CHAPTER 3

A GENETIC MAP OF THE *Yd2* REGION OF BARLEY CHROMOSOME 3

3.1 Introduction

Disease resistance genes used in crop breeding often originate from wild species or non-cultivated accessions possessing poor agronomic traits. The introduction (introgression) of these genes into the genetic backgrounds of agronomically adapted cultivars by backcrossing produces resistant cultivars which differ from the recurrent parent only for a small chromosome segment containing the resistance gene (Young *et al.*, 1988). These resistant cultivars and the recurrent parent cultivars from which they were derived are commonly referred to as pairs of Nearly-Isogenic Lines or NILs. Many pairs of NILs exist for disease resistance genes in crop species, and can be used to identify molecular markers tightly linked to these genes (Young *et al.*, 1988). Bulked segregant analysis (Michelmore *et al.*, 1991) provides an alternative to the use of NILs for the identification of markers closely linked to genes of interest.

A number of mapping programs have been undertaken in cereals to identify molecular markers closely linked to genes that provide resistance against viruses, fungi and nematodes (Hinze *et al.*, 1991; Ronald *et al.*, 1992; Schüller *et al.*, 1992; Görg *et al.*, 1993; Graner and Bauer, 1993; Eastwood *et al.*, 1994; Williams *et al.*, 1994). These markers can be used to assist in the selection of these genes during breeding programs (Tanksley *et al.*, 1989; Gebhart and Salamini, 1992) and can also be used to initiate programs for the isolation of these genes by a map-based approach (Tanksley, 1995). In recent years, a number of disease resistance genes have been isolated from plants by map-based approaches and by other strategies (Johal and Briggs, 1992; Martin *et al.*, 1993; Bent *et al.*, 1994; Jones *et al.*, 1994; ; Mindrinos *et al.*, 1994; Whitham *et al.*, 1994; Lawrence *et al.*, 1995; Grant *et al.*, 1995). The analysis of these genes is helping us to understand the biochemical and genetic basis of naturally occurring disease resistance in plants (Staskawicz *et al.*, 1995; Ellis *et al.*, 1995), and provides the opportunity to produce new resistant crop cultivars by genetic transformation (Staskawicz *et al.*, 1995).

The Yd_2 gene on barley chromosome 3 (Rasmusson and Schaller, 1959; Section 1.4) provides resistance against barley yellow dwarf virus (BYDV), which is regarded

as the most damaging viral pathogen of cereals worldwide (Plumb, 1983). The *Yd2* gene was discovered in Ethiopian barley accessions which possess poor agronomic traits (Comeau *et al.*, 1990). Hence, BYDV resistant barley cultivars containing *Yd2* have been bred by backcrossing (Schaller and Chim, 1969a, 1969b; Schaller *et al.*, 1973, 1977, 1979; Vertigan, 1979), which has resulted in the availability of NILs in a number of genetic backgrounds.

Identifying plants containing *Yd2* from breeding populations is difficult, and relies on the availability of appropriate aphid vectors and BYDV isolates. The degree of effectiveness and dominance of *Yd2* varies with the growth conditions and the genetic background (Catherall and Hayes, 1970; Catherall *et al.*, 1970). In addition, symptoms of phosphorus and nitrogen deficiency can resemble those of BYDV infection (Paliwal and Comeau, 1987) and could complicate the diagnosis of BYDV resistance in the field. The difficulty of using BYDV resistance assays to determine *Yd2* genotype prompted Holloway and Heath (1992) to search for a protein marker for *Yd2* which could provide breeders with a more convenient means of selecting for this gene. By analysing total protein from *Yd2* NILs by two dimensional gel electrophoresis, Holloway and Heath (1992) identified a *Yd2*-associated 40 kDa protein that showed allelic variation in iso-electric point. The more acidic form of the protein (pI = 7.0) was found in all of the *Yd2*-containing barley lines, while the more basic form of the protein (pI = 7.2) was found in all of the non-*Yd2* barleys. This association with the *Yd2* gene was maintained in all pairs of NILs examined, suggesting that the gene encoding this protein was closely linked to *Yd2*.

In 1992, a collaborative research project was began between this laboratory (University of Adelaide), the laboratory of Peter Holloway (Victorian Department of Agriculture, Burnley, Victoria) and the laboratory of Richard Simpson (Institute for Biomolecular Engineering, Parkville, Victoria) to isolate, characterise and map the gene encoding the protein marker for *Yd2* identified by Holloway and Heath (1992). Chris Ford (University of Adelaide) succeeded in obtaining partial amino acid sequence of the protein which was subsequently used to design a pair of oligonucleotide primers for

the PCR amplification of a portion of the gene. The isolation and sequence analysis of alleles of the gene from Yd_2 and non-Yd_2 barleys will be described in a future publication (Ford *et al.*, 1996, manuscript in preparation).

In this study, RFLP markers closely linked to Yd_2 are identified using NILs, and used to construct a genetic map of the Yd_2 region of barley chromosome 3. RFLP probes used for this exercise include ones previously mapped to the Triticeae chromosome group 3, and an RFLP probe derived from the protein marker gene described by Holloway and Heath (1992). It is envisaged that these mapped RFLP markers could be used to assist breeders in selecting for Yd_2 and to initiate a program to isolate Yd_2 by a map-based approach.

3.2 Materials and methods

3.2.1 Acknowledgments

The author is sincerely grateful to Mark Sorrells, Mike Gale, Koichiro Tsunewaki, Bikram Gill, Andris Kleinhofs, Peter Langridge and Chris Ford for their kind donations of RFLP clones, to Peter Sharp and his staff at the Australian Triticeae Mapping Initiative clone collection for assisting in the distribution of these clones, and to the Grains Research and Development Corporation for financing the ATMI clone collection. The author would also like to thank the Rafiq Islam, Wayne Vertigan, Mandy Jenkin, and Michael Mackay and his staff at the Australian Winter Cereals Collection for generously supplying seed.

Nicholas Paltridge performed all of the Southern hybridisations involving the probes KSUF2, KSUA3, PSR543, PSR570, PSR754, ABG396, ABG398, ABG399, ABG703, AWPh7d and AWPhA12a, using membranes made by the author. All other experimental work presented in this chapter was performed by the author.

3.2.2 Plant lines

Seed of the barley lines and cultivars used in this exercise were provided by Mandy Jenkin, formerly of the University of Adelaide (CI 1179, CM67 and CI 2376),

Wayne Vertigan of the Tasmanian Department of Agriculture (Shannon, Proctor and CI 3208-1), the Australian Winter Cereal Collection at Tamworth, NSW (Atlas 57, Atlas 68, Turk and CI 3920-1), Rafiq Islam of the University of Adelaide (Betzes), or were obtained from the barley seed collection at the University of Adelaide (Atlas, Atlas 46, Hanna, Lion, California Mariout and Club Mariout). Rafiq Islam also supplied seed of the wheat cultivar Chinese Spring and Chinese Spring wheat-Betzes barley disomic and ditelosomic addition lines (Islam *et al.* 1981; Islam, 1983).

3.2.3 RFLP clones

Information regarding the type and source of the 78 RFLP clones used in this study is provided in Table 3.1. The AWPh clones and the YLP clone were obtained directly from their developers, while all other clones were obtained through the Australian Triticeae Mapping Initiative RFLP clone collection at the University of Sydney. All of the clones except YLP had previously been located to the Triticeae chromosome group 3 as a result of genetic mapping programs in barley, *T. tauschii*, or wheat (Liu and Tsunewaki 1991; Heun *et al.* 1991; Gill *et al.* 1991; Devos and Gale, 1993b; Kleinhofs *et al.* 1993; Nelson *et al.*, 1995b), or chromosome addition line analysis (Peter Sharp and Peter Langridge, personal communication). The YLP clone developed by Chris Ford (Ford *et al.*, 1996, manuscript in preparation) is a 680 bp partial-length cDNA clone derived from the gene encoding the protein marker for Yd_2, described by Holloway and Heath (1992).

RFLP clones were received as stab cultures, as bacterial colonies on solid media, or as plasmid DNA. Plasmids were used to transform *E. coli* strain DH5α (Stratagene, USA) by electroporation as described in Section 2.2.3. Liquid cultures of each clone were grown and used to make mini-preparations of plasmid DNA (Sections 2.2.4) and 15% glycerol stocks (Sambrook *et al.*, 1989) for storage of the bacteria at -80°C.

Table 3.1. RFLP clones used in this study

clone prefix	clone type	source	no.
BCD	barley cDNA	M Sorrells, Cornell University	16
CDO	oat cDNA	M. Sorrells, Cornell University	13
WG	wheat genomic DNA	M. Sorrells, Cornell University	8
PSR	wheat cDNA†	M. Gale, John Innes Institute, UK	9
Tag	wheat genomic DNA	K. Tsunewaki, Kyoto University	12
KSU	*T. tauschii* genomic DNA	B. Gill, Kansas State University	13
ABG	barley genomic DNA	A. Kleinhofs, Washington State Univ.	4
AWPh	*Phalaris coerulescens* Desf. cDNA	P. Langridge, University of Adelaide	2
YLP	barley cDNA	C. Ford, University of Adelaide	1

†except PSR543, which is a wheat genomic DNA clone

3.2.4 The identification of RFLPs closely linked to *Yd2*

The BYDV resistant barley cultivars CM67 (Schaller and Chin, 1969b), Atlas68 (Schaller and Chin, 1969a) and Shannon (Symes 1979; Vertigan 1979) are the result of backcrossing programs used to introgress *Yd2* into the genetic backgrounds of the agronomically adapted cultivars California Mariout, Atlas and Proctor, respectively. As such, these cultivars represent three pairs of NILs for *Yd2*. Genes for aleurone colour, awn morphology and resistance to other pathogens were also introgressed into the California Mariout and Atlas backgrounds to produce CM67 and Atlas68. Hence, these two cultivar pairs are nearly-isogenic for these genes as well as for *Yd2*.

