CHAPTER 6

RFLP MARKER COLLINEARITY BETWEEN RICE CHROMOSOME 1 AND THE *Yd*$_2$ REGION OF BARLEY CHROMOSOME 3

6.1 Introduction

The evolution of members of the grass (Gramineae) family from a common ancestral species has involved considerable expansion and contraction in genome size, due mainly to the amplification and deletion of repetitive DNA sequences. These changes in genome size have been accompanied by very little change in the content of genes and other low copy sequences (reviewed in Section 1.5). Low copy sequences in the genomes of different grass species which are related by their descent from single ancestral sequence (orthologous) usually show enough sequence homology to enable their identification by a single probe. This has allowed common sets of probes to be used to map othologous sequences in different grasses as RFLP loci. These comparative RFLP mapping studies have shown that the order of low copy sequences along the chromosomes has also been considerably conserved during the evolution of the grasses (Section 1.5.3).

At 4.2×10^8 bp, the genome of rice is one of the smallest grass genomes known (Arumuganathan and Earle, 1991). In comparison, the genomes of economically important cereals such as maize, barley, and hexaploid wheat are approximately 6, 12, and 38 times larger, respectively (Arumuganathan and Earle, 1991). In the small genome of rice, the physical distances separating low copy sequences are expected to be much shorter than those separating sequences orthologous to these in larger cereal genomes. Therefore, it may be possible to use rice genomic DNA clones to assist in the map-based isolation of genes of interest from other cereals, by using them to bridge the large physical distances separating the target genes from the closest markers (Moore *et al.*, 1993; Kurata *et al.* 1994b).

In Chapter 3 it was estimated that a marker density of approximately 120 markers per cM would be required to identify markers suitable for the isolation of a Yd_2-containing barley genomic DNA clone. In Chapter 4, techniques for the generation of the large numbers of markers required for the map-based isolation of Yd_2 were discussed, and one of these was evaluated. An alternative to these techniques would be to identify a large insert genomic DNA clone using markers most closely flanking Yd_2,

and to use subclones to generate RFLP markers close to Yd_2. However, this strategy could only be used if the collinearity between orthologous sequences in rice and barley extends to sequences very close to Yd_2.

The aim of this chapter is to determine the extent of collinearity between sequences close to Yd_2 in barley and their orthologous counterparts in rice, in order to assess the feasibility of using rice to assist in the map-based isolation of Yd_2. This is investigated in two ways. Firstly, probes mapped close to Yd_2 in Chapter 3 are mapped in rice using a segregating F_2 population created especially for this purpose. Secondly, the location of RFLP markers mapped close to Yd_2 in Chapter 3 are compared to the positions of RFLPs mapped in rice by other researchers using the same probes.

6.2 Materials and methods

6.2.1 Acknowledgments

The author is sincerely grateful to Laurie Lewin for his generous donation of F_2 rice seeds.

The extraction of genomic DNA from the Doongara × Hungarian #1 F_2 individuals, and the preparation of membranes for the RFLP analysis of these DNA samples were performed in collaboration with Nick Paltridge.

6.2.2 Rice lines and F_2 rice seed

F_2 rice seed derived from the crosses Doongara × Hungarian #1, Hungarian #1 × YRM34, and Doongara × Inga, and seed of the parent lines of these crosses, were kindly supplied by Laurie Lewin (New South Wales Department of Agriculture, Yanco Agricultural Institute, Australia). YRM34, Hungarian #1 and Inga are Japonica type rice lines, while Doongara is derived from a cross between an Indica rice line and a Japonica rice line, and resembles a Japonica type most closely (Laurie Lewin, personal communication).

6.2.3 RFLP analysis of rice F_2 populations

The general procedures used for RFLP analysis were those outlined in Section 2.2.6. To identify RFLPs which were potentially mappable in each of the three available rice F_2 populations, genomic DNA of the four parent lines Doongara, Hungarian #1, YRM34 and Inga was digested separately with a range of restriction enzymes and subjected to RFLP analysis with probes mapped close to *Yd*$_2$ in Chapter 3. Probes KSUH7 and TAG538 were also tested. These two probes detected *Yd*$_2$-associated polymorphisms in Chapter 3 but could not be mapped due to a lack of polymorphism in the Proctor × Shannon F_2 mapping population. Each probe was tested with six 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, *Hin*dIII, *Hae*III, *Hin*fI, *Hpa*II, *Kpn*I, *Nco*I, *Nde*I, *Rsa*I, *Sau*3AI, *Taq*I and *Xba*I.

Segregation analysis of 56 Doongara × Hungarian #1 F_2 individuals was performed using the computer program MAPMAKER (Lander *et al.* 1987), as described in Section 2.2.7.

6.3 Results

6.3.1 Results of the screen for RFLPs

The results of the search for RFLPs between the parents of the three rice crosses are shown in Table 6.1. All of the probes produced hybridisation patterns containing clear bands, except for the wheat genomic DNA probes TAG538 and TAG223 which showed no apparent hybridisation to rice DNA. Of the 14 probes showing clear hybridisation to rice DNA, the numbers that identified RFLPs between the parents of the Doongara × Hungarian #1, Hungarian #1 × YRM34, and Doongara × Inga crosses were eight, seven and four, respectively. The maximum number of loci that each probe detected in the rice genome was determined on the basis of the numbers of hybridising restriction fragments observed (Table 6.1).

Table 6.1. Results of the screen for RFLPs between the parent lines of three rice crosses

probe	no. of rest. enzymes tested	maximum number of loci detected in rice (and barley) †	restriction enzymes that detected RFLPs¥		
			Doongara × Hungarian #1	**Hungarian #1 × YRM34**	**Doongara × Inga**
TAG223	6	0 (1)	no clear hybridisation		
BCD127	20	1 (1)	none	none	none
BCD134	20	1 (1)	none	none	none
AWPh7d	14	3 (3)	*Nde*I, ***Hpa*II**, *Ban*II, *Alu*I, ***Rsa*I**, *Taq*I, *Hinf*I	*Hpa*II, *Hinf*I	not tested
YLP	20	1 (1)	***Cla*I**	*Cla*I	none
WG889	6	5 (5)	none	none	none
BCD828	20	2 (2)	*Ban*II, *Dra*I, ***Eco*RV**, *Nco*I, *Rsa*I, *Sau*3AI	none	none
BCD263	6	2 (1)	*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, ***Hin*dIII**	*Bam*HI	*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII
TAG80	6	3 (1)	none	none	none
BCD809	20	2 (2)	*Bst*NI, *Dra*I, *Eco*RI, ***Eco*RV**, *Hpa*II, *Xba*I	*Bst*NI, *Dra*I, *Eco*R1, *Eco*RV, *Xba*I	none
PSR156	20	1 (1)	none	none	none
WG178	6	1 or 0 (1)	hybridised weakly to Hungarian #1 DNA	hybridised weakly to Hungarian #1 DNA	*Bam*HI, *Dra*I, *Eco*RI, *Eco*RV
KSUG59	6	1 (1)	none	none	none
CDO718	6	2 (2)	***Eco*RV**, *Xba*I	*Eco*RV, *Xba*I	*Xba*I
KSUH7	6	3 ()	*Eco*RI, *Eco*RV, ***Hin*dIII**	*Eco*RV	*Eco*RI, *Hin*dIII
TAG538	6	0 (1)	no clear hybridisation		
Total no. of probes that detected RFLPs			**8**	**7**	**4**

†The maximum number of loci detected equals the minimum number of restriction fragments detected using any one restriction enzyme. Maximum number of loci detected by each probe in barley in Chapter 3 are shown in brackets.

¥Restriction enzymes used for mapping in the Doongara × Hungarian F_2 population are shown in bold.

6.3.2 RFLP map construction using the Doongara × Hungarian #1 F_2 population

As the greatest number of polymorphic probes were found for the Doongara × Hungarian #1 cross, the F_2 family resulting from this cross was the one chosen for mapping. The probes that detected polymorphism between Doongara and Hungarian #1 were used to characterise 56 F_2 individuals from this family, using the restriction enzymes indicated in Table 6.1.

Out of the eight polymorphic probes, only KSUH7 and WG178 could not be used for mapping RFLP loci. Hybridisation of the probe KSUH7 was too weak to enable the segregating restriction fragments to be scored accurately. The probe WG178 hybridised with less intensity to the DNA of Hungarian #1. However, the hybridisation intensities of this probe to individuals heterozygous or homozygous for this polymorphism were not distinct enough to enable this polymorphism to be confidently scored in the mapping population.

The results obtained by mapping RFLPs in rice are illustrated in Figure 6.1. Loci mapped using the probes AWPh7d, YLP, BCD263 and BCD809 were linked to one another (LOD > 3), and were used to produce a genetic map (Figure 6.1). The order of these linked loci was the same as the order of the RFLP loci mapped on barley chromosome 3 using the same probes in Chapter 3. AWPh7d was used to characterise the mapping population for a *Rsa*I polymorphism (all 56 F_2 individuals) as well as a *Hpa*II polymorphism (10 F_2 individuals[4]). Only one recombination event was observed between these two RFLPs, indicating that they were positioned at distinct but closely linked loci (Figure 6.1). The probe BCD263 revealed two *Hin*dIII polymorphisms. One of these mapped within the main linkage group, while the other segregated independently. The RFLP loci mapped using CDO718 and BCD828 were not linked to the main group or to one another (LOD < 2).

