



**TOWARDS CLONING Yd_2 , A BARLEY
RESISTANCE GENE TO BARLEY YELLOW
DWARF VIRUS**

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by

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Summary

The Yd_2 gene in barley provides protection against barley yellow dwarf luteovirus (BYDV), the most economically devastating virus of cereals worldwide. The aim of this thesis was to investigate strategies to enable the Yd_2 gene to be cloned. These strategies included a map-based approach to cloning Yd_2 in barley, a syntenous map-based approach to cloning a Yd_2 orthologue in rice and the use of PCR primers designed to amplify conserved regions of resistance genes in an attempt to amplify a candidate Yd_2 gene.

Essential for cloning Yd_2 was the development of a resistance assay system to reliably characterise Yd_2 genotypes. Aphids reared on 3-week old oat plants grown at 22°C days and 18°C nights were highly viruliferous resulting in 100% infection rates for barley cultivars tested. Infected Yd_2 and non- Yd_2 barley cultivars were able to be reliably distinguished as early as 4 weeks post infection based on symptom expression. F₂ progeny homozygous-positive for Yd_2 were resistant and distinguishable from other F₂ progeny. Cultivars heterozygous and homozygous-minus for Yd_2 were susceptible and unable to be distinguished from each other. Resistance assays were required to be performed on the F₃ progeny of these F₂ individuals to distinguish between homozygous and heterozygous susceptible F₂ individuals.

A quantitative dot-blot assay relating to viral titre levels in infected plants was developed to quickly distinguish between resistant and susceptible genotypes of different Yd_2 sources. Significant differences between viral titre in both shoot and root material were evident between non- Yd_2 and Yd_2 genotypes, with the differences being even more pronounced in the root material. The dot-blot assay was able to effectively distinguish between resistant and susceptible individuals 5 days post infection from a range of barley cultivars differing in Yd_2 status. This assay was unable to distinguish between homozygous and heterozygous susceptible individuals. Therefore, dot-blot analysis was performed on the F₃ progeny of F₂ individuals to identify homozygous susceptible and heterozygous susceptible F₂ individuals. In combination with the qualitative resistance screening assay this quantitative assay was used to help genotype individuals for Yd_2 in our mapping populations.

To undertake map-based cloning in barley, genetic linkage maps of the *Yd₂* region were constructed using 106 F₂ individuals of the Proctor (*Yd₂-minus*) × Shannon (*Yd₂-plus*) cross and 572 F₂ individuals of the Atlas (*Yd₂-minus*) × Atlas68 (*Yd₂-plus*) cross. These maps contain 29 molecular markers, the *Yd₂* gene and the centromere. Twelve of these markers are within 1cM of *Yd₂*. From the Atlas × Atlas68 cross, 18 F₂ individuals were carrying a recombination event within a genetic interval of 1.6cM between two *Yd₂* flanking loci *YLM* and *Xmwg952*. F₃ progeny derived from these F₂ individuals which were homozygous for the recombinant chromosome were identified and characterised for use in bulked segregant analysis and fine scale mapping of the *Yd₂* region.

Pulsed field gel electrophoresis (PFGE) was used to establish the relationship between genetic and physical distance in the region of *Yd₂*. Physical mapping of the *Yd₂* region revealed a physical linkage between the two loci *Xbcd134* and *Ylp*. Because these loci are separated by 0.5cM on the Proctor × Shannon genetic map, a relationship of physical to genetic distance around this immediate area could be calculated as 1cM being less than to 360kb. This relationship suggested that there was a good chance of identifying a single genomic clone with our co-segregating marker *Ylp* which may also contain the *Yd₂* gene.

A rice yeast artificial chromosome (YAC) library established by the Japanese Rice Genome Project was utilised as a tool for cloning the *Yd₂* gene. Two YACs from an area of rice Chromosome 1 syntenous with the *Yd₂* region of barley Chromosome 3 were identified. A physical linkage of 9kb between two loci *XrYlp* and *Xrc122*, syntenous with the loci *Ylp* and *Xc122* which co-segregate with the *Yd₂* gene in barley was established in rice. The YAC contig of this area of the rice genome however, was not continuous. Other molecular markers in close genetic proximity to *Yd₂* with the exception of *Xwg889* from both our barley maps could not be placed on the rice YAC clones identified as syntenous with *Yd₂* region of barley. With the possibilities of microsynteny breakdown, a favourable relationship between genetic and physical distance in the region of barley *Yd₂* and the development of a publicly available large genomic insert barley library, a syntenous map-based approach in rice was not continued.

Barley bacterial artificial chromosome (BAC) filters were obtained, screened and a number of barley BACs from the *Yd₂* region were identified. Two BACs were identified as encompassing the *Ylp* locus which co-segregates with *Yd₂*, 5 BACs were identified as encompassing the *Xbcd134* loci, a loci 0.5cM proximal to *Yd₂* and 4 BACs identified as encompassing the *YLM* loci, 0.7cM distal of *Yd₂*. A partial contig over the *Yd₂* gene was established with BACs positive for the *Ylp* locus showing a small overlap with 4 of the 5 BACs positive for the locus *Xbcd134*. No physical linkage was identified between the BACs positive for *YLM* and *Ylp*, and it was concluded that *YLM* may be too distal to *Yd₂* to begin a chromosome walk from this side. All other markers identified within 2cM of *Yd₂* on the genetic maps were absent from these clones. Based on this physical information it was concluded that a number of chromosome walks using end-clones from the BACs positive for *Ylp* and distal to *Xbcd134* may complete a contig spanning the *Yd₂* region.

Concurrent with the map-based approaches in barley and rice, a strategy aimed at cloning *Yd₂* using PCR primers shown to amplify conserved areas of resistance genes was undertaken. In order to amplify possible *Yd₂* candidates, primers were targeted to the template of a rice YAC identified from the syntenous region of rice Chromosome 1, at barley Near Isogenic Lines (NILs) and also to resistant and susceptible bulks derived from critical recombinants identified from the Atlas × Atlas68 mapping population. While an number of the products amplified were identified as closely linked genetically or physically to *Yd₂*, sequence information of these products showed no identity to resistant gene analogues. However, a number of the products cloned from rice showed low identity matches to genes or transcripts which could be considered potential candidates for *Yd₂*. Northern analysis using these clones as probes however, detected no mRNA in infected or noninfected barley cultivars.

Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference is made in the text.

I consent to this thesis being made available for photocopying and loan.

Brendon James King
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Barley yellow dwarf virus (BYDV) is the most economically damaging virus of cereals worldwide (Plumb, 1983). Johnstone (1995) estimated reductions in the value of cereal harvests due to BYDV in Australia alone to be worth over \$US100 million annually. The Yd_2 resistance gene, originally discovered in wild Ethiopian barley (Rasmusson and Schaller, 1959), has been introduced into a number of barley lines and has provided durable resistance to the most prevalent isolates of this virus during the past 40 years (Burnett *et al.*, 1995). Unlike the majority of resistance genes currently cloned, the defense mechanism of the host plant is not a hypersensitive response (Section 1.5.2). Resistance genes which elicit a hypersensitive response recognize the pathogen and prevent its further entry into the plant by sudden cell death, thereby completely stopping the pathogen's colonisation of the host (Stakman, 1915; Goodman and Novacky, 1994). However, this puts enormous selection pressure on the pathogen for the selection of a mutant strain to overcome the resistance, eventually leading to a break down of the resistance (Salomon, 1999).

In contrast, the Yd_2 gene does not prevent the virus from spreading systemically from the point of infection, rather it appears to act by reducing the rate of replication of the virus in the vascular tissue of the plant (Ranieri *et al.*, 1993). Therefore, the host plant allows the growth of the pathogen. Because the Yd_2 resistance gene does not completely stop the growth of the viral pathogen but tolerates it in a reduced presence, there is less selection pressure on the pathogen to overcome this resistance. This has resulted in the gene retaining its effectiveness in the field for the last 40 years. The cloning and characterisation of the Yd_2 resistance gene may identify a new class of resistance gene with a novel mode of action. Such a discovery may give us insight into targeting specific plant genes involved in host-pathogen interactions for the creation of novel and durable plant resistances to other pathogens. This is especially important considering the continual break down of resistance genes to invading pathogens.

A major research effort was begun in 1991 in the laboratory of Professor Robert. H. Symons to isolate and characterise the Yd_2 gene, in a hope to understand the molecular basis of the host-pathogen interaction. The work presented in this thesis is a continuation of the early molecular

genetic analysis of the Yd_2 region which was commenced in this laboratory with Nick Collins in 1992 and continued by Nick Paltridge in 1994. The specific aim of the work presented in this thesis was the continuation of this work so as to enable the eventual isolation of the Yd_2 gene. Ultimately, it is hoped that the findings of this study will lead to an improved understanding of pathogen resistance in plants.

1.2 General features of Barley Yellow Dwarf Virus (BYDV)

Barley yellow dwarf virus (BYDV) was first recognized as a serious pathogen of cereals during a severe epidemic of the disease in California in 1951 (Oswald and Houston, 1953a). Since then it has become regarded as the most damaging viral pathogen of cereal crops worldwide (Plumb, 1983). This section describes the general features of the viruses, their transmission and vector relationships, host range and symptomatology, effects on hosts, distribution, economic importance and control measures.

1.2.1 BYDV, a suite of viruses

BYDVs are single icosahedral particles which range in size from 25-30nm and comprise a capsid protein monomer of 24kDa (Rochow and Brakke, 1964; Plumb, 1992). They have a single stranded, positive sense RNA genome of approximately 5600 nucleotides containing six open reading frames, and their genomic RNA's are not polyadenylated. They are obligately transmitted by aphids which precludes mechanical inoculation (Miller *et al.*, 1995). Their absorbance maxima ranging from 261 to 258nm, their minima 261 to 238nm (Proll *et al.*, 1985).

BYDVs have been described as a continuous, often overlapping range of luteoviruses, only some of which are closely related serologically and share common aphid vectors (Irwin and Thresh, 1990). In general five distinct isolates of BYDV are recognised. These were identified by Rochow (1969), and Johnson and Rochow (1972), who showed that BYDVs collected in the state of New York, USA, could be classified into isolates according to the efficiency with which they are transmitted via each of four aphid vector species (Table 1.1). Each isolate was named with an acronym based on the initial letters of its principal vector species. These five isolates were used

Table 1.1 The relative efficiency of transmission of five BYDV isolates by four aphid species†

Aphid species	BYDV Isolate				
	MAV	PAV	SGV	RPV	RMV
<i>Rhopalosiphum padi</i> L.	+	+++	-	+++	-
<i>Rhopalosiphum maidis</i> Fitch	-	-	-	-	+++
<i>Sitobion avenae</i> Fabr.	+++	++	-	-	-
<i>Schizaphis graminum</i> Rodani	+	+	++	+	-

†Data were obtained from an experiment by Johnson and Rochow (1972), involving BYDV isolates collected in New York. The transmission efficiency for each virus-aphid combination is based on the percentage of 36 infected plants that became infected.

+++ = 90 to 100%, ++ = 70 to 89%; + = 35 to 69%; - = 0 to 34%

as antigens to produce polyclonal and monoclonal antibodies and used to assign BYDV isolates from wide geographic areas to one of the five BYDV serotypes.

In the classification of luteoviruses agreed upon by the International Committee on Taxonomy of Viruses (Randles and Rathjen, 1995), the five isolates of BYDV defined by Rochow (1969) and Johnson and Rochow (1972) are placed into two subgroups. This grouping is based on a range of criteria, including serology (Aapola and Rochow, 1971; Rochow and Carmichael, 1979), ultrastructural symptomatology (Gill and Chong, 1979b), synergy and cross protection between isolates within the host, (Halstead and Gill, 1971; Rochow, 1975; Wen *et al.*, 1991), and the size and number of double stranded RNAs found in infected plant tissues (Gildow *et al.*, 1983). PAV, MAV, and SGV make up Subgroup I, RPV and RMV make up Subgroup II.

