CHAPTER 4

THE POTENTIAL OF GENETICALLY DIRECTED REPRESENTATIONAL DIFFERENCE ANALYSIS TO GENERATE RFLP MARKERS CLOSE TO THE *Yd*$_2$ GENE

4.1 Introduction

In Chapter 3, a genetic map of a portion of barley chromosome 3 was constructed, containing RFLP markers and the *Yd2* gene for barley yellow dwarf (BYDV) resistance. As discussed in Chapter 3, a vast improvement of the marker density and resolution of this map will be required to identify molecular markers close enough to *Yd2* to enable the isolation of the *Yd2* gene by a map-based approach. RFLP loci used to construct the map of the *Yd2* region were detected using RFLP clones which had been developed by other researchers for use in other grass mapping programs. Although it may be possible to obtain further clones for mapping RFLPs close to *Yd2* from other researchers, the number of markers that can be obtained in this manner is dependent on the researchers' generosity and the extent of their mapping programs. Therefore, it is likely that further markers will need to be obtained by other means in order to achieve the marker density required for the isolation of *Yd2*.

Markers in the vicinity of a gene of interest can be obtained by randomly generating markers such as RFLPs and RAPDs, and sreening these for close linkage to the gene using nearly-isogenic lines (Young *et al.*, 1988) or bulked segregant analysis (Michelmore *et al.*, 1991). Genetically Directed Representational Difference Analysis, or GDRDA (Lisitsyn *et al.*, 1994) differs from these procedures in that it employs subtractive hybridisation rather than random screening to identify markers closely linked to a gene. GDRDA has been used to generate RFLP markers closely linked to the four mutant loci in mice (Lisitsyn *et al.*, 1994) and the *H25* gene for hessian fly larvae resistance in rye (Delaney *et al.*, 1995b).

GDRDA is a variation of the method Representational Difference Analysis (RDA) for detecting genetic differences between two complex genomes (Lisitsyn *et al.* 1993). In GDRDA, the RDA procedure is performed on two closely related lines or groups of lines differing for a chromosome segment containing a gene of interest in order to specifically target RFLPs close to the gene. Genetic material suitable for use in GDRDA, originally described for use in mice, includes congenic strains (equivalent to

nearly-isogenic lines in plants) or two groups of appropriately selected individuals from a segregating population (the equivalent of bulked segregant analysis).

Two genomic DNA samples, one designated as the driver and the other designated as the tester, serve as the starting material for GDRDA. To begin, the driver and tester DNA samples are cut separately using a restriction enzyme with a six bp recognition sequence (eg. *Bam*HI), and an adaptor consisting of one 24-base oligonucleotide and one 12-base oligonucleotide ligated onto the sticky ends of the restriction fragments. The DNA is then used as template in PCR reactions, using the 24-base oligonucleotide as primer. Before the PCR is performed, an end-filling step is performed to produce sequences at the ends of the restriction fragments that the primer can anneal to (Figure 4.1). Of the restriction fragments present in the original digestions (≤ 25 kbp), only the smaller (≤ 2.0 kbp) restriction fragments are present in the resulting amplicons, due to greater efficiency with which the PCR amplifies the smaller DNA fragments from a mixture. Consequently, sequences located on small restriction fragments in the tester digest and on large restriction fragments in the driver digest (or absent in the driver genomic DNA) are present only in the tester amplicon. Three to four rounds of subtractive hybridisation are performed in order to purify these polymorphic sequences from the tester amplicon (Figure 4.2). When used as RFLP probes, the GDRDA products should detect RFLPs between the original driver and tester digests. Because of the nature of the driver and tester DNAs, these RFLPs should also be located close to the gene of interest.

RFLP subtraction is another technique that can be used to generate libraries of clones detecting RFLPs between two similar mixtures of genomic DNA (Rosenberg *et al.*, 1994). This technique works on the same principals as GDRDA, and only differs in regard to details of the experimental protocol. Although the use of RFLP subtraction to target markers close to genes of interest has not been reported, the potential of the technique to be used in this way has been recognised (Rosenberg *et al.*, 1994).

The GDRDA and RFLP subtraction procedures may have potential to assist in the project to isolate *Yd*$_2$ by a map-based approach, by providing a means of generating

Figure 4.1. End-filling process used in GDRDA

Adaptors used in GDRDA are composed of one 12-base oligonucleotide and one 24-base oligonucleotide. In this figure, the adaptor is derived from the oligonucleotides RBam12 and RBam24 described by Lisitsyn *et al.* (1993), and is designed to be compatible with the sticky ends of *Bam*HI restriction fragments. Due to the absence of a phosphate group at the 5´ ends or the oligonucleotides, only the 24-base oligonucleotide becomes covalently bound to the restriction fragments in the ligation reactions. An incubation at 72°C is used to melt off the 12-base oligonucleotide, and the single stranded ends of the restriction fragments filled in using *Taq* DNA polymerase. This end-filling process provides sequences that the 24 bp oligonucleotide can anneal to during the subsequenct PCR in which this oligonucleotide is used as primer. Abbreviations: A=Adenine; T=Thimidine; G=Guanosine; C=Cytosine; N=any one of the four nucleotides A, T, G or C.

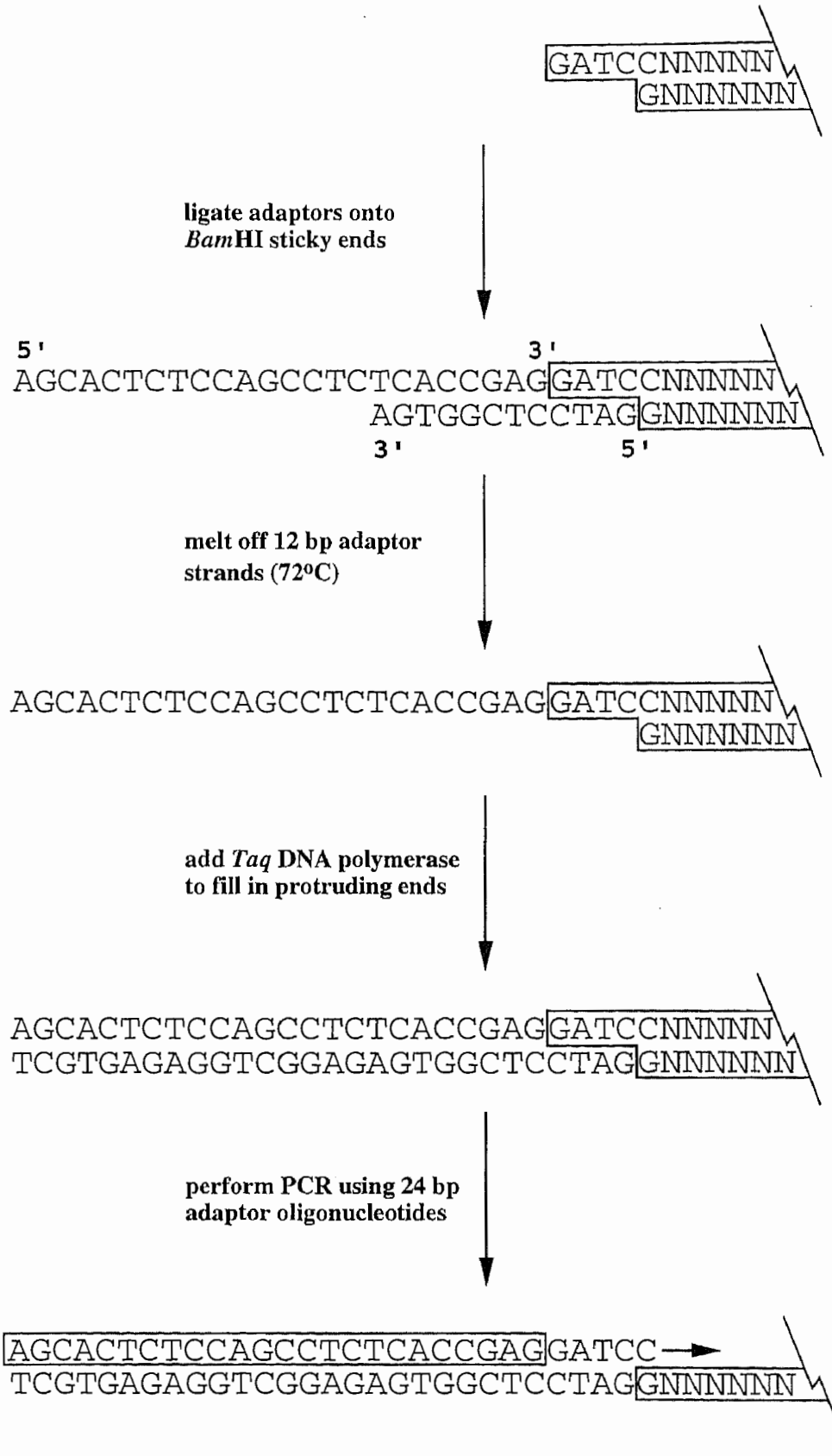
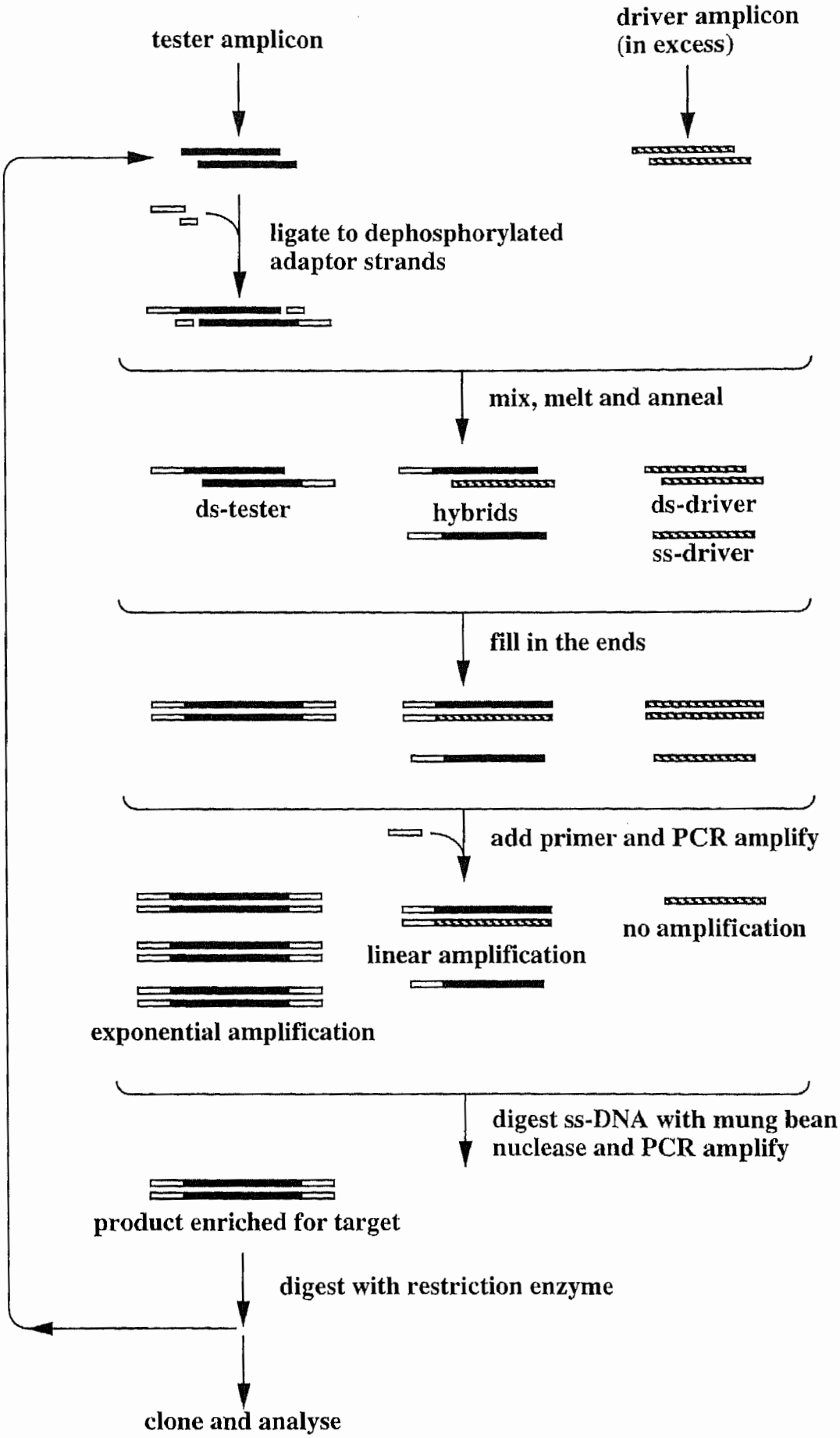