The three BYDV resistant barley cultivars and the barleys from which they were derived were analysed with a range of RFLP probes, using the procedures for RFLP analysis outlined in Section 2.2.6. Each probe was used to analyse the DNA of the

barleys, cut separately with the restriction enzymes *Dra*I, *Eco*RI, *Hin*dIII and *Xba*I. A number of probes were also tested using some or all of the following restriction enzymes: *Alu*I, *Bam*HI, *Ban*II, *Bcl*I, *Bst*NI, *Cla*I, *Eco*RV, *Hae*III, *Hinf*I, *Hpa*II, *Kpn*I, *Nco*I, *Nde*I, *Rsa*I, *Sau*3AI and *Taq*I. Those RFLPs showing associations with *Yd*$_2$ were likely to be closely linked to *Yd*$_2$ and were chosen for segregation analysis.

3.2.5 Segregation analysis

Segregation analysis was performed using a mapping population of 106 F_2 individuals derived from a single cross between the non-*Yd*$_2$ barley cultivar Proctor and the *Yd*$_2$-containing barley cultivar Shannon. The DNA used in the RFLP analysis of each F_2 plant was extracted from tissue of the F_2 plant, or from tissue pooled from at least 12 of its F_3 progeny. The *Yd*$_2$ genotypes of selected individuals from the mapping population were determined by BYDV resistance assays as described in Section 3.2.7. Linkage analysis was performed using the computer program MAPMAKER (Lander *et al.*, 1987), as described in Section 2.2.7.

3.2.6 Wheat-barley disomic and ditelosomic addition line analysis

Wheat-barley disomic and ditelosomic addition lines (Islam *et al.* 1981; Islam, 1983) were used to verify that RFLP loci were located on the chromosome 3, and to locate RFLPs to the long or the short arm of barley chromosome 3. For probes that detected multiple restriction fragments in barley, care was taken to base locus positions on restriction fragments corresponding to those mapped.

3.2.7 BYDV resistance assays

To determine the *Yd*$_2$ genotype of F_2 individuals from the Proctor × Shannon mapping population, 12 to 30 F_3 or F_4 progeny derived from each F_2 plant were infected with the PAV_{adel} isolate of BYDV-PAV_{adel}, as described in Section 2.2.9. Infected plants were grown in a glasshouse at 20 to 25°C, or outside during May to September. Families were classified as resistant, susceptible or segregating for

resistance, by comparing the stunting within these families with that observed in infected Proctor and Shannon control plants. BYDV northern dot blot hybridisation analysis using a probe derived from the RNA genome of a BYDV-PAV isolate (Section 2.2.10) was used to confirm that BYDV infection had occurred.

3.3 Results

3.3.1 The identification of RFLPs closely linked to *Yd2* using three sets of nearly-isogenic barley lines

To identify RFLPs closely linked to *Yd2*, the DNA of three sets of barley lines nearly-isogenic for *Yd2* were subject to Southern hybridisation analysis using a range of restriction enzymes and RFLP probes. All of the probes had previously been mapped to Triticeae chromosome group 3, except for the YLP probe. In each nearly-isogenic barley set, RFLPs associated with *Yd2* consisted of a restriction fragment in the BYDV resistant cultivar derived from the Ethiopian accession used as *Yd2* donor, while RFLPs which were not associated with *Yd2* consisted of a restriction fragment in the BYDV resistant cultivar which was derived from one of the BYDV susceptible barley lines.

Tables 3.2 and 3.3 summarise the results of this exercise. A probe could only be tested for its association with *Yd2* in a set of nearly-isogenic barley lines if it identified a restriction fragment in the *Yd2* donor barley which was different in size to those identified in the BYDV susceptible barley cultivars. Of the 73 probes that produced a clear hybridisation patterns, 42, 36 and 41 identified such informative polymorphisms for the CM67, Atlas68 and Shannon sets of barleys, respectively. Of these probes, the numbers that identified *Yd2*-associated RFLPs in the CM67, Atlas68 and Shannon sets of nearly-isogenic barleys was 6, 8 and 25, respectively. All of these probes detected single *Yd2*-associated RFLPs, except for BCD339, WG180 and KSUH7, which identified two *Yd2*- associated RFLPs each.

Table 3.2. RFLP data for the 31 RFLP probes that identified *Yd*$_2$-associated RFLPs

RFLP probe	no. of rest. enzymes tested	max. no. of loci detected†	*Yd*$_2$-associated RFLPs detected§		
			CM67 set	Atlas68 set	Shannon set
BCD127	4	1	no	?	yes
BCD134	4	1	no	?	yes
BCD263	4	1	no	?	yes
BCD339¥	6	7	no (RFLP 1) yes (RFLP 2)	yes (RFLP 1) ? (RFLP 2)	yes (RFLP 1) no (RFLP 2)
BCD809	4	1	?	no	yes
BCD828	5	2	?	yes	?
BCD1127	5	2	?	yes	yes
CDO395	4	1	no	yes	no
CDO419	4	5	?	no	yes
CDO718	4	2	no	no	yes
CDO1396	4	2	yes	no	no
WG178	4	1	no	no	yes
WG180¥	4	12	yes (RFLP 1) no (RFLP 2)	no (RFLP 1) ? (RFLP2)	? (RFLP 1) yes (RFLP 2)
WG184	4	10	?	?	yes
WG889	4	3	no	?	yes
KSUA3	5	6	no	?	yes
KSUG59	4	1	?	?	yes
KSUH7¥	4	7	? (RFLP 1) yes (RFLP 2)	yes (RFLP 1) no (RFLP 2)	? (RFLP 1) no (RFLP 2)
PSR116	4	1	no	?	yes
PSR156	4	1	no	no	yes
PSR543	4	1	no	?	yes
Tag80	4	1	?	no	yes
Tag221	4	1	no	no	yes
Tag223	4	2	?	yes	yes
Tag485	4	1	?	no	yes

Table 3.2 (continued)

RFLP probe	no. of rest. enzymes tested	max. no. of loci detected†	*Yd2*-associated RFLPs detected§		
			CM67 set	Atlas68 set	Shannon set
Tag538	4	2	**yes**	**yes**	?
Tag718	4	1	**yes**	?	no
ABG396	4	1	no	no	**yes**
ABG703	5	2	no	no	**yes**
AWPh7d	4	3	?	?	**yes**
YLP	20‡	1	no	**yes**	**yes**
number of probes detecting informative RFLPs:			**21**	**20**	**29**
number of probes detecting *Yd2*-associated RFLPs:			**6**	**8**	**25**

†The maximum number of loci each probe detected in barley was defined as the minimum number of restriction fragments detected for any one restriction enzyme.

§RFLP probes failed to identify an informative polymorphism in a barley set (?), or identified an RFLP which was either *Yd2*-associated (**yes**) or not *Yd2*-associated (no). For each probe which may detect multiple loci in barley, the data presented for the different sets was for the same RFLP, unless otherwise stated. RFLPs consisting of unexpected, unique restriction fragments in the BYDV resistant barley are marked here with a '?', and are not counted as informative polymorphisms.

¥The RFLP probes BCD339, WG180 and KSUH7 each detected two *Yd2*-associated polymorphisms. RFLPs identifying the same loci in the different barley sets were identified on the basis of hybridisation intensity and the size of the restriction fragments, and are indicated as 'RFLP 1' or 'RFLP 2'.

‡Only five restriction enzymes were used to analyse CM67 and Atlas68 using the YLP probe.

Table 3.3. RFLP data for RFLP probes which did not identify *Yd_2*-associated RFLPs

RFLP probe	no. of rest. enzymes tested	max. no. of loci detected†	informative polymorphisms§		
			CM67 set	Atlas68 set	Shannon set
BCD102	4	3	-	-	+
BCD115‡	4	4	+	-	+
BCD147‡	4	4	+	+	-
BCD358	4	1	-	-	-
BCD451‡	4	3	+	+	+
BCD452	4	1	-	+	-
BCD589	4	1	+	-	-
BCD782	4	1	-	-	-
BCD927‡	4	1	+	+	-
CDO105‡	4	2	+	+	-
CDO118	4	3	+	-	-
CDO455	4	1	+	-	-
CDO474‡	4	6	+	+	+
CDO534	4	1	-	-	-
CDO684	no clear hybridisation pattern				
CDO920	12¥	1	-	-	-
CDO1174	4	4	-	-	+
CDO1406	no clear hybridisation pattern				
WG110	4	1	+	+	+
WG177	4	1	-	-	-
WG405‡	4	5	+	+	+
WG940	4	1	+	-	+
KSUB8	4	1	-	-	-
KSUD4	no clear hybridisation pattern				
KSUD7‡	4	4	+	+	-
KSUD19	4	4	+	-	-
KSUD24	no clear hybridisation pattern				
KSUE2‡	4	2	+	+	+
KSUG36	2	3	-	+	-

Table 3.3 (continued)

RFLP probe	no. of rest. enzymes tested	max. no. of loci detected†	informative polymorphisms§		
			CM67 set	Atlas68 set	Shannon set
KSUG62	4	1	-	-	-
KSUH15	no clear hybridisation pattern				
KSUI32‡	4	8	+	+	+
PSR123	4	1	+	+	-
PSR394	14¥	2	-	-	-
PSR570	6	1	-	-	-
PSR578	4	1	-	+	-
PSR754	6	1	-	-	-
PSR902	20¥	1	-	-	-
Tag118	4	1	-	-	-
Tag221	4	1	+	+	-
Tag577	no clear hybridisation pattern				
Tag637	4	1	-	-	-
Tag645	4	1	-	-	-
Tag683‡	4	3	-	+	+
Tag747	6	1	-	-	-
ABG398	4	1	+	-	-
ABG399	4	1	+	-	+
AWPhA12a	4	3	+	-	-
Total number of probes detecting informative RFLPs:			**21**	**16**	**12**

†The maximum number of loci each probe detected in barley was defined as the minimum number of restriction fragments detected for any one restriction enzyme.