Recently, based on sequence data it has been shown that the genome organisation of Subgroup I differs from the genomic organisation of Subgroup II (Miller *et al.*, 1995). The degree of similarity between the nucleotide sequences of the coat proteins of the different isolates, as shown by coat protein sequence comparisons, supports the classification of subgroups (Domier *et al.*, 1994). However, the differences between the subgroups are so great they are considered by some to be two separate viruses (Miller *et al.*, 1995). Genome organisation, serology and nucleotide sequence are more similar between Subgroup II and Beet Western Yellows Virus (BWYV) and Potato Leafroll Virus (PLRV), which infect dicotyledons, than to BYDV Subgroup I (Rochow and Duffus, 1978; Domier *et al.* 1994; Miller *et al.*, 1995).

Four serologically distinct isolates of BYDV have been identified in Australia which infect cereals and grasses throughout the country. These include the MAV-like, PAV-like, RMV-like and RPV-like isolates. While PAV-like, RPV-like and RMV-like have conventional vector-like relationships, the Australian MAV-like isolates have *Rhopalosiphum padi* as a major vector species. PAV-like isolates are the most common in Australia (Sward and Lister, 1988).

1.2.2 BYDV transmission and vector relationships

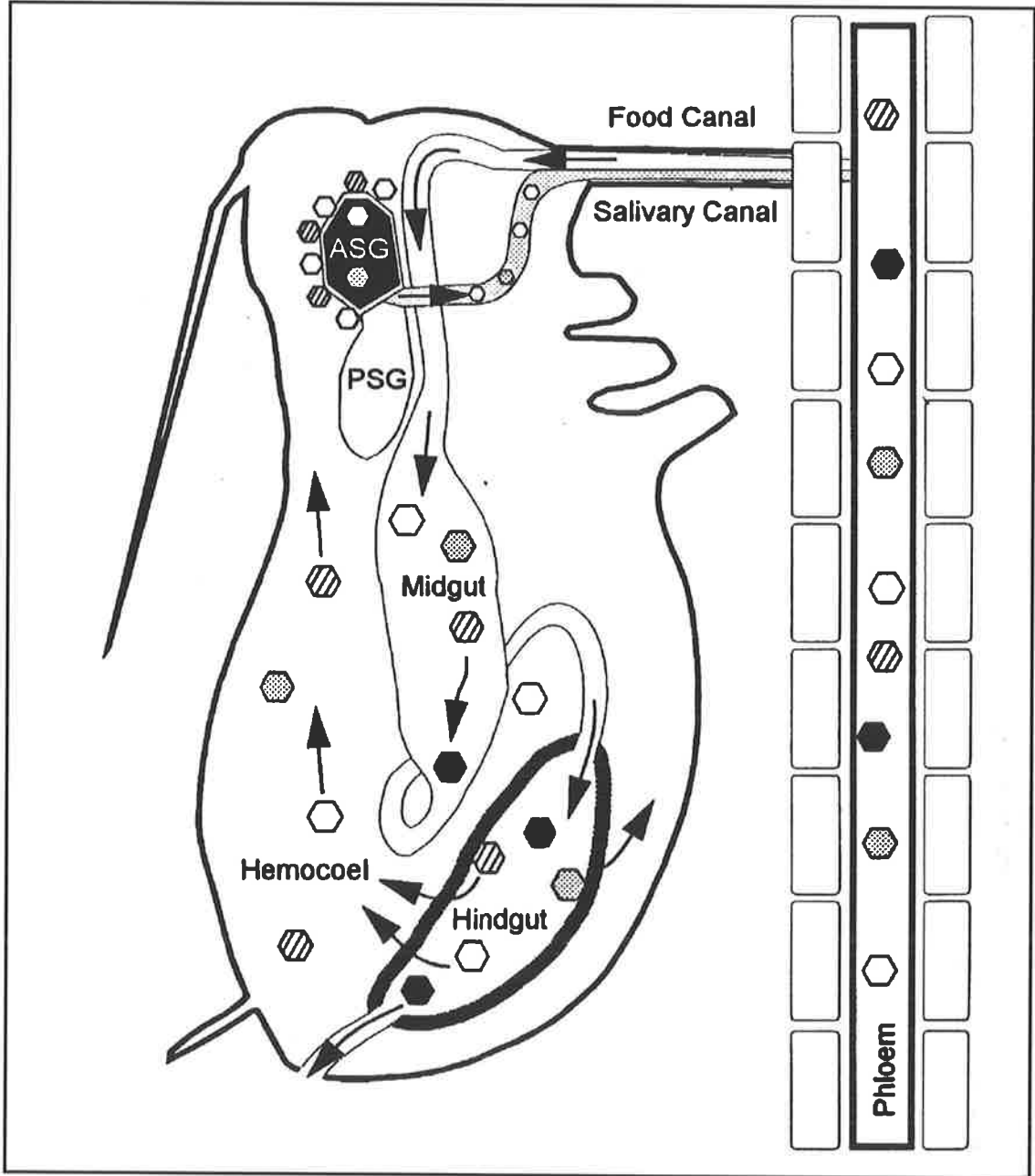
Many species of aphid infect grasses, including cereal crops. To date, twenty-five have been reported as vectors of BYDV, with the *Rhopalosiphum* (genus) species the most frequently reported vectors of BYDV world-wide. They are holocyclic, utilising *Prunus* or *Pomae* as primary hosts and summer hosts are usually Poacea (Power and Gray, 1995). Other common aphid vectors of BYDV include *Sitobion avenae*, *Macrosiphum euphorbiae*, *Schizaphis graminum*, *Metopolophium dirhodum*, *Sipha elegans*, *Diuraphis noxia* and *Diuraphis frequens* (Halbert and Voegtlin, 1995). With the exception of one aphid-isolate combination (*Rhopalosiphum maidis*-MAV), all the other aphid species are able to transmit each of the five isolates of BYDV. However, each aphid vector has different transmission efficiencies for the five BYDV isolates of BYDV (Rochow and Muller, 1971) (Table 1.1).

BYDVs are transmitted by aphids in a circulative, nonpropagative manner (Gildow, 1987). The ingested virus is actively transported by receptor-mediated endocytosis across gut epithelial cells and released into the haemocoel of the aphid (Gildow, 1993). Peiffer *et al.*, 1997, showed that the accessory salivary gland (ASG) basal laminae and plasmalemma contain specific components which are independently involved in the recognition and transmission of luteoviruses. The virus is transmitted only if the virions traverse the ASG cells into the salivary canal. Where the virus exits along with salivary secretions during feeding (Figure 1.1)(Gildow, 1987).

Low viral titre levels in host plants have been associated with lower levels of transmission efficiency (Pereira *et al.*, 1991; Gray *et al.*, 1994; Guo *et al.*, 1997). Pereira *et al.*, 1991 showed by experiments in which aphids fed on virus preparations through a Parafilm membrane, a strong correlation existed between virus concentration and efficiency of acquisition and transmission for both efficient and inefficient vectors. It was concluded that an overriding factor in virus acquisition *in vivo* is the availability of the virus in the phloem from which ingestion is taking place. Therefore, conditions affecting viral titre are important considerations for maximizing transmission of BYDV by aphid vectors. Factors reported in the literature affecting viral titre include cultivar type, plant age, plant material, time after infection, and temperature (Catherall *et al.*, 1970; Jones and Catherall, 1970).

Figure 1.1 The circulative route of BYDVs through aphids. (Reproduced from Power and Gray in "40 years of Progress")

All BYDV strains can be ingested from phloem into the aphids alimentary canal and arrive in the hindgut intact. The hindgut epithelium is the first transmission barrier; most BYDV strains can bind to hindgut epithelial cells and be transported into the hemocoel, but some strains are excluded (black hexagons). BYDV strains acquired into the hemocoel must migrate to the accessory salivary gland (ASG). The basal lamina of the ASG may selectively filter BYDV strains or it may act to concentrate virions, thereby increasing the efficiency of transport into the ASG. Strains (dotted hexagons) not concentrated at the ASG may be transported into the ASG if they encounter the ASG, but the efficiency of transmission is low. BYDV strains may be concentrated at the ASG, but be prevented from entering the ASG by an inability to bind to the ASG plasmalemma and initiate endocytosis (stripped hexagons). Efficiently transmitted strains are concentrated at the ASG and efficiently transported into the ASG and salivary canal (white hexagons).



Apart from the availability of the virus in the source plants, the length of the acquisition access period (AAP) is of major importance in determining the overall transmission efficiency of a population of vectors (Gray *et al.*, 1991). Persistently transmitted viruses can be acquired in a very brief period of time, i.e., <30min, presumably limited by the time it takes for the vector to locate and begin feeding on phloem tissue. But on average, efficient transmission clearly requires a more extended AAP. Phloem contact is required for the acquisition of any luteovirus by an aphid, and the longer the AAP, the greater amount of virus detected in the aphid (Paliwal and Sinha, 1970).

Acquisition efficiency may depend more on the length of time the aphid spends acquiring the virus than on the amount of virus in the infected sap. Virus must be transported from the ingested sap across the hindgut membrane into aphid hemocoel by receptor-mediated endocytosis (Gildow, 1987). A majority of the acquired virus is voided in the aphid honeydew suggesting that one limiting factor is the rate at which virus can be transported across the hindgut membrane. Peiffer *et al.* (1997), showed that accessory salivary gland (ASG) basal laminae and plasmalemma contain specific components which are independently involved in the recognition and transmission of luteoviruses. Increasing the concentration of virus would not necessarily increase the efficiency of this if the limiting factors are these recognition components. Increasing the length of time that the virus moves through the hindgut by increasing the phloem feeding period would provide a continuous supply of virus to be transported across the hindgut membrane as receptors became available to accept and initiate transport of virus particles (Gray *et al.*, 1991).

1.2.3 Host range and symptomatology of BYDV

The suite of viruses collectively known as BYDV are restricted in host range to the Poaceae, which includes more than 150 species in 5 of the 6 subfamilies of Poaceae in 11 of the 25 tribes (Gould and Shaw, 1983). All the major cereal crops including barley, maize, oats, rye, wheat and rice are hosts as are annual and perennial lawn, pasture, weed and range grasses, thereby providing BYDVs with a variety of host species (Oswald and Houston, 1951; Watson and Mulligan, 1960).

The symptoms of BYDV were first described by Bruehl (1961) and Rochow (1961). Symptom expression is dependent on many variables (Plum, 1983). It can be affected by host species, cultivar type, age of the plant when infected, the isolate and dosage of the virus, the physiological condition of the plant at the time of infection, and weather conditions. For example, oat leaves may turn orange or red while barley turns yellow when infected by BYDV.

The most common symptom of BYDV infection is stunting caused by reduced internode elongation. Other common symptoms include inhibition of root formation and leaf elongation, reduced tillering, suppressed heading and leaf discoloration. Diagnosis of BYDV infection on symptoms alone is risky and can be confused with moisture stress or nitrogen and phosphorous deficiency. Therefore, diagnostic methods are needed to confirm BYDV infection (Burnett, 1984).

1.2.4 Anatomical and cytological effects of BYDV on hosts

The cytopathological effects of BYDV on its hosts were first studied by Esau (1957). Light microscopy of infected host species revealed necrotic obliteration of sieve elements, nearby parenchyma and companion cells in both the large and small vascular bundles of the leaf. The earlier the infection the more severe the obliteration. At later stages of plant development, such as tillering and stem extension, BYDV infection results in only moderate vascular disruption.

The first electron microscopic cytological study of barley infected with BYDV transmitted by *R. padi*, and sampled two weeks after inoculation found 24nm particles in the phloem cells of young and old leaves and roots (Jenson, 1969). Eweida *et al.* (1988) who studied oats infected with a PAV-like virus showed that virus-like particles first appeared in the root phloem cells 4 days after inoculation. Nuclear disruption and virus induced vesicles appeared after 5-6 days, callose deposition after 10 days, and cell necrosis after 13 days.