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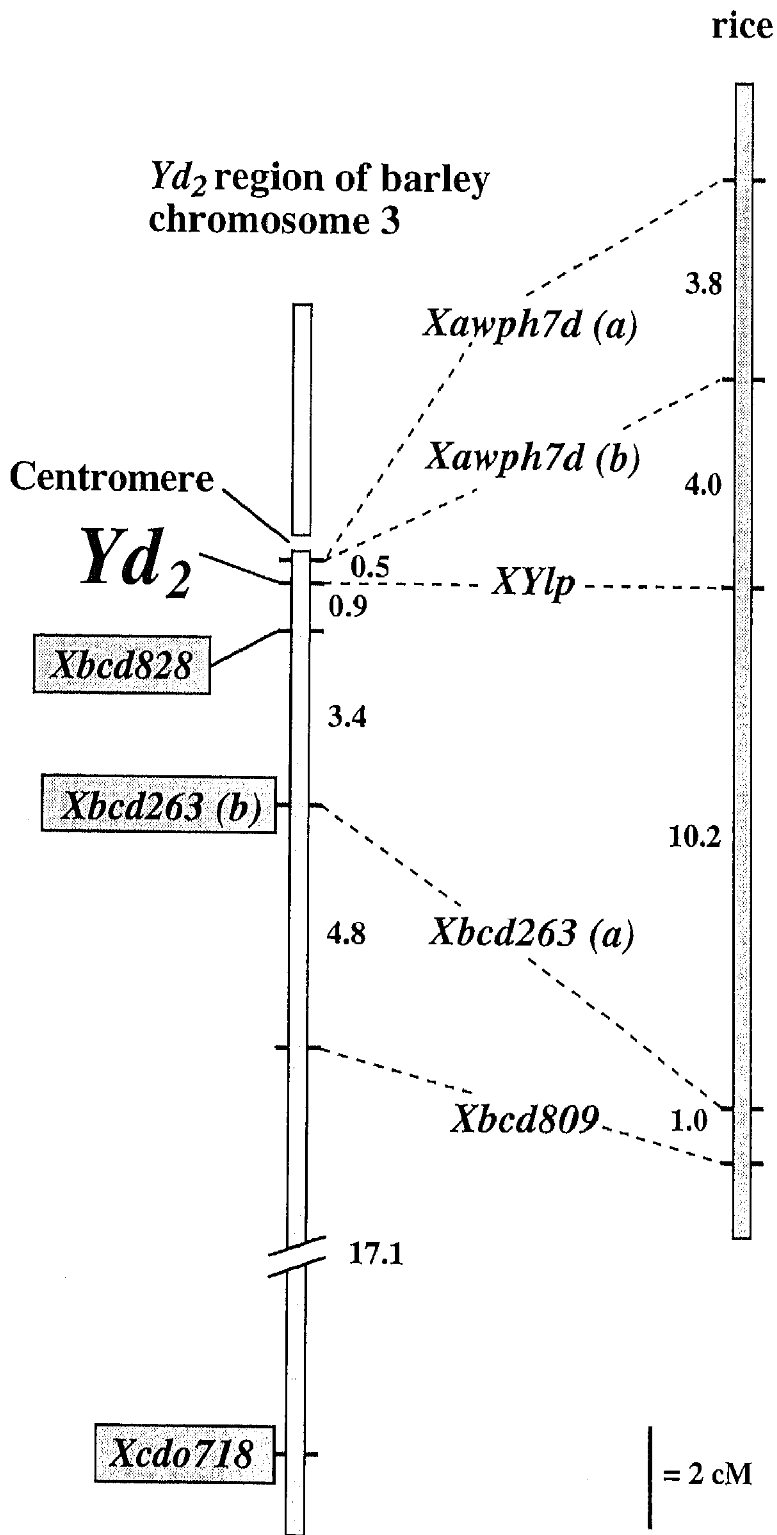
[4]These individuals included all four recombinants for the interval between the *XYlp* locus and the *Rsa*I RFLP detected using AWPh7d.

Fig 6.1. Positions of RFLP loci mapped in rice using the Doongara × Hungarian #1 F_2 population, using probes mapped close to Yd_2 on barley chromosome 3

Shown here are the locations of RFLPs mapped in rice using the Doongara × Hungarian #1 F_2 population, and the locations of RFLPs mapped close to Yd_2 on barley chromosome 3 using the same probes. The map of the Yd_2 region is adapted from Figure 3.2 (Chapter 3). Six probes were used to map eight RFLP loci in rice, five of which mapped to a single linkage group. The loci that did not map to this linkage group are boxed. The probe AWPh7d was used to map separate loci using the restriction enzymes *Hpa*II (locus a) and *Rsa*I (locus b). BCD263 was used to map two loci using *Hin*dIII (loci a and b). Distances are in cM.


rice
Yd2 region of barley chromosome 3
Centromere
Yd2
Xawph7d (a)
Xawph7d (b)
XYlp
Xbcd828
Xbcd263 (b)
Xbcd263 (a)
Xbcd809
Xcdo718
0.5
0.9
3.4
4.8
17.1
3.8
4.0
10.2
1.0
= 2 cM

6.3.3 RFLP marker collinearity between the map of the Yd_2 region of barley and published maps of rice chromosome 1

The RFLP probes BCD134, BCD828, CDO718, PSR116 and PSR156 were mapped close to Yd_2 on barley chromosome 3 in Chapter 3, and had also been used by Causse *et al.* (1994) and Kurata *et al.* (1994a) to map RFLP loci in rice. All of these RFLP loci were mapped close to the middle of rice chromosome 1, except for the locus mapped by Causse *et al.* (1994) to rice chromosome 2 using the probe CDO718 (Figure 6.2).

6.4 Discussion

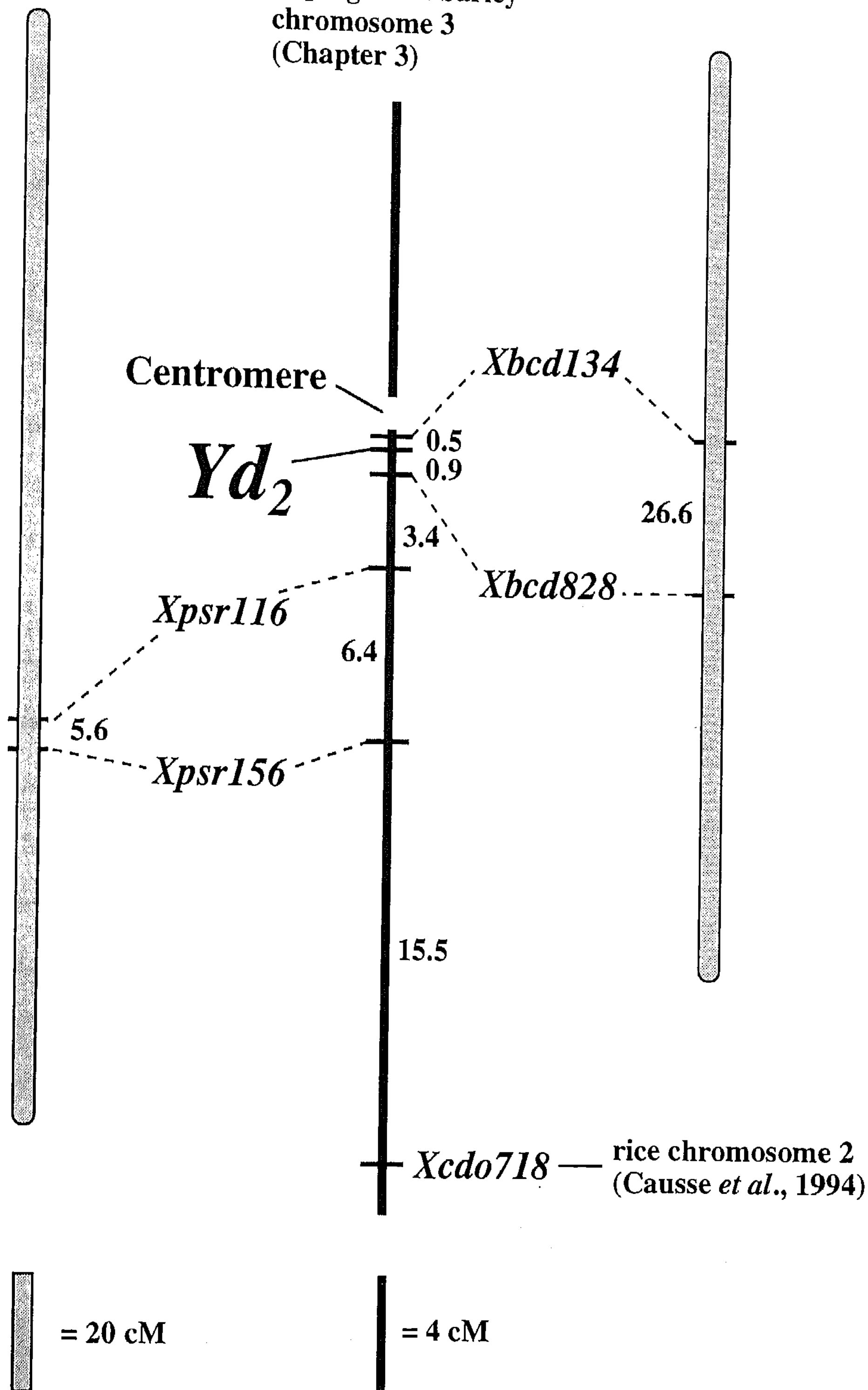
6.4.1 Which region of the rice genome contains sequences orthologous to those close to Yd_2 on barley chromosome 3?

In Chapter 3, a genetic map of the Yd_2 region of barley chromosome 3 was constructed, containing RFLP markers and the Yd_2 gene for BYDV resistance. In this study, the RFLP probes used for mapping the Yd_2 region were used to locate RFLPs in rice, using a segregating F_2 population derived from a cross between the rice lines Doongara and Hungarian #1. Of the eight loci mapped using the Doongara × Hungarian #1 F_2 population, five mapped close together, in the same order as the loci mapped close to Yd_2 using the same probes. This demonstrates that rice sequences orthologous to those close to Yd_2 in barley are mainly confined to one region of the rice genome.

Five probes previously used to map RFLP loci close to the Yd_2 gene on barley chromosome 3 (Chapter 3) had been used by Causse *et al.* (1994) and Kurata *et al.* (1994a) to map RFLP loci in rice. Out of these five probes, four were used to map loci close to the middle of rice chromosome 1. This implies that the main region of the rice genome with orthology to the Yd_2 region of barley corresponds to the middle of rice chromosome 1. In the comparative mapping studies by Kurata *et al.* (1994b) and Ahn *et al.* (1993), extensive collinearly was observed between RFLP markers located on rice chromosome 1 and wheat homoeologous chromosome group 3. Chromosomes from wheat homoeologous chromosome group 3 also show a high degree of RFLP marker

Fig 6.2. The location of RFLP markers mapped in rice by other researchers, using probes mapped close to Yd_2 in Chapter 3

Shown here are the locations of RFLP loci mapped in rice by Causse *et al.* (1994) and Kurata *et al.* (1994a) using probes that had been used to construct the map of the Yd_2 region of barley chromosome 3 in Chapter 3. All of these loci in rice were mapped to rice chromosome 1, except for the locus mapped by Causse *et al.* (1994) to rice chromosome 2 using the probe CDO718. The shaded rods represent the full-length maps of rice chromosome 1 by Causse *et al.* (1994) and Kurata *et al.* (1994a), and are presented in a different scale to the map of the Yd_2 region. The dashes between the maps indicate loci detected using the same probes. Distances are in cM.



rice chromosome 1
(Kurata et al., 1994a)
Yd2 region of barley
chromosome 3
(Chapter 3)
rice chromosome 1
(Causse et al., 1994)
Centromere
Xbcd134
0.5
0.9
26.6
3.4
Xbcd828
Xpsr116
6.4
5.6
Xpsr156
15.5
Xcdo718
rice chromosome 2
(Causse et al., 1994)
= 20 cM
= 4 cM

collinearity with barley chromosome 3 (Nelson *et al.*, 1995b). Therefore, the studies of Kurata *et al.* (1994b) and Ahn *et al.* (1993) provide indirect evidence that rice sequences orthologous to those on barley chromosome 3 are mainly located on rice chromosome 1. The map constructed in rice using the Doongara × Hungarian #1 F_2 population can therefore be tentatively assigned to rice chromosome 1, and will hereafter be referred to as the putative rice chromosome 1 linkage group.