GATCCNNNNN
GNNNNNN
ligate adaptors onto
*Bam*HI sticky ends
5'
3'
AGCACTCTCCAGCCTCTCACCGAG
GATCCNNNNN
AGTGGCTCCTAG
GNNNNNN
3'
5'
melt off 12 bp adaptor
strands (72°C)
AGCACTCTCCAGCCTCTCACCGAG
GATCCNNNNN
GNNNNNN
add *Taq* DNA polymerase
to fill in protruding ends
AGCACTCTCCAGCCTCTCACCGAG
GATCCNNNNN
TCGTGAGAGGTCGGAGAGTGGCTCCTAG
GNNNNNN
perform PCR using 24 bp
adaptor oligonucleotides
AGCACTCTCCAGCCTCTCACCGAG
GATCC
TCGTGAGAGGTCGGAGAGTGGCTCCTAG
GNNNNNN

Figure 4.2. Hybridisation and selective PCR amplification stages of GDRDA

This figure is reproduced from Lisitsyn *et al.* (1993) to illustrate the hybridisation and selective PCR amplification stages of GDRDA. Firstly, driver and tester amplicons are digested to cleave off adaptor sequences, and a second set of adaptors ligated onto the tester DNA. The driver DNA is then combined with the tester DNA, with the driver in excess. The DNA mixture is denatured and allowed to reanneal. The single-stranded ends of the reannealed DNA fragments are filled in as shown in Figure 4.1, and the DNA used as template in a PCR reaction using the 24-base oligonucleotide from the second adaptor as primer. Only those reannealed DNA fragments composed of two tester strands have sequences complimentary to the primer at both ends, and are amplified exponentially. Those fragments containing only one tester strand have one end complimentary to the primer and are amplified linearly, while those fragments composed only of driver strands are not amplified at all. The resulting amplified DNA is therefore enriched for sequences present in the tester amplicon and absent in the driver amplicon. Poorly hybridising sequences represented by single stranded fragments at the end of the hybridisation are degraded using mung bean nuclease. The adaptor sequences in the amplified products are exchanged for a third adaptor, and the hybridisation and selective amplification process performed two or three more times to further enrich for the target sequences. Producs of the GDRDA are cloned and analysed. Abbreviations: ss=single stranded; ds=double stranded.


tester amplicon
driver amplicon
(in excess)
ligate to dephosphorylated
adaptor strands
mix, melt and anneal
ds-tester
hybrids
ds-driver
ss-driver
fill in the ends
add primer and PCR amplify
no amplification
linear amplification
exponential amplification
digest ss-DNA with mung bean
nuclease and PCR amplify
product enriched for target
digest with restriction enzyme
clone and analyse

further RFLP markers closely linked to this gene. In this chapter, the potential of GDRDA to be used to generate further RFLP markers close to *Yd2* is assessed.

4.2 Materials and methods

4.2.1 Experimental design

Two separate GDRDA experiments were performed to generate RFLP markers closely linked to *Yd2*. The DNA used as the driver and tracer in these experiments was extracted from the pooled tissue of appropriate individuals derived from the Proctor × Shannon F_2 mapping population used in Chapter 3 (Figure 4.3). Both experiments targeted a small *Yd2*-containing section of barley chromosome 3 represented by the area above the RFLP marker *Xabg396* on the map constructed in Chapter 3. Experiments A and B were reciprocal experiments, designed to isolate DNA sequences from the parent cultivars Shannon and Proctor, respectively.

4.2.2 General GDRDA procedures

The GDRDA procedures used were essentially the same as those outlined for RDA by Lisitsyn *et al.* (1993). A detailed RDA protocol was kindly sent to us by Eric Lander (Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, USA), and was followed closely in this study, except for some minor details.

All centrifugation steps were performed at top speed (14,000 rpm) in an Eppendorf 5415 C bench centrifuge, unless otherwise stated. To carry out phenol:chloroform:iso-amyl alcohol (25:24:1) extractions, the DNA solution was combined with an equal volume of this mixture, the tubes shaken vigorously for two min, centrifugation carried out for 10 min, and the aqueous phase transferred to a new tube. Nucleic acid precipitations were performed by combining the solution with 0.1 volume of NaOAc, and 2.0 volumes of EtOH or 1.0 volume of iso-propyl alcohol. Precipitated nucleic acid was pelleted by centrifugation for 10 min, and the pellet

Figure 4.3. Genetic material used to derive driver and tester DNAs

This figure illustrates the genotypes of the individuals derived from the Proctor (BYDV susceptible) × Shannon (BYDV resistant) F_2 mapping population used as to derive the driver and tester amplicons for the two GDRDA experiments. The resistant tester DNA was extracted from eight F_3 progeny seedlings of an F_2 plant of the genotype shown here[1]. The susceptible tester DNA was extracted from eight F_4 progeny of an F_3 individual with the genotype shown . Each of the driver DNA samples were extracted from 28 F_3 seedlings derived from six or seven F_2 plants which were either all homozygous for the Proctor alleles of the RFLP markers *XksuG59* to *Xglk223* (susceptible driver) or all homozygous for the Shannon alleles of these RFLP loci (resistant driver). The susceptible and resistant driver pools are shown as heterozygous for *Xcdo718*, as some of the individuals used to construct these pools were heterozygous for this RFLP locus.

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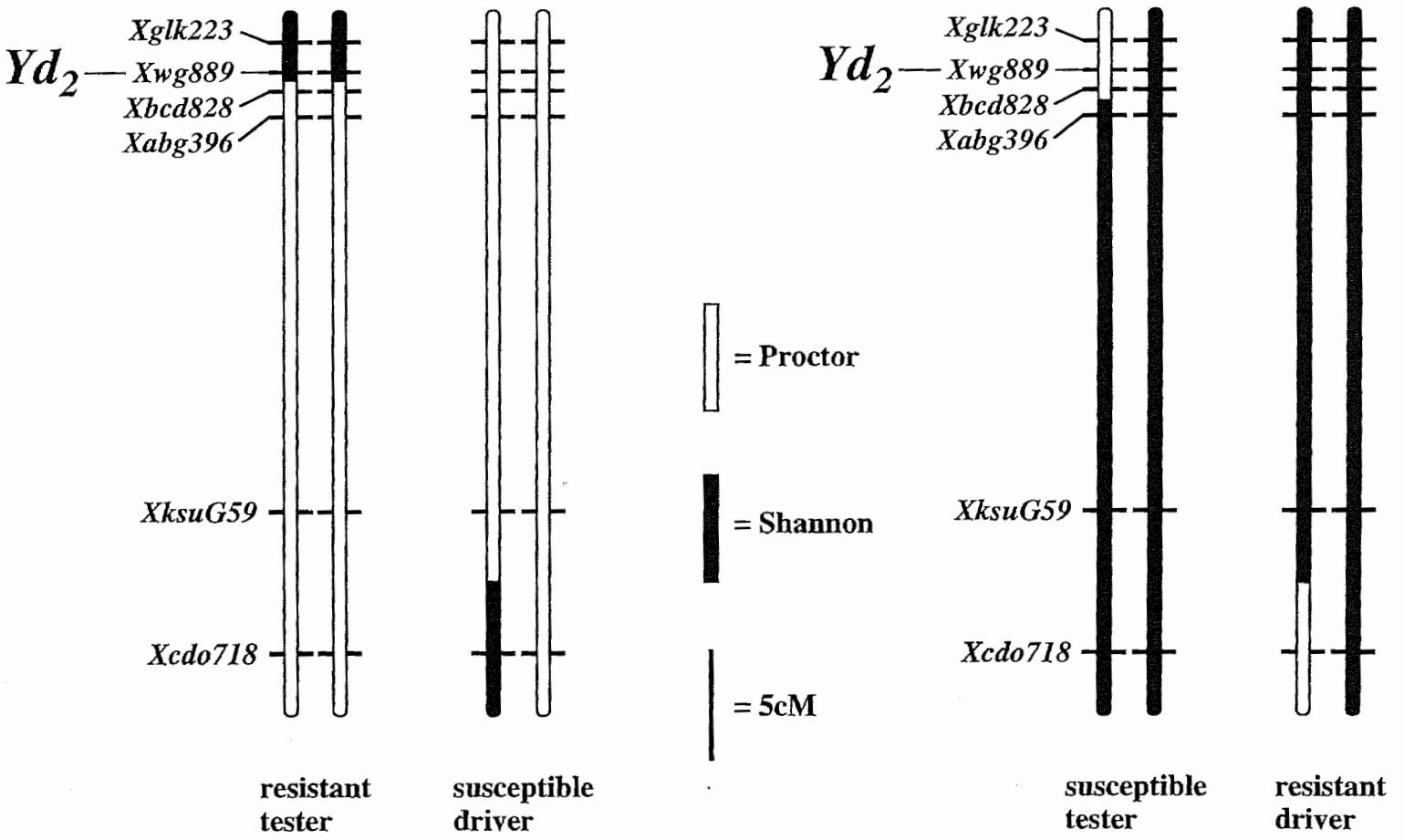
[1]This F_3 plant was derived from an F_2 plant containing the recombinant chromosome shown here plus a non-recombinant chromosome containing only Proctor RFLP alleles. The F_3 plant was found to be homozygous for the Shannon allele for the RFLP marker *Xwg889*, and so was presumed to have the genotype shown here.