Gill and Chong (1975, 1976, 1979a, 1979b, 1981) using oats performed a series of cytopathological investigations using different strains of BYDVs transmitted specifically and non specifically by different aphid species. They noted significant differences between the

cytopathology of a vector non-specific (PAV-like) and of an RPV-like strain and proposed that BYDV strains be classified into two subgroups based on cytopathology (Gill and Chong, 1979b). These subgroups were based principally on three criteria:

- 1) Interactions of the virus with the nucleus: Subgroup 1 strains distorted the nuclear outline followed by an accumulation of densely staining material while for subgroup 2 strains the nucleus retained a normal outline but the heterochromatin slowly disintegrated.
- 2) Site of first occurrence of virus progeny: Virus progeny of subgroup 1 first appeared in the cytoplasm, while subgroup 2 viral progeny were first seen in association with the nucleus.
- 3) Appearance of membranes, vesicles, and fibrils: Subgroup 1 strains induced single membrane-bound vesicles fibrils, while subgroup 2 strains induced double membrane-bound vesicles containing fibrils.

The classifications into subgroups based on these cytopathological changes correlate or support the classification of the BYDV isolates based on serological data.

1.2.5 Physiological effects of BYDV on hosts

These changes in plant anatomy, most notably phloem disruption, lead to reduced translocation. This results in carbohydrate accumulation, which in turn increases dry weight, inhibits photosynthesis by feedback, reduces chlorophyll content and increases respiration. While total fresh weight is reduced, there is a dramatic increase in percentage dry weight (Jenson, 1968). Reduced transpiration and increased stomatal resistance can be measured as early as three days after infection. Increases in peroxidase activity and a decrease in catalase activity have also been observed in infected plants (Orlob and Arny, 1961).

BYDV infection can make plants more susceptible to secondary infections such as root rot diseases (Comeau and Pelletier, 1976; Sward and Kollmorgen, 1990). However, it can also have the opposite effect and decrease secondary infection. Infection by BYDV increases soluble carbohydrate in leaves but causes a decrease in nitrogen. Therefore, due to the increases in water

soluble carbohydrate in the leaf, BYDV infection subsequently decreased infection by leaf blotch and net blotch resulting in increased resistance (reviewed in Plum, 1983). BYDV infection can also affect cold tolerance. Andrews and Paliwal (1983), observed a reduction in ice tolerance in BYDV infected plants.

1.2.6 Distribution and economic importance

Cereal grains are grown throughout the world, and pasture, range and wild grasses are ubiquitous. As the Poaceae provide BYDVs with a wide host range, they can be spread efficiently by a range of aphid vectors. Therefore, it is not surprising that BYDV is of global economic importance. Since the first reported case of BYDV in cereals in California by Oswald and Houston (1951), epiphytotics have been reported in many parts of the world (Conti *et al.*, 1990). BYDV causes the greatest economic damage in bread wheat (*Triticum aestivum* L.), durum wheat (*Triticum turgidum* L. var. *durum*), barley (*Hordeum vulgare* L.) and hexaploid oat (*A. sativa* L. and *A. byzantina*). Although significant yield reductions also occur in maize (*Zea mays* L.), rice (*Oryza sativa* L.) and triticale (*X Triticosecale* Wittmack). The extent and severity of BYDV damage to cereal crops is determined by a number of factors, including the climate, the isolate and the aphid vectors (Irwin and Thresh, 1990; Plumb, 1992).

In North America severe epidemics of BYDV occur every five to eight years (Paliwal and Comeau, 1987; Hewings and Eastmen, 1995). Epidemics usually follow abnormally warm wet winters which encourage the build up of BYDV aphid vector populations and the growth of grass weeds and volunteer cereals which act as reservoirs for BYDVs (Oswald and Houston, 1953; Plumb, 1983; Irwin and Thresh, 1990). Hewings and Eastman (1995) calculated that in the USA in 1989 a hypothetical 5% loss would result in crop losses valued at \$847 million for corn, \$387 million for wheat, \$48 million for barley and \$28 million for other cereals. Global yield losses are difficult to calculate due to insufficient information. Average yield losses attributable to natural BYDV infection range between 11 and 33%. However, in some areas losses of up to 68% have been reported (reviewed in Miller and Rasochova, 1997).

1.2.7 Control of BYDV damage

A number of strategies are currently used to minimise crop losses caused by BYDV infection. Yield losses caused by BYDV are higher, the younger plants are when infected. In addition the ease by which plants are infected decreases with age (Doodson and Saunders, 1970). Consequently, if BYDV infection can be avoided when crops are young, yield loss is substantially decreased. Therefore, avoiding BYDV by changing sowing dates to avoid the most concentrated flights of viruliferous aphids depends upon local knowledge of disease epidemiology and a reliable disease forecasting (A'Brook, 1981; McGrath *et al.*, 1987; Pons and Aldajes, 1990).

A reliable forecasting system is also necessary to prevent prophylactic spraying (Derron and Goy 1988). In the absence of reliable forecasting there is a tendency to minimise risk through routine application of insecticides to eliminate the aphid vectors. In the UK this costs in excess of 10 million pounds per annum and is environmentally damaging (Mann *et al.*, 1996). Holmes *et al.* (1992) identified the need for considerable in-crop monitoring to reliably identify times when risk of infection is high. Harrington *et al.* (1994) suggested that advances in understanding the basis of the spread of the disease could be made by establishing the nature of key meteorological events.

Strategic monitoring of aphid populations has the potential to improve the timing of insecticide application for better control and reduced pesticide usage. Sensitive detection methods such as reverse transcriptase polymerase chain reaction (RT-PCR) rather than enzyme-linked immunosorbent assay (ELISA) need to be used to determine if aphid vectors are viruliferous (Canning *et al.*, 1996). Other aspects of avoiding infection depend upon breaking the cycle of aphid survival and virus acquisition and the elimination of field weeds and nearby grasses which serve as reservoirs for BYDV and its vector aphids between the growing seasons (Osler *et al.*, 1980; Plum, 1983; Kendall, 1986; Kendall *et al.*, 1991).

Success in cereal aphid control by biological vectors is often greatest where the vector aphid is introduced in the absence of their natural enemies. In South America, the introduction of several

aphid predators and parasites has largely replaced the need for pesticide application (Zúñiga, 1990). In New Zealand, populations of *M. dirhodum* were diminished when the Hymenopterous parasite, *Aphidius rhopalosiphi*, was introduced (Stufkens and Farrell, 1987) giving an estimated annual benefit of \$3.5M (US) (Grundy, 1990).

Genetically modified cereal and pasture grasses offer the opportunity for new sources of resistance to BYDV. The biolistic transformation of the coat protein gene of some isolates of BYDV in oats and barley has resulted in transformants with high levels of resistance (McGrath *et al.*, 1987). While Koev *et al.*, (1998) demonstrated that oat plants transformed with the 5' half of the BYDV-PAV genome, which includes the RNA polymerase gene, resulted in resistant transformants.

However, the use of cereal cultivars which are resistant to BYDV is an effective and cheap means of minimising losses caused by BYDV (Plumb and Johnstone, 1995). Furthermore, it is less environmentally damaging than the application of insecticides. (Plum, 1992).

1.3 Pathogen resistance in plants

Plant diseases cause billions of dollars in lost harvest annually, and in some instances, these losses have severe consequences for humans (Agrios, 1988; Schumann, 1991). One of the most convenient, inexpensive, and environmentally sound ways to control plant disease is to utilise disease resistant varieties (Agrios, 1988). Plant breeders for many years have utilised wild relatives of crop plants as a source for new resistance (*R*) genes, and they have become very adept at introgressing *R* genes into elite varieties. However, a continual battle is fought between the resistance genes and the pathogen, with the pathogen often evolving and overcoming the resistance. The advent of modern molecular genetic techniques is helping us gain a better understanding of the interaction between host and pathogen at the molecular level and to point us to new directions for the durable control of plant pathogens using natural and engineered plant resistance. This section reviews induced resistance, the genetics of plant pathogen resistance, molecular characterisation of pathogen resistance genes in plants, defense signal transduction,

other mechanisms of plant disease resistance apart from induced resistance and engineering of disease resistance.

1.3.1 Induced resistance

Plants possess resistance against a wide range of pathogens, including viruses, bacteria, fungi and nematodes. In many instances, plants have been found to mount an active resistance response to pathogen infection, manifested as changes in the visible appearance of the infected tissue or as detectable increases in the production of particular chemicals or proteins. Visible responses of plants to pathogen infection include suberisation and the production of callose deposits which provide physical barriers to infection (Bostock and Stermer, 1989). A widely occurring reaction to pathogen infection in plants is the hypersensitive response (HR). The HR was defined by Stakman (1915), and more recently by Goodman and Novacky (1994) as a rapid death of plant cells associated with disease resistance. It occurs in resistant plants in response to pathogenic viruses, bacteria, fungi and nematodes, and is associated with a multitude of biochemical processes that make these dead cells, and the adjacent living cells, an inhospitable environment for microbes (Kombrink and Somssich, 1995). Which of these processes actually causes the cessation of pathogen growth has rarely been unequivocally proven, and it probably varies among different plant-pathogen combinations (Heath, 1997).

Defenses include the modification of plant cell walls, the accumulation of potentially antimicrobial molecules such as pathogenesis-related (PR) proteins (chitinase, glucanase) or phytoalexins (Kombrink and Somssich, 1995), and the eventually autofluorescence and browning of the dead cell due to the accumulation of oxidized compounds (Nicholson and Hammerschmidt, 1992). The upregulation of "defense genes" that code for antimicrobial molecules or the enzymes that produce them is not restricted to the cells destined to die, since this occurs in adjacent living cells. In addition, the HR typically induces systemic changes throughout the plant including resistance to a variety of previously compatible pathogens (Kombrink and Somssich, 1995).

1.3.2 The genetics of plant pathogen resistance

The HR typically occurs in "gene-for-gene" plant pathogen interaction in which resistance in the host cultivars is controlled by pathogen-specific resistance (*R*) genes that have to be matched by avirulence (*Avr*) genes in the pathogen. Unless the avirulence gene in the pathogen corresponds to the resistance allele in the host, the resistance response does not occur and the pathogen grows in the host. Resistance systems such as these are referred to as gene-for-gene systems (Flor *et al.*, 1956; Keen, 1990).

The ability of a plant to recognise and resist specific races of a pathogen may be conferred by allelic forms of a single *R* gene, or by closely linked resistance genes of different specificity. These two types of resistance loci are exemplified by the *L* and *M* loci for resistance to flax rust fungus *Melampsora lini* (Ehrenb.) Lév in flax (*Linum usitatissimum* L.). The results of classical genetic studies suggest that the 13 specificities at the *L* locus are allelic, and that the seven specificities at the *M* locus are provided by at least four distinct but closely linked genes (Flor, 1965; Mayo and Shepherd, 1980; Islam *et al.*, 1989). Recently, evidence in support of the proposed complexity of the *M* and *L* loci has been obtained by molecular analysis (Ellis *et al.*, 1995; Anderson *et al.*, 1997). Other examples of multiple genes or alleles at single plant disease resistance loci are reviewed by Pryor and Ellis (1993).

Multiple closely linked disease resistance genes with different specificities are likely to have arisen by the duplication of a single gene, followed by sequence divergence of these genes by mutation. Once the gene has been duplicated, further copies of these genes may be produced by unequal crossing over (Ellis *et al.*, 1995). Unequal crossing over between tandemly arranged resistance genes may also be responsible for generating resistance genes with new specificities (Richter *et al.*, 1995).