6.4.2 Implications of probes that detect multiple loci

The probe BCD263 detected a locus within the putative rice chromosome 1 linkage group, but was also found to detect a second, completely unlinked locus in rice. Similarly, the probe BCD828 detected an RFLP in the Doongara × Hungarian F_2 mapping population which was not linked to the putative rice chromosome 1 linkage group, but was used by Causse *et al.* (1994) to map an RFLP close to the middle of rice chromosome 1. The loci mapped using BCD263 and BCD828 in barley therefore appear to have orthologous counterparts in other regions of the rice genome as well as on rice chromosome 1.

The probe CDO718 detected an RFLP in the Doongara × Hungarian F_2 population which was not located within the putative rice chromosome 1 linkage group. This probe may hybridise to two loci in the rice genome, judging from the number of restriction fragments it detected in digests of rice genomic DNA (Table 6.1). Therefore, it is possible that this probe detected a second locus on rice chromosome 1 which was monomorphic and therefore not mappable in the Doongara × Hungarian F_2 population. For this reason, the result obtained using CDO718 does not necessarily imply a breakdown in orthology between barley chromosome 3 and rice chromosome 1. Causse *et al.* (1994) used CDO718 to map an RFLP locus on rice chromosome 2. This chromosome 2 locus may have been the same as the RFLP locus mapped in the Doongara × Hungarian F_2 population using CDO718.

The probe AWPh7d was used to map one locus in barley, but detected two closely linked RFLP loci in the putative rice chromosome 1 linkage group. In Chapter

3, it was shown that this probe may detect 3 loci in barley, judging from the number of restriction fragments it hybridised to (Table 6.1). Therefore, this probe may have hybridised to a second locus in the *Yd*$_2$ region which was not mapped in the Proctor × Shannon F_2 barley mapping population in Chapter 3. Because of this possibility, the occurrence of two closely linked loci in rice with homology to AWPh7d does not necessarily imply that a duplication within rice chromosome 1 or a deletion within barley chromosome 3 occurred after the divergence of rice and barley from a common anscestor.

Overall, the comparative RFLP mapping data presented here are consistent with there being complete collinearity of orthologous sequences between the *Yd*$_2$ region of barley and rice chromosome 1. The data obtained using some of the RFLP probes may at first glance appear to imply a breakdown in this collinearity. However, these results can be attributed to the fact that each of these probes may detect more than one locus in barley or rice.

6.4.3 Using the RFLP marker collinearity between barley and rice to obtain further RFLP markers close to *Yd*$_2$ in barley

In this chapter, a number of RFLP markers showed conserved order in barley and rice, including markers which were located to within 1.0 cM of *Yd*$_2$ and to both sides of it. This suggests that no translocations, large deletions or other major chromosomal rearrangements occurred close to the point corresponding to the *Yd*$_2$ locus during the evolution of barley and rice from a common ancestor. Kilian *et al.* (1995) and Dunford *et al.* (1995), demonstrated small-scale conservation in RFLP marker linkage for a number regions of the rice and barley genomes, by showing that markers located within 1.0 cM of one another in rice were also closely linked in barley. The conserved RFLP marker linkage shown in this study is therefore likely to extend to sequences immediately surrounding the *Yd*$_2$ locus. Overall, there would appear to be adequate conservation in RFLP marker collinearity between the *Yd*$_2$ region of barley

and the corresponding region of the rice genome to allow rice to be used to generate further markers close to Yd_2.

The total length of the detailed rice genetic map constructed by Kurata *et al.* (1994a) is 1,575 cM, and the physical size of the rice genome is approximately 4.2 x 10^8 bp (Arumuganathan and Earle, 1991), indicating that the rice genome has an average ratio of physical to genetic distance of approximately 270 kbp/cM. The average insert size of plant genomic DNA libraries constructed using YAC or BAC vectors is approximately 150 kbp (Woo *et al.*, 1994, and references therein). Therefore, it should be possible to use two markers which flank Yd_2 in barley and which map within 0.5 cM of one another in rice to identify YAC or BAC rice genomic DNA clones suitable for the generation of further RFLP markers close to Yd_2. There is a good chance that a pair of markers with these properties exists among those markers already mapped relative to Yd_2 in barley, judging from the expected density of these markers in rice (Compare the map of the Yd_2 region in Figure 3.2, Chapter 3, with the map of the putative rice chromosome 1 linkage group in Figure 6.1.). To test if these existing markers may be useful in this strategy, markers which were not resolved from Yd_2 in Chapter 3 could be mapped to a higher resolution to identify the pair of markers which most closely flank Yd_2. This pair of markers could then be used to perform physical mapping in rice to determine if they are located within 150 kbp of one another in the rice genome and therefore suitable for screening a YAC or BAC rice genomic DNA library.

Once a large insert rice genomic DNA clone containing sequences orthologous to those spanning Yd_2 in barley is obtained, it could provide an excellent source of RFLP markers mapping very close to Yd_2. However, initially, further markers may need to be obtained by other means and mapped in the Yd_2 region before a pair of markers suitable for the isolation of such a clone can be identified. Approaches to obtaining further markers for mapping close to Yd_2 were discussed in Chapters 3 and 4. One of these approaches was to use published maps of barley and other Triticeae species to identify probes likely to detect RFLPs close to Yd_2. Given the RFLP marker collinearly

demonstrated in this chapter between barley and rice, it should also be possible to exploit published maps of rice in a similar fashion.

The molecular genetic maps of rice constructed by Causse *et al.* (1994) and Kurata *et al.* (1994a) are among the most extensive maps constructed for any cereal species, containing an average of one marker for every 1.1 cM and 2.1 cM, respectively. As such, these maps may represent a potentially rich source of RFLP markers for mapping close to *Yd*$_2$ in barley. Some of the RFLP markers on these maps have also been mapped relative to *Yd*$_2$ in barley (Figure 6.2), and could serve as reference points to assist in the selection of probes likely to be of most use. RFLP clones used in the construction of the rice map of Kurata *et al.* (1994a) are currently being used in this laboratory to map RFLPs relative to *Yd*$_2$ in barley.

At the Rice Genome Research Program (RGP) in Tsukuba, Japan, RFLP probes used in the construction of the rice map of Kurata *et al.* (1994a) are being used to screen libraries of rice genomic DNA YAC clones in order to construct a map of overlapping YAC clones spanning each of the rice chromosomes (Umehara *et al.*, 1995). In theory, this contig map could be used to identify rice YAC clones spanning any point located on the rice map of Kurata *et al.* (1994a). Similarly, the accurate localisation of the point corresponding to the *Yd*$_2$ locus on the rice map of Kurata *et al.* (1994a) may enable the identification of a rice YAC clone suitable for the generation of further RFLP markers for mapping close to *Yd*$_2$ in barley. This strategy would obviate the need to screen a YAC library for the sole purpose of identifying such a clone. The point corresponding to *Yd*$_2$ on the rice map of Kurata *et al.* (1994a) could be accurately located by mapping further RFLP markers from this map relative to *Yd*$_2$ in barley. Most of the cDNA clones used to map RFLP markers on this map of rice have been sequenced (Kurata *et al.*, 1994a). Therefore, the sequencing of the barley, wheat, oat and *Phalaris coerulescens* cDNA clones used to map RFLPs relative to *Yd*$_2$ in barley may enable the identification of orthologous RFLP loci on the rice map of Kurata *et al.* (1994a). This may provide another means of accurately localising the point corresponding to the *Yd*$_2$ locus on this map of rice.

6.4.4 Differences in the rate of recombination between orthologous loci in rice and barley

The two loci identified by the probes AWPh7d and BCD263 in barley were positioned close to the centromere on the long arm of barley chromosome 3 where the rate of recombination is relatively low (Chapter 3). The total genetic length of the rice and barley genomes are similar, at 1,575 cM and 1,433 cM, respectively (Kurata *et al.*, 1994a; Graner *et al.*, 1994). However, the rate of recombination observed between orthologous RFLP loci identified in rice and barley using the probes PHBM7d and BCD263 was approximately three times greater in rice than in barley, as indicated by the genetic distances shown in Figure 6.1 (These recombination rates were significantly different at P = 0.01, using a χ^2 contingency test.). Therefore, the interval delimited by these probes in rice did not show the same reduction in recombination rate that is characteristic of this interval in barley. This is consistent with the observation made by Kurata *et al.* (1994b) that chromosomes of rice recombine more evenly along their length compared to wheat chromosomes which show decreased recombination close to the centromeres.

Due to the difference in the rates of recombination observed in the Yd_2 region of barley chromosome 3 and the orthologous region of the rice genome, it is likely that RFLP markers close to Yd_2 could be genetically resolved with greater efficiently in rice than in barley. Furthermore, it should also be possible to use a rice mapping population to to help identify the pair of markers most closely flanking the Yd_2 locus. However, scoring the Yd_2 locus in such a mapping population will only be possible if a BYDV resistance gene orthologous to Yd_2 can be found in rice. The possibility of identifying and utilising such a BYDV resistance gene is considered in detail in the next chapter.

CHAPTER 7

THE RELATIONSHIP BETWEEN *Yd2* AND A BYDV RESISTANCE GENE IN RICE

7.1 Introduction

Probes derived from cDNA sequences readily cross hybridise to the genomes of different species from the Gramineae, suggesting these species possess a similar repertoire of genes inherited from a common ancestral grass species (reviewed in Section 1.5.2). Evidence accumulating from comparative mapping studies suggests that many orthologous genes provide similar functions in different grass species. For example, genes controlling similar morphological traits or enzyme activities have been located to regions of conserved linkage in the genomes of rice, wheat and maize (Ahn *et al.*, 1993). Three regions of conserved linkage in oat, wheat, barley, maize and rice have been found to contain genes influencing heading date (Van Deynze *et al.*, 1995b). In addition, genes providing resistance to rust fungus (*Puccinia* sp.) exist in regions of conserved linkage in oat, wheat, and *T. tauschii* (Van Deynze *et al.*, 1995a; Van Deynze *et al.*, 1995b). This suggests that alleles providing pathogen resistance (as opposed to pathogen susceptibility) may exist for orthologous loci in more than one cereal species.

Rice has a number of features that makes it particularly amenable to map-based gene isolation. The rice genome is one of the smallest grass genomes known, at approximately 4.2×10^8 bp per haploid nucleus (Arumuganathan and Earle, 1991), and has a correspondingly low repetitive sequence content of about 50% (Deshpande and Ranjekar, 1980). The rice genome has a total genetic length that is comparable to the genomes of other cereals such as barley, which are physically larger (Kurata *et al.*, 1994a; Graner *et al.*, 1994). Therefore, the average ratio of physical to genetic distance in the rice genome of about 270 kbp/cM, calculated in Chapter 6, is relatively low. Rice has also been subject to considerable molecular and genetic characterisation. The rice maps by Causse *et al.* (1994) and Kurata *et al.* (1994a) are the among the most extensive genetic maps constructed for any cereal species. In addition, the maps of contiguous rice genomic DNA YAC clones constructed in rice by Umehara *et al.* (1995) could be of considerable assistance in the map-based isolation of genes from rice. Relatively efficient procedures for genetically transforming rice are available (Li

et al., 1993; Hiei *et al.*, 1994). Transformation could therefore be used to identify a target gene within a relatively large physically defined genomic region in the final stages of a map-based gene isolation program in rice.