Experiment A **Experiment B**

washed in 70% EtOH, dried and dissolved in the specified volume of milliQ H_2O. All procedures were performed at room temperature unless otherwise stated.

To estimate DNA concentrations, small amounts of the samples were subject to mini agarose gel electrophoresis (Section 2.2.5) alongside varying known amounts of a standard barley DNA preparation, cut with the restriction enzyme *Sau*3AI or *Bam*HI. The standard barley DNA was purified from barley leaves using the CsCl procedure described by Sambrook *et al.* (1989), and quantified by UV absorbance spectroscopy at λ=260 nm (Sambrook *et al.*, 1989). Carrier t-RNA in the samples did not interfere with the estimation of the DNA concentration, as its molecular weight was lower that of the DNA in the samples.

4.2.3 Plant growth and tissue collection

Seed coats showing visible blackening due to fungal infection were removed prior to surface sterilisation. To surface sterilise the seeds, they were soaked in a solution of 2.0% ($^{w}/_{v}$) NaOCl and 0.01% Tween20 for 15 min with frequent shaking, rinsed briefly in 70% EtOH, and then rinsed in sterile milliQ H_2O. Seeds were sown on perlite in light proof plastic containers previously swabbed with 70% ethanol. Seven days after sowing, seedling leaf tissue from each tester or driver planting was harvested and transferred to a sterile 10 ml polypropylene tube. Each tester and driver tissue sample was handled with a fresh sterile scalpel blade and a fresh pair of latex gloves. Each tissue sample was frozen by placing the tube into liquid nitrogen and then crushed to a powder using a spatula which had previously been flamed red hot and allowed to cool. Tubes of powdered leaf tissue were stored at -80°C until the tissue was required.

4.2.4 Preparation of *Bam*HI cut DNA

Nucleic acid samples used to make driver and tester amplicons were prepared from the powdered frozen leaf tissue using the barley genomic DNA extraction procedures outlined in Section 2.2.6.1, except that RNase A was omitted from the nucleic acid resuspension buffer. Ten µl of each nucleic acid sample was digested with

30 U restriction enzyme *Bam*HI (Promega) for three hr at 37°C, in a reaction containing 1.0 mg/ml BSA and the buffer supplied with the enzyme. Digestion reactions were adjusted to a volume of 700 µl with the addition of milliQ H_2O, extracted with phenol:chloroform:isoamyl alcohol (25:24:1), and the nucleic acid EtOH precipitated, washed, dried and dissolved in 30 µl milliQ H_2O. The concentration of each digested DNA sample was determined, and ranged from 0.18 to 0.24 mg/ml.

4.2.5 Adaptor ligation

Adaptors were attached to 1.0 µg of each *Bam*HI restricted DNA sample in 30 µl ligation reactions containing 0.93 nM of each of the oligonucleotide primers R Bam24 (5′-AGC ACT CTC CAG CCT CTC ACC GAG-3′) and R Bam12 (5′-GAT CCT CGG TGA-3′), 6.0 Weiss units of T4 DNA ligase (Bresatec, Australia), and 1 × ligation buffer (10 mM $MgCl_2$, 10 mM DTT, 1.0 mM ATP, 25 mg/ml BSA, 50 mM Tris-HCl pH 7.8). Before the ligase was added, the adaptors were annealed to the ends of the restriction fragments by gradually cooling the reactions from 55°C to 4.0°C over one hr. After the addition of the ligase, the reactions were incubated at 16°C for 15 to 20 hr.

4.2.6 Generation of driver and tracer amplicons by PCR

Each of the two tubes used for the generation of each tester amplicon and each of the ten tubes used to generate each driver amplicon contained (in 400 µl) 80 ng DNA with ligated adaptors, 10 mM β-mercaptoethanol, 3.0 mM $MgCl_2$, 0.3 mM (each) dATP, dTTP, dCTP and dGTP, 1.0 µM R Bam24 primer and 1 × PCR buffer (16 mM $(NH_4)_2SO_4$, 0.1 mg/ml BSA, 67 mM Tris-HCl pH 8.8). The reactions were incubated at 72°C for three min to melt away the 12-mer oligonucleotide from the adaptors, 15 U of *Taq* DNA polymerase added to each tube, and the reactions incubated for a further five min at 72°C to fill in 5′ protruding ends. Reactions were overlaid with mineral oil and subject to 20 cycles (each consisting of 95°C for 1 min and 72°C for 3 min), followed by an incubation at 72°C for 10 min, in a Perkin Elmer Cetus DNA Thermal Cycler.

Duplicated PCR reactions were combined, the mineral oil removed, and the reactions extracted with phenol:chloroform:iso-amyl alcohol (25:24:1). The amplified DNA was EtOH precipitated, washed, dried and resuspended in 100 µl (drivers) or 20 µl (testers) milliQ H_2O. The concentrations of the driver and tester amplicons were determined, and were found to be between 0.8 and 1.5 mg/ml.

4.2.7 Cleavage of adaptors from driver and tester amplicons

To cleave away the adaptor sequences from the driver and tester amplicon DNA fragments, the DNA preparations were digested at 37°C for three hr, in a minimal volume containing *Bam*HI at a rate of 10 U per µg of DNA, 0.1 mg/ml BSA, and the 1 × buffer recommended by the manufacturer of the enzyme. To minimise the concentration of glycerol in these digestion reactions, highly concentrated *Bam*HI (80 U/µl) was obtained from Promega (USA) for this task. Each amplicon digestion was adjusted to a volume of 2.0 ml (drivers) or 0.5 ml (testers) with milliQ H_2O and extracted with phenol:chloroform:iso-amyl alcohol (25:24:1). The DNA was EtOH precipitated, washed, dried and resuspended in 100 µl (drivers) or 20 µl (testers) milliQ H_2O.

4.2.8 Exchange of adaptors on tester DNA fragments

Each digested tester DNA sample was subject to mini-agarose gel electrophoresis in gels made from 2.0% low melting point agarose (BRL, USA). Prior to electrophoresing, the gel boxes were washed with 1.0 M NaOH, 50% EtOH solution and rinsed thoroughly in H_2O to eliminate foreign DNA which could have otherwise contaminated the DNA. After running the gel at 100 V in TAE buffer at 4°C, a gel slice containing the highest molecular weight DNA was excised, leaving behind a prominent band of lower molecular weight DNA that evidently contained adaptor sequences. DNA was purified from the gel slice by Quiagen tip-20 chromatography (Quiagen Inc, USA), using the supplier's protocol for the removal of adaptor fragments. The final eluate of 2.0 ml was combined with 20 µg *E. coli* t-RNA (Fraction 4, Sigma) and the

nucleic acid precipitated at -80°C for five min using iso-propyl alcohol. Samples were entrifiged at 4.0°C for 20 min, the nucleic acid pellets washed carefully in ice cold 70% EtOH, dried and resuspended in 20 µl milliQ H_2O. The concentration of the tester DNA with the adaptors removed was determined, and 1.0 µg ligated to the second set of adaptors in reactions containing the oligonucleotides J Bam12 (5′-GAT CCG TTC ATG-3′) and J Bam24 (5′-ACC GAC GTC GAC TAT CCA TGA ACG-3′), using the conditions specified in Section 4.2.5. Ligations were otherwise performed as specified in Section 4.2.5.

4.2.9 The first hybridisation and selective amplification

For each experiment, 50 µg of driver DNA was mixed with 0.5 µg of tester DNA ligated to the second set of adaptors, and the DNA solution adjusted to a volume of 500 µl with milliQ H_2O. The DNA was EtOH precipitated, washed, dried and resuspended in 4.5 µl hybridisation buffer (3.0 mM EDTA, 30 mM HEPES, pH 8.3). The solution was overlaid with mineral oil, heated at 97°C for four min to denature the DNA, combined with 1.2 ml of 5.0 M NaCl, and incubated at 67°C for 20 hr to allow hybridisation to take place.

Following hybridisation, the mixture was combined with 400 µl milliQ H_2O, and 40 µl used as template in each of two 400 µl PCR mixtures containing the oligonucleotide J Bam24 and the other reaction components specified in Section 4.2.6. The 5′ protruding ends were filled in and the mixture subject to 10 cycles of PCR amplification as described for the original generation of the driver and tester amplicons in Section 4.2.6. The mineral oil was removed, the reactions extracted with phenol:chloroform:iso-amyl alcohol (25:24:1), the DNA EtOH precipitated, dried and resuspended in 35 µl milliQ H_2O. To digest non hybridised fragments, the DNA was incubated with 30 U mung bean nuclease (New England Biolab, Australia) at 30°C for 30 min, in 40 µl reactions containing the buffer supplied with the enzyme. The reactions were combined with 160 µl of 50 mM Tris-HCl, pH 9.0, heated at 97°C for 10 min to inactivate the enzyme, and 40 µl of the mixture used as template in each of two

400 µl PCR reactions containing J Bam24 as primer. PCR was performed for 18 cycles as before, except that no end-filling step was used. The amount of PCR product in 10 µl of PCR reaction was estimated, and if was not greater than 0.2 mg/ml, a further 10 U of *Taq* DNA polymerase was added to each reaction and another three PCR cycles performed. The PCR reactions were extracted with phenol:chloroform:iso-amyl alcohol (25:24:1), and the DNA EtOH precipitated, dried and resuspended in 20 µl milliQ H_2O.