§Each probe either detected an informative polymorphism (+) or did not detect an informative polymorphism (-) in each of the barley sets.

‡The informative RFLPs detected by each of these probes in the different barley sets may or may not represent the same RFLP loci.

¥The CM67 and Atlas68 sets of barleys were screened using only 5 to 6 restriction enzymes

The YLP probe derived from the gene encoding a protein marker for *Yd*$_2$ (Holloway and Heath, 1992) is one example of a probe that detected a *Yd*$_2$-associated polymorphism. This *Hae*III polymorphism was *Yd*$_2$-associated in the Atlas68 and Shannon sets of barleys but not associated with *Yd*$_2$ in the CM67 set of barleys (Figure 3.1). This contrasts to the observation by Holloway and Heath (1992) that the protein polymorphism is associated with *Yd*$_2$ in all three sets of nearly-isogenic barleys. This discrepancy could be explained by genetic heterogeneity in CI 2376, the Ethiopian *Yd*$_2$ donor barley line used to breed CM67. More specifically, the CI 2376 selections used in this study and by Holloway and Heath (1992) could have differed with respect to their genotypes at this locus. The polymorphic restriction fragments identified by YLP using *Hae*III showed at least five different sizes which varied by about 20 to 100 bp among the barley cultivars examined (Figure 3.1). This polymorphism may have been due to a microsatellite located close to the probe hybridisation site, as microsatellite loci typically show series of multiple alleles differing by insertion and deletions of 100 bp or less (Saghai Maroof *et al.*, 1994; Becker and Heun, 1995).

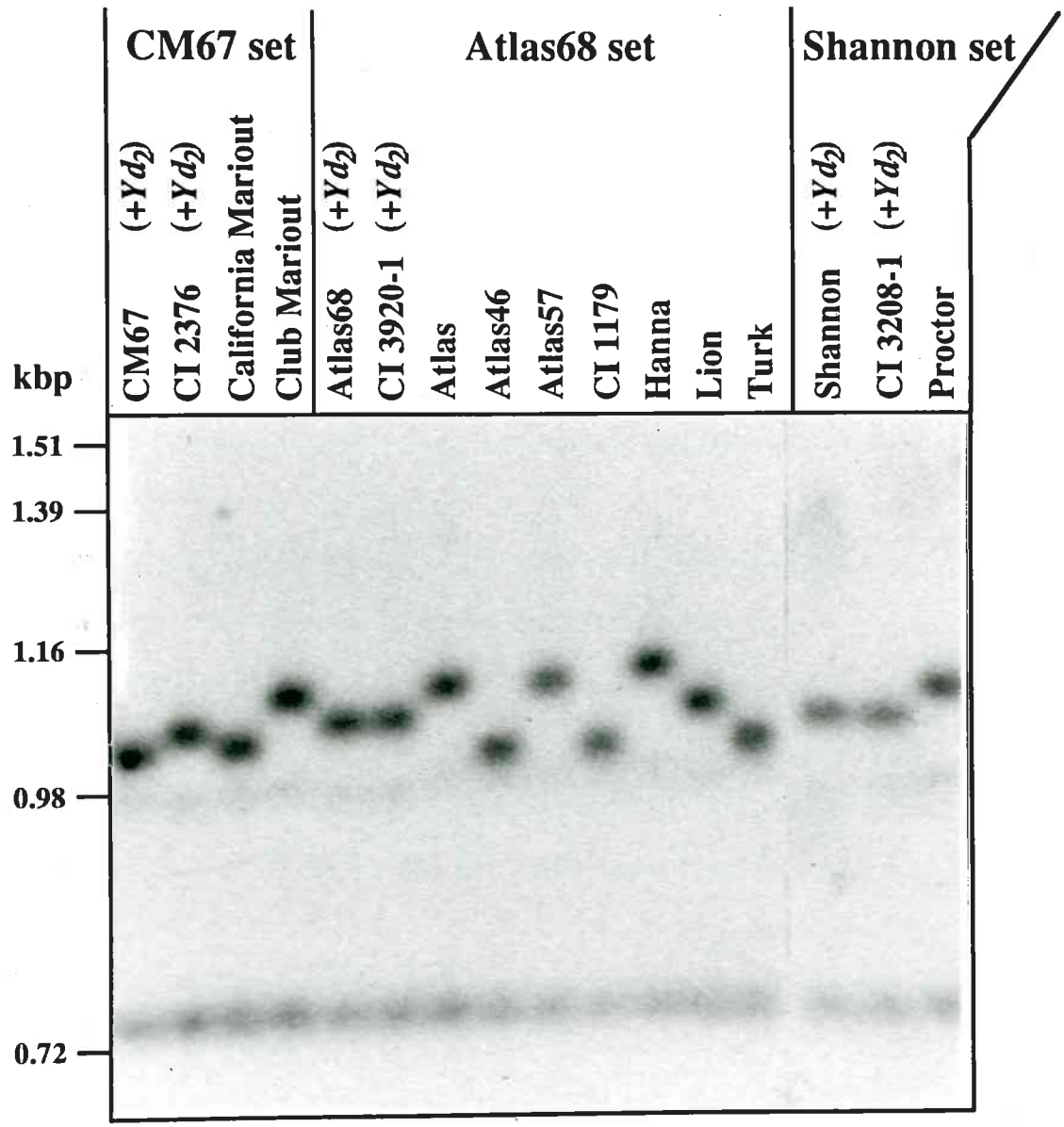
Yd$_2$-associated RFLPs are likely to be positioned on the Ethiopian *Yd*$_2$-containing chromosome segments in the resistant barley cultivars, and are therefore likely to be genetically closely linked to *Yd*$_2$. The greatest number of *Yd*$_2$-associated polymorphisms were detected using the Shannon set of nearly-isogenic barleys, which contained only one BYDV susceptible barley cultivar, Proctor. Therefore, a cross between Shannon and Proctor was chosen to produce an F_2 mapping population for segregation analysis.

3.3.2 Map construction

Probes used to analyse the Proctor × Shannon F_2 mapping population were those that detected *Yd*$_2$-associated polymorphisms in the Shannon set of barleys, plus BCD828, which identified a unique restriction fragment in Shannon. The probes PSR116 and BCD263 detected loci that perfectly cosegregated in the Proctor × Shannon F_2 population, and showed identical hybridisation patterns for a number of barley lines

Figure 3.1. *Yd*$_2$-associated RFLPs detected using the YLP probe

The figure shows the hybridisation pattern of the YLP probe to the DNA of three sets of nearly-isogenic barley lines cut with the restriction enzyme *Hae*III. Each set comprises a BYDV-resistant, *Yd*$_2$-containing cultivar (CM67, Atlas68 or Shannon), the Ethiopian accession used as the source of the *Yd*$_2$ gene in the breeding the resistant cultivar (CI 2376, CI 3920-1 and CI 3208-1), and the BYDV susceptible (non-*Yd*$_2$) cultivar(s) used as the *Yd*$_2$ acceptors in the breeding of the resistant cultivar. The *Hae*III RFLP is associated with *Yd*$_2$ in the Atlas68 and Shannon sets of nearly-isogenic barley lines, but not associated with *Yd*$_2$ in the CM67 set of barley lines. DNA of the cultivar Lion was introduced into Atlas68 via Atlas57 (Schaller and Chim, 1969a). Because the *XYlp* locus in Atlas68 was not derived from Atlas57, this locus in Atlas68 was not derived from Lion, despite the fact that Lion and Atlas68 contained the same-sized restriction fragment. The high level of polymorphism and the presence of small insertions or deletions at this locus suggests that it contains a microsatellite. Size markers were derived from SPP-1 DNA cut with *Eco*RI.


CM67 set
Atlas68 set
Shannon set
CM67 (+Yd2)
CI 2376 (+Yd2)
California Mariout
Club Mariout
Atlas68 (+Yd2)
CI 3920-1 (+Yd2)
Atlas
Atlas46
Atlas57
CI 1179
Hanna
Lion
Turk
Shannon (+Yd2)
CI 3208-1 (+Yd2)
Proctor
kbp
1.51
1.39
1.16
0.98
0.72

and restriction enzymes. It was therefore concluded that the wheat cDNA clone PSR116 and the barley cDNA clone BCD263 originated from orthologous genes. Similarly, the barley cDNA clones BCD828 and BCD1127, showed identical hybridisation patterns for a number of barley varieties and restriction enzymes, and were considered to be derived from the same barley gene. Accordingly, BCD828, but not BCD1127 was used in the analysis of the mapping population. All other RFLP probes used to analyse the mapping population showed unique hybridisation patterns, and therefore detected distinct loci. Polymorphisms detected by the probes WG180, WG184, Tag221, Tag485 and BCD339 could not be confidently scored in the mapping population, due to the fact that each of these probes showed either weak hybridisation, hybridisation to large numbers of restriction fragments, or cross hybridisation to repetitive DNA sequences in the barley genome. Altogether, 19 unique RFLP loci could be mapped using the Proctor × Shannon F_2 population.