1.3.3 Molecular characterisation of pathogen resistance genes in plants

The isolation of plant pathogen resistance genes is a necessary step toward gaining a complete understanding of the molecular basis of resistance gene action in plants. The first resistance gene

isolated was the *Hm1* gene, which provides resistance against the fungus *Cochliobolus carbonum* Nelson race 1 in maize by producing an enzyme that degrades the disease causing toxin produced by this fungus (Johal and Briggs, 1992). Unfortunately, studies of *Hm1* did not suggest a structure or function for classically defined *R* genes because the toxin-degrading strategy of *Hm1* does not involve pathogen *Avr* genes, induction of hypersensitive plant cell death, or other hallmarks of gene-for gene interactions.

However, the majority of resistance genes which have been subsequently isolated mediate race-specific resistance involving HR (Table 1.2). Despite the fact that these genes are from diverse plant species with specificity for a wide variety of viral, bacterial and fungal pathogens, sequence analysis revealed that *R* genes often encode structurally similar proteins suggesting that these genes may act by similar mechanisms. The majority of these genes belong either to a class encoding a stretch of leucine rich repeats (LRRs) with a putative nucleotide binding site (NBS) (Table 1.2), or to a class that encodes a LRR domain without a NBS (Jones *et al.*, 1994; Dixon *et al.*, 1996; Thomas *et al.*, 1997; Table 1.2). The two other classes each have only one known representative and are characterised by proteins containing LRRs and a serine-threonine protein kinase domain without a NBS (Song *et al.*, 1995), or by proteins containing a protein kinase domain without a NBS or LRRs (Martin *et al.*, 1993) (Table 1.2).

LRRs are multiple, serial repeats of a motif~24 amino acids in length (Kobe and Deisenhofer, 1994). LRRs contain leucines or other hydrophobic residues at regular intervals and can also contain regularly spaced prolines and asparagines. LRR domains of proteins from yeast, *Drosophila*, humans and other species are known to participate in protein-protein interactions (reviewed in Kobe and Deisenhofer, 1994) and may therefore, be involved in the perception of the avirulence protein or the activation of regulatory proteins in signal transduction which begin the defense response. Two subgroups within the NBS-LRR class have been recognised by the presence or absence of an amino-terminal region (TIR domain) with amino acid similarity and predicted structural similarity (Baker *et al.*, 1997; Parker *et al.*, 1997; Rock *et al.*, 1998) to the cytoplasmic signalling domains of the Toll and interleukin-1 (IL-1) receptor. The first subgroup (TIR-NBS-LRR) includes the *N*, *L6* (reviewed in Hammond-Kosack and Jones, 1997) *M*, (Anderson *et al.*, 1997) and *RPP5* resistance (Parker *et al.*, 1997). The second subgroup which

Table 1.2 Cloned plant resistance genes showing common protein structural motifs

R Gene^a	Plant	Pathogen	Structure^b	Reference
<i>Pto</i>	Tomato	<i>Pseudomonas syringae pv tomato</i>	Protein kinase	Martin <i>et al.</i> (1993)
<i>Xa21</i>	Rice	<i>Xanthomonas campestris pv oryzae</i>	LRR, protein kinase	Song <i>et al.</i> (1995)
<i>RPS2</i>	<i>Arabidopsis</i>	<i>Pseudomonas syringae pv tomato</i>	LRR-NBS-LZ	Bent <i>et al.</i> (1994); Mindrinos <i>et al.</i> (1994)
<i>RPM1</i>	<i>Arabidopsis</i>	<i>Pseudomonas syringae pv maculicola</i>	LRR-NBS-LZ	Grant <i>et al.</i> (1995)
<i>Prf</i>	Tomato	<i>Pseudomonas syringae pv tomato</i>	LRR-NBS-LZ	Salmeron <i>et al.</i> (1996)
<i>Mi (Meu1)</i>	Tomato	<i>Macrosiphum euphorbiae</i> and <i>Meloidogyne incognita</i>	LRR-NBS-LZ	Milligan <i>et al.</i> (1998); Rossi <i>et al.</i> (1998)
<i>RPP8</i>	<i>Arabidopsis</i>	<i>Peronospora parasitica</i>	LRR-NBS-LZ	McDowell <i>et al.</i> (1998)
<i>Rx2</i>	Potato	Potato virus X	LRR-NBS-LZ	Bendahmane <i>et al.</i> (2000)
<i>N</i>	Tobacco	<i>Tobacco mosaic virus</i>	LRR-NBS	Whitham <i>et al.</i> (1994)
<i>L6</i>	Flax	<i>Melampsora lini</i>	LRR-NBS	Lawrence <i>et al.</i> (1995)
<i>M</i>	Flax	<i>Melampsora lini</i>	LRR-NBS	Anderson <i>et al.</i> (1997)
<i>Dm3</i>	Lettuce	<i>Bremia lactucae</i>	LRR-NBS	Okubara <i>et al.</i> (1997)
<i>RPP5</i>	<i>Arabidopsis</i>	<i>Peronospora parasitica</i>	LRR-NBS	Parker <i>et al.</i> (1997)
<i>RPP1</i>	<i>Arabidopsis</i>	<i>Peronospora parasitica</i>	LRR-NBS	Botella <i>et al.</i> (1998)
<i>Xa1</i>	Rice	<i>Xanthomonas oryzae pv oryzae</i>	LRR-NBS	Yoshimura <i>et al.</i> (1998)
<i>I2C</i>	Tomato	<i>Fusarium oxysporum f.sp. lycopersici</i> race 2	LRR-NBS	Simons <i>et al.</i> (1998)
<i>PiB</i>	Rice	<i>Magnaporthe grisea</i>	LRR-NBS	Wang-ZiXuan <i>et al.</i> (1999)
<i>Cf-9</i>	Tomato	<i>Cladosporium fulvum</i>	LRR	Jones <i>et al.</i> (1994)
<i>Cf-2</i>	Tomato	<i>Cladosporium fulvum</i>	LRR	Dixon <i>et al.</i> (1996)
<i>Cf-4</i>	Tomato	<i>Cladosporium fulvum</i>	LRR	Thomas <i>et al.</i> (1997)

^a This list includes race-specific R genes

^b Structure refers to protein structure motifs recognisable in the derived amino acid sequences of the listed genes: leucine-rich repeat (LRR; Kobe and Deisenhofer, 1994), nucleotide binding site (NBS; Saraste *et al.*, 1990), and leucine zipper (LZ; Alber, 1992).

lacks the TIR region are predicted to contain an amino-terminal leucine zipper (LZ), a motif frequently involved in protein dimerisation (Table 1.2). Whether this motif is functional in the dimerisation of *R* proteins has not yet been reported.

The presence of the highly conserved NBS domain in some *R* gene products suggests that nucleotide triphosphate binding is essential for the functioning of these proteins. However, the mechanistic role of NBS domains in the activation of plant defense remains unknown. One possibility is that nucleotide triphosphate binding may alter the interaction between *R* genes products and other members of the defense signal transduction, activating kinases or G proteins (reviewed in Hammond-Kosack and Jones, 1997). Recently clear and extensive amino acid sequence similarity in the NBS domain, including motifs that were previously thought to be common only to NBS-LRR proteins, has been detected in nematode CED-4 and mammalian Apaf-1 proteins-activators of the apoptotic proteases CED-3 and caspase-9 respectively (Chinnaiyan *et al.*, 1997; Van der Biezen and Jones, 1998). The similarity between this response and animal apoptosis has often been remarked upon (reviewed in Heath, 1998), so this structural similarity between CED-4 and Apaf-1 is intriguing.

The cloning and characterisation of *Pto* (Martin *et al.*, 1993) demonstrated the central role of kinase-mediated signal transduction in gene-for-gene plant disease resistance. Modulation of phosphorylation state is one of the most common mechanisms that living organisms use to control protein activity (Pawson, 1994). The serine/threonine kinase capacity possessed by *Pto* and *Xa21* could clearly facilitate downstream signalling.

1.3.4 Resistance genes and defense signal transduction

Most of the *R* genes which have been cloned so far seem to be involved in signal transduction pathways (Baker *et al.*, 1997; Feuillet *et al.*, 1997; Hammond-Kosack and Jones, 1997). Historically, plant pathologists have placed considerable importance on *R* genes, but it must be remembered that their discovery reflects the fact that they segregate during crosses with susceptible plants in which the *R* allele is missing (Flor, 1971). With the advent of molecular genetics, it is becoming apparent that *R* genes are part of a battery of genes involved in signalling

and defense expression, most of which are present in all genotypes of a plant species. This fact presumably explains why disease resistance, and the HR, can be expressed in nonhost plants in the absence of pathogen-specific *R* genes (Heath, 1997).

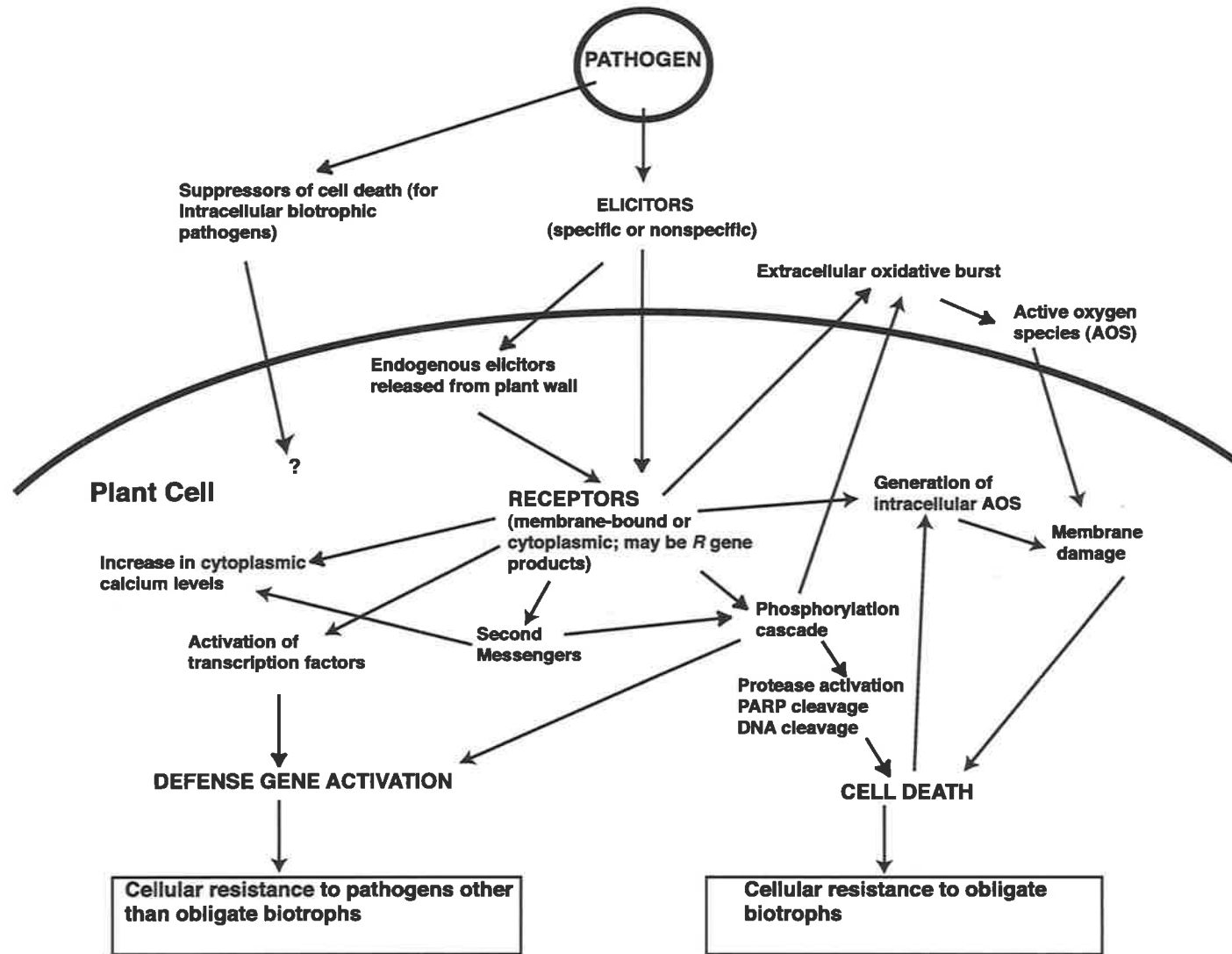
One of the currently best studied *R* genes is *Pto*, which confers resistance to bacterial speck in genotypes of tomato. This gene codes for a cytoplasmic serine/threonine protein kinase which, after binding to the bacterial *Avr* gene product, interacts with a family of transcription factors that bind a cis-element found in genes that code for Pr proteins (Zhou *et al.*, 1997). Significantly, the *Pto* kinase also interacts with a different gene product (another serine/threonine kinase) that is involved in triggering hypersensitive cell death (Zhou *et al.*, 1995). This separation of defense gene activation from cell death is now being reported in a number of situations other than in HR controlled by *Pto* (Buschges *et al.*, 1997; Hunt *et al.*, 1997; Perez *et al.*, 1997). Such results suggest that, although there may be 'cross-talk' between signal transduction pathways, the one(s) that lead to cell death become separated at some point from the one(s) which lead to defense activation (Figure 1.2).