In Chapter 7, the rate of recombination between RFLP markers flanking the Yd_2 gene was found to be lower than between orthologous RFLP loci in the corresponding region of the rice genome. Therefore, high resolution genetic mapping of RFLP markers close to the Yd_2 locus may be performed with greater efficiency in rice than in barley. However, the scoring of the Yd_2 locus in a segregating rice mapping population will only be possible if a BYDV resistance gene can be identified in rice which is orthologous to Yd_2. By enabling markers to be resolved with the Yd_2 locus in rice, such a gene could be of assistance in the map-based isolation of the Yd_2 gene from barley.

Due to the characteristics that make rice amenable to map-based gene isolation, the isolation of a BYDV resistance gene in rice that is orthologous to Yd_2 could be much simpler than the map-based isolation of the Yd_2 gene from barley. In addition, the greater rate of recombination expected around this gene could be directly exploited in such a project. A BYDV resistance gene in rice that is orthologous to Yd_2 would be expected to show sequence homology to the Yd_2 gene, in view of the hybridisation that most cDNA probes from barley show to the rice genome, and *visa versa* (Causse *et al.*, 1994; Van Deynze *et al.*, 1995c). Therefore, upon isolating the BYDV resistance gene from rice, it is likely that it could be used to screen a barley cDNA library to identify clones corresponding to the Yd_2 gene. This approach to isolating Yd_2 may be easier than the those considered in the previous chapters.

The incompletely dominant gene described by Baldi *et al.* (1991) is the only BYDV resistance gene that has been reported in rice. The gene was derived from the Italian rice variety Vialone Nano, and is presently being used in rice breeding in Italy, where BYDV causes severe yield losses in rice crops (Baldi *et al.*, 1991). The chromosomal location of this BYDV resistance gene is unknown.

In Chapter 6, RFLP marker collinearity was observed between the Yd_2 region of barley chromosome 3 and a region of the rice genome, most probably corresponding to

the central portion of rice chromosome 1. In this chapter, probes used to detect RFLP loci close to Yd_2 in barley are tested for linkage to the BYDV resistance gene described by Baldi *et al.* (1991) in rice, in order to determine if this gene is located in this region of conserved linkage. This information is sought in order to determine whether the BYDV resistance gene in rice is orthologous to Yd_2, and therefore of potential use in the map-based isolation of the Yd_2 gene.

7.2 Materials and methods

7.2.1 Acknowledgments

The author is grateful to Anna Callegarin and her colleagues for their generous donation of BYDV resistant rice lines which provided the basis for this study.

7.2.2 Rice cultivars and lines

Seeds of 36 BYDV resistant rice breeding lines and the four cultivars from which they were derived were supplied by Anna Callegarin (National Centre for Rice Research in Milano, Italy). Each breeding line had been determined to be homozygous for the BYDV resistance gene described by Baldi *et al.* (1991), and was highly inbred (A. Callegarin, personal communication). Descriptions of these lines are provided in Table 7.1. The breeders' reference numbers for these lines are listed in Appendix B.

Table 7.1. Descriptions of BYDV resistant lines imported from Italy

lines were selected from the F_4 generation (or beyond) of the following crosses†	no. of lines supplied
Cripto × **Naville**	17
(Cripto × **Veneria**) × Cripto	11
Radon × **Veneria**	8

†BYDV resistant parent cultivars are shown in bold.

7.2.3 RFLP analysis

The general procedures used for RFLP analysis were those outlined in Section 2.2.6. DNA for the analysis of each rice cultivar or line was extracted from the leaves of 10 or more plants grown from the original seeds imported from Italy, or from 10 or more plants grown from the next generation of seed resulting from self pollination. The DNA of the rice cultivars Cripto, Naville, Veneria and Radon were cut separately with a range of restriction enzymes and subject to Southern hybridisation analysis using the following probes mapped close to Yd_2 in barley in Chapter 3: BCD127, AWPh7d, BCD134, YLP, BCD828, BCD263, TAG80, BCD809 and PSR156. The restriction enzymes used in this analysis were *Alu*I, *Bam*HI, *Ban*2, *Bst*NI, *Cla*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Hpa*II, *Kpn*I, *Mbo*I, *Nco*I, *Nde*I, *Rsa*I, *Sau*3AI, *Taq*I and *Xba*I.

RFLPs identified between these cultivars were used to characterise the respective BYDV resistant breeding lines. The RFLP data was then analysed to determine whether the RFLPs showed genetic linkage to one another or to the BYDV resistance gene. Due to the highly inbred nature of the lines, they were treated as recombinant inbred lines (Burr and Burr, 1991) for the purpose of linkage analysis. In other words, the lines were assumed to contain two identical sets of chromosomes, unless shown otherwise. The number of recombinant chromosomes was assumed to follow a binomial distribution, and an observed proportion of recombinant chromosomes significantly smaller than 0.5 ($P < 0.10$) taken to indicate linkage.

7.3 Results

7.3.1 The identity of RFLPs detected between the parents of the BYDV resistant rice lines

Of the nine probes used to screen for RFLPs between the parents of BYDV resistant rice breeding lines, only AWPh7d, BCD263 and PSR156 identified polymorphisms. These polymorphisms are summarised in Table 7.2. The probe AWPh7d identified an identical *Hpa*II RFLP in the Cripto-Naville and Cripto-Veneria

pair of parents (Figure 7.1), as did the probe PSR156. Similarly, BCD263 identified the same *Bam*HI polymorphism for the Cripto-Veneria and Radon-Veneria pairs of parents.

The *Hpa*II RFLP identified by AWPh7d showed a different banding pattern to the *Hpa*II RFLP mapped to the putative rice chromosome 1 linkage group in the previous chapter using the Doongara × Hungarian #1 F_2 mapping population. However, because one polymorphic restriction fragment was common to both of these RFLPs (Figure 7.1), these two polymorphisms must represent the same locus.

The probe BCD263 was shown in the previous chapter to identify two loci in rice, only one of which was located within the putative rice chromosome 1 linkage group. These loci were mapped using *Hin*dIII, whereas the polymorphisms identified in the parents of the BYDV resistant rice lines was detected using other restriction enzymes. Therefore, it was not known whether the RFLPs detected using BCD263 in this study corresponded to the locus in the putative rice chromosome 1 linkage group or to the second unlinked locus.

PSR156 was shown in the previous chapter to hybridise to a single locus in the rice genome (Table 6.1).Therefore, the *Hpa*II RFLP detected by this probe in the parents of the BYDV resistant rice lines must represent the locus that Kurata *et al.* (1994a) mapped to the middle of rice chromosome 1 using PSR156 (Figure 6.2, Chapter 6).

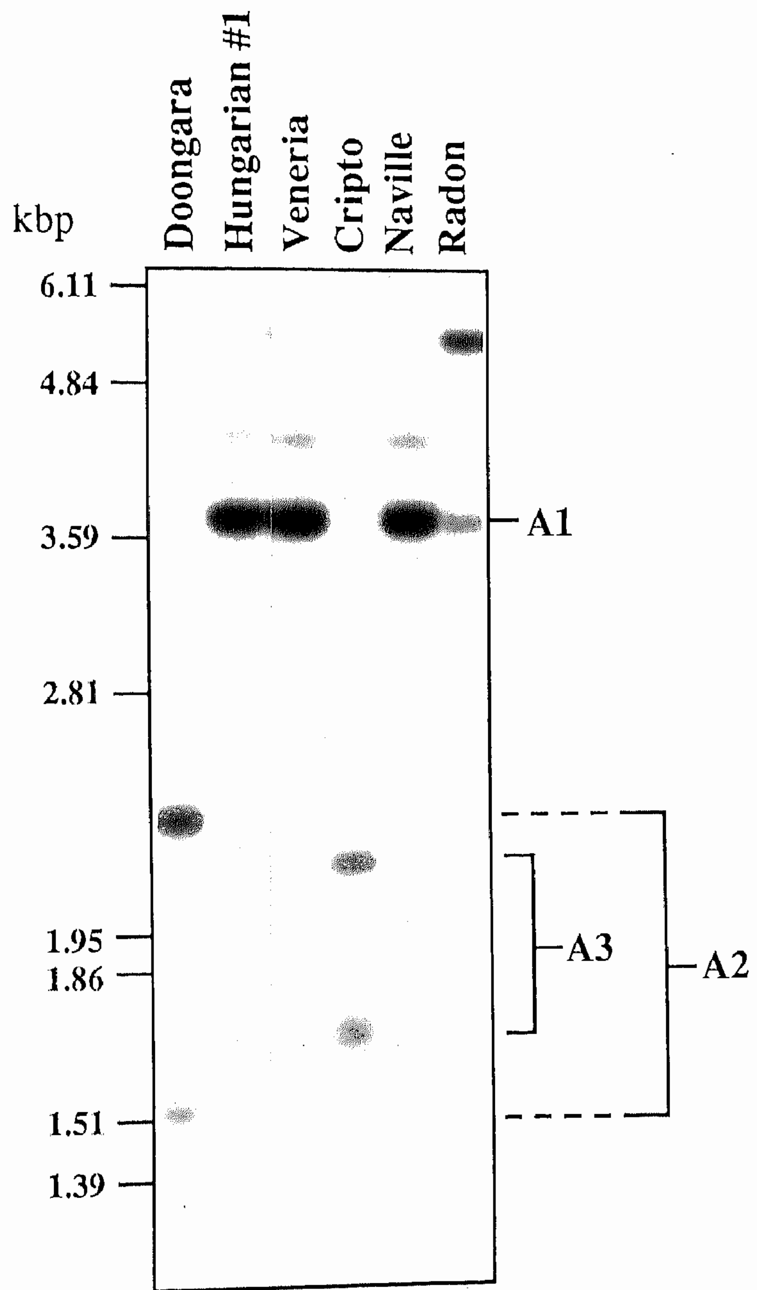
Table 7.2. RFLPs identified between the parents of three rice crosses

crosses used to derive the BYDV resistant lines	probe†		
	AWPh7d	BCD263	PSR156
Cripto × Naville	***Hpa*II**	none	***Hpa*II**
(Cripto × Veneria) × Cripto	***Hpa*II**	***Bam*HI** *Kpn*I *Taq*I	***Hpa*II**
Radon × Veneria	none	***Bam*HI** ***Eco*RV** *Nde*I	none

†Restriction enzymes showing polymorphism between the parents of each cross are shown. The restriction enzymes in bold indicate the RFLPs used to characterise the respective BYDV resistant lines.