To conserve time and resources, the mapping population of 106 Proctor × Shannon F_2 individuals were characterised for the RFLP loci and the *Yd2* gene in two stages. In the first stage, 50 F_2 individuals from the mapping population were genotyped for the 19 RFLPs, using the restriction enzymes shown in Table 3.4. All of the RFLP loci except *Xcdo419* showed linkage to one another at a LOD threshold value of 3.0. The twenty-six F_2 individuals resulting from recombination between the linked RFLP markers were genotyped for *Yd2* using BYDV resistance assays (Section 3.3.3), and consequently, the *Yd2* gene was located within this linkage group. In the second stage, the remaining 56 F_2 individuals from the mapping population were genotyped for the 10 RFLP loci found to be the closest to *Yd2*. These loci comprised *Xglk223* and *Xpsr116/Xbcd263* and all loci positioned between them (Figure 3.2). Eight of these F_2 individuals were found to have resulted from recombination between *Xglk223* and *Xpsr116/Xbcd263*, and were genotyped for *Yd2*. The combined mapping data from both stages was used to produce the genetic map of the *Yd2* region shown in Figure 3.2.

Figure 3.2. A genetic map of the *Yd*$_2$ region of barley chromosome 3

The genetic map of the *Yd*$_2$ region of barley chromosome 3 was constructed using the Proctor × Shannon F_2 mapping population and wheat-barley ditelosomic addition line analysis. The centromere is located between *Xbcd127* and the locus group *Xbcd134*, *XksuA3* and *Xawph7d*, even though these four RFLP loci perfectly cosegregated in the mapping population. All other loci that perfectly cosegregated are separated by commas. The RFLP loci *Xpsr116* and *Xbcd263* are presented as one locus *Xpsr116/Xbcd263*, as the probes PSR116 and BCD263 were derived from orthologous genes (see Section 3.3.2). Distances are in cM. The inset illustrates the position and size of the map relative to the total genetic map of barley chromosome 3 constructed by Graner *et al.* (1994).

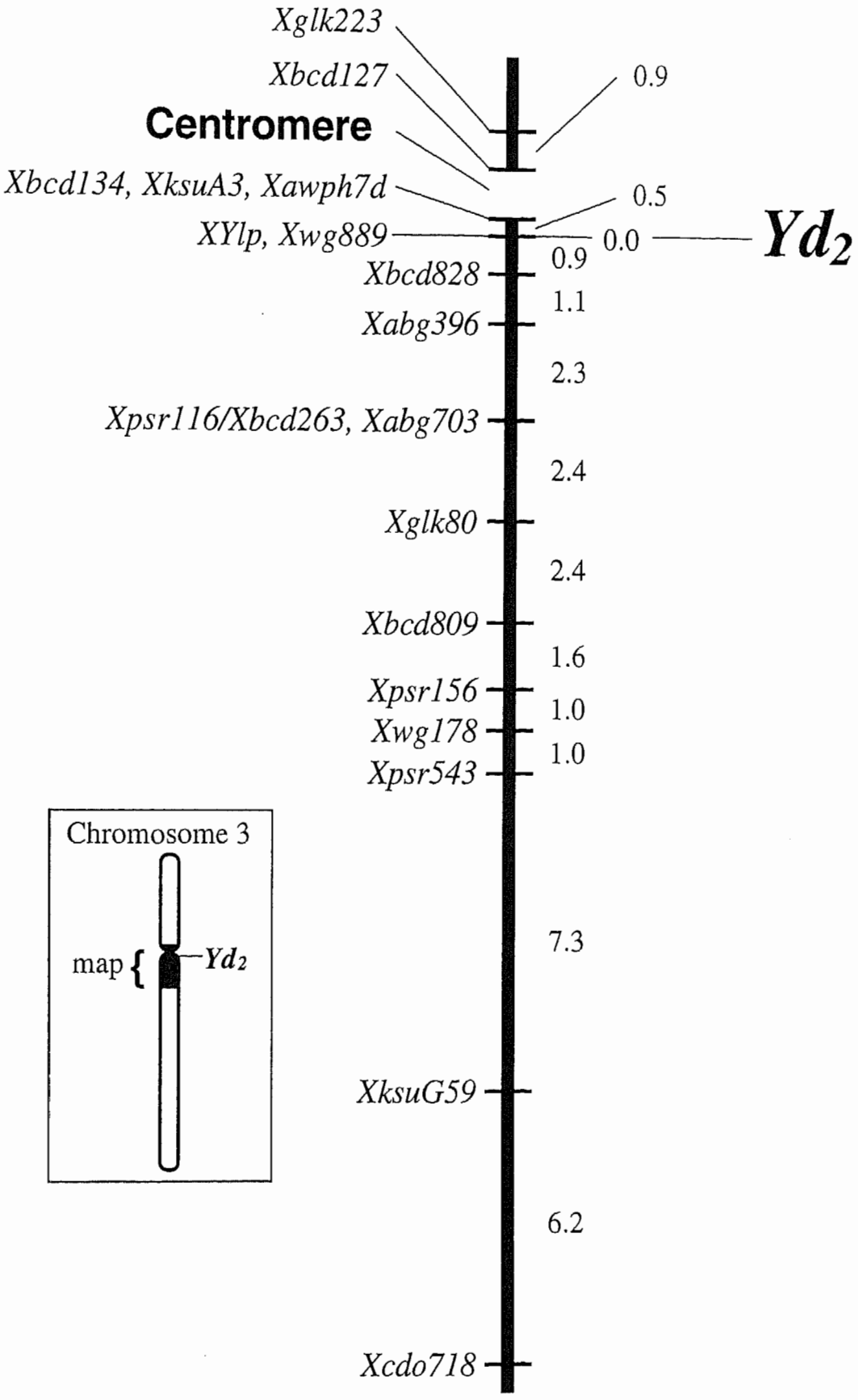

Xglk223
Xbcd127
Centromere
Xbcd134, XksuA3, Xawph7d
XYlp, Xwg889
Xbcd828
Xabg396
Xpsr116/Xbcd263, Xabg703
Xglk80
Xbcd809
Xpsr156
Xwg178
Xpsr543
XksuG59
Xcdo718
0.9
0.5
0.0
0.9
1.1
2.3
2.4
2.4
1.6
1.0
1.0
7.3
6.2
Yd2
Chromosome 3
map
Yd2

Table 3.4. Restriction enzymes used for mapping RFLP loci in the Proctor × Shannon F_2 mapping population, and the results of the wheat-barley ditelosomic addition line analysis

RFLP locus	Restriction enzyme used for mapping	Chromosome arm location§
Xglk223	*Dra*I	3HS
Xbcd127	*Eco*RI	3HS
Xbcd134	*Eco*RI	3HL
XksuA3	*Bam*HI	3HL
Xawph7d	*Bam*HI	3HL
XYlp	*Hae*III	3HL
Xwg889	*Dra*I	3HL
Xbcd828	*Bam*HI	3HL
Xabg396	*Xba*I	n. d.
Xpsr116/Xbcd263†	*Xba*I	n. d.
Xabg703	*Hin*dIII	3HL
Xglk80	*Xba*I	n. d.
Xbcd809	*Hin*dIII	n. d.
Xpsr156	*Xba*I	n. d.
Xwg178	*Eco*RI	n. d.
Xpsr543	*Xba*I	n. d.
XksuG59	*Dra*I	n. d.
Xcdo718	*Xba*I	n. d.
Xcdo419	*Hin*dIII	n. d.

†*Xpsr116* and *Xbcd263* represent the same RFLP loci (see text).

§RFLP loci were located to the short arm of barley chromosome 3 (3HS), the long arm of barley chromosome 3 (3HL), or the chromosome arm location of the RFLP loci were not determined (n. d.).

The analysis of 9 RFLP markers using Chinese spring wheat-Betzes barley disomic and ditelosomic addition lines (Islam *et al.*, 1981; Islam, 1983) showed that the map of the Yd_2 region spanned the centromere of barley chromosome 3. Each of the 9 probes, indicated in Table 3.4, hybridised to restriction fragments which were present in

the barley cultivar Betzes and the 3H disomic addition line but absent in the wheat cultivar Chinese Spring, confirming that the map in Figure 3.2 represented a portion of barley chromosome 3. The absence of the restriction fragments from either the 3HS or 3HL ditelosomic addition lines allowed the localisation of seven of the RFLP loci to the long arm and three RFLP loci to the short arm (Table 3.4). This enabled the centromere to be located between the RFLP locus *Xbcd127* and the RFLP loci *Xbcd134*, *XksuA3* and *Xawph7d* as shown in Figure 3.2. According to the position of Yd_2 relative to these RFLP loci, Yd_2 is located 0.5 cM from the centromere on the long arm of barley chromosome 3. Two examples of the Southern hybridisation patterns obtained in the analysis of the wheat-barley disomic and ditelosomic addition lines are shown in Figure 3.3.