Current models suggest that the HR involves a G-protein-mediated oxidative burst that generates extracellular active oxygen species (AOS) as a consequence of nonspecific or specific (*Avr* gene products) elicitors binding to plant receptors (Wojtaszek, 1997) (Figure 1.2). In the case of specific elicitors, these receptors may be the *R* gene products (Chandra *et al.*, 1996). However, oxidative bursts do not necessarily lead to cell death (Baker and Orlandi, 1995) suggesting that AOS may act as another signal, rather than be the direct cause of the HR (Figure 1.2).

A number of genes required for the function of a number of specific plant pathogen resistance genes have been identified using mutagenesis (Torp and Jørgensen, 1986; Salmeron *et al.*, 1994; Hammond-Kosack *et al.*, 1994; Freialdenhoven *et al.*, 1994, 1996). These genes were identified by generating susceptible mutants in lines containing the respective resistance genes and by showing that these mutations mapped to loci which were distinct from the resistance genes. The future isolation and analysis of these genes will be essential for understanding the processes that are activated upon perception of the pathogen by the resistance gene product. Furthermore,

Figure 1.2 The Hypersensitive Response (Adapted from Heath,1998)

An overview, based on currently available data, of the processes that accompany, and/or may lead to, the hypersensitive response (HR) within a resistant plant cell. It is likely that different receptor-ligand combinations activate different combinations of these processes. The possibility that cell death during some examples of the HR is caused by directly toxic pathogen products is not illustrated. Also ignored are the signals released by the dying cell that activate defense genes in neighbouring, living cells or trigger systematic acquired resistance in the plant.



combining these mutations with other resistance genes in the same host species will indicate whether one or several resistance mechanisms are operating (Freialdenhoven *et al.*, 1994).

1.3.5 Other mechanisms of disease resistance

Not all pathogen resistance in plants can be attributed to an active defense response mounted by the host upon infection. One example is the resistance to certain fungal diseases conferred by the presence of enzymes in the plant tissues which degrade disease causing toxins produced by the fungi, or by the absence of receptors for these toxins (Pryor and Ellis, 1993).

In some forms of virus resistance, the virus is prevented from spreading from the point of infection in the absence of HR (Fraser, 1986). This type of resistance is provided by the *Tm-2* resistance gene in tomato to tobacco mosaic virus (TMV). TMV is not prevented from replicating in individual protoplasts derived from *Tm-2*-containing plants (Stobbs and MacNeill, 1980). Furthermore, the analysis of *Tm-2* resistance-breaking strains of TMV has shown that the 30-kDa viral protein normally required for cell to cell movement within the host confers susceptibility to the resistance gene, indicating that *Tm-2*-mediated resistance acts directly on this protein to prevent movement of the virus from one cell to another (Meshi *et al.*, 1989). In other plant virus resistance systems such as resistance to tomato aspermy virus in cucumber, the virus is allowed to replicate within the inoculated leaf but is prevented from spreading to other parts of the plant (Dufour *et al.*, 1989). In such cases the resistance appears to be acting by preventing the long distance virus movement within the host vasculature, rather than by preventing cell to cell movement.

Another type of virus resistance is exemplified by the *Tm-1* gene for TMV resistance in tomato, which limits virus titre (but not movement) in infected plants and in protoplasts. The 130 kDa and 180 kDa replicase proteins of TMV have been shown to confer susceptibility to the *Tm-1*-mediated resistance, suggesting that host factors directly interfere with the replication of the viral RNA genome (Meshi *et al.*, 1988). Host factors could conceivably interfere with other processes involved in virus multiplication, such as the translation of viral proteins or the assembly of virus particles (Fraser, 1986).

1.3.6 Engineering of disease resistance

The availability of cloned *R* genes now opens up possibilities for addition of new *R* genes to a plant line by genetic transformation therefore, opening up a powerful new capability of transferring *R* genes across species barriers and of deploying *R* genes that have been modified by rational design. A landmark goal in the engineering of plants for improved disease resistance has been to transfer resistance between species by genetic transformation with an *R* gene. This has been achieved with the transfer of *Pto* to tobacco (Rommens *et al.*, 1995; Thilmony *et al.*, 1995). Clearly, the product of an *R* gene must be compatible with other components of the defense apparatus in order to function, and transfer of *R* genes between species cannot be expected to impart resistance in all cases.

The most exciting approach toward engineering improved resistance to disease may be the creation of new *R* genes in the laboratory. Initially, it may be most effective to swap domains between *R* genes that are otherwise unmodified. This approach was recently demonstrated with the highly similar *Fen* and *Pto* genes, when genes with the opposite ligand specificity were created by domain swapping (Rommens *et al.*, 1995). More refined rational design strategies are likely to become prominent as more is learned about the molecular basis of pathogen recognition and the subsequent induction of defense cascades. The challenge here will be to not simply generate new resistance specificities but to synthesise *R* genes that detect a broad range of pathogen genotypes.

Two strategies in particular are being pursued that use *R* genes to engineer broader and more durable disease resistance. One is to identify pathogen traits that contribute to virulence (eg. Kearney and Staskawicz, 1990) and then to focus on the cloning or design of *R* genes that confer recognition based on those traits. A second, more complex strategy involves the generation of plants that express a pathogen *Avr* (such as *Avr9*) gene under the control of a heterologous, infection-inducible promoter (De Wit, 1992; Hammond-Kosack *et al.*, 1994). If the plant carries the corresponding *R* gene (such as *Cf-9*) it will respond with an HR in the region in which expression of the *Avr* gene has been triggered (Hammond-Kosack *et al.*, 1994) The second strategy has several advantages. First, pathogen specificity is very broad (resistance is effective

against any pathogen that induces expression of the chosen promoter). Second, the multifactorial defense response by the *R* gene is more effective than is the expression of a single defense gene such as a chitinase. Third, defense is theoretically expressed only in infected tissues.

While newly designed *R* genes are released into the field it will be essential to consider older plant breeding and cultivar release strategies that could enhance the durability of disease control. Such strategies may include the pyramiding in a single genotype multiple *R* genes with specificity for a given pathogen (to exploit the minimal likelihood that the pathogen would simultaneously lose the corresponding *Avr* genes), release of varieties with different *R* genes in alternative growing seasons, or the planting of mixed resistant and susceptible seed or susceptible refuge plots so that avirulent pathogen genotypes remain predominant over rare virulent strains in the local pathogen population.

1.4 Sources of host plant resistance to BYDV

Breeding for tolerance or resistance to control BYDV has the important advantage of being sustainable, environmentally friendly and not excessively costly (Burnett *et al.*, 1995). Numerous sources of host plant resistance to BYDV have been observed in both cultivated cereals and wild grasses. Searches for BYDV resistance have been most intensively undertaken in wheat, barley and oats, and screening programs to identify BYDV resistant cereal lines have been conducted in over 15 countries (reviewed by Burnett, 1990). The following sections describe sources of resistance in crop plants, their wild relatives and their genetic basis where it is known.

1.4.1 Resistance in Barley

High levels of resistance have been found in barley, with the main source of resistance, the *Yd₂* gene, originating from a number of Ethiopian barleys (Rasmussen and Schaller, 1959). This gene has provided a durable resistance in barley to BYDV over the last 40 years and is reviewed in detail in Section 1.5.

A recessive gene named *yd1* was identified in the commercial barley cultivar Rojo (Suneson, 1955). However, the level of resistance provided was low and this gene has not been widely used in breeding programs (Burnett *et al.*, 1995). Numerous sources of BYDV resistance other than *Yd₂* associated resistance have been found among winter barley and are in use in commercial cultivars such as Surry, Henry, Monroe and Maury (Starling *et al.*, 1980a,b,c,d; Grafton *et al.*, 1982; Burnett *et al.*, 1995). In a screen of the worldwide barley collection, the Chinese barley line C.I.1113 was found to be BYDV resistant (Schaller *et al.*, 1963), although the genetic basis of the resistance in this line has not been determined. Other sources of BYDV resistance in barley have been identified by Damsteegt and Bruehl (1964). Schaller (1984) concluded that resistance appears to exist in cultivated barley to provide suitable parental material for most cultivated areas. However, it was suggested that accessions that have exhibited resistance at one site need to be tested for resistance at a range of sites where consistent infection takes place (Burnett *et al.*, 1995).

1.4.2 Resistance in Oats

Sufficiently high levels of BYDV resistance suitable for breeding purposes have been found in oat (Burnett *et al.*, 1995). Inheritance studies have suggested that BYDV resistance in different oat lines may be controlled by 1, 2, 3 or 4 genes. (Landry *et al.*, 1984; McKenzie *et al.*, 1985; Qualset *et al.*, 1990). However, a single major gene providing a high level of BYDV resistance has not been identified in oat. Minor genes for BYDV resistance in oat can be combined to produce levels of resistance higher than those provided by the individual genes alone (Comeau and Dubuc, 1978; Brown and Jedlinski, 1978; Kolb *et al.*, 1991). Therefore, the breeding of highly resistant oat lines involves the selection of individuals with the highest BYDV resistance levels from populations showing quantitative variation in resistance levels (Qualset *et al.*, 1990). Burnett *et al.*, (1995) lists 32 oat cultivars known to exhibit resistance to BYDV. Resistance in oat cultivars has proven to be durable and effective over a wide geographic range (Jedlinski, 1984).

1.4.3 Resistance in Wheat

Overall the best sources of BYDV resistance in wheat are less effective than BYDV resistance in barley and oat (Burnett *et al.*, 1995). Two sources of BYDV resistance in wheat are present in the spring wheat cultivar Anza and the wheat line NS879/4. In Anza, the BYDV resistance is provided by the incompletely dominant gene *Bdv1*, located on Chromosome 7D (Singh, 1993; Singh *et al.*, 1993). BYDV resistance from other sources in wheat have shown quantitative inheritance, and are therefore likely to be controlled by multiple genes (Cisar *et al.*, 1982; Qualset, 1992). As in oat, BYDV resistance genes in wheat have additive effects when combined (Qualset *et al.*, 1973).

Due to the poor BYDV resistance in wheat, the potential to introduce BYDV resistance from other grass species has received much attention. Relatives of wheat which show resistance to BYDV include species of the genera *Lophopyron*, *Thinopyron*, *Agropyron*, *Elymus* and *Elytrigia* (Sharma *et al.*, 1984, 1989; Larkin *et al.*, 1990; Banks *et al.*, 1995). McGuire and Qualset (1990) using disomic addition lines of barley Chromosome 3, which carries the *Yd₂* gene, transferred *Yd₂* to Chinese Spring Wheat. The disomic addition to wheat conferred only a small improvement in BYDV resistance, but this was expected as *Yd₂* is incompletely dominant. Improvement of the expression of *Yd₂* in wheat substitution lines is being investigated.