4.2.10 Subsequent hybridisation and amplication steps

The J-adaptor sequences were cleaved away from the amplified difference products by digestion with *Bam*HI, as in Section 4.2.7. The digests were adjusted to a volume of 0.5 ml with milliQ H_2O, extracted with phenol:chloroform:iso-amyl alcohol, the DNA EtOH precipitated, washed with 70% EtOH, dried and resuspended in 20 µl milliQ H_2O. The DNA concentration was estimated, and 100 ng ligated to N-adaptors in ligation reactions containing the oligonucleotides N Bam24 (5′-AGG CAA CTG TGC TAT CCG AGG GAG-3′) and N Bam12 (5′-GAT CCT CCC TCG-3′) as described in Section 4.2.5. The DNA product with adaptors attached (50 ng) was then hybridised to 50 µg of driver DNA, and the DNA subject to selective PCR amplification as in Section 4.2.9.

The N-adaptor sequences in the amplified DNA were exchanged for the J-adaptors and 100 pg used in the third hybridisation as before. DNA from the third hybridisation was subject to a 23 cycles of PCR amplification following the mung bean nuclease treatment.

4.2.11 Cloning the GDRDA products

The DNA recovered by PCR after the third hybridisation was extracted with phenol:chloroform:iso-amyl alcohol (25:24:1), EtOH precipitated, washed, dried and resuspended in 20 µl milliQ H_2O. The adaptor sequences were then cut from the difference products by *Bam*HI digestion and removed by preparative low melting point

gel electrophoresis and Quiagen tip-20 chromatography as described in Sections 4.2.7 and 4.2.8, and the DNA finally resuspended in 100 µl milliQ H_2O.

Two µg of the plasmid cloning vector pBluescript SK- (Stratagene) was digested with 10 U *Bam*HI for three hr at 37°C, in 20 µl reactions containing 1.0 mg/ml BSA and the buffer supplied with the enzyme. The *Bam*HI cut plasmid was then 5′ dephosphorylated for 90 min at 37°C, in reactions made by combining the restriction digestion with 2.0 µl of 1.0 U/µl calf intestinal alkaline phosphatase (Boehringer-Mannheim), 3.0 µl 10 × buffer supplied with the enzyme, and 5.0 µl milliQ H_2O. Vector DNA was then gel-purified using the Bresa-Clean kit (Bresatec, Australia), according to the manufacturer's instructions, and quantified by subjecting a small amount of the DNA preparation to mini agarose gel electrophoresis alongside known amounts of SPP-1 DNA cut with *Eco*R1.

RDA products from each experiment were ligated to the dephosphorylated plasmid vector, in reactions of 15 µl volume containing 30 ng *Bam*HI cut dephosphorylated vector, 3.0 or 9.0 ng of RDA product with adaptors removed, 0.3 Weiss units of T4 DNA ligase (Bresatec, Australia), and 1 × ligation buffer (10 mM $MgCl_2$, 10 mM DTT, 1.0 mM ATP, 25 µg/ml BSA and 50 mM Tris-HCl, pH 7.8). Following incubation of the ligation reactions at 16°C for 12 to 20 hr, 10 µg *E. coli* t-RNA was added, the volume adjusted to 100 µl with milliQ H_2O, and the nucleic acid EtOH precipitated, dried, washed in 70% EtOH, and resuspended in 10 µl milliQ H_2O. One µl of ligated DNA solution was used to transform *E. coli* strain DH5α (Stratagene, USA) by electroporation, as described in Section 2.2.3.

4.2.12 Purification and analysis of cloned DNA inserts

PCR reactions of 50 µl were constructed, containing 3.0 mM $MgCl_2$, 0.2 mM (each) dATP, dTTP, dCTP and dGTP, 0.25 µM of each of the oligonucleotide primers RSP (5′- ACA GGA AAC AGC TAT GAC CAT G -3′) and -40P (5′-CAG GGT TTT CCC AGT CAC GAC-3′), 0.5 U *Taq* DNA polymerase (Bresatec, Australia), and the buffer supplied with the enzyme. Single colonies of transformed bacteria were

transfered to each PCR reaction using a sterile toothpick, and the reactions incubated at 94°C for five min to lyse the bacteria. The tubes were then subject to 25 cycles (each consisting of 30 sec at 95°C, 30 sec at 55°C and 90 sec at 72°C), and a final cycle of 10 min at 72°C, in a PTC-150 Mini Cycler (MJ Research, USA). PCR-amplified DNA inserts were gel-purified and used to make radioactively labelled probes as described in Sections 2.2.6.3 and 2.2.6.4.

The probes were used to analyse *Bam*HI cut barley genomic DNA, using the methods for Southern hybridisation analysis outlined in Section 2.2.6. The four barley genomic DNA preparations used to derive the driver and tester amplicons were analysed initially. Those probes that showed expected hybridisation patterns were then used to analyse individuals from the Proctor × Shannon F_2 mapping population used in Chapter 3.

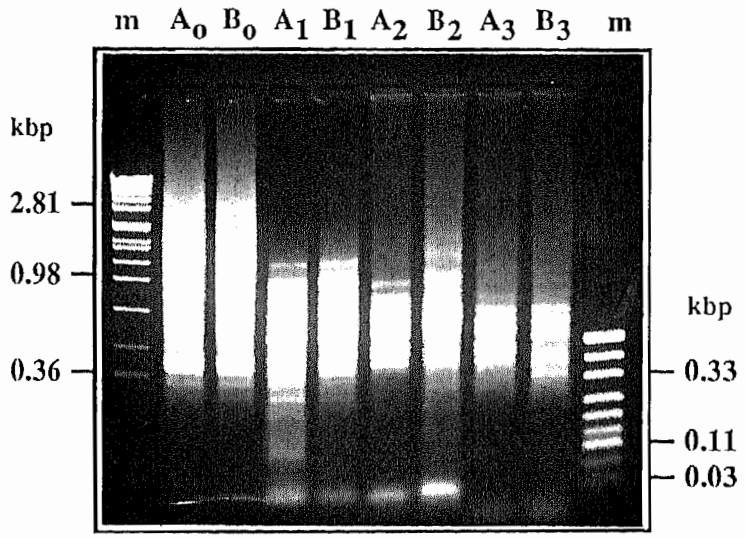
4.3 Results

Figure 4.4 illustrates the appearance of the DNAs recovered during the two RDA experiments. Driver and tester DNA amplicons contained fragments visible as bands on a mini-agarose gel which presumably corresponded to repetitive sequences in the barley genome. With subsequent rounds of subtractive hybridisation, these bands disappeared and were replaced by other prominent fragments. The DNA mixtures recovered after the third round of subtraction in each experiment consisted of about seven prominent fragments. Similar changes were observed in RDA generated DNA derived from mouse DNA (Lisitsyn *et al.*, 1994).

The six DNA clones from Experiment A and the five clones from Experiment B chosen for RFLP analysis contained uniquely sized cloned inserts (not shown). The probes were firstly used to analyse *Bam*HI digestions of the original barley genomic DNA preparations used to derive the driver and tracer amplicons. The six probes from Experiment A showed expected hybridisation patterns after one to three days of exposure, hybridising to a low molecular weight restriction fragment in the tester and up to three high molecular weight restriction fragments in the driver. Each of these

Figure 4.4. The appearance of the DNAs recovered at various stages of the two RDA experiments

DNA samples (approximately 0.5 μg of each) taken at various stages of the GDRDA experiments A and B were run together on a 1.5% mini agarose gel for comparison. Included were the two original tester DNA amplicons (A_0 and B_0), and the DNAs amplified after the first, second and third rounds of subtractive hybridisation (A_1 and B_1, A_2 and B_2, and A_3 and B_3, respectively). Bands in the original tester samples representing repetitive sequences in the barley genome disappeared after the first subtractive hybridisation step as other DNA fragments became prominent. The size markers were derived from SPP-1 DNA digested with *Eco*RI.


m
A_0 B_0 A_1 B_1 A_2 B_2 A_3 B_3
m
kbp
2.81
0.98
0.36
kbp
0.33
0.11
0.03

probes gave a distinct hybridisation pattern, confirming that these probes were unique. Each of the five probes analysed from Experiment B hybridised to highly repetitive DNA sequences, as indicated by hybridisation signals which were spread over a large restriction fragment size range and visible after only several hours of exposure. Examples of the hybridisation patterns of probes obtained in each of the two RDA experiments are shown in Figure 4.5.