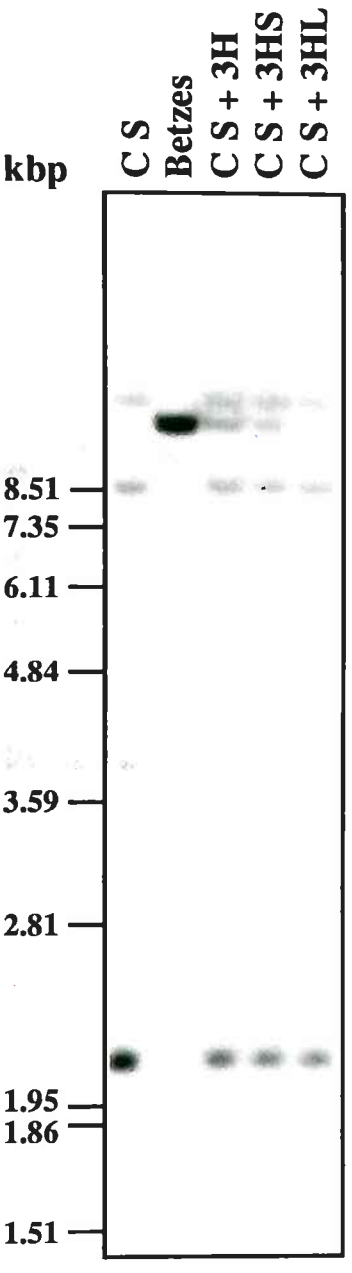
3.3.3 BYDV resistance assays

Individuals from the Proctor × Shannon F_2 mapping population were genotyped for Yd_2 by comparing the level of stunting of BYDV infected progeny of these individuals with the level of stunting obtained in infected plants of the parent cultivars Proctor and Shannon. The fresh shoot weights of uninfected Proctor and Shannon individuals were not significantly different (Figure 3.4). However, upon BYDV infection, Proctor individuals attained fresh shoot weights which were significantly less the fresh shoot weights of Shannon individuals (Figure 3.4). The BYDV resistance from the Yd_2-containing parent cultivar Shannon was therefore effective in limiting the degree of stunting caused by the PAV_{adel} isolate of BYDV. This is consistent with previous observations which had shown Yd_2 to be effective against PAV isolates of BYDV (Scaria *et al.*, 1985; Herrera and Plumb, 1989). In addition, BYDV infection resulted in more extensive leaf yellowing in Proctor than in Shannon (not shown).

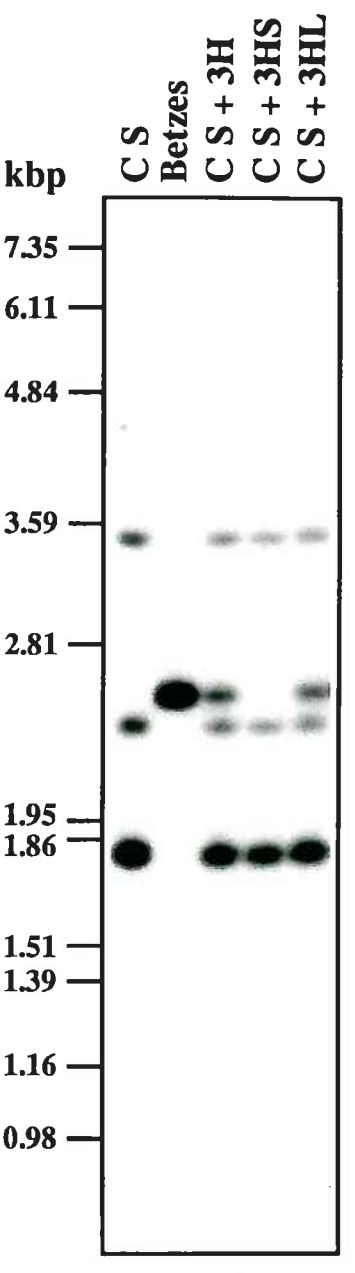
Thirty-four recombinant individuals from the Proctor × Shannon F_2 mapping population were genotyped for Yd_2 by BYDV infecting 12 to 36 F_3 or F4 progeny of each F_2 individual. Families were classed as resistant, susceptible or segregating for BYDV resistance, defining the Yd_2 genotypes of the corresponding F_2 individuals as

Figure 3.3. Wheat-barley disomic and ditelosomic addition lines analysis of the probes BCD127 and BCD134

The probes BCD127 and BCD134 were used to analyse *Eco*RI cut DNA of wheat-barley disomic and ditelosomic addition lines derived from Betzes barley and Chinese spring wheat. Each of the probes detected one band in Betzes barley which was present in the addition line containing chromosome 3 from Betzes barley (CS+3H), but absent in Chinese Spring wheat, confirming that these probes detected loci on barley chromosome 3. The presence or absence of these fragments in the ditelosomic addition lines containing the short arm of barley chromosome 3 (CS+3HS) or the long arm of barley chromosome 3 (CS+3HL) was used to assign BCD127 to the short arm and BCD134 to the long arm. Because each of these probes detect only one locus in barley, these chromosome arm positions correspond to the RFLP loci *Xbcd127* and *Xbcd134* mapped using these probes in the Proctor × Shannon F_2 population. Size markers were derived from SPP-1 DNA cut with *Eco*RI.



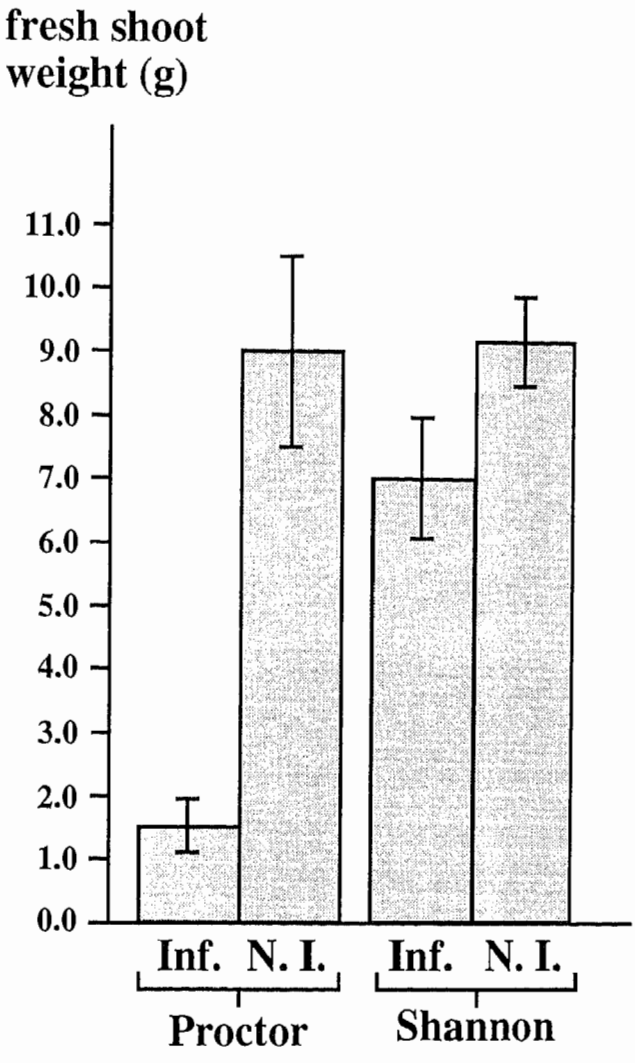
probe: BCD127



probe: BCD134

Figure 3.4. The effect of the *Yd_2* gene on the fresh shoot weight of BYDV infected barley plants

Eight seedlings of the barley cultivars Proctor and Shannon were infected with BYDV-PAV_{adel} one week after sowing, and the fresh shoot weights of the plants measured four weeks after infection. For comparison, the fresh shoot weights of eight virus-free plants of each cultivar were measured at the same stage. The graph shows the mean and standard deviation of these fresh weights. The fresh weights of the virus-free plants of these two cultivars did not differ significantly (according to a t-test; $P > 0.5$). However, in the presence of the virus, the fresh shoot weights of Proctor plants were significantly less than those of Shannon ($P < 0.5$), demonstrating that the *Yd_2* gene in Shannon provided protection against stunting caused by BYDV-PAV_{adel} infection.


fresh shoot
weight (g)
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Inf.
N. I.
Inf.
N. I.
Proctor
Shannon

Yd2/Yd2, -/- and *Yd2*/-, respectively. Individuals from BYDV susceptible families were as stunted as infected Proctor individuals, while those from the BYDV resistant families resembled infected Shannon individuals by showing very little stunting. Families segregating for BYDV resistance contained two recognisable classes of individuals (resistant and susceptible). Catherall and Hayes (1970) showed that the expression of BYDV resistance by a given *Yd2* allele could be codominant or recessive, depending on the growth conditions and background genotype. Under the conditions used here, BYDV resistance was expressed in a recessive fashion, as resistant and susceptible individuals occurred in segregating families in a ratio of approximately 1:3 (not shown).

BYDV northern dot blot hybridisation analysis was used to monitor infection by BYDV-PAV_{adel} throughout the resistance assays. It was not known if the Australian PAV isolate of BYDV used to derive the probe (Miller *et al.*, 1988; Young *et al.* 1991) was the same as BYDV-PAV_{adel}, another Australian BYDV isolate. However, the probe was shown to diagnose BYDV-PAV_{adel} infection satisfactorily (Figure 3.5). All plants not expressing obvious symptoms were tested by BYDV northern dot blot hybridisation analysis, and all of these were found to be infected. Therefore, the procedures used for BYDV infection were sufficient to achieve an infection rate of 100%.

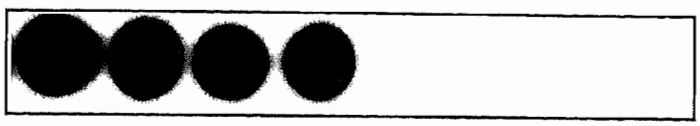
3.3.4 Delimitation of the Ethiopian *Yd2*-containing introgressed chromosome segments in CM67, Atlas68 and Shannon

The RFLPs on the map shown in Figure 3.2 represent loci in the barley cultivar Shannon which were derived from CI 3208-1, the Ethiopian barley accession used as a *Yd2* donor in the breeding of Shannon. Therefore, the 27.6 cM region spanned by the map represents the minimum extent of the chromosome segment introgressed with *Yd2* into the genetic background of the BYDV susceptible cultivar Proctor during the breeding of Shannon.

