.

Acknowledgements We thank Dr. Andreas Graner (IPK, Gatersleben, Germany) for kindly donating the RFLP probes; Drs. Rogier ten Hoopen (IPK, Gatersleben, Germany) and Christine Morris (Christchurch School of Medicine, New Zealand) for help and advice in the preparation of the digitized images of chromosomes; Mrs. Merle Forbes and Dr. Matthew Cromey (Crop & Food Research, New Zealand) for technical assistance and pathological testing, respectively.

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