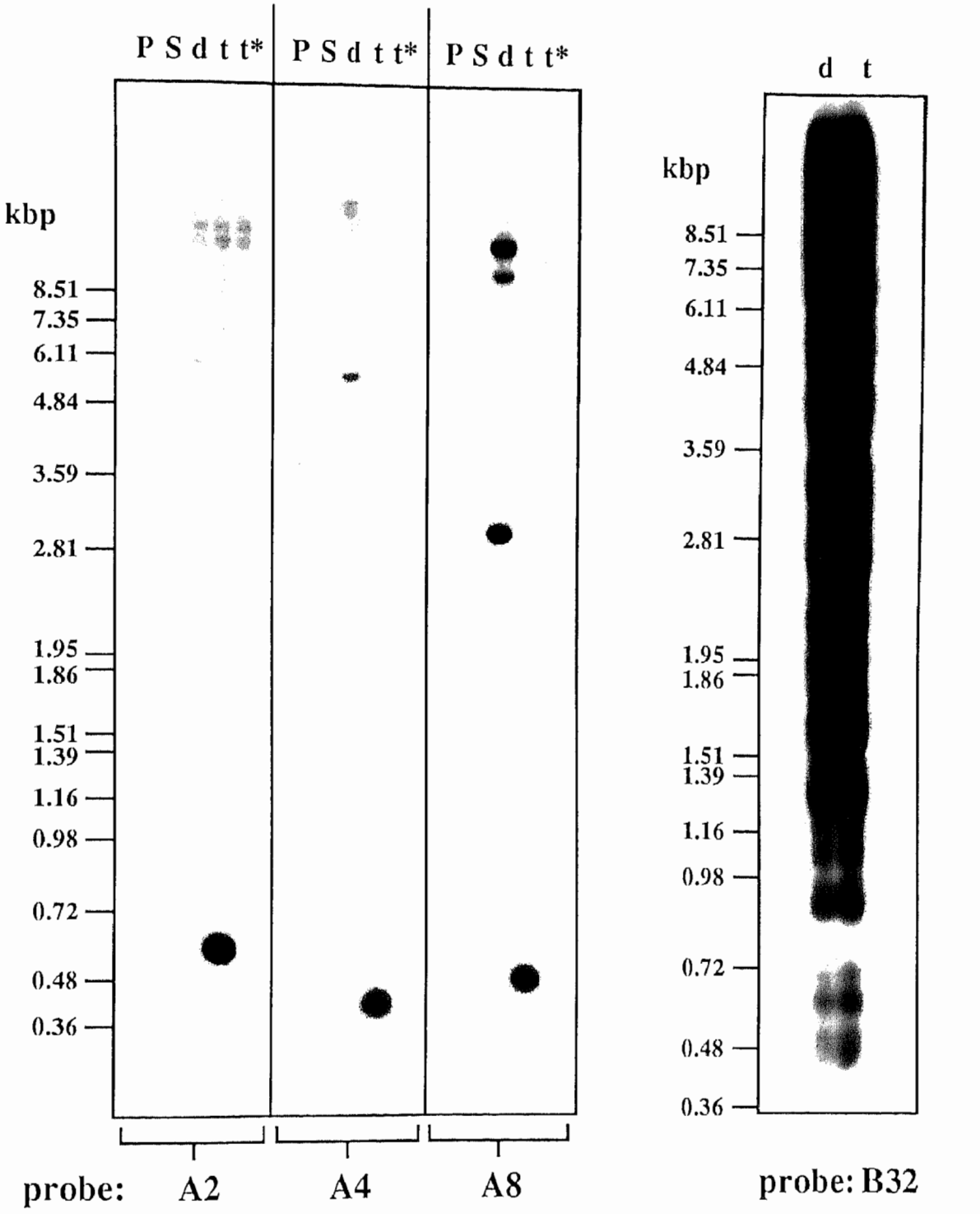
The probes from Experiment A were used to probe *Bam*HI cut DNA of individuals from the Proctor × Shannon F_2 mapping population with the intention of mapping these probes. However, these probes showed no hybridisation to the DNA of these F_2 individuals (not shown). To help determine the origin of these probe sequences, further seedlings which were of the same genotype as those used to derive the tester amplicon for Experiment A were sown, and their DNA analysed using four of the probes. The probes failed to hybridise to these DNA samples, and to DNA extracted from Proctor and Shannon seedlings (Figure 4.5). It was apparent from these observations that the probes from Experiment A were not derived from the barley genome.

4.4 Discussion

The ability of GDRDA to identify polymorphic RFLP markers by subtractive hybridisation makes it an attractive procedure for the generation of markers suitable for use in the map based-isolation of a gene of interest. As markers are mapped close to the gene, appropriate individuals from the mapping population may be selected for use in GDRDA experiments to target a progressively smaller area of the chromosome surrounding the gene. Because the markers from these chromosomal regions are selected by subtractive hybridisation, no increase in the difficulty of the procedure is expected with the decrease in the size of the chromosome segment being targeted. In contrast, other procedures involving the screening of randomly generated markers would become increasingly difficult as markers more closely linked to the gene are sought.

Figure 4.5. Hybridisation patterns obtained using four GDRDA probes

*Bam*HI digested genomic DNA of barley seedlings were subject to Southern hybridisation analysis using three probes generated in Experiment A (probes A2, A4 and A8), and one probe generated in RDA experiment B (probe B32). Each of the three probes from Experiment A hybridised to a low molecular weight restriction fragment in the DNA sample originally used to make the tester (t), and to one or more high molecular weight restriction fragments in the DNA sample originally used to make the driver DNA (d). These probes did not clearly hybridise to DNA of the cultivars Proctor (P) and Shannon (S) used to derive the genetic material for these RDA experiments, or to the DNA of seedlings of the same genotype as the seedlings used to derive the tester for Experiment A (t*) grown at a later stage. Therefore, the probes from Experiment A did not appear to be derived from the barley genome. The probe B32 showed a hybridisation pattern typical of a repetitive DNA sequence after an overnight exposure. Size markers were derived from SPP-1 DNA digested with *Eco*RI.



Experiment A

Experiment B

Due to the potential advantages of GDRDA, it was thought that it may be useful as a means of obtaining the further markers required for the map-based isolation of the *Yd2* gene. To test if GDRDA could be used in this way, two experiments were performed to target a 4.0 cM region of barley chromosome 3 containing *Yd2*, using appropriate genetic material derived from the Proctor × Shannon F_2 mapping population used in Chapter 3. The first of these experiments yielded probes that failed to hybridise to barley genomic DNA, while the probes analysed from the second experiment hybridised to repetitive sequences in the barley genome. The GDRDA procedure was therefore found to be ineffective in generating further RFLP markers close to *Yd2*.

In each of the experiments described here, the GDRDA products contained about seven prominent DNA fragments. Most of the prominent fragments were expected to be among the 11 analysed cloned DNA inserts, due to the high frequency with which they were expected to occur in the libraries of the GDRDA products. Lisitsyn *et al.* (1994) found that most probes corresponding to fragments other than the prominent GDRDA products hybridised to repetitive sequences or to non polymorphic restriction fragments. Therefore, further screening of the two libraries of GDRDA products is unlikely to yield any useful clones.

The GDRDA experiments performed by Delaney *et al.* (1995b) were designed to generate RFLP markers linked to the *H25* gene for hessian fly larvae resistance on the long arm of rye chromosome 1. To target markers linked to this gene, DNA of wheat lines with and without the long arm of rye chromosome 1 was used to derive the driver and tester amplicons. The probes obtained from one of these experiments were similar to those obtained here in Experiment B, in that they hybridised to repetitive genomic DNA sequences. In another experiment by Delaney *et al.* (1995b), about a half of the probes failed to hybridise to wheat or rye genomic DNA. Therefore, these probes had similar properties to the probes obtained in Experiment A which failed to hybridise to barley genomic DNA.

Delaney *et al.* (1995b) concluded that the most likely source of the non hybridising probe sequences was the DNA of insects or pathogens present on the plants used as the source of the DNA used in their GDRDA experiments. To avoid this problem, they grew DNA source plants in a growth chamber instead of a glasshouse. This strategy appeared to be successful, as all probes analysed from these subsequent experiments hybridised to wheat or rye DNA. In the GDRDA experiments reported here, the barley seedlings used to prepare DNA were grown on fresh perlite in enclosed containers for only one week, from seeds which had been given a surface sterilisation treatment. Despite these precautions, all of the probes obtained from Experiment A failed to hybridise to barley genomic DNA, and were presumably derived from pathogens which were present on the seedlings used to derive the tester amplicons. Therefore, pathogens were a more persistent problem in these experiments than in the experiments of Delaney *et al.* (1995b). If the experiments in barley were to be repeated, it would be wise adopt different growth conditions. One option may be to grow the seedlings from surface sterilised seeds on tissue culture media in clear sterile containers as described by Wolter *et al.* (1993). These conditions may be more sterile than those used here, and may enable the visual identification and elimination of those seedlings infected by pathogens.

While the two GDRDA experiments in barley failed to generate any useful RFLP markers close to the *Yd2* gene, the three experiments by Delaney *et al.* (1995b) yielded five useful RFLP probes that detected a total of seven RFLP loci on the long arm of rye chromosome 1. There are two potential reasons why Delaney *et al.* (1995b) experienced a greater level of success with GDRDA. Firstly, they targeted markers from a whole chromosome arm, whereas the experiments reported here were designed to generate markers from a much smaller chromosome section, comprising 4.0 cM of barley chromosome 3. Secondly, markers detecting interspecific polymorphism between wheat and rye were sought by Delaney *et al.* (1995b), whereas markers detecting intraspecific polymorphism between two different barley cultivars were targeted in the RDA experiments described here. Therefore, it is highly probable that

GRDA detectable polymorphisms targeted by Delaney *et al.* (1995b) were much more numerous than those targeted close to *Yd2*.

The work in this chapter suggests that the GDRDA procedure may not be effective in identifying intervarietal polymorphisms in small chromosome regions containing genes of interest. Such polymorphisms potentially identifiable by GDRDA may be so rare that the DNA of pathogens present on the DNA source plants may be amplified at their expense. In light of this, it would be important to use growth conditions for the DNA source plants which are absolutely sterile during future efforts to generate markers close to *Yd2* using GDRDA. Although Delaney *et al.* (1995b) generated RFLP markers close to the *H25* gene by GDRDA, the closest of these markers was still at least 2.2 cM from the gene, and was no closer to the gene than a conventionally generated RFLP marker that they obtained from an RFLP map. Because of these limitations, it seems unlikely that GDRDA could be used to generate the large number of markers necessary for the map-based isolation of the *Yd2* gene.

AFLPs are a type of molecular genetic marker which can be generated and screened for polymorphism in very large numbers (Zabeau and Vos, 1993; Vos *et al.*, 1995). Recently, AFLPs were used to construct a very high resolution genetic map of a region of the tomato genome containing the *Cf-9* gene for resistance to *Cladosporium fulvum* (Thomas *et al.*, 1995). After preliminary mapping of the gene, AFLPs located closer to the gene were successfully targeted by screening for appropriate polymorphisms between pools of individuals resulting from recombination close to the *Cf-9* gene. The success Thomas *et al.* (1995) experienced in targetting AFLPs located on a very small chromosome segment may have been partly due to the ability of AFLPs to reveal a wide variety of DNA polymorphisms, including single base substitutions and small or large insertion-deletion events. Consequently, AFLPs allow a much larger number of polymorphisms to be targeted than GDRDA, which only has the potential to detect the presence and absence of sequences within small restriction fragments. As such, the AFLP technique may provide a more effective means of obtaining further markers close to *Yd2* than GDRDA. Nevertheless, in light of the observations made in

this exercise, it would be wise to take care to eliminate pathogens from the DNA source plants when any small chromosome region is being targeted using AFLPs, GDRDA, or any other method.