Using the map of the *Yd2* region and the results of the polymorphism screen, the extent of the chromosome segments in CM67 and Atlas68 derived from the respective

Figure 3.5. The use of northern dot blot hybridisation analysis for the detection of BYDV-PAV$_{adel}$ in barley

A radioactively-labelled DNA probe derived from the genome of an Australian PAV isolate of BYDV (Miller *et al.*, 1988; Young *et al.*, 1991) was used to probe a membrane containing total nucleic acid samples from barley infected with BYDV-PAV$_{adel}$ (I) and nucleic acid from virus-free plants (NI). The probe showed clear hybridisation to the nucleic acid of plants infected with BYDV-PAV$_{adel}$, but showed no apparent hybridisation to the nucleic acid of virus-free plants. Therefore, this procedure can be effectively used to identify barley plants infected with BYDV-PAV$_{adel}$.



I I I I NI NI NI NI

Yd$_2$ donor barley lines CI 2376 and CI 3920-1 was determined (Figure 3.6). The section of chromosome 3 from CI 3920-1 in Atlas68 was found to extend from a point between the RFLP loci *Xabg396* and *Xbcd828* on the long arm, to a point distal to *Xglk223* on the short arm. The CI 3920-1 chromosome segment in Atlas68 must therefore be greater than 3.4 cM in length. The location of this introgressed chromosome segment above the RFLP locus *Xabg396* was consistent with the position of *Yd*$_2$ close to the centromere determined by segregation analysis. None of the mapped RFLP loci in CM67 could be shown to be derived from CI 2376. However, the chromosome segment in CM67 from CI 2376 could be defined by on the basis of the mapped location of the *Yd*$_2$ gene, and the RFLP loci in CM67 which were shown to be not derived from CI 2376. The introgressed chromosome segment was thereby determined to lie in the 0.5 cM interval between *XksuA3* and *XYlp*, or in the 2.0 cM interval between *XYlp* and *Xabg396* (Figure 3.6).

3.3.5 Comparison of the map of the *Yd*$_2$ region with other maps of Triticeae group 3 chromosomes

The RFLP clones used in this study were obtained from a number of different mapping programs. As a result, the RFLP markers on the map in Figure 3.2, with the exceptions of *Xbcd127*, *Xawph7d*, *Xwg889* and *XYlp*, could be found on published genetic maps of barley, wheat, rye and *T. tauschii*. Table 3.5 indicates the RFLP markers common to these maps and the map of the *Yd*$_2$ region of barley chromosome 3 created in this study. RFLP markers from the *Yd*$_2$ region were positioned on chromosome 3 in the barley maps, and on chromosomes belonging to Triticeae group 3 in the maps of the other species. Almost all of the markers were positioned close to the centromere and in the same order that they were mapped in this study. The only exception was the marker *XksuG59*, which was the distal most marker mapped to the long arm of wheat chromosome 3D by Nelson *et al.* (1995b).

Figure 3.6. The extent of the Ethiopian *Yd2*-containing introgressed chromosome segments in CM67 and Atlas68

Table 3.2 shows whether or not RFLP markers in the *Yd2*-containing barley cultivars CM67 and Atlas68 were derived from the respective *Yd2* donor barleys CI 2376 and CI 3920-1. This information is reproduced here in the context of the map of the *Yd2* region (Figure 3.2), in order to show the extent of the introgressed segments of barley chromosome 3 from the *Yd2* donor barleys in CM67 and Atlas68. RFLP loci in CM67 or Atlas68 were derived from the *Yd2* donor barley (yes) or not derived from the *Yd2* donor barley (no), or the origins of the loci in CM67 or Atlas68 were not determined (?). The introgressed chromosome segment in CM67 from CI 2376 is located in one of the two shaded regions representing intervals of 0.5 cM and 2.0 cM. The introgressed chromosome segment from CI 3920-1 in Atlas68 (shaded) may extend beyond the mapped region on the short arm, and therefore represents an interval of at least 3.4 cM. The total 26.7 cM mapped region of barley chromosome 3 represents the minimum extent of the introgressed chromosome from the Ethiopian barley CI 3208-in the cultivar Shannon. Distances between RFLP loci are shown in cM.

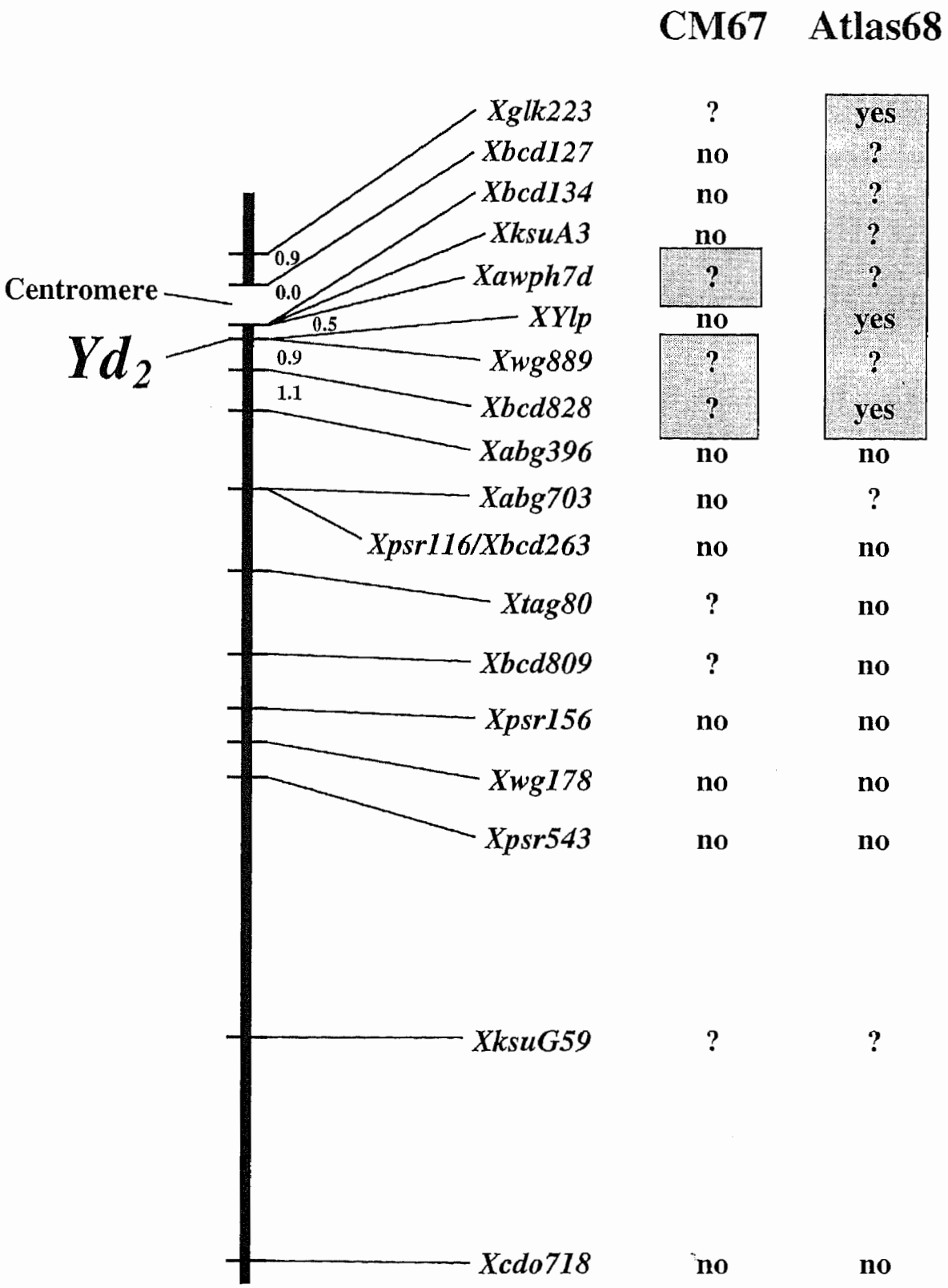

CM67
Atlas68
Xglk223
Xbcd127
Xbcd134
XksuA3
Xawph7d
XYlp
Xwg889
Xbcd828
Xabg396
Xabg703
Xpsr116/Xbcd263
Xtag80
Xbcd809
Xpsr156
Xwg178
Xpsr543
XksuG59
Xcdo718
Centromere
Yd2
0.9
0.0
0.5
0.9
1.1
? yes
no ?
no ?
no ?
? ?
no yes
? ?
? yes
no no
no ?
no no
? no
? no
no no
no no
no no
? ?
no no