Figure 7.1. *Hpa*II polymorphisms identified by the probe AWPh7d in rice

Shown here are the hybridisation patterns of the probe AWPh7d to various rice lines. The RFLP mapped using AWPh7d in the Doongara × Hungarian #1 F_2 population in Chapter 6 consisted of a single hybridising restriction fragment for one allele (A_1), and a pair of restriction fragments for the other allele (A_2). Lines derived from the Cripto × Naville cross and (Cripto × Veneria) × Cripto backcross segregated for the A_1 allele from the BYDV resistant parents Naville and Veneria, and for an allele from the BYDV susceptible parent Cripto (A_3) consisting of two restriction fragments which were a different size to those representing the A_2 allele in Doongara. The A_1/A_2 polymorphism represented the same locus as the A_1/A_3 polymorphism, due to the presence of the common A_1 restriction fragment. Molecular weight markers were derived from SPP-1 DNA cut with *Eco*R1.



kbp
Doongara
Hungarian #1
Veneria
Cripto
Naville
Radon
6.11
4.84
3.59
2.81
1.95
1.86
1.51
1.39
A1
A3
A2

7.3.2 Linkage analysis using the BYDV resistant rice lines

The probes detecting polymorphism between the parent rice cultivars were used to characterise the respective BYDV resistant lines, using the restriction enzymes indicated in Table 7.2. All of the 36 BYDV resistant lines were homozygous for the RFLPs tested, except for seven of the lines which were heterozygous for one RFLP locus and homozygous for the others. This high level of homozygosity was expected from the inbred nature of the lines (Table 7.1). The *Bam*HI and *Eco*RV polymorphisms identified by the probe BCD263 perfectly cosegregated in the lines derived from the Radon × Veneria cross, and were assumed to represent the same locus.

Table 7.3 shows the results of the linkage analysis. All of the lines derived from the (Cripto × Veneria) × Cripto backcross were homozygous for the Cripto alleles at the RFLP loci identified by BCD263 and PSR156. However, it was unclear whether the association observed between these loci was a result of genetic linkage or a bias toward alleles from the recurrent parent Cripto. Otherwise, no significant genetic linkage was detected between any of the RFLP loci, or between any of the RFLP loci and the BYDV resistance gene ($P > 0.10$).

Table 7.3 Linkage data obtained using the BYDV resistant rice breeding lines

cross from which BYDV resistant lines were derived	loci tested for linkage†	number of recombinant chromosomes§
Cripto × Naville	*Xawph7d*-BYDV^R	10/19
	Xpsr156-BYDV^R	9/18
	Xawph7d-*Xpsr156*	7/20‡
(Cripto × Veneria) × Cripto	*Xawph7d*-BYDV^R	7/14
	Xbcd263-BYDV^R	11/11
	Xpsr156-BYDV^R	11/11
	Xawph7d-*Xbcd263*	7/14
	Xawph7d-*Xpsr156*	7/14
	Xbcd263-*Xpsr156*	0/11¥
Radon × Veneria	*Xbcd263*-BYDV^R	4/10‡

†Loci preceded by an *X* = RFLP loci; BYDV^R = the BYDV resistance locus.

§The numbers of chromosomes with the recombinant genotypes are shown as a fraction of the total number of chromosomes observed. One chromosome was counted for every line homozygous for both RFLP loci, and two chromosomes were counted for every line heterozygous for one RFLP locus and homozygous for the other. All lines were assumed to be homozygous resistant for the BYDV resistance locus.

¥The association between *Xbcd263* and *Xpsr156* is not necessarily meaningful (see Section 7.3.2).

‡Fraction of recombinant chromosomes is not significantly smaller than 0.5 ($P > 0.10$).

7.4. Discussion

7.4.1 Is the BYDV resistance gene in rice orthologous to Yd_2?

In this exercise, the BYDV resistance gene described by Baldi *et al.* (1991) was tested for genetic linkage to three RFLP loci detected using probes mapped close to the Yd_2 gene in barley in Chapter 3. The first of these RFLP loci, detected using the probe AWPh7d, had been mapped in the previous chapter to a region of the rice genome shown to contain sequences orthologous to those close to Yd_2 in barley. By comparing the map of the Yd_2 region with published maps of rice, the region of the rice genome

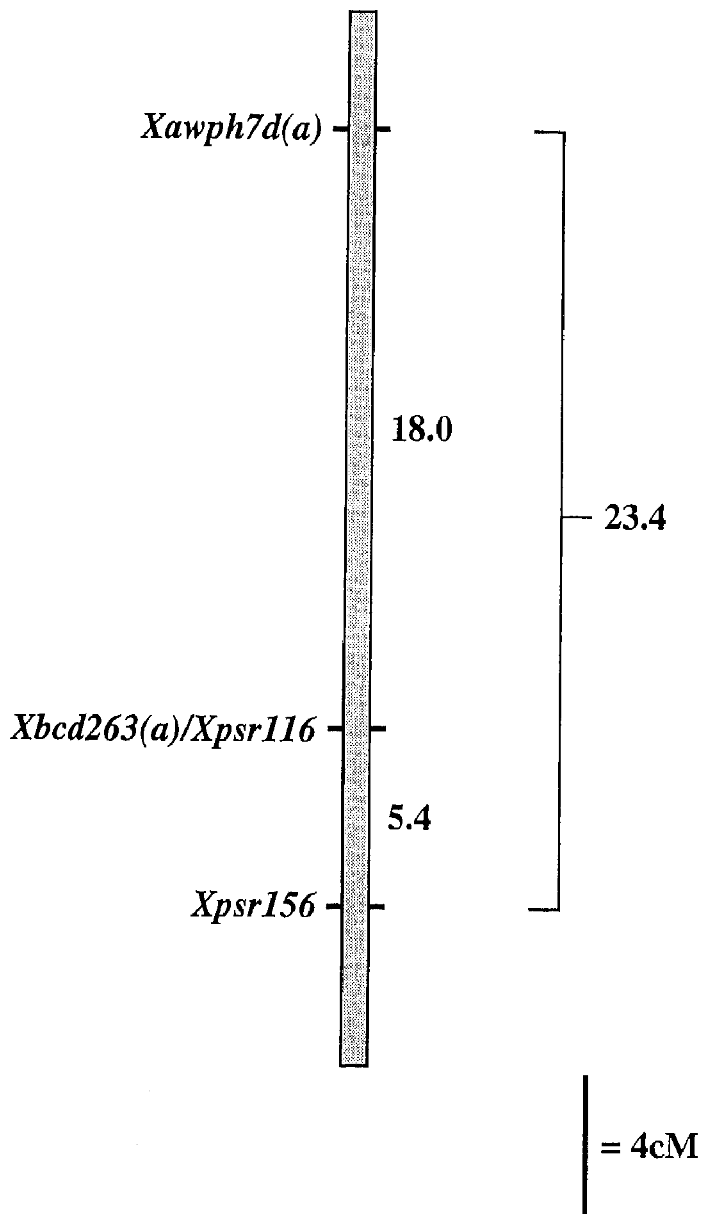
showing conserved linkage with the *Yd*$_2$ region had been tentatively identified as the central portion of rice chromosome 1. The second RFLP locus tested was detected using PSR156 and had been previously mapped to the middle of rice chromosome by Kurata *et al.* (1994a). The third RFLP locus was detected using the probe BCD263, which had been shown in the previous chapter to detect two unlinked loci in rice, only one of which was located to the putative rice chromosome 1 linkage group. However, it could not be determined which of these two loci corresponded to the locus analysed in this exercise. In summary, two of the loci used in the analysis were known to be located in a region of the rice genome showing conserved linkage with the *Yd*$_2$ region of barley, while the third may or may not have been located in the same region.

The genetic distance between the RFLP loci analysed in rice using the probes AWPh7d and PSR156 can be predicted to be about 23.4 cM, based on mapping data generated in Chapters 3 and 5, and by Kurata *et al.* (1994a). Figure 7.2 shows how this estimation was made. There are a number of factors which may have contributed to the failure to detect linkage between these two loci in this study. These include the relatively large genetic distance separating these two loci and the relatively small number of individuals used in the linkage analysis. In addition, the generations of backcrossing and inbreeding used to derive these rice lines would have provided extra opportunities for recombination to occur. As a result, linkage between these markers would have been more difficult to detect than in an F_2 mapping population.

In Chapter 3, the probes AWPh7d and PSR156 were shown to detect loci flanking the *Yd*$_2$ gene on barley chromosome 3. Therefore, in view of the RFLP marker collinearity shown between rice and barley in Chapter 6, a gene that is orthologous to *Yd*$_2$ in rice would be expected to be located between the loci identified by these probes. Linkage was not detected between these two loci in rice, which were expected to be located 23.4 cM from one another. However, linkage between these RFLP loci and a gene that is orthologous to *Yd*$_2$ would be expected to be much easier to detect, as the genetic distance separating such a gene from one of these markers must be less than half this distance (< 11.7 cM). In this study, no genetic linkage was detected between

Figure 7.2. The estimation of the genetic distance between the RFLP loci detected by AWPh7d and PSR156 in rice

The distance between the RFLP markers detected by AWPh7d and PSR156 in the BYDV resistant rice lines was predicted to be 23.4 cM. The diagram shows how this value was obtained. The RFLP locus *Xawph7d(a)* mapped in Chapter 6 (Figure 6.1) is the same locus detected using AWPh7d in this study (see Figure 7.1). The RFLP probes BCD263 and PSR116 were shown in Chapter 3 to be derived from orthologous genes in barley and wheat. Therefore, the *Xpsr116* locus mapped by Kurata *et al.* (1994a) on rice chromosome 1 is expected to be the same as the *Xbcd263(a)* locus mapped in Chapter 6. PSR156 was shown in Chapter 6 to detect a single locus in rice (Table 6.1). This locus (*Xpsr156*) was mapped on rice chromosome 1 by Kurata *et al.* (1994a). The linear order of the three loci is expected to be the same as the order of the loci identified by the same probes on barley chromosome 3 in Chapter 3. The *Xawph7d(a)-Xbcd263(a)* distance was determined in Chapter 6, while the *Xpsr116-Xpsr156* distance was determined by Kurata *et al.* (1994a).



Xawph7d(a)
18.0
23.4
Xbcd263(a)/Xpsr116
5.4
Xpsr156
= 4cM

either of these two RFLPs and the BYDV resistance gene described by Baldi *et al.* (1991). This strongly suggests that the BYDV resistance gene is not located in this interval of rice chromosome 1, and that it is not orthologous to Yd_2. The failure to detect linkage between this gene and the RFLP locus detected using BCD263 is less informative, as it was not known if this RFLP was positioned on chromosome 1 or elsewhere in the rice genome.

Further information is required before it can be definitely concluded that the BYDV resistance gene in rice is not orthologous to Yd_2. The use of a segregating F_2 rice mapping population consisting of 100 or more individuals would enable the detection of distant linkages more effectively than the lines used in this study. Markers located closer to Yd_2 could also be used in such an exercise, as they would show a much clearer association with the gene that is orthologous to Yd_2.