1.4.4 Resistance in Rye and Triticale

Among the cultivated cereals, rye is the most resistant to BYDV (Oswald and Houston, 1953). Resistance has been associated with rye chromosomes 1R and 2R in the triticale (a synthetic hybrid between rye and wheat) cultivars Muskox and Nord Kivu, respectively (Nkongolo *et al.*, 1992). Collin *et al.*, 1990 suggested that resistance in a number of triticale lines is controlled by a small number of genes.

1.4.5 Resistance in Rice

A screen of rice cultivars grown in Italy identified a number of BYDV resistant lines (Moletti *et al.*, 1979). Resistance in these lines was subsequently shown to be controlled by one gene, derived from the cultivar Vialone Nero (Baldi *et al.*, 1990; Baldi *et al.*, 1991). The resistance provided by this gene ranged from dominant to incompletely dominant.

1.4.6 Resistance in other Poaceae

Several other grass species have been screened for resistance to BYDV, with promising results. Sharma *et al.* (1984, 1989) and Shukle *et al.* (1987) identified several species of wheatgrass which showed no evidence of BYDV infection when tested by ELISA. Larkin *et al.* (1990) list a number of sources of resistance including *Thinopyrum*, *Agropyron*, *Elymus*, and *Elytrigia*. Banks *et al.* (1992) found that resistances from *Thinopyrum intermedium* and *Agropyron pulcherrimum* in wheat backgrounds were effective against three BYDV-RPV isolates. *Yd₂* resistance was effective against only two of these isolates.

Xin *et al.* (1988) identified a 56 chromosome amphidiploid of *Triticum aestivum*-*Thinopyrum intermedium* which ELISA assays indicated low viral levels and therefore good resistance. An amphiploid derived from a hybrid between *Lophopyrum elongatum* (a diploid E genome wheatgrass), and Chinese Spring wheat appears to be immune to BYDV in field tests in California and single substituted chromosomes showed the resistance to be mainly on two chromosomes (McGuire and Qualset, 1990).

1.5 *Yd₂*, a resistance gene to BYDV

The *Yd₂* gene discovered in Ethiopian barley by Rasmusson and Schaller (1959) has provided a durable resistance over the past 40 years to the most prevalent isolates of BYDV. The following sections review its discovery and characterisation, the nature and expression of its resistance, and its use throughout the world in breeding programs.

1.5.1 The discovery and characterisation of Yd_2

In a screen of over 6000 entries in the worldwide barley collection, a number of Ethiopian lines were found to exhibit resistance to BYDV. Subsequently, the inheritance of this resistance in 16 of the most resistant lines was determined (Rasmusson and Schaller, 1959; Schaller *et al.*, 1963; Damsteegt and Bruell, 1964; Schaller *et al.*, 1964). It was found that resistance from each of the Ethiopian barleys segregated as an incompletely dominant monogenic trait in families derived from crosses between the resistant barleys and susceptible barleys. Crosses between pairs of resistant lines yielded no susceptible individuals in over 3000 F₂ and F₃ progeny. This suggested that resistance in the different lines was controlled by the same gene or a number of very tightly linked genes (Rasmusson and Schaller, 1959; Schaller *et al.*, 1964). For simplicity, the BYDV resistance from the different Ethiopian lines is commonly referred to as being controlled by a single gene, named Yd_2 (Rasmusson and Schaller, 1959). Schaller *et al.* (1964) localised Yd_2 to barley Chromosome 3, by detecting linkage between Yd_2 and five morphological markers, a scald resistance locus and two translocation break points known to be located on this chromosome.

1.5.2 The nature and expression of Yd_2 -mediated BYDV resistance

The effects of Yd_2 have been well characterised by comparing closely related barley lines with and without Yd_2 . Unlike most other resistance genes, the defense mechanism of the host plant is not a hypersensitive response. ELISA studies have shown that Yd_2 limits the level of virus accumulation in infected tissues (Skaria *et al.*, 1985; Pereira and Lister, 1989; Larkin *et al.*, 1991; Ranieri *et al.*, 1993; Makkouk *et al.*, 1994). However, Yd_2 does not prevent the virus from spreading systemically from the point of infection, rather it appears to act by reducing the rate of replication of the virus in the phloem (Ranieri *et al.*, 1993). Measurements of viral titre have shown BYDV-PAV accumulation is significantly reduced by Yd_2 in the roots and shoots, whereas, the accumulation of BYDV-MAV appears to be reduced in the roots only (Skaria *et al.*, 1985; Ranieri *et al.*, 1993). Interestingly, while Yd_2 is effective against the MAV and PAV-like isolates of BYDV subgroup I, it is not effective against isolates of subgroup II (Skaria *et al.*, 1985; Baltenberger *et al.*, 1987; Pereira and Lister, 1989; Herrera and Plumb, 1989; Ranieri *et al.*, 1993; Makkouk *et al.*, 1994).

The effectiveness of Yd_2 -mediated BYDV resistance is strongly influenced by growth rate and can be seen when the Yd_2 gene is introduced into genetic backgrounds of barley lines which differ in their rate of maturity. When Yd_2 was bred into the genetic background of a slow maturing barley line from the Ethiopian barley C.I. 3208-2, the resistance was ineffective against BYDV (Jones and Catherall, 1970). Jones and Catherall (1970) demonstrated that in homozygous resistance lines selected from a segregating population, the level of BYDV resistance was significantly correlated with the rate at which these lines grew under virus-free conditions. Growth conditions also have a similar effect. For example, Catherall *et al.* (1970) and Jones and Catherall (1970) showed that Yd_2 -containing plants were less BYDV resistant when grown slowly in cool conditions than when grown rapidly in warm conditions. They suggested that the slow growth rate may reduce the effectiveness of Yd_2 by allowing virus multiplication to keep pace with plant growth, allowing the virus to reach a greater concentration in the tissues.

Rasmusson and Schaller (1959) and Damsteegt and Bruehl (1964) found the level of BYDV resistance in F₁ or F₂ plants heterozygous for Yd_2 to be somewhere in between that of the Yd_2 and non- Yd_2 parent used. However, the expression of BYDV resistance in Yd_2 heterozygotes was highly variable, varying from almost full resistance to almost full susceptibility. Because the resistance was never observed to be completely dominant or completely recessive, the Yd_2 gene was referred to as being incompletely dominant. Catherall *et al.*, (1970) reported observing complete dominance and complete recessiveness of BYDV resistance in Yd_2 heterozygotes. The dominance related to the level of resistance in the Ethiopian parent barley used as the source of Yd_2 , with BYDV resistance from the most resistant Ethiopian parents being dominant and the resistance from the least resistant parent being recessive. The dominance of Yd_2 is also influenced by growth conditions. For example, the Yd_2 gene from Ethiopian barley C.I. 1237 was co-dominant when plants were grown in a warm glasshouse, and recessive when grown in a cool glasshouse (Catherall *et al.*, 1970). Catherall *et al.* 1970 reasoned that the variation in effectiveness and dominance shown by Yd_2 may be due to differences between the Yd_2 alleles present in the Ethiopian barleys. However, they conceded that these differences may have also been due to other genes from the Ethiopian barleys modifying the BYDV resistance levels in the heterozygotes.

1.5.3 The use of Yd_2 in breeding

Five different Ethiopian barleys have been used in the breeding of over 16 Yd_2 -containing, BYDV resistant cultivars. The effectiveness of Yd_2 in reducing BYDV induced grain yield losses has been firmly established by conducting field trials of Yd_2 and non- Yd_2 cultivars in areas that experience high levels of natural BYDV infection (McLean *et al.*, 1984; Qualset *et al.*, 1990). The Yd_2 gene provides resistance against the PAV and MAV isolates of BYDV, which are generally the most prevalent BYDV isolates found in cereal growing regions around the world (Lister and Ranieri, 1995). Although reports of severe BYDV damage in Yd_2 containing barley cultivars have been rare (Qualset *et al.*, 1990), combining Yd_2 with other BYDV resistance genes may provide cultivars with resistance to a broader range of BYDV isolates (Qualset *et al.*, 1990; Burnett *et al.*, 1995). With the recent development in this laboratory of two PCR markers closely linked to Yd_2 , the incorporation of Yd_2 into the background of agronomically important cultivars will be expedited (Ford *et al.*, 1998., Paltridge *et al.*, 1998).

1.6 Linkage mapping in the cereals

Detailed genetic linkage maps are fundamental tools for studies on selection, identification and organisation of plant genomes. DNA markers that are shown to be genetically linked to a trait of interest can be used for gene cloning, and trait introgression into plant breeding programs. This section reviews the developments in marker technology, methods for identifying mappable DNA polymorphisms closely linked to genes of interest, mapping populations used for linkage analysis and comparative mapping in the cereals.

1.6.1 Developments in marker technology

The first linkage maps in cereals were constructed using polymorphic morphological, biochemical and cytogenetic loci (Søgaard and von Wettstein-Knowles, 1987; Milne and McIntosh, 1990; Coe *et al.*, 1990). However, recent advances in molecular biology have seen the development of a variety of new genetic markers which are based on polymorphisms

detected at the DNA level. It is these methods which are now routinely used to identify markers for linkage analysis in cereal genomes.

The first DNA-based markers to be developed were restriction fragment length polymorphisms (RFLPs), which are detected after genomic DNA is restriction digested, gel electrophoresed, transferred to a nylon membrane and hybridised to a labelled DNA probe (Botstein *et al.*, 1980; Tanksley *et al.*, 1989). The most comprehensive cereal linkage maps which have been published are dominated by this marker type. Examples of such maps include those constructed in barley by Graner *et al.* (1991), Heun *et al.* (1991), Kleinhofs *et al.* (1993), Graner *et al.* (1994) and Langridge *et al.* (1995).

However, additional DNA marker systems have been developed in recent years which have also been used to construct linkage maps of the cereal genomes. They all rely on the polymerase chain reaction (PCR) to amplify polymorphic DNA sequences, which are characterised by their differential mobilities during agarose or polyacrylamide gel electrophoresis. The main marker types developed for linkage analyses are Simple Sequence Repeat polymorphisms or microsatellites (SSRs; Tautz, 1989), Random Amplified Polymorphic DNAs (RAPDs; Williams *et al.*, 1990) and Amplified Fragment Length Polymorphism (AFLPs; Zabeau and Vos, 1993; Vos *et al.*, 1995).

Briefly, SSRs were the first of these developed, and are based on the amplification of short and highly polymorphic repetitive sequences which are dispersed throughout the genome. RAPDs were developed at about the same time. This marker type is generated through the random amplification of genomic sequences by short DNA primers of arbitrary sequence. Most recently, the AFLP marker system was devised. AFLP analysis begins with the restriction digestion of genomic DNA and the ligation of DNA adapters of known sequence onto the ends of restriction fragments. PCR primers complementary to the adapters and carrying two or three selective nucleotides at the 3' end then allow the amplification of subsets of the restriction fragments, which are screened for polymorphism.