CHAPTER 5

USING MORPHOLOGICAL MARKERS TO FACILITATE HIGH RESOLUTION GENETIC MAPPING OF THE Yd_2 LOCUS

5.1 Introduction

As discussed in the previous two chapters, the genetic map of the *Yd2* region of barley chromosome 3 (Chapter 3) will need to be improved in two ways to identify markers which may be suitable for the identification of large insert barley genomic clones containing the *Yd2* gene. Firstly, the density of molecular markers in the vicinity of *Yd2* will need to be increased to approximately 120 markers per cM. Secondly, the genetic resolution will need to be increased to enable the identification of the two markers most closely flanking *Yd2*. As discussed in Chapter 3, adequate resolution may be achieved by increasing the size of the mapping population to about 6,000 F_2 individuals.

The characterisation of thousands of F_2 individuals using molecular markers is likely to be an extremely time consuming and expensive task, involving the extraction of DNA from each individual. One approach that allows large mapping populations to be analysed more efficiently involves the use of two markers that have visible phenotypes, located on either side of the gene. In mapping populations segregating for the gene of interest and the pair of flanking markers, individuals resulting from recombination close to the gene can be identified visually. Only these recombinants would need to be analysed for the intervening molecular markers and the gene of interest, thus avoiding the detailed analysis of large numbers of uninformative non-recombinant individuals.

Pairs of markers for visible traits have been used to facilitate high resolution genetic mapping of at least two disease resistance loci. The markers *polymitotic 1* and *yellow kernel 1* (controlling male-sterility and kernel colour, respectively) were used to identify recombinants for a 3.0 cM interval of maize chromosome 6 containing the *Mdm1* gene for maize dwarf mosaic virus resistance, among 7,650 F_2 individuals (Simcox *et al.*, 1995). Similarly, the markers *eceriferum 2* and *apetala 2* (controlling leaf wax production and flower morphology, respectively) were used to identify recombinants for a region of *A. thaliana* chromosome 4 containing the *RPS2* gene for

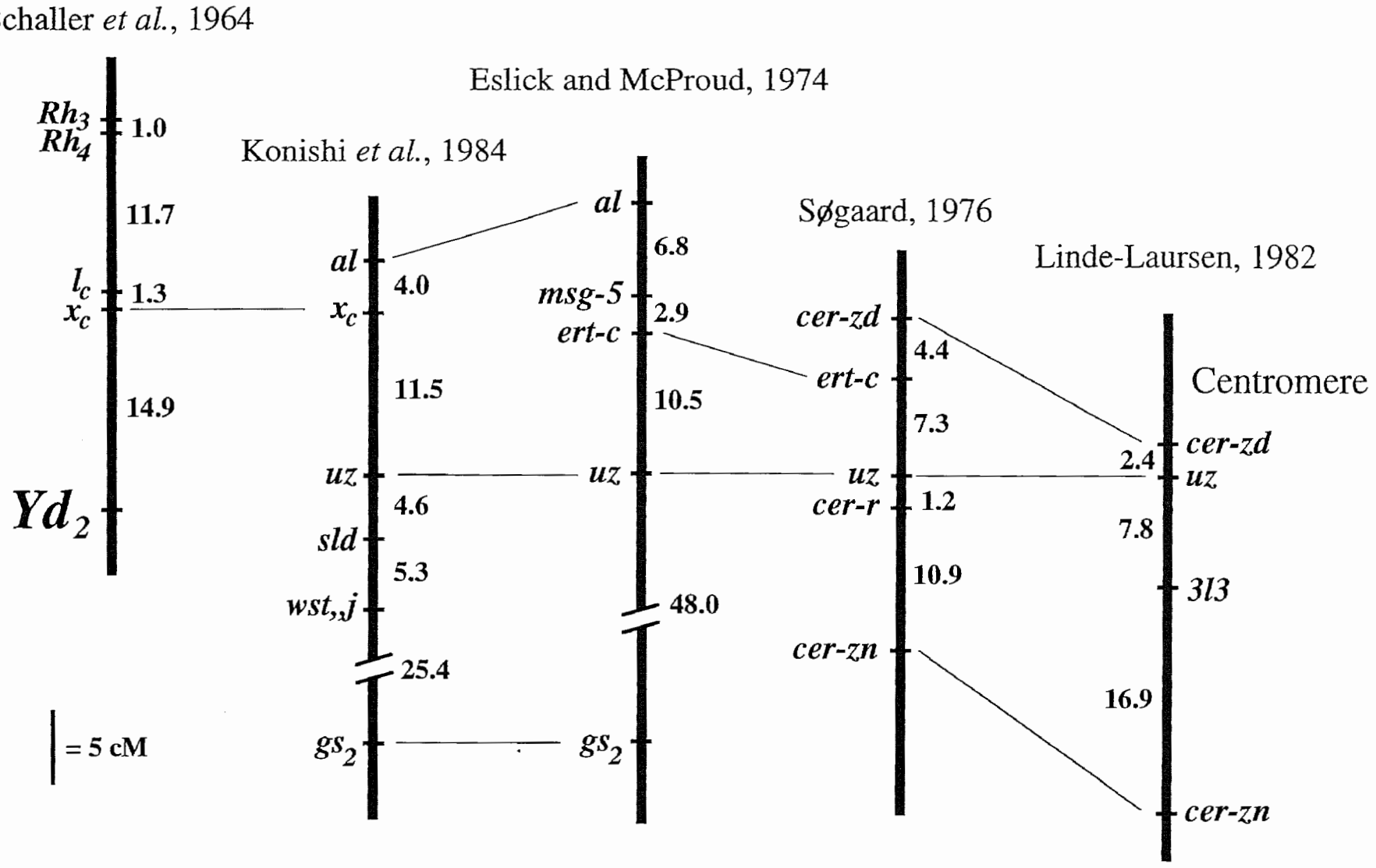
resistance to *Pseudomonas syringae*, from a population of 5,000 F_2 individuals (Mindrinos *et al.*, 1994).

Schaller *et al.* (1964) mapped *Yd2* relative to a number of markers for visible traits on barley chromosome 3 (Figure 5.1). However, all of these markers were located to one side of *Yd2*. To identify a pair of markers flanking *Yd2*, the genetic map constructed by Schaller *et al.* (1964) can be compared to other maps of markers controlling visible traits, using common markers as reference points (Figure 5.1). Based on the comparison between the map of Schaller *et al.* (1964) and the map of Konishi *et al.* (1984), it appears as though the two markers *uzu dwarf* (*uz*) and *white stripe,,j* (*wst,,j*) closely flank the *Yd2* locus. The recessive allele at the *uz* locus is found in many Japanese barleys and results in a dwarf growth habit, dense heads and a 50% reduction in coleoptile length (Takahashi, 1951). The *wst,,j* mutation was induced in the Japanese *uzu dwarf* barley variety Akashinriki using ethyl methanesulfonate, and expresses a leaf striping phenotype at temperatures below 20°C, in a recessive fashion (Konishi *et al.*, 1984). These markers are located only 10 cM from one another, can be scored at the early seedling stage, and are available together in coupling phase in the mutant barley line produced by Konishi *et al.* (1984). As such, these two markers appeared to be ideal for facilitating high resolution genetic mapping of the *Yd2* locus.

Mapping data obtained from different crosses cannot be used to reliably predict the order of closely linked markers. Therefore, the placement of *Yd2* between the morphological markers *uz* and *wst,,j* is uncertain. The aim of this chapter is to determine the location of these two morphological markers relative to RFLP markers mapped close to *Yd2* in Chapter 3, and thereby determine if the morphological markers do indeed flank *Yd2*.

Figure 5.1. Comparison of the map containing *Yd*$_2$ by Schaller *et al.* (1964) with other maps of visually scorable markers

The genetic map of a portion of barley chromosome 3 containing *Yd*$_2$ (Schaller *et al.*, 1964) was aligned to other maps of this chromosome region using markers common to these maps (indicated by lines connecting the maps). Schaller *et al.* (1964) reported close linkage between *Yd*$_2$ and *uz*, although they did not report the frequency of recombination between these loci due to the small size of their mapping population. This linkage between *Yd*$_2$ and *uz* was the basis for orienting the map of Schaller *et al.* (1964) as shown. Linde-Laursen (1982) showed that the markers on their map were located on the long arm, with the centromere proximal to *cer-zd*. Therefore, the long arm is located toward the bottom of the figure. All of the markers shown here can be scored with the naked eye, except for *3I3*, which is a chromosome C-band. All distance are in cM. For this comparison, recombination fractions presented by Schaller *et al.* (1964), Eslick and McProud (1974), Søgaard (1976) and Linde-Laursen (1982) were converted to cM using the Kosambi mapping function (Kosambi, 1944).


Schaller *et al.*, 1964
Eslick and McProud, 1974
Konishi *et al.*, 1984
Søgaard, 1976
Linde-Laursen, 1982
Rh_3
Rh_4
1.0
11.7
l_c
1.3
x_c
14.9
Yd_2
= 5 cM
al
4.0
x_c
11.5
uz
4.6
sld
5.3
wst,,j
25.4
gs_2
al
6.8
msg-5
2.9
ert-c
10.5
uz
48.0
gs_2
cer-zd
4.4
ert-c
7.3
uz
cer-r
1.2
10.9
cer-zn
Centromere
2.4
cer-zd
uz
7.8
3l3
16.9
cer-zn

5.2 Materials and methods

5.2.1 Acknowledgments

The author is sincerely grateful to K. Sato and the Barley Germplasm Centre at Okayama University for the supply of the mutant barley line OUM231. The author would also like to thank Reg Lance, David Sparrow and Ken Shepherd for their advice on potentially useful morphological markers in barley. The assistance given by the late Peter Ellis in performing barley crosses and in the importation of the OUM231 line through quarantine was also greatly appreciated.

5.2.2 Source of plant lines

The Ethiopian barley accessions CI 3208-1 and CI 3920-1 were obtained from Wayne Vertigan of the Tasmanian Department of Agriculture, and from the Australian Winter Cereals Collection at Tamworth, NSW, Australia, respectively. The mutant barley line OUM231, containing the recessive alleles at the marker loci *uz* and *wst,,j* (Konishi *et al.*, 1984) was kindly provided by K. Sato at the Barley Germplasm Centre at Okayama University, Japan.