7.4.2 Prospects

Overall, the data obtained in this study strongly suggest that the BYDV resistance gene described by Baldi *et al.* (1991) is not orthologous to Yd_2. Because a BYDV resistance gene that is orthologous to Yd_2 would be an asset in a project to isolate the Yd_2 gene, it may be worthwhile conducting searches for additional sources of BYDV resistance in rice for the purpose of identifying such a gene. In the study of Causse *et al.* (1994), 87% of barley cDNAs were shown to hybridise clearly to the genomic DNA of rice. This suggests that most genes in barley have orthologous counterparts in rice and that a rice gene orthologous to Yd_2 is likely to exist. However, it is possible that such a gene will never be identified on the basis of BYDV resistance, as all lines of rice may possess alleles for BYDV susceptibility (as opposed to alleles for BYDV resistance). Mutants of plant pathogen resistance genes have been obtained with no reported pleiotropic effects (Lawrence *et al.*, 1995; Jones *et al.*, 1994; Whitham *et al.*, 1994; Freialdenhoven *et al.*, 1996), suggesting that many resistance genes are not essential to the health of the host under pathogen-free conditions. Therefore, it is also possible that a gene orthologous to Yd_2 could be absent in rice as a result of a non-lethal

deletion event occurring after the evolutionary divergence of rice and barley from a common ancestor. For these reasons, further searches for a BYDV resistance gene orthologous to *Yd2* in rice may be unsuccessful.

Although the BYDV resistance gene studied here is unlikely to be of assistance in the isolation of *Yd2*, it may represent an appropriate target for positional cloning in its own right. Its isolation and subsequent characterisation would provide knowledge of the basis of naturally occurring resistance to BYDV in cereals, and may also enable the production of BYDV resistant rice lines by genetic transformation. The first necessary step in the isolation of this gene would be to determine its location in the rice genome. Clues that may help locate this gene can be obtained from the positions of other BYDV resistance genes in cereals. For example, BYDV resistance has been shown to be controlled by genes on homoeologous chromosome groups 2 and 7 in wheat, rye and *Thinopyrum intermedium* (Brettell *et al.*, 1988; Nkongolo *et al.*, 1992; Singh, 1993; Sharma *et al.*, 1995; Larkin *et al.*, 1995). These Triticeae chromosome groups show orthology to chromosomes 2, 4, 6, 7 and 8 of rice (Van Deynze *et al.*, 1995c). Hence, it is possible that the BYDV resistance gene in rice is orthologous to one of these BYDV resistance genes from the Triticeae and therefore located on one of the above mentioned rice chromosomes.

CHAPTER 8

THE CHARACTERISATION OF FURTHER BYDV RESISTANCE SOURCES IN BARLEY

8.1 Introduction

Suneson (1955) identified a recessive gene for BYDV resistance in the barley cultivar Rojo. Because this gene was found to confer lower levels of resistance than the Yd_2 gene from a number of Ethiopian barleys, Rasmusson and Schaller (1959) concluded that it was different from Yd_2, and named it *yd1*. The genetic experiments performed by Catherall *et al.* (1970) suggested that different Ethiopian barleys may possess alleles of Yd_2 that vary in their effectiveness and dominance (discussed in Section 1.4.3). Considering this possible variability between Yd_2 resistance alleles, it is conceivable that *yd1* may actually represent an allele of the Yd_2 locus. The *yd1*-containing cultivar Rojo is not known to have Ethiopian barleys in its pedigree. Therefore, if *yd1* was found to be an allele of Yd_2, it would provide an exception to the Yd_2 resistance alleles known so far, which are all of Ethiopian origin.

Single genes have also been found to control the moderate levels of BYDV resistance observed in the Algerian accession CI 1179 and the Chinese accession CI 4228 (Damsteegt and Bruehl, 1964). The chromosome locations of the BYDV resistance genes in these barleys are unknown.

Of the 117 barleys found to be the most BYDV resistant in the screen of a worldwide barley collection by Schaller *et al.* (1963), the accession CI 1113 from China was the only one that was not of Ethiopian origin or a hybrid with an Ethiopian parent. Although some of the most resistant Ethiopian accessions were subject to subsequent genetic tests and found to contain the Yd_2 gene, the resistance from CI 1113 was not analysed genetically. Another barley reported to contain a level of BYDV resistance as high as Yd_2-containing barleys is the cultivar Post (Grafton *et al.*, 1982; Habekuβ and Lehmann, 1991). Moderate levels of resistance were also reported in the cultivars Perry, Hannchen and Gem, and in the accession CI 6306 from Uruguay (Damsteegt and Bruehl, 1964; Grafton *et al.*, 1982; Habekuβ and Lehmann, 1991). The genetic basis of the BYDV resistance in CI 1113, Perry, Hannchen, Gem and CI 6306 is not known. Other non-Ethiopian sources of BYDV resistance that are known in barley (summarised in Section 1.3.2) have also not been characterised genetically.

The Ethiopian barley CI 5791 was found to possess high levels of BYDV resistance (Gill *et al.*, 1969), but has not been tested genetically to determine if it contains Yd_2. Lines produced in breeding programs using this accession were found to differ in their susceptibility to RPV-like and RMV-like isolates of BYDV (Gill and Buchannon, 1972). Some of the lines were found to be resistant to only one of these isolates, suggesting that CI 5791 may contain separate genes for BYDV-RPV and BYDV-RMV resistance. The Yd_2 gene from Ethiopian barleys has been found to be ineffective against the RPV and RMV isolates of BYDV (Ranieri *et al.*, 1993). Therefore, if the resistance to one of these isolates in CI 5791 is found to be controlled by the Yd_2 locus, such a finding would provide a contrast to the specificity of known Yd_2 alleles.

BYDV resistance in barley is measured in a quantitative fashion, in terms of the degree of symptom development or virus multiplication, rather than in a qualitative fashion, such as resistance involving the hypersensitive response. The quantitative nature of BYDV resistance, combined with the codominant expression of Yd_2-mediated resistance, would make conventional tests for allelesm between Yd_2 and genes conferring only moderate levels of resistance very difficult. However, the RFLP markers located close to the Yd_2 locus in Chapter 3 provide the opportunity to use a alternative scheme which is likely to be much easier and more effective. Briefly, the BYDV resistant barley is crossed to a susceptible line or cultivar, and the F_2 progeny analysed for both the RFLP marker and BYDV resistance. Cosegregation of the resistance with the RFLP marker would provide strong evidence that the segregating resistance gene is allelic to Yd_2.

The following work forms the beginning of a study which aims to use the RFLP markers mapped close to Yd_2 in Chapter 3 to determine if BYDV resistance genes in the above mentioned barleys are allelic to Yd_2. Information regarding the BYDV resistance in most of these barleys has been based solely on observations made in the field where unknown isolates of BYDV were present. Therefore, the barleys are tested to determine if their BYDV resistance can be detected using the procedure commonly

used in our laboratory for assaying Yd_2-mediated resistance, involving infection with BYDV-PAV$_{adel}$ in the glasshouse. Some of these barleys such as Post are winter cultivars which require a cold treatment (vernalisation) in order to flower. The BYDV resistance in these winter barleys is tested to determine if vernalisation is required to express the resistance in these barleys, or whether the plants can be assayed under more convenient conditions. Finally, a search for RFLPs suitable for the genetic analysis of these resistance sources is undertaken.

8.2 Materials and methods

8.2.1 Barley cultivars and lines

Ten barley lines and cultivars previously reported to possess resistance to BYDV were chosen for analysis. These barleys are listed in Table 8.1, together with brief descriptions or their origin, their reported BYDV resistance and the genetic basis of their resistance where known.

Seed of the accessions CI 3208-1, CI 2376, CI 3920-1 and CI 1179, and the barley cultivars Shannon, Atlas68, Proctor and Atlas were obtained from the sources indicated in Chapter 3. Seed of the remaining barleys were obtained from the Australian Winter Cereal Collection at Tamworth, NSW (Post, Perry, CI 5791 and CI 1113) and the barley seed collection at the Waite Institute, University of Adelaide (Rojo, Hannchen, CI 4228, CI 6306 and Gem).

Table 8.1. BYDV resistant barleys used in this study

barley	description	level of resistance	single gene control?	authority
CI 1179	Algerian accession	moderate	yes	a
CI 4228	Chinese accession	moderate	yes	a
CI 6306	accession from Uruguay	moderate	unknown	a
Hannchen	USA cultivar	moderate	unknown	a, b
Gem	USA cultivar	moderate	unknown	a
Rojo	USA cultivar	moderate	yes	c
Post	USA cultivar	high	unknown	d, e
Perry	USA cultivar	moderate	unknown	d, e
CI 1113	Chinese accession	high	unknown	f
CI 5791	Ethiopian accession	high	unknown	g, h

[a]Damsteegt and Bruehl (1964) [b]Oswald and Houston (1953a) [c]Suneson (1955)
[d]Grafton *et al.* (1982) [e]Habekuβ and Lehmann (1991) [f]Schaller *et al.* (1963)
[g]Gill *et al.* (1969) [h]Gill and Buchannon (1972)

8.2.2 BYDV resistance assays

The procedures used for the BYDV resistance assays were the same as those outlined in Section 2.2.9, with some modifications. For comparison, the ten barleys being evaluated were assayed alongside the Yd_2-containing barleys Atlas68, Shannon, CI 2376, CI 3920-1 and CI 3208-1, and the non-Yd_2, BYDV-susceptible cultivars Atlas and Proctor. For each barley cultivar or line, sixteen seedlings were grown from seeds germinated in the glasshouse. Seven days after sowing, eight of the seedlings were infected with BYDV-PAV_{adel} by allowing viruliferous *Rhopalosiphum padi* aphids to feed on them for two days, as described in Section 2.2.9. The other eight seedlings were left uninfested and served as uninfected controls. This infection procedure had been shown in Chapter 3 to result in the infection of 100% of barley seedlings with BYDV-

PAV_{adel}. Therefore, dot blot hybridisation was not used to confirm infection in this study.

The accessions CI 4228 and CI 1179, and the winter barley cultivars Post and Perry do not flower unless given a vernalisation treatment. The vernalisation treatment used in this study involved germinating the seeds on perlite at 4.0°C for four weeks before transplanting the seedlings into soil in the glasshouse. This procedure had been found by the author to be sufficient to induce flowering in these four barleys. For each of these four barleys, sixteen seeds were sown at 4.0°C, and four weeks later, half of the seedlings infected with BYDV-PAV_{adel} as above. At the end of the cold treatment, seedlings were the same size as seven day-old seedlings grown in the glasshouse. Seed was germinated in the glasshouse three weeks after sowing seed at 4.0°C so that vernalised and non-vernalised seedlings of each barley could be infected at the same time.

Infected and uninfected plants were grown in a glasshouse at 18 to 25°C. Four weeks after infection, the above-ground parts of each plant were removed and their fresh weights determined. The percentage reduction in fresh shoot weight due to BYDV infection was used as the measure of BYDV resistance for each barley cultivar or line. Visual symptoms of BYDV infection were also observed.