Table 3.5. RFLP markers common to the genetic map of the *Yd*$_2$ region and other Triticeae chromosome 3 maps

map	number of RFLP markers on map	markers common to the map of the *Yd*$_2$ region
barley chromosome 3 (Heun *et al.*, 1991)	14	*Xwg178*
barley chromosome 3 (Kleinhofs *et al.*, 1993)	44	*XksuA3* *Xbcd828* *Xabg396* *Xabg703* *Xpsr156*
barley chromosome 3 (Graner *et al.*, 1994)	70	*Xbcd828* *Xbcd809*
wheat chromosome 3B (Liu and Tsunewaki, 1991)	11	*Xglk223* *Xglk80*
wheat chromosome 3B (Devos and Gale, 1993b)	14	*Xpsr156*
wheat chromosome 3D (Devos and Gale, 1993b)	20	*Xpsr116* *Xpsr156* *Xpsr543*
wheat chromosome 3A (Nelson *et al.*, 1995)	58	*Xbcd828*
wheat chromosome 3B (Nelson *et al.*, 1995)	62	*Xbcd809* *Xcdo718*
wheat chromosome 3D (Nelson *et al.*, 1995)	40	*Xbcd134* *XksuG59*
T. tauschii chromosome 3 (Gill *et al.*, 1991)	21	*XksuG59*
T. tauschii chromosome 3 (Lagudah *et al.*, 1991)	8	*Xpsr156*
T. tauschii chromosome 3 (Gill *et al.*, 1993)	53	*Xbcd828* *Xwg178* *XksuG59*
rye chromosome 3 (Devos and Gale, 1993)	19	*Xpsr116* *Xpsr156* *Xpsr543*

The barley maps of Kleinhofs *et al.* (1993) and Graner *et al.* (1994) were constructed using doubled haploid F_2 mapping populations derived from the crosses Steptoe × Morex and Igri × Franka, respectively. Each of these maps contain two or more markers common to the map of the *Yd2* region constructed using the Proctor × Shannon F_2 mapping population. As such, these maps provided the opportunity to compare the rates of recombination observed in the *Yd2* region for three different crosses. The two markers *Xbcd828* and *Xbcd809* were positioned 8.4 cM apart by Graner *et al.* (1994), which is similar to the distance of 8.2 cM obtained in this study. However, on the map constructed by Kleinhofs *et al.* (1993), the distance between the RFLP loci *XksuA3* and *Xpsr156* is 28.9 cM, which is nearly three times greater than the distance of 11.2 cM determined in this study. The numbers of recombinant and non-recombinant chromosomes observed for the *XksuA3-Xpsr156* interval in the Steptoe × Morex F_2 population were obtained from the Grain-Genes data base on the Internet, and were compared with the corresponding numbers observed in the Proctor × Shannon F_2 mapping population using a χ^2 contingency test. However, the differences in the recombination rate for these two crosses were not significant ($P > 0.10$).

The 210 cM long genetic map of barley chromosome 3 by Graner *et al.* (1994) contains 73 markers, and as such, is the most extensive genetic map of barley chromosome 3 published. The value of 210 cM is therefore the closest available estimate of the total genetic length of barley chromosome 3, and shows that the 27.6 cM map of the *Yd2* region spans approximately 12% of the total genetic length of barley chromosome 3. The location and extent of the region of the region of barley chromosome 3 mapped in this study is illustrated in Figure 3.2.

3.4 Discussion

3.4.1 The value of the markers mapped close to *Yd2* in barley breeding

Considering the difficulties associated with performing BYDV resistance assays in the field (see Introduction), molecular markers closely linked to *Yd2* may provide breeders with a more convenient means of selecting for the *Yd2* gene. Typically, BYDV resistant barley cultivars have been produced using backcrossing programs designed to introgress the *Yd2* gene into genetic backgrounds of agronomically adapted barley cultivars. Up to seven backcrosses have been used in these breeding programs (Schaller and Chim, 1969a, 1969b; Schaller *et al.*, 1973, 1977, 1979; Vertigan, 1979). To expect a greater than 95% chance of retaining the *Yd2* gene throughout a breeding program of seven backcrosses using marker aided selection, the marker would need to be located within 0.64 cM of *Yd2*[2]. On this basis, the six RFLP markers mapped closest to *Yd2* in this study (Figure 3.3) would be expected to enable the selection of *Yd2* throughout such a breeding program.

The extent of the *Yd2*-containing introgressed chromosome segments observed in the cultivars CM67, Atlas68 and Shannon (Figure 3.6) indicates that the six RFLP markers mapped closest to *Yd2* would have been effective in maintaining the *Yd2* gene throughout the six backcrosses used to breed Atlas68 (Schaller *et al.*, 1969a), or the four backcrosses used to breed Shannon (Vertigan, 1979), if these markers had been used for selection instead of BYDV resistance assays. However, during the seven backcrosses used to breed CM67 (Schaller and Chim, 1969b), the RFLP markers *XksuA3*, *Xbcd134*,

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[2] This figure was obtained by assuming that the number of recombinant chromosomes observed follows a binomial distribution, with the number of sample units = no. of opportunities for recombination to occur during the breeding program, and the probability of recombination occurring at each opportunity = the genetic distance between the loci (expressed as a recombination fraction). The number of opportunities for recombination during a breeding program of seven backcrosses was taken to be eight, counting one for each of the seven backcross, and one for the generation of inbreeding that must be used to make the cultivar homozygous for *Yd2*. Because of the small distances involved, recombination fraction can be regarded as equivalent to genetic distance in cM.

Xbcd127 and *XYlp*, would not have been effective in selecting for Yd_2, despite the fact that these markers were mapped within 0.64 cM of Yd_2. Recombination between these markers and the Yd_2 gene during the breeding of CM67 may have been due to a particularly high rate of recombination between the parents of this cultivar, or to intentional selection by the breeders for a return to the genetic background of the recurrent parent. This observation shows that in practice, the selection of a single marker located within 0.64 cM of Yd_2 may not be sufficient for the maintenance of Yd_2 during seven or more backcrosses. In light of this, it is recommended that the closest possible marker to Yd_2 be used for selection, and that the Yd_2 status of the resulting line be confirmed using a BYDV resistance assay. Alternatively, the presence of Yd_2 could be ensured by simultaneously selecting for two RFLP markers that closely flank Yd_2. The map of the Yd_2 region contains a number of markers located to either side of Yd_2 that could be used for such a selection strategy.

Comeau *et al.* (1990) observed close genetic linkage between Yd_2 and a number of undesirable traits in Ethiopian barleys, including excessive height, severe lodging, and reduced yield and grain quality under virus-free growing conditions. The linkage between these traits and Yd_2 has prevented the release of a Yd_2-containing cultivar by the barley breeding program at Quebec, Canada, despite 20 years of effort (Comeau, 1994b). It is possible that the molecular markers identified in this study could be used to help break these linkages. Large segregating populations could be screened using RFLP markers close to Yd_2 to identify individuals resulting from recombination events very close to and to either side of Yd_2. These recombinants could then be crossed to derive Yd_2-containing lines possessing adequate agronomic characteristics.

Holloway and Heath (1992) identified a protein encoded for by a gene which was likely to be closely linked to Yd_2, and proposed that it could be used by breeders to assist in the selection of Yd_2. In this study, a probe derived from this gene was used to map the RFLP locus *XYlp*. In the mapping population of 106 Proctor × Shannon F_2 individuals, *XYlp* cosegregated perfectly with the Yd_2 gene, and was thereby likely

(95% chance) to be located within 1.4 cM of Yd_2[3]. This placement of *XYlp* is consistent with the estimation by Holloway and Heath (1992) that the gene for the protein marker is located within 9.0 cM of Yd_2. The close proximity of *XYlp* to Yd_2 also confirms that this locus may be a useful marker for Yd_2. An added virtue of the *XYlp* marker is that it appears to be associated with a microsatellite. Due to the high levels of polymorphism shown by microsatellites (Saghai Maroof *et al.*, 1994; Becker and Heun, 1995; Röder *et al.*, 1995), the *XYlp* locus is more likely to be polymorphic for any given breeder's cross than most other RFLP loci. Furthermore, polymorphisms at microsatellite loci are amenable to detection using PCR-based assays (Saghai Maroof *et al.*, 1994; Becker and Heun, 1995). A number of simple procedures are available for preparing large numbers of plant DNA samples suitable for PCR analysis (Wang *et al.*, 1993; Williams and Ronald, 1994; Steiner *et al.*, 1995). Therefore, a PCR-based assay for the detection of the *XYlp* locus may provide a more convenient means of selecting for Yd_2 in large breeding populations than an RFLP assay. The findings of this study therefore provide further evidence that this marker locus may be useful to breeders, as originally suggested by Holloway and Heath (1992). However, an RFLP or PCR based assay for the locus is likely to be more appropriate than the protein-based assay proposed by Holloway and Heath (1992).

As a result of this study, RFLP markers were identified which have potential to be used to assist in the selection of Yd_2 or to break the linkage between Yd_2 and agronomically undesirable traits. The need for such markers as aids in the production of new BYDV resistant barley cultivars has been expressed by eight barley breeding groups from around the world, who have actively sought the results of the work described here (Appendix A).

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[3]Calculated by assuming that the number of recombinant chromosomes follows a binomial distribution, with the number of sample units = the number of chromosomes observed in the mapping population of 106 individuals (212 chromosomes), and the probability of recombination occurring at each opportunity = the genetic distance between the loci (expressed as a recombination fraction). Because of the small distances involved, recombination fraction can be regarded as equivalent to genetic distance in cM.

3.4.2 Implications of the RFLP map for the map-based isolation of *Yd2*

Apart from its usefulness to breeders, the genetic map of the *Yd2* region of barley chromosome 3 described here could be used to initiate a project to isolate the *Yd2* gene by a map-based approach. As the isolation of the *Yd2* gene is a goal we would like to achieve in this laboratory, it is of interest to us to assess the usefulness of the map generated thus far in such a project.