5.2.3 Morphological marker analysis

Crosses were made between the non-Yd_2 mutant barley line OUM231 (*uz*, *uz*; *wst,,j*, *wst,,j*) and the two Yd_2-containing Ethiopian barleys CI 3920-1 and CI 3208-1 (*Uz*, *Uz*; *Wst,,j*, *Wst,,j*). The F_2 seeds derived from the two crosses were germinated on perlite at 4.0°C and the seedlings scored for coleoptile length and leaf colour after four weeks. The frequency of recombination between *uz* and *wst,,j* was calculated using the product ratio and converting it to recombination fraction using the tables of Stevens (1939). A number of long white or short green recombinant seedlings were selected for RFLP analysis and the rest of the seedlings discarded. The selected recombinants were transplanted into pots containing soil, and grown in a glasshouse at 18 to 25°C where *wst,,j*, *wst,,j* seedlings grew into healthy green plants. Twenty or more F_3 seeds from each selected F_2 recombinant were germinated at 4.0°C as above, in order to determine

if the recombinants were heterozygous or homozygous for the dominant allele at the second morphological marker locus.

5.2.4 RFLP analysis

Procedures used for RFLP analysis were those outlined in Section 2.2.6. Each of the RFLP probes used to construct the map of the *Yd2* region in Chapter 3 (except for YLP) were firstly used to screen for polymorphism between the parents of the OUM231 × 3920-1 and OUM231 × CI 3208-1 crosses. Each probe was tested with four or more of the following restriction enzymes: *Alu*I, *Bam*HI, *Ban*II, *Bcl*I, *Bst*NI, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Hin*fI, *Hpa*II, *Kpn*I, *Nco*I, *Nde*I, *Rsa*I, *Sau*3AI, *Taq*I and *Xba*I. Probes showing polymorphisms were then used to characterise the selected *uz-wst1,j* F_2 recombinants derived from these crosses. The combined RFLP and morphological marker data were analysed to determine the positions of the two morphological markers relative to the RFLP loci.

5.3 Results

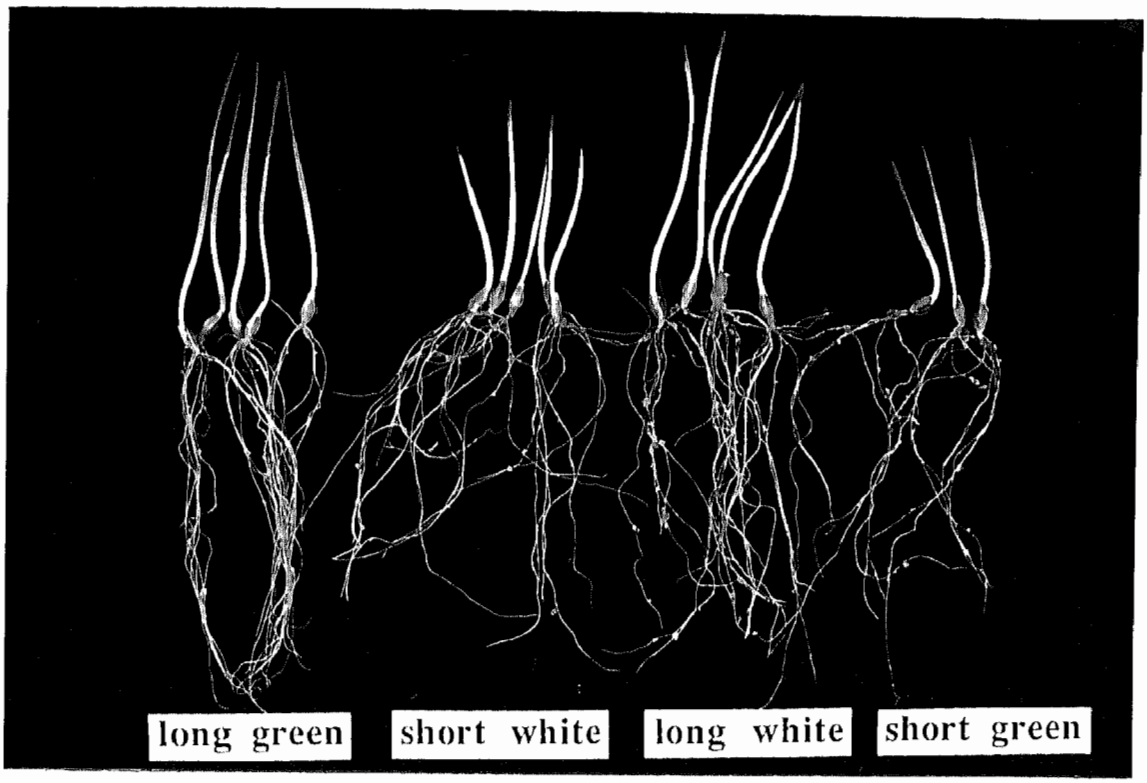
5.3.1 Morphological marker analysis

As expected, the F_2 families derived from the crosses OUM231 × 3920-1 and OUM231 × CI 3208-1 segregated for leaf colour and coleoptile length, which are traits controlled by the markers *wst1,j* and *uz*, respectively. Figure 5.2 illustrates the appearance of F_2 seedlings from one of these crosses. These F_2 families were also expected to be segregating for the *Yd2* gene, although BYDV resistance assays were not performed to confirm this. The F_2 populations were made to segregate for *Yd2* so that they could used to facilitate high genetic resolution mapping of the *Yd2* locus if the morphological markers were subsequently shown to flank *Yd2*.

The results of the morphological marker analysis are shown in Table 5.1. In total, 306 F_2 seedlings derived from the OUM231 × CI 3920-1 cross, and 390 F_2 seedlings derived from the OUM231 × CI 3208-1 cross were scored for leaf colour and coleoptile length. The numbers of F_2 individuals showing the recessive or dominant

Figure 5.2. Appearance of seedlings from an F_2 family segregating for *uz* and *wst,,j*

Seedlings from the CI 3208-1 × OUM231 F_2 family are shown here to illustrate the four phenotype classes with respect the morphological markers *uz* and *wst,,j*. These seedlings were grown at 4.0°C for four weeks to express the temperature-sensitive *wst,,j* phenotype. The recessive *uz* marker reduces the coleoptile length, while the recessive *wst,,j* mutation results in white leaves at a low temperature. The parental phenotype classes are represented by the seedlings with long coleoptiles and green leaves (long green) or short coleoptiles and white leaves (short white), while the recombinant phenotype classes are represented by the seedlings with long coleoptiles and white leaves (long white) or short coleoptiles and green leaves (short green).


long green
short white
long white
short green

trait for each of the two morphological marker loci did not differ significantly from the 1:3 ratio expected for monogenic segregation ($P > 0.10$). However, the phenotype class sizes observed for the two markers differed significantly from those expected for two independently segregating loci ($P < 0.01$), confirming that these two markers were linked. The rate of recombination between *uz* and *wst,,j* was 10.5 +/- 2.2% for the OUM231 × CI 3920-1 cross, and 8.6 +/- 1.5% for the OUM231 × CI 3208-1 cross. These values are consistent with the 10.0 +/- 1.1% recombination fraction observed between these two markers by Konishi *et al.* (1984).

Table 5.1. Linkage analysis of the two morphological markers *uz* and *wst,,j*

phenotype class†	no. of seedlings	
	OUM231 × CI 3920-1	OUM231 × CI 3208-1
long green	212	257
short white	63	99
long white	13	16
short green	18	18
Total:	306	390
long:short χ^2 (3:1):	0.353¥	5.20¥
green:white χ^2 (3:1):	0.004¥	4.19¥
long green: long white: short green: short white χ^2 (9:3:3:1):	23.0***	34.1***
recombination rate +/- standard deviation:	10.5 +/- 2.2%	8.6 +/- 1.5%

†long/short = seedlings with long or short coleoptiles, green/white = seedlings with green or white leaves

¥not significant ($P > 0.10$) *** significant at ($P < 0.01$)

In total, 25 *uz-wst,,j* recombinants were selected for RFLP analysis. These comprised six long white and seven short green seedlings from the OUM231 × CI 3920-1 F_2 family, and four long white and eight short green seedlings from the OUM231 × CI 3208-1 F_2 family. Progeny testing showed all of the long coleoptile F_2 recombinants to be heterozygous at the *uz* locus and all of the green F_2 recombinants to be heterozygous at the *wst,,j* locus. This suggested that each of the selected F_2 recombinants contained only one recombinant chromosome for the *uz-wst,,j* interval.

5.3.2 RFLP analysis of *uz-wst,,j* recombinants

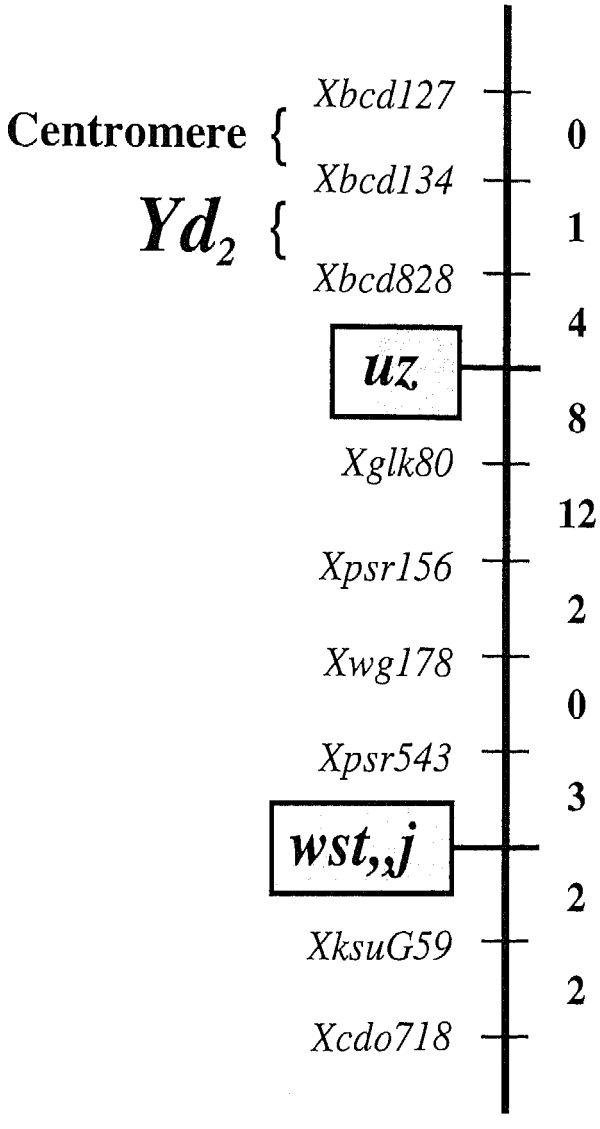
Eighteen probes used to map RFLP loci close to Yd_2 in Chapter 3 were used to screen for RFLPs between the parents of the crosses OUM231 × CI 3920-1 and OUM231 × CI 3208-1, using a range of restriction enzymes. Each probe detected polymorphism for at least one of the crosses, except for the probes TAG223, WG889, PSR116 and BCD263, which failed to detect polymorphism. Out of the 14 polymorphic probes, nine were chosen for the analysis of the *uz-wst,,j* F_2 recombinants, using the restriction enzymes indicated in Table 5.2. The *Hae*III polymorphism detected in the recombinants by the single locus probe BCD134 was different to the *Eco*RI polymorphism used to map this probe in the Proctor × Shannon mapping population (Chapter 3). Otherwise, the polymorphisms chosen to analyse the recombinants were the same as those mapped using these probes in Chapter 3.