8.2.3 Search for RFLPs between the BYDV resistant barleys and the susceptible cultivars Proctor and Atlas

Barley lines that showed the highest levels of BYDV-PAV_{adel} resistance in the BYDV resistance assays were subject to RFLP analysis alongside the non-Yd_2, BYDV-susceptible barley cultivars Atlas and Proctor, in order to identify RFLPs suitable for genetically analysing the resistance from these lines. The following probes were chosen for screening, and had been mapped close to Yd_2 in Chapter 3: TAG223, AWPh7d, BCD134, BCD127, YLP, BCD828, PSR116, TAG80, and BCD809. The restriction enzymes used in the analysis were *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I. The general procedures used for the RFLP analysis were those outlined in Section 2.2.6.

8.3 Results

8.3.1 BYDV resistance assays

The results of the resistance assay are shown in Table 8.2. Vernalisation was found to decrease the level of BYDV resistance in all four of the winter barleys CI 1179, CI 4228, Post and Perry. The decrease in the percentage stunting due to vernalisation ranged from 11% (CI 4228) to 42% (CI 1179). Vernalisation also altered the resistance ranking among these barleys, with CI 1179 being the most resistant without vernalisation, and Perry being the most resistant with vernalisation.

The Ethiopian, Yd_2-containing accessions CI 3208-1 and CI 2376 were the only barleys to not show significant stunting upon infection with BYDV-PAV_{adel} ($P < 0.10$). Of the lines being evaluated, CI 1179, Rojo, Perry (not vernalised) and Hannchen showed less stunting than the Yd_2-containing cultivars Shannon and Atlas68. When not vernalised, Post and CI 4228 also displayed resistance, although this resistance was not as high as in the Yd_2-containing cultivars. CI 6306 and CI 5791 were moderately susceptible, showing 62% and 70% stunting, respectively. CI 1113 and Gem were stunted more than the non-Yd_2, BYDV-susceptible cultivars Proctor and Shannon, and were therefore highly susceptible.

The severity of leaf yellowing was generally in accordance with the levels of stunting observed in each barley. The highly resistant barleys CI 3920-1, CI 3208-1, CI 2376 and Shannon displayed no visible symptoms, while the susceptible barleys Proctor, Atlas and Gem showed extensive leaf yellowing and serrated leaf margins. The other barleys showed mild leaf yellowing. In Post, Perry, CI 4228, CI 1179, CI 6306 and Atlas68, yellowing occurred in stripes, while in CI 5791 and CI 1113, it was manifested in stripes and patches. In Hannchen, only the leaf tips were yellowed, and in Rojo, only the first (inoculated) leaf showed yellowing.

Table 8.2. BYDV resistance assay data

barley line or cultivar‡	fresh shoot weight (mean +/- standard deviation, in g)		% stunting	t†
	infected	uninfected		
CI 3208-1 (+Yd_2)	10.8 +/- 1.6	10.1 +/- 1.9	-7	0.80
CI 3920-1 (+Yd_2)	8.6 +/- 1.2	9.6 +/- 1.4	10	1.53*
CI 2376 (+Yd_2)	10.0 +/- 0.9	11.2 +/- 2.4	11	1.32
CI 1179 (n. v.)	9.0 +/- 1.2	10.3 +/- 1.1	13	2.25**
Rojo	8.0 +/- 1.0	9.7 +/- 1.7	18	2.44**
Perry (n. v.)	7.7 +/- 1.2	9.5 +/- 1.7	19	2.44**
Hannchen	8.6 +/- 1.8	11.1 +/- 1.4	23	3.1***
Shannon (+Yd_2)	7.0 +/- 1.0	9.1 +/- 0.7	23	4.88***
Atlas 68 (+Yd_2)	7.8 +/- 1.0	10.3 +/- 1.2	24	4.53***
Post (n. v.)	7.0 +/- 0.7	10.1 +/- 2.6	31	3.26***
CI 4228 (n. v.)	5.0 +/- 2.6	7.8 +/- 1.0	36	2.84***
Perry (vern.)	3.0 +/- 1.3	4.7 +/- 1.3	36	2.62**
CI 4228 (vern.)	4.3 +/- 1.9	8.1 +/- 1.0	47	5.01***
Post (vern.)	2.8 +/- 1.0	5.3 +/- 1.3	47	4.31***
CI 1179 (vern.)	2.3 +/- 1.7	5.1 +/- 2.0	55	3.02***
CI 6306	4.2 +/- 0.8	11.0 +/- 1.2	62	13.30***
CI 5791	2.9 +/- 2.6	9.6 +/- 1.2	70	14.12***
Proctor (-Yd_2)	1.5 +/- 0.4	9.0 +/- 1.5	83	13.69***
CI 1113	0.8 +/- 0.1	9.5 +/- 1.3	92	18.34***
Atlas (-Yd_2)	0.8 +/- 0.3	9.5 +/- 0.8	92	19.83***
Gem	0.6 +/- 0.1	9.9 +/- 1.5	94	17.50***

‡Varieties were either known to contain Yd_2 (+Yd_2) or known not to contain Yd_2 (-Yd_2). Resistance assays were performed on vernalised (vern.) and non vernalised (n. v.) plants of winter barley lines.

†The t-statistic was calculated to determine if the mean infected shoot weight was significantly less than the mean uninfected shoot weight. *$P < 0.100$; **$P < 0.025$; ***$P < 0.010$.

8.3.2 RFLP analysis of BYDV resistance sources

Among the ten barleys that were investigated, CI 1179, Rojo, Perry, Hannchen, Post and CI 4228 were found to be the most resistant to BYDV-PAV$_{adel}$, and were the

barleys screened for RFLPs with the BYDV-susceptible, Yd_2-containing cultivars Proctor and Atlas. Table 8.3 shows the results of the polymorphism screen. Of the nine probes screened, only BCD127 and YLP failed to detect polymorphism. Polymorphisms were detected for every combination of resistant and susceptible barley. Although the probes TAG223 AWPh7d and BCD828 may detect multiple loci in barley (Chapter 3), the polymorphisms these probes detected here were generally the same as those mapped close to Yd_2 using these probes in Chapter 3 (Table 8.3). The polymorphisms detected using each of the other six probes must have also represented the loci mapped close to Yd_2 in Chapter 3, as these probes each detect single loci in barley (Chapter 3).

Table 8.3. RFLPs between BYDV susceptible and resistant barleys†

resistant barley	**polymorphisms†**	
	Atlas	**Proctor**
CI 1179	BCD134 (*Eco*RI) BCD828 (*Bam*HI‡, *Eco*RI) PSR116 (*Eco*RI)	BCD828 (*Bam*HI‡, *Eco*RI) PSR116 (*Eco*RI, *Eco*RV)
Rojo	BCD134 (*Eco*RI)	PSR116 (*Eco*RV)
Perry	TAG223 (*Dra*I‡) AWPh7d (*Bam*HI‡)	TAG223 (*Dra*I‡) BCD134 (*Eco*RI) AWPh7d (*Bam*HI‡) PSR116 (*Eco*RV)
Hannchen	AWPh7d (*Bam*HI‡) PSR116 (*Dra*I) TAG80 (*Dra*I, *Eco*RV, XbaI)	BCD134 (*Eco*RI) AWPh7d (*Bam*HI‡) PSR116 (*Dra*I, *Eco*RV) TAG80 (*Dra*I, *Eco*RV, *Xba*I)
Post	TAG223 (*Dra*I‡) AWPh7d (*Bam*HI‡) BCD809 (*Dra*I, *Eco*RI, *Hin*dIII, *Xba*I)	TAG223 (*Dra*I‡) BCD134 (*Eco*RI) AWPh7d (*Bam*HI‡) PSR116 (*Eco*RV)
CI 4228	TAG223 (*Dra*I‡) AWPh7d (*Bam*HI‡) BCD828 (*Eco*RV)	TAG223 (*Dra*I‡) BCD134 (*Eco*RI) AWPh7d (*Bam*HI‡) BCD828 (*Eco*RV) PSR116 (*Eco*RV)

†Restriction enzymes revealing polymorphism are shown in brackets

‡For the multiple locus probes TAG223, AWPh7d and BCD828, these polymorphisms are the same as those mapped close to Yd_2 in Chapter 3.

8.4 Discussion

8.4.1 Levels of BYDV resistance

In this study, ten barleys previously reported to possess moderate to high levels of BYDV resistance were tested for resistance to the PAV_{adel} isolate of BYDV in the glasshouse, using a procedure employed in our laboratory for assaying plants for *Yd*$_2$-mediated resistance. Except for Gem and CI 1113, all of these barleys displayed higher levels of resistance than the BYDV-susceptible, non-*Yd*$_2$ cultivars Proctor and Atlas. Therefore, in F_2 families derived from crosses between the BYDV resistant barleys and Proctor or Atlas, it should be possible to use this resistance assay procedure to observe the segregation of resistance, provided that this resistance is controlled by one or two genes. Segregation may be difficult to observe in crosses involving the accessions CI 6306 and CI 5791, as these accessions show only a low level of resistance. However, the use of Atlas rather than Proctor as the susceptible parent may make the segregation clearer, as Atlas is the more susceptible of these two cultivars.

Because vernalisation is a natural part of the life cycle of the winter barleys Post, Perry, CI 1179 and CI 4228, these barleys may be expected to express higher levels of BYDV resistance when vernalised than when not vernalised. However, it was shown here that the opposite was true. Evidently, changes in the physiology and gene expression brought about by a cold treatment was not necessary for the function of the BYDV resistance in these barleys. This result also indicates that segregating populations used to study the inheritance of the resistance from these barleys can be conveniently grown directly from seed sown in pots, rather than from seeds germinated at 4.0^{o}C for four weeks.

Uninfected control plants of the barleys CI 1179, Perry and Post attained approximately half the fresh shoot weight of plants of the same genotype grown from unvernalised seedlings (Table 8.2). This showed that the cold treatment or the transplantation process resulted in a slower growth rate in these barleys. Cold growth conditions resulting in slow growth have been found to be accompanied by diminished levels of *Yd*$_2$-mediated resistance (Catherall *et al.*, 1970; Jones and Catherall, 1970).

Therefore, the slower growth may have been responsible for reducing the levels of resistance in the vernalised winter barleys. The cold treatment did not appear to reduce the growth rate of uninfected control plants of CI 4228. Therefore, there are no obvious explanations for the reduction in BYDV resistance resulting from the cold treatment in this barley accession.

The resistance assay performed in this study gave some unexpected results. Firstly, CI 1179, Hannchen, Rojo and Perry were more resistant than the Yd_2-containing barley Atlas68, even though these barleys had previously been shown to be less resistant than Yd_2 containing barleys (Rasmusson and Schaller, 1959; Damsteegt and Bruehl, 1963; Habekuβ and Lehmann, 1991). Secondly, Perry was found to be more resistant than Post, even though the reverse had been found to be the case in other studies (Grafton *et al.*, 1982; Habekuβ and Lehmann, 1991). Thirdly, the Chinese accession CI 1113, reported to be highly resistant (Schaller *et al.*, 1963), and the commercial cultivar Gem, reported to be possess a low level BYDV resistance (Damsteegt and Bruehl, 1964) were both shown here to be highly susceptible. The reasons for these inconsistencies are unknown. However, possible causes include differences between the growth conditions, virus strains and methods of measuring resistance used in the different studies.