The relative strengths and weaknesses of these four main marker systems have been assessed (Lin *et al.*, 1996; Powell *et al.*, 1996; Russell *et al.*, 1997). The main strengths of RFLPs are that they are highly reproducible and are usually co-dominant, allowing heterozygotes to be distinguished from either homozygote and therefore, providing the maximum amount of genetic information in segregating F₂ populations. In addition, they are able to be readily transferred between different crosses and even different species, since hybridisation will still occur between imperfectly matched sequences. However, RFLPs do have the significant disadvantage of being relatively time-consuming to develop and analyse, without producing a great many mappable polymorphisms. SSRs are also relatively time-consuming to develop, but are simple to assay subsequently, and have the advantages of co-dominance and of detecting the highest levels of polymorphism between different genotypes. RAPDs are simple to develop and analyse and enable the analysis of multiple loci in a single experiment (*i.e.*, have a high multiplex ratio). They are, however, usually dominant and reproducibility between laboratories is a problem. Finally, AFLPs are characterised by a very high multiplex ratio, enabling a great many loci to be scanned for polymorphism at once however, they are not easily transferred between different crosses and the preparation of template for analysis is laborious.

Given their different strengths and weaknesses, the different marker types are most suited to different applications. RFLPs are certainly most appropriate for transfer between different maps, and SSRs are most suitable for identifying a few informative polymorphisms in closely related lines which can be simply assayed in large populations. Finally, AFLPs show the greatest potential for the rapid generation of large numbers of markers in a particular cross (Thomas *et al.*, 1995; Powell *et al.*, 1996). Since the advent of AFLP analysis, RAPDs appear to have been little used in the cereals.

1.6.2 Methods for identifying mappable DNA polymorphisms closely linked to genes of interest

There have been two main approaches developed for the identification of DNA markers closely linked to a gene of interest in plants. The first of these was devised by Young *et al.* (1988), and involves screening pairs of nearly isogenic lines (NILs) for polymorphism. In theory, these

lines differ only in the presence or absence of the target gene and a small region of flanking DNA. Thus, any polymorphisms identified between the two lines should be derived from the targeted region, and should map close to the target region in segregating populations derived from these NILs. The backcross method of breeding used to introgress resistance genes into adapted crop cultivars commonly produces pairs of NILs, which differ only for the small chromosome segment carrying the resistance gene. As a consequence, NIL analysis has been used to identify molecular markers closely linked to a number of different disease resistance genes in a variety of different crop species, including wheat (Williams *et al.*, 1994; Schachermayr *et al.*, 1995) and barley (Hinze *et al.*, 1991).

The second approach developed for the identification of markers linked to a gene of interest is bulked segregant analysis (Michelmore *et al.*, 1991). In this approach, two bulked DNA samples are assembled from a population segregating for a trait of interest. Each bulk contains individuals that are identical for the trait of interest, but segregating at all unlinked loci. Thus, the only consistent differences between bulks should be in regions closely linked to the gene of interest. Bulked segregant analysis has been successfully used to identify markers linked to several different resistance genes in crop species, including barley (Barua *et al.*, 1993; Borokova *et al.*, 1995). A variant of bulked segregant analysis was developed by Giovannoni *et al.* (1991). In this approach, pools are designed to carry opposing alleles of a genetically defined interval, rather than simply to differ for a given trait or defined point in the genome. Clearly, a more comprehensive analysis of the genotype of each segregant is required before such well defined pools can be assembled.

1.6.3 Mapping populations used for linkage analysis

Traditionally, linkage analyses in barley have been conducted using segregating F₂, F₃ or backcross populations. However, in barley, doubled haploid populations are now used extensively (Devaux *et al.*, 1995). These are developed by culturing the haploid androgenic structures of F₁ individuals of the desired cross, rendering them diploid and regenerating whole plants from these doubled haploid cells. This generates a population of individuals with fixed genotype. This is the great strength of doubled haploid populations: the population can be

maintained and distributed indefinitely simply by maintaining and distributing seed stocks. They are, however, time consuming and expensive to generate. Where large mapping populations have been required for the generation of high resolution maps, it is still usual to use a segregating population generated by natural means (for example, DeScenzo *et al.*, 1994, and Simons *et al.*, 1997).

1.6.4 Comparative mapping in the cereals

The various species which make up today's cereals have probably evolved from a common ancestor over the past 60 million years (Crepet and Feldman, 1991). Despite this common ancestry, the genomes of different cereals vary considerably in size. The genomes of barley and hexaploid bread wheat are among the largest cereal genomes (5×10^9 base-pairs and 1.7×10^{10} base-pairs per haploid nucleus, respectively), while those of some other cereals are around an order of magnitude smaller; for example, the rice (*Oryza sativa*) genome measures only 4×10^8 base-pairs per haploid nucleus, and is more in keeping with the sizes of the genomes of other crop plants (Moore *et al.*, 1993). Most of the additional DNA in the large genome cereals is thought to consist of amplified repetitive DNA, which evolves and diverges rapidly with speciation (for review, see Moore *et al.*, 1993).

The application of molecular markers, particularly RFLPs, in cereal genetics has shed new light on the ancestry and genetic relatedness of the different members of the grass family (for review, see Devos and Gale, 1997). Firstly, it was recognised that a majority of cDNA sequences cloned from one cereal species would cross-hybridise at moderate stringency to homologous sequences in the genomes of other cereal species (Hulbert *et al.*, 1990; Wang *et al.*, 1992; Causse *et al.*, 1994; Kurata *et al.*, 1994b; Van Deynze *et al.*, 1995a, b). This implied that gene content had been largely conserved among the cereals during their evolution.

The high levels of conservation in gene content then raised the question of whether gene order would also be largely conserved amongst the cereals. Initial investigations were concentrated within the Triticeae (wheat, barley and rye) and Andropogonodae (for example, maize and sorghum) tribes, but were later extended across tribe boundaries, to include species as diverse as

rice, sugar cane, oats and millet (for review, see Devos and Gale, 1997). The picture which emerged was that gene order within the grasses is largely conserved, with breaks in colinearity representing points where chromosomal translocations and other rearrangements have taken place during the evolutionary process. This led to the development of integrated cereal maps, the most recent of which (Devos and Gale, 1997), includes species belonging to 6 different tribes and three different subfamilies. Three distinct genome classes are presented, by rice, a member of the Bambusoideae subfamily, by oats and the Triticeae crops of the Pooideae subfamily, and by several members of the Panicoideae subfamily, for example maize and sorghum.

At the time the present work commenced, synteny between the different members of the Triticeae tribe was well recognised. The different members of the tribe were known to share a basic chromosome number of seven and a high degree of RFLP marker colinearity had been observed between them. This had led to the construction of consensus maps of the seven Triticeae chromosomes (Devos and Gale, 1993; Nelson *et al.*, 1995a, b; Van Deynze *et al.*, 1995a,b). The great potential of the maps for application in special purpose mapping populations — for example, for the identification of a suite of markers likely to map to a chromosomal region of interest — had been identified by these authors but had not yet been realised.

1.7 Methods for the isolation of plant genes

A number of approaches have been used to isolate genes of interest from plant genomes. If the gene product can be identified either as a protein with a characteristic biochemical activity, or as a messenger RNA with a tissue-specific pattern of expression, the product can be purified. Information derived from the product can then be used to isolate the encoding gene. However, more often researchers have no information about the gene product or its pattern of expression, and have to adopt other approaches to isolate the gene of interest (Schulze-Lefert, 1995 and Tanksley *et al.*, 1995).

Recently, there has been much progress in the development of strategies to discover new plant genes. These developments are derived from four experimental approaches: firstly, genetic mapping in plants and the associated ability to use map-based gene isolation strategies; secondly, transposon/T-DNA tagging which allows the direct isolation of a gene; thirdly, protein-protein interactions that permit the isolation of multiple genes contributing to a single pathway or metabolic process; and finally, bioinformatics/genomics - particularly the development and use of large EST databases, search software, and methods to quickly analyse gene expression in response to environmental stresses or during specific developmental stages. The following sections will review recent progress in these four areas which are permitting the isolation of many new plant genes.

1.7.1 Genetic mapping and map-based cloning

Genetic maps based upon large numbers of cloned DNA markers are now available for all major crop plants, and several model plant species (<http://probe.nalusda.gov>). These maps have revealed that related species often have highly conserved gene order along extensive segments of their chromosomes (Bennetzen and Freeling, 1993; Paterson *et al.*, 1995, Havukkala, 1996; Lagercrantz *et al.*, 1996). Conservation of gene order is perhaps most compelling among the grasses, where synteny has been found among segments of the rice, corn, wheat, sorghum, oats and barley genomes (as reviewed in Section 1.6.4). Such synteny allows cross-referencing among plant genomes (Bennetzen and Freeling, 1993; Paterson *et al.*, 1995, Havukkala, 1996; Lagercrantz *et al.*, 1996).

DNA markers have proven useful for following the inheritance of genetic loci in segregating populations. Originally, DNA markers consisted of RFLPs (restriction fragment length polymorphisms detected by hybridisation of genomic DNA with random genomic clones or cDNAs), or PCR-based markers such as random amplified polymorphic DNAs (RAPDs). Recently, other PCR-based strategies have been developed, such as the amplification of restriction fragments attached to small DNA linkers (AFLPs)(Vos *et al.*, 1995), amplification of simple-sequence repeats (SSR) (Taramino and Tingey, 1996) or the amplification of spacer regions between these repeats (inter simple sequence repeats, ISSR) (Kantety *et al.*, 1995) (as

reviewed in Section 1.6.1). These high-throughput marker strategies in combination with near isogenic lines or bulk segregant analysis now allow a specific region of the genome to be saturated with DNA markers. AFLPS in particular have proven to be integral to the isolation of several resistance (*R*) genes, including the *Mlo* gene from barley and the *Rx* gene from potato (Buschges *et al.*, 1997; Bendahmane *et al.*, 1997).

Map-based cloning involves the identification of closely linked DNA markers to the trait of interest, and the use of these markers to isolate a genomic clone that spans the target locus. The identification of very closely linked markers accelerates the direct isolation of a large insert genomic clone spanning the target locus without the need for chromosome walking (Tanksley and Couch, 1997). Such "chromosome landing" greatly simplifies gene isolation by eliminating the difficult procedure of isolating overlapping clones spanning a region. The availability of extensive physical maps of the rice and *Arabidopsis* genomes are further simplifying map based cloning in these species (Sommerville 1996; Monna *et al.*, 1997; Nakamura *et al.*, 1997).

In the past, an impediment to map-based cloning has been the relative difficult procedure of constructing and manipulating the large insert genomic clones, yeast artificial chromosomes (YACs). More recently, the development of bacterial artificial chromosome (BAC) vectors have allowed the construction of large BAC libraries from many crop species (Zhang *et al.*, 1996; <http://www.genome.clemson.edu>; <http://hbz.tamu.edu>). As it is easier to isolate large amounts of DNA from BAC clones than from YAC clones, the subsequent isolation of end-clones and subclones for complementation is expedited.

The genomic clone is used to identify candidate clones by sequencing, complementation of a mutant plant with subclones, or by the use of the clone as a hybridisation probe to isolate corresponding cDNA clones (Buschges *et al.*, 1997; Seki *et al.*, 1997). Complementation of a mutant phenotype by transformation of a clone carrying the putative target locus, or comparison with multiple alleles, provides the definitive proof that the target gene has been cloned. The recent development of a BAC vector system that can be used directly in *Agrobacterium* transformation promises to facilitate this often time-consuming step (Adam *et al.*, 1997; Hamilton, 1997). In the syntenic cereal genomes where there is high conservation of expressed

sequences and little conservation of repetitive sequences, it is feasible to identify potential genes by hybridisation of BAC/YAC from a related species to a cDNA or genomic library of the target species (Avramova *et al.*, 1996; Chen *et al.*, 1997).