In the first stage of a map-based gene isolation program, the molecular marker mapping closest to the gene of interest is used to identify a clone from a genomic DNA library. If this clone does not contain the gene, then a series of overlapping genomic DNA clones can be identified by chromosome walking until one containing the gene is found. Repetitive genomic DNA sequences hamper chromosome walking. Therefore, for organisms such as barley which have genomes of high repetitive sequence content, it is advisable to minimise the number of chromosome walking steps, or to avoid chromosome walking altogether (Tanksley, 1995). For this reason, we would prefer to identify markers which are close enough to *Yd2* to enable the identification of a barley genomic DNA clone containing *Yd2* in one step without chromosome walking.

In map-based gene cloning programs, genomic DNA clones used to bridge the gap between the closest marker and a target gene are most frequently obtained from libraries with large inserts, created using Yeast Artificial Chromosome, or YAC vectors (Burke *et al.*, 1987) or Bacterial Artificial Chromosome, or BAC vectors (Shizuya *et al.*, 1992). Although these vectors are capable of cloning DNA fragments larger than 300 kbp, the majority of YAC and BAC libraries constructed in plants have average insert sizes of around 150 kbp (Grill and Somerville, 1991; Martin *et al.*, 1992; Kleine *et al.*, 1993; Woo *et al.*, 1994; Wang *et al.*, 1995). Therefore, for a marker to have a reasonable chance of identifying a YAC or BAC barley genomic DNA clone containing *Yd2*, the physical distance separating the marker and *Yd2* should be no greater than 150 kbp.

Genetic distance cannot be used to accurately calculate physical distance in plant genomes (Schulze-Lefert, 1995). To ensure that the closest flanking markers are located within 150 kbp of *Yd2* and of potential use in screening a library for a *Yd2*-containing barley genomic DNA clone, the physical distance separating these markers will need to be determined empirically by physical mapping (Ganal *et al.*, 1989; Ronald *et al.*, 1992; Wu and Tanksley, 1993). This would involve the digestion of barley genomic DNA with restriction enzymes that cut barley DNA rarely, the resolution of the large restriction fragments by pulsed field gradient gel electrophoresis (Schwartz and Cantor, 1984), and the Southern analysis of the fractionated restriction fragments (Southern, 1975). If the two RFLP probes flanking *Yd2* are found to hybridise to a single DNA fragment smaller than 300 kbp, then at least one of these probes must be closer to *Yd2* than 150 kbp.

Although physical mapping will ultimately be required to determine whether markers mapped in this exercise are located sufficiently close to *Yd2* to be useful in library screening, a rough indication of the likelihood of this can be obtained from the genetic distances observed between these markers.

In this study, the *Yd2* gene was located 0.5 cM from the centromere on the long arm of barley chromosome 3. The location of *Yd2* has important implications for an attempt to isolate *Yd2* by a map-based approach, as relatively low rates of recombination have been encountered in this chromosome region by Linde-Laursen (1982). In the study of Linde-Laursen (1982), chromosome C-bands observed by microscopy to divide the long arm of barley chromosome 3 into three segments of approximately equal length were mapped as genetic markers in an F_2 population. Although the total genetic length of the long arm of barley chromosome 3 is approximately 134 cM (Graner *et al.*, 1994), the genetic lengths of the proximal and middle thirds of this chromosome arm were observed to be 0.0 cM and 12 cM, respectively. This demonstrates that recombination in this chromosome arm is confined mostly to the distal region. A relatively low rate of recombination close to the centromere has also been shown for a number of chromosomes in wheat and rye, and

may be a general feature of chromosomes in these cereal species (Dvořák and Chen, 1984; Lawrence and Appels, 1986; Kota *et al.*, 1993; Hohmann *et al.*, 1994; Delaney *et al.*, 1995a). The barley genome has an average ratio of physical to genetic distance of approximately 3.5×10^6 bp/cM, based on a total genetic length of 1,400 cM (Graner *et al.*, 1994) and a physical size of 4.9×10^9 bp (Arumuganathan and Earle, 1991). However, considering the findings of Linde-Laursen (1982), the ratio of genetic to physical distance in the *Yd2* region can be conservatively estimated to be five times greater than the average for the barley genome, or 1.8×10^7 bp/cM. Markers located within 0.0083 cM of *Yd2* would therefore be appropriate candidates for use in physical mapping.

In this exercise, the two RFLP markers *XYlp* and *Xwg889* perfectly cosegregated with the *Yd2* gene in the mapping population of 106 F_2 individuals. Based on this observation, the chance that each of these markers are located within 0.0083 cM of *Yd2* is only 1.7% (see Footnote 3). Therefore, it is highly unlikely that these markers will be shown to be suitable for the identification of a *Yd2*-containing clone. The identification of markers suitable for physical mapping and library screening will most probably require the addition of greater numbers of markers to the map of the *Yd2* region, and an improvement of the genetic resolution of the map to identify those markers flanking *Yd2* most closely.

3.4.3 Improvement of the genetic map of the *Yd2* region

The density of markers in the 2.0 cM surrounding *Yd2* on the map of the *Yd2* region is approximately 2.6 markers/cM. An increase in this marker concentration to about 120 markers/cM will provide a marker for every 0.0083 cM, on average, and will thereby present a good chance of identifying a marker sufficiently close to *Yd2* to be useful in physical mapping and subsequent screening of a barley genomic DNA library for a *Yd2* containing clone.

RFLP loci mapped relative to *Yd2* on barley chromosome 3 in this study were mapped by other researchers in similar positions, on chromosomes belonging to

Triticeae group 3 in wheat, rye and *T. tauschii*. These data and the comparative mapping data presented Nelson *et al.* (1995b) reflect a general conservation in chromosome structure between members of the Triticeae tribe. Other comparative mapping studies have also demonstrated RFLP marker collinearity between Triticeae chromosomes and chromosomes of cereal species from other tribes, such as maize, oat and rice, although this collinearity is generally broken at a greater number of points than the collinearity observed within the Triticeae (Section 1.5.3).

Regions of known marker collinearity between the genomes of cereals can be used to identify markers likely to map close to Yd_2 in barley from the genetic maps of other cereal species. A number of RFLP markers on the map of the Yd_2 region also occur on maps of rice and oat (Kurata *et al.*, 1994a; Causse *et al.*, 1994; Van Deynze *et al.*, 1995b) as well as on maps of Triticeae species (Table 3.5), and can be used as reference points to assist in the selection of the most appropriate markers from these maps. At present, 38 additional RFLP clones predicted to map close to Yd_2 have been obtained from the cereal mapping group at Cornell University, and from the Rice Genome Research Program in Tsukuba, Japan. Currently these clones are being mapped relative to Yd_2 in this laboratory. Twelve RFLP clones mapped to the central region of barley chromosome 3 by Graner *et al.* (1994) have also been obtained for mapping close to Yd_2.

RFLP markers that can be obtained from cereal mapping programs represent a finite resource. Therefore, it is likely that molecular markers will also need to be obtained by other means in order to obtain the marker density required for the map-based isolation of Yd_2. One technique that may be effective in generating these additional markers is Genetically Directed Representational Difference Analysis, which based on subtractive hybridisation (Delaney *et al.*, 1995b). This technique is assessed in Chapter 4, where other alternative methods for marker generation are also discussed. Another possible approach to generating further markers close to Yd_2 makes use of the small size of the rice genome and marker collinearity between the genomes of rice and barley. In Chapter 6, this approach is described in detail, and its feasibility is evaluated.

When a sufficient density of RFLP markers in the *Yd2* region are obtained, a high degree of genetic resolution will be required to identify the two markers that most closely flank *Yd2*. Expansion of the mapping population to 6,000 F_2 individuals will provide a recombination event for every 0.0083 cM, on average, and would therefore present a good chance of ordering markers spaced 150 kbp apart. The analysis of such a large mapping population would involve a considerable investment of time and resources. Therefore, it would be worthwhile considering strategies that could make this task easier. A potential strategy that could facilitate high-resolution genetic mapping of the *Yd2* locus involves the use of morphological markers flanking the gene. This approach is explored in Chapter 5, where alternatives to this approach are also discussed.

In this study, the rates of recombination observed in the *Yd2* region for the Proctor × Shannon cross and the crosses used for mapping by Kleinhofs *et al.* (1993) and Graner *et al.* (1994) were not found to be significantly different. Despite this, barley crosses showing significant differences in recombination rates for a particular chromosome interval have been previously identified (Säll, 1990; Säll *et al.*, 1990). Therefore, it may be possible to identify a cross between a *Yd2* and non-*Yd2* barley line that shows a higher rate of recombination in the vicinity of *Yd2* than the Proctor × Shannon cross, and to use this cross to map the *Yd2* region with greater efficiency.

When a barley genomic clone spanning the *Yd2* locus is obtained, genes within it could be identified by direct selection techniques (Lovett, 1994). Rice is estimated to contain approximately 30,000 genes (Yamamoto *et al.*, 1995), and hybridisation studies using cDNA probes indicate that rice contains a similar number of genes to other cereals (Section 1.5.2). Non methylated CpG and CpXpG sites are less concentrated towards the centromeres of cereal chromosomes, suggesting that genes are more sparsely distributed there (Moore *et al.*, 1993). Considering this, and the size of the barley genome (4.9×10^9 bp; Arumuganathan and Earle, 1991), each 150 kbp in the vicinity of *Yd2* would be expected to contain fewer than 0.92 genes, on average. A 150 kbp YAC or BAC genomic DNA clone spanning the *Yd2* locus is therefore unlikely to

contain a large number of candidate genes which would otherwise need to be distinguished from *Yd2* by high resolution genetic mapping. It is conceivable that the *Yd2* gene could be identified from among such a small number of candidate genes by genetic transformation. Therefore, once a genomic DNA clone containing *Yd2* is obtained, no further genetic mapping is likely to be required.