8.4.2 The genetic analysis of non-Ethiopian resistance sources

Among the ten barleys evaluated, CI 1179, Rojo, Perry, Hannchen, Post and CI 4228 were found to express the highest degree of resistance to BYDV-PAV_{adel}. A strategy for testing for allelism between Yd_2 and the BYDV resistance genes in these barleys involves crossing these barleys to the BYDV susceptible barleys Proctor or Atlas, and testing if the resistance in the resulting F_2 families cosegregates with RFLP markers located close to the Yd_2 locus.

In this exercise, RFLPs were detected for each combination of resistant and susceptible barleys, using probes mapped close to Yd_2 in Chapter 3. The only polymorphism detected for the Proctor-Rojo pair of barleys was identified using the

probe PSR116, and had been mapped 4.3 cM from *Yd*$_2$ in Chapter 3. For each other combination of resistant and susceptible barleys, polymorphisms representing RFLP loci located within 0.9 cM of *Yd*$_2$ were detected. In addition, polymorphic loci on both sides of the *Yd*$_2$ locus were identified in most of the resistant and susceptible barley combinations. The use of these flanking markers together would allow F_2 plants to be scored for the *Yd*$_2$ locus with almost 100% confidence. Overall, the RFLPs identified will be sufficient to facilitate tests for allelism between *Yd*$_2$ and the BYDV resistance genes present in the six most resistant barleys.

The BYDV resistance from the barleys CI 1179, Rojo, and CI 1179 had previously been shown to be controlled by single genes (Suneson, 1955; Damsteegt and Bruehl, 1964). However, the number of BYDV resistance genes in Hannchen, Post and Perry is unknown, and may be more than one. If this is the case, analysis of the resistance genes from these winter barleys is likely to require the analysis of families from the F_3 generation (or beyond) of crosses between these barleys and susceptible barleys, in order to obtain clear segregation of resistance.

The BYDV susceptible barleys Proctor and Atlas have been crossed to CI 1179, Rojo, Perry, Hannchen, Post and CI 4228, and the F_2 seed collected. Unfortunately, time did not allow the analysis of these F_2 families by the author. However, this work may be resumed in our laboratory at a later date. If BYDV resistance in any of the six non-Ethiopian barleys is found to be due to alleles at the *Yd*$_2$ locus, these *Yd*$_2$ alleles may show some interesting differences to known *Yd*$_2$ resistance alleles from Ethiopia. These may include variations in the level of effectiveness, and differences in the specificity of the resistance against different BYDV isolates. Once an Ethiopian *Yd*$_2$ resistance allele is isolated, it could be used to isolate non-Ethiopian alleles by homology. The non-Ethiopian *Yd*$_2$ alleles could then be analysed at the molecular level to determine the basis of any such differences from Ethiopian *Yd*$_2$ alleles.

The production of a plant cultivar containing more than one resistance gene for a particular pathogen has been proposed as a strategy for providing durable resistance (Nelson, 1978). Therefore, although the *Yd*$_2$ gene has been found to provide barley

cultivars with effective levels of BYDV resistance for over 30 years, combining other resistance genes with Yd_2 may reduce the chance of resistance-breaking strains of BYDV becoming more common. BYDV resistance genes which are unlinked to the Yd_2 locus and therefore suitable for the combining with Yd_2 may be identified if this study is continued. Further work may also show such genes to be resistant against isolates that are not affected by the Yd_2 gene. Combining these genes with Yd_2 may therefore provide barley cultivars with resistance against a broader range of BYDV isolates.

CHAPTER 9

PROSPECTS

9.1 Prospects for the map-based isolation of the *Yd_2* gene

As a result of the work described in this thesis, considerable progress has been made toward the map-based isolation of the *Yd_2* gene for BYDV resistance. With regard to isolating this gene, the most significant achievements were the construction of a genetic map of the *Yd_2* region of barley chromosome 3 and the demonstration of RFLP marker collinearity between the *Yd_2* region and a central region of rice chromosome 1. Currently Nicholas Paltridge and Brendon King (both PhD students) in this laboratory are continuing efforts to isolate the *Yd_2* gene by a map-based approach. Much of this work has been made possible by the information and resources generated in this study, and by recent advances made by other researchers in the fields of cereal genome analysis and molecular marker generation. This continuing work in our laboratory has been alluded to in the individual chapters of this thesis, and involves the following:

♦ The exploitation of genetic maps of barley and other cereals to obtain further RFLP markers for mapping close to *Yd_2*. RFLP markers common to these maps and the map of the *Yd_2* region have been used to identify those markers most likely to be useful for this purpose.

♦ The use of appropriately selected genetic material derived from the Proctor × Shannon F_2 mapping population (Chapter 3) to screen AFLP markers for close linkage to the *Yd_2* gene. A number of AFLP markers tightly linked to *Yd_2* have already been identified in this way.

♦ The identification of a cross that provides a higher rate of recombination around the *Yd_2* locus than the Proctor × Shannon cross used for mapping in this study. Already a cross has been found that shows three times the recombination rate in this region. This cross is presently being used to construct a larger mapping population to help resolve markers closest to *Yd_2*.

♦ The mapping of further morphological markers relative to *Yd_2* in order to identify a pair of flanking markers which could be used to facilitate high resolution genetic mapping close to *Yd_2*. Appropriate morphological markers for this analysis were chosen on the basis of the finding made in this study (Chapter 5), that *Yd_2* is located proximal to the *uz* morphological marker.

♦ The localisation of the point corresponding to the *Yd_2* locus on the map of rice chromosome 1 by Kurata *et al.* (1994). Once this point is located in rice, it should be possible to use the map of contiguous rice genomic DNA YAC clones being constructed by Umehara *et al.* (1995) to identify a clone suitable for generating RFLP markers close to *Yd_2* in barley. The point corresponding to *Yd_2* on the map of Kurata *et al.* (1994) is being identified by mapping RFLP clones from this map relative to *Yd_2* in barley, and by sequencing genes mapped close to *Yd_2* in barley in order to identify orthologous loci on the rice map.

Overall, the author is satisfied that good progress is being made, and that a barley genomic DNA clone containing the *Yd_2* gene will be identified in the near future. Once such a clone is obtained, genes within it could be identified using direct cDNA selection systems (Lovett, 1994). It is envisaged that the *Yd_2* gene will then be found by genetically transforming a BYDV susceptible barley cultivar with these candidate genes and observing which one provides BYDV resistance.

9.2 Structure and function of the *Yd_2* gene

Plant pathogen resistance genes isolated so far were discussed in Section 1.2.4. Except for one gene that encodes a fungal toxin degrading enzyme, all of the plant pathogen resistance genes isolated so far control a resistance response involving HR. The predicted products of these HR resistance genes share common motifs, which suggest that they act by similar mechanisms. However, because *Yd_2* does not involve HR, its isolation and sequence determination may lead to the characterisation of a completely different resistance mechanism.

Genetic studies have been unable to determine whether the Yd_2 locus consists of a single resistance gene or a number of closely linked BYDV resistance genes, although the structure of the Yd_2 locus is commonly thought of in terms of the first model by default (Rasmusson and Schaller, 1959; Damsteegt and Bruehl, 1964; Catherall *et al.*, 1970). Once a resistance allele from the Yd_2 locus is isolated, the number of restriction fragments that it is found to hybridise to at the Yd_2 locus could be used to help resolve this issue. Such an approach has been used to confirm the previously suspected structural organisation of the *L* and *M* rust resistance loci in flax (Ellis *et al.*, 1995).

Once a Yd_2 allele from a particular BYDV resistant Ethiopian barley is isolated, it could be used to isolate other Yd_2 alleles (from one gene or from a number of duplicated genes) for susceptibility and resistance, by virtue of the sequence homology expected among these alleles. Sequencing alleles from the Yd_2 locus may indicate which regions of the protein product are important in conferring resistance versus susceptibility, and may identify protein domains that are responsible for the differences in the effectiveness of different resistance alleles. The characterisation of the specificity and effectiveness of additional Yd_2 resistance alleles from Ethiopian and non-Ethiopian barleys may be useful in providing a basis for these structure-function studies.

9.3 Other genes involved in BYDV resistance in cereals

Genes required for the function of a number of plant pathogen resistance genes have been identified via mutagenesis (Torp and Jørgensen, 1986; Salmeron *et al.*, 1994; Hammond-Kosack *et al.*, 1994; Freialdenhoven *et al.*, 1994; Freialdenhoven *et al.*, 1996). In a similar fashion, genes required for the Yd_2-mediated BYDV resistance could be identified by generating BYDV susceptible mutants of a Yd_2-containing barley. The number of mutant genes identified which are unlinked to Yd_2 would provide an indication of the number of host factors required for the BYDV resistance mechanism. The isolation of these genes, in addition to Yd_2, would be essential for gaining a complete understanding of the basis of Yd_2-mediated BYDV resistance. On the other hand, if mutations at the Yd_2 locus are the only ones that can be identified in a mutant

search, this would suggest that *Yd_2* is the only gene involved in *Yd_2*-mediated resistance, and that the *Yd_2* gene product directly interferes with BYDV replication or movement within barley.

The isolation of other BYDV resistance genes in cereals and the genes required for their function will provide a more complete knowledge of the molecular basis of naturally occurring BYDV resistance in cereals. BYDV resistance genes which could be the subject of gene isolation programs include the BYDV resistance gene investigated in rice in Chapter 7 (Baldi *et al.*, 1991) and BYDV resistance genes known to exist on homoeologous chromosome groups 2 and 7 in wheat, rye and *T. intermedium* (Brettell *et al.*, 1988; Nkongolo *et al.*, 1992; Singh, 1993; Sharma *et al.*, 1995; Larkin *et al.*, 1995). The continuation of the genetic study described in Chapter 8 may also demonstrate the occurrence of BYDV resistance genes in barley which are distinct from *Yd_2* and suitable for gene cloning efforts.

The isolation of BYDV resistance genes such as *Yd_2* may provide the opportunity to produce BYDV resistant cereal cultivars by genetic transformation (Larkin and Young, 1995). Furthermore, the elucidation of the mechanisms of BYDV resistance gene function may allow the rational design of artificial BYDV resistance genes for use in cereal improvement. RFLP markers identified in this study are presently being used to assist in the selection of the *Yd_2* gene in barley breeding programs (Appendix A). In addition, by contributing to an effort to isolate the *Yd_2* gene by a map-based approach, the work described in this thesis may ultimately help achieve the long term goal of improving the BYDV resistance of cereals by genetic engineering. Overall, it is hoped that this work will eventually help reduce the significant damage caused by this serious pathogen of cereals worldwide.