The combined RFLP and morphological marker data showed that the *uz* locus was positioned between RFLP markers *Xglk80* and *Xbcd828*, and that the *wst,,j* locus was positioned between *XksuG59* and *Xpsr543*. These was the only possible positions for the morphological marker loci, assuming that no chromosomes resulting from double recombination events were present in the recombinants.

Figure 5.3 indicates the number of recombinants observed for each interval between the RFLP and morphological markers. As expected, each of the 25 F_2 recombinants contained one chromosome resulting from single recombination event in the *uz-wst,,j* interval. Recombination outside this interval was also observed in a

Figure 5.3. The numbers of F_2 recombinants observed for each interval between the RFLP and morphological marker loci

The analysis of 25 recombinants for the *uz-wst,,j* interval enabled the morphological markers *uz* and *wst,,j* to be positioned relative to the RFLP markers, as shown here. The RFLP loci detected in the recombinants were the same as those mapped in Chapter 3 using the same probes, based on hybridisation patterns and the number of loci that each probe detects in the barley genome (Section 5.3.2). The order of these RFLP loci and the relative positions of the *Yd2* gene and the centromere were determined in Chapter 3. The numbers to the right of the figure represent the number of recombinant chromosomes observed for each interval in the *uz-wst,,j* recombinants. These numbers represent the combined data obtained from the OUM231 × CI 3920-1 and OUM231 × CI 3208-1 F_2 recombinants.


Centromere
Yd2
Xbcd127
Xbcd134
Xbcd828
uz
Xglk80
Xpsr156
Xwg178
Xpsr543
wst,,j
XksuG59
Xcdo718
0
1
4
8
12
2
0
3
2
2

number of the recombinants, allowing the identification of the RFLP markers most closely flanking the morphological marker pair.

In Chapter 3, the *Yd*$_2$ gene was located proximal to the RFLP marker *Xbcd828*. However, in this exercise, both of the morphological markers *uz* and *wst,,j* were located distal to this RFLP marker. Therefore, this pair of morphological markers do not flank *Yd*$_2$.

Table 5.2. RFLPs used in the analysis of the *uz-wst,,j* recombinants

RFLP probe	no. of restriction enzymes screened	restriction enzymes showing RFLPs†	
		OUM231 × CI 3920-1	OUM231 × CI 3208-1
BCD127	4	none	***Eco*RI**
BCD134	13	none	***Hae*III**, *Bcl*I
BCD828	5	***Bam*HI**, *Eco*RI, *Hin*dIII, *Xba*I	***Bam*HI**, *Eco*RI, *Hin*dIII, *Xba*I
TAG80	4	***Xba*I**	***Xba*I**
PSR156	4	*Dra*I, *Eco*RI, *Hin*dIII, ***Xba*I**	*Dra*I, *Eco*RI, *Hin*dIII, ***Xba*I**
WG178	4	***Eco*RI**	***Eco*RI**
PSR543	5	*Dra*I, *Eco*RI, *Eco*RV, ***Xba*I**	*Dra*I, *Eco*RI, ***Xba*I**
KSUG59	4	***Dra*I**, *Hin*dIII	***Dra*I**, *Hin*dIII
CDO718	4	*Xba*I	***Xba*I**

†Restriction enzymes found to identify polymorphism for each of the crosses are listed. The restriction enzymes used in the analysis of the recombinants from the respective F_2 populations are indicated in bold.

5.4 Discussion

The analysis of a large F_2 mapping population consisting of thousands of individuals is a prerequisite for the isolation of the *Yd2* gene by a map based approach (Chapter 3). The analysis of a mapping population of this size is likely to be extremely tedious. However, morphological markers flanking *Yd2* could greatly assist in this task, by enabling the visual identification of informative recombinants from the mapping population. Initially, the morphological markers *uz* and *wst,,j* appeared to be ideally suited to this strategy. These markers were located only 10 cM from one another, were available together in coupling phase in mutant barley lines, could be scored easily at the early seedling stage, and were likely to flank *Yd2*, based on comparisons made between previously constructed genetic maps. However, in this chapter, it was shown that both of these morphological markers were in fact located distal to *Yd2*. Therefore, the *uz*, *wst,,j* marker pair cannot be used to facilitate the high resolution genetic mapping of the *Yd2* locus.

The actual order of the *uz*, *wst,,j* and *Yd2* loci determined in this study is not consistent with the order of these loci suggested by the comparison of the genetic maps of Schaller *et al.* (1964) and Konishi *et al.* (1984) shown in Figure 5.1. This discrepancy may have been partly due to differences in the rates of recombination for the different crosses used to construct these maps. Evidence that such differences in recombination rate occur between different crosses can be seen by comparing the maps shown in Figure 5.1. For example, the genetic distance between the markers *cer-zd* and *uz* is more than four times greater on the map of Søgaard (1976) than on the map of Linde-Laursen (1982).

Dixon *et al.* (1995) used the markers *thiaminless* and *yellow virescent* (controlling endogenous thiamine production and leaf colour, respectively) to visually identify F_2 individuals resulting from recombination close to the *Cf-2* gene for *Cladosporium fulvum* resistance in tomato. However, each of the two morphological markers were used independently to select for recombination to one side of *Yd2* in F_2 populations segregating either for *thiaminless* and *Cf-2*, or for *yellow virescent* and *Cf-*

2. Therefore, the identification of the recombinants relied on the ability to score both the *Cf-2* gene and the adjacent markers efficiently in the F_2 generation. Based on the author's experience obtained during the work described in Chapter 3, the determination of the Yd_2 genotype of single BYDV infected plants from a segregating population is unreliable. Therefore, it is unlikely that a marker such as *uz* could be used on its own to identify recombination events to one side of the Yd_2 gene. On this basis, a strategy for selecting for recombination close to Yd_2 must involve a single mapping population, segregating for the Yd_2 gene and a pair of flanking markers.

Most of the genetic maps shown in Figure 5.1 contain the *uz* marker. In this study, Yd_2 was located proximal to *uz*. Therefore, this information can be used to assist in the identification of further markers from these maps which may be of potential use in facilitating the high resolution genetic mapping of the Yd_2 locus. On the map by Søgaard (1976) the markers *eceriferum-zd* (*cer-zd*), controlling epidermal wax production, and *erectoides-c* (*ert-c*), controlling ear density, are both located proximal to *uz*. Genetic lines containing these two markers and have been obtained from Penny von Wettstein-Knowles (Carlsberg Laboratory, Denmark). Currently these markers are being mapped relative to Yd_2 in this laboratory using a similar procedure to the one used in this study. If these markers are found to be located proximal to Yd_2 then they could be used together with *uz* to enable the identification of individuals resulting from recombination close to Yd_2.

Molecular genetic markers detected using the polymerase chain reaction (PCR) are more suited to the analysis of large numbers of individuals than RFLP markers. This is mainly because PCR analysis can be performed using much smaller quantities of DNA. Consequently, relatively simple and rapid procedures can be used to prepare plant genomic DNA samples for PCR analysis (Wang *et al.*, 1993; Williams and Ronald, 1994; Steiner *et al.*, 1995). PCR-based markers were used to facilitate the high resolution genetic mapping of the *Cf-9* gene for resistance to *C. fulvum* in tomato, by enabling the efficient identification of informative recombinants from the mapping population of 626 F_2 individuals (Thomas *et al.*, 1995). Similarly, a pair of PCR

markers flanking *Yd2* could be used to select for recombination around the *Yd2* locus if a suitable pair of morphological markers cannot be found for this purpose.

RFLP markers can be converted into PCR-based, Sequence-Tagged-Site (STS) or Cleaved Amplified Polymorphic Sequence (CAPS) genetic markers (Olson *et al.*, 1989; Tragoonrung *et al.*, 1992; Konieczny and Ausubel, 1993; Talbert *et al.*, 1994). This involves sequencing the ends of the RFLP clones and designing pairs of primers for the specific PCR amplification of the intervening genomic sequences. The amplified DNA fragments may show insertion-deletion polymorphisms visible after agarose gel electrophoresis. Digestion with restriction enzymes can also be performed prior to electrophoresis to reveal RFLPs within the fragments. In Chapter 3, a number of RFLP markers were mapped very close to the *Yd2* gene and to either side of it. These RFLP markers would provide an ideal basis for the development of a pair of PCR markers that could be used to facilitate the high resolution genetic mapping of the *Yd2* locus if such a pair of markers were required.

Overall, there appears to be good potential for the development of a pair of morphological or PCR-based markers as tools to enable the identification of individuals resulting from recombination close to the *Yd2* gene. Such a pair of markers would be extremely valuable in a project to isolate the *Yd2* gene by a map-based approach, as it would enable a very large mapping population to be analysed more efficiently. If a pair of markers are to be developed to identify recombinants, priority should be given to a pair of morphological markers, as these could be scored with greater ease.