1.7.2 PCR approach based on conserved domains of Resistance genes

Genetic linkage maps on which agronomically important loci have been located are proving useful for mapping expressed sequence tags (ESTs) or PCR products encoding motifs characteristic of certain classes of gene products. Recently, this strategy was used to isolate and map DNA sequences with homology to resistant (*R*) genes from plants (Kananzin *et al.*, 1996; Leister *et al.*, 1996; Yu *et al.*, 1996 and Botella *et al.*, 1997). *R* genes encode various motifs including leucine-rich repeats, nucleotide binding sites, protein kinase domains, interleukin-1 receptor homologies, and other short conserved sequences with unknown functions. To isolate homologues of *R* genes these features were targeted by degenerate PCR or EST databases searches. The resulting sequences were placed on genetic maps to determine whether they are located near known *R* loci. This approach has successfully identified over 100 *R* gene homologs from potato, soybean and *Arabidopsis* (Kananzin *et al.*, 1996; Leister *et al.*, 1996; Yu *et al.*, 1996 and Botella *et al.*, 1997). However, it is important to note that close linkage or even co-segregation with a particular *R* locus is not sufficient evidence that the candidate gene is responsible for disease resistance. This requires analysis of mutant alleles or complementation of the mutant phenotype by transformation of the cloned gene into a mutant plant.

1.7.3 Transposons/T-DNAs

Gene identification based on gene inactivation by insertion of a transposon or T-DNA into an open reading frame or regulatory sequence continues to be used successfully in many plant species (Bhatt *et al.*, 1996; Cooley *et al.*, 1996; Azpiroz-Leehan *et al.*, 1997; Gray *et al.*, 1997). A particularly powerful approach, one which can shed light on gene function, is the use of large populations of maize that contain active Mutator (Mu) transposons (Gray *et al.*, 1997). Mu transposons are especially amenable to gene identification because they have high rates of mutation, insert randomly throughout the genome rather than preferentially to linked sites, and

are stable once inserted. However, such a 'site-selected' strategy requires knowledge of the gene sequence in order to develop PCR primers.

A related strategy has recently been developed to aid in the characterisation of unknown genes that carry a Mu insertion (Gray *et al.*, 1997). Previously, such Mu-tagged alleles were identified by co-segregation of a Mu-hybridising restriction fragment with the mutation. However, determining co-segregation is often difficult, because Mu can be present in many copies in the genome thus complicating analysis by hybridisation. A new approach termed 'amplification of mutagenised sites' (AIMS) incorporates the AFLP approach of adding DNA linkers to restriction fragments followed by PCR with linked-targeted primers and Mu-specific primers (Gray *et al.*, 1997). Mu-tagged fragments that differ by as little as a few base pairs can be visualised by this method, and products that co-segregate with the mutation are used as probes for further mapping and cDNA isolation. This clever combination of techniques promises to greatly simplify identification of tagged alleles and should be applicable in any plant species where routine insertional activation of the transposon is possible.

The maize activator/dissociation (Ac/Ds) transposon system is also functional in species such as *Arabidopsis*, tobacco, petunia and tomato. In these species Ac/Ds elements behave as they do in maize and preferentially transpose to linked sites on the chromosome in these species. Therefore, for gene identification by transposon inactivation it is an advantage if an Ac/Ds insertion site is near a target gene. Potential limitations to heterologous transposon tagging are, the relatively low efficiency of transformation in some plant species which makes generation of large transposon-tagged populations difficult and, in *Arabidopsis*, the observation that Ac shows reduced levels of activity. A clearer understanding of the host factors involved in controlling transposon activity, may improve their utility in gene identification (Javis *et al.*, 1997).

1.7.4 Protein-protein interaction cloning

Gene isolation methods often rely on the ability to detect a mutant phenotype in the plant. However, genes that are indispensable or that give no discernible phenotype when mutated may be overlooked by such approaches. One approach to overcome this problem is to use protein-

protein interactions as a basis for gene isolation (McNabb and Guarente, 1996). This approach relies on the fact that protein-protein interactions are fundamental to many cellular processes, including signal transduction, transcription, and metabolism. The yeast two-hybrid system relies on the physical interaction of a 'bait' protein fused to a DNA binding domain with a 'prey' protein fused to a transcription activation domain. Interaction of the prey and bait protein activates expression of a reporter gene by localising the transcriptional activation domain near the cognate promoter genes (Fields and Sternglanz, 1994). New genes have been discovered by using 'interaction hunts' involving a characterised bait protein and a cDNA library fused to the transcriptional activation domain. Using this approach genes have been isolated that play a role in signal transduction (Zhou *et al.*, 1995; De Veylder *et al.*, 1997; Zhou *et al.*, 1997), transcription (Schultz and Quatrano, 1997) and a metabolic pathway (Wedel *et al.*, 1997).

Because protein-protein interaction screens often give rise to many candidate interactors, it is important that strict criteria be developed for testing the biological significance of putative protein partners. Such criteria include testing specificity of the interaction by substituting a closely related but non-functional partner protein in the assay (Zhou *et al.*, 1995; Zhou *et al.*, 1997) and demonstrating interaction *in vivo* by co-immunoprecipitation or other biochemical techniques (Wedel *et al.*, 1997). Ultimately, the most convincing evidence for biological significance is to show that perturbation of the candidate gene expression by mutagenesis, antisense, or overexpression, alters the phenotype under study.

1.7.5 Genomics/bioinformatics

Large scale sequencing of cDNA and genomic clones and the analysis of databases that contain this information underlie many new gene discovery approaches (Delsney *et al.*, 1997; Cutter and Somerville, 1996; Somerville *et al.*, 1997). A clear match to an anonymous cDNA or previously characterised gene in the database simplifies the identification of transcribed regions within large inserts of BAC or YAC clones, candidate transposon-tagged genes, or genes encoding specific protein-protein interactors.

To date, large numbers of cDNA sequences are publicly available for rice and *Arabidopsis* and numerous EST projects are underway for other model organisms and economic species (see <http://www.probe.nalusda.gov>, and dbEST database at <http://www.ncbi.nlm.gov/>). Large cDNA databases for corn, soybean, and probably other crops have been developed by private industry but, to date have not been released publicly. Refinements in optimizing sequence information in the *Arabidopsis* database and in search software have accelerated progress in identifying large numbers of genes based on specific motifs (Botella *et al.*, 1997; Cutter and Somerville, 1997).

The pattern of gene expression in different tissues and developmental stages, and in response to specific stresses may provide insight into gene function and thus, play an important role in the identification of genes. Recently, there has been exciting progress in the development of new techniques to analyse gene expression on a genome-wide basis. Two promising methods are hybridisation to micro arrays of characterised cDNAs (Schena, 1996; Schena *et al.*, 1996; Derisi *et al.*, 1997), and serial analysis of gene expression (Zhang *et al.*, 1997). The serial analysis of gene expression technique has been used to analyse gene expression differences between cancerous and normal human cells (Zhang *et al.*, 1997). From an examination of over 300,000 transcripts, 500 were identified that were differentially expressed. A similar approach to examine gene expression in plants during pathogen infection or environmental stresses, for example, may identify a number of new genes which may play a role in the plants response to these events.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Materials

Materials used in this study are listed below, together with the suppliers' names. All chemicals used for *in vitro* use were at least analytical grade in standard. Solutions were prepared under sterile conditions using MilliQ H₂O, and autoclaved when appropriate. Descriptions of RFLP clones and genetic material used in this study can be found in the individual chapters.

Chemicals: • bovine serum albumen (BSA) fraction V, spermidine, ampicillin, kanomycin, salmon sperm DNA, N-(2-hydroxyethyl) piperazine-N'-(2-ethane-sulfonic acid (HEPES), tris (hydroxymethyl) amino-methane (trizma base), ethidium bromide, polyvinyl pyrrolidone (PVP, 40,000 molecular weight), *E. coli* t-RNA, salmon sperm DNA, dithiothreitol (DTT): Sigma Chemicals (USA). • dextran sulphate, ficoll 400: Pharmacia (USA) • phenol: Wako Industries (Japan). • NaCl, NaOH, Na₂EDTA, MgCl₂, potassium acetate (KOAc), sodium acetate (NaOAc), urea, sucrose, glucose, ethanol (EtOH), iso-propyl alcohol, iso-amyl alcohol, chloroform, bromophenol blue, HCl, glacial acetic acid, sodium dodecyl sulphate (SDS): BDH. • xylene cyanol: Ajax Chemicals.

Enzymes: • pancreatic RNase A: Sigma (USA). • *Taq* DNA polymerase, Klenow fragment (large fragment of *E. coli* DNA polymerase I): Bresatec (Australia). • restriction enzymes: Bresatec (Australia), Boehringer Mannheim (Germany), New England Biolabs and Promega (USA) • PNK T4 kinase: Geneworks (Australia).

Oligodeoxyribonucleotides: Synthetic oligodeoxyribonucleotides were made on an Applied Biosystems (USA) Model 380B DNA synthesizer by Neil Shirley in the Department of Plant Science, University of Adelaide. Oligonucleotides were purified by ion exchange HPLC using a MonoQ column (Pharmacia, USA).

Nucleotides and Radionucleotides: Ultrapure nucleotide triphosphates (NTPs) and deoxynucleotide triphosphates (dNTPs) were obtained from Pharmacia. γ -³²P-dATP (10 μ Ci/ μ l), α -³²P-dCTP (10 μ Ci/ μ l), α -³²P-dUTP (10 μ Ci/ μ l) were obtained from Bresatec (Australia).

Molecular weight markers, and cloning vectors: • SPPI DNA cut with *EcoRI*, λ DNA cut with *HindIII*, and pUC19 DNA cut with *HpaII*: Bresatec (Australia).

High Range Lambda Ladder PFG marker #340 and Low Range Lambda Ladder PFG marker #350: New England Biolabs (USA).

- pBluescript SK-: Stratagene (USA).
- pGem®-T Vector Kit: Promega (USA).

Bacterial media ingredients: bacto-agar, bacto-tryptone and yeast extract: Difco Laboratories (USA).

Agaroses: • low melting point agarose: BRL (USA). • LE agarose, Analytical Grade: Promega (USA). • NuSeive GTG grade: FMC Bioproducts (USA). • SeaKem® LE agarose: FMC Bioproducts (USA).

Computer programs: MAPMAKER (Lander *et al.*, 1987).

Chromatography matrix: Bio-Gel P-10: Bio-Rad (USA).

Bacterial strains: *Escherichia coli* DH5 α : Stratagene (USA).

Kits: • Bresa-Clean: Bresatec (Australia). • pGem®-T Vector Kit: Promega (USA). • Quiagen tip-20: Quiagen (Germany).

2.2 Methods

Methods were carried out according to standard procedures (eg. Sambrook *et al.*, 1989) or using manufacturers specifications, except where indicated. Methods used routinely throughout this study are described below. Specific methods that were used only in particular parts of this study are described in the individual chapters.

2.2.1 Plant growth conditions

Potting soils were prepared by the plant growth facility at the Waite campus of the University of Adelaide. Unless otherwise stated, recycled soil made from discarded soil and plant material was used. UC (University of California) soil mix consisting of four parts washed river sand and three parts (dry volume) moss peat was used when a more consistent soil was required. Both types of soil were steam treated to reduce the viability of contaminating seeds and micro-organisms. Unless otherwise stated, plants were grown in 13 to 25cm pots in the glasshouse, at 18 to 25°C.

2.2.2 Growth of bacteria

Cultures of *E. coli* bacteria were grown overnight at 37°C, using solid or liquid media. Solid media was prepared by dissolving bacteriological agar (1.5% w/v) in boiling LB broth (1.0% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1.0% (w/v) NaCl, pH 7.0). Liquid cultures were grown in 10ml tubes containing 3.0ml LB broth or 2YT broth (1.6% (w/v) bacto-tryptone, 1.0% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.0). Each liquid culture was inoculated using single bacterial colony or a scraping from a frozen glycerol culture, and grown on a rotator. The antibiotics ampicillin or kanomycin were added to bacterial growth media at concentrations of 100µg/ml or 50µg/ml when needed.

2.2.3 Transformation of *E. coli* with plasmids by electroporation

Procedures used to prepare electrocompetent cells were those supplied with the Gene-Pulser (Bio-Rad, USA). One ml LB culture inoculated using 10ml of an overnight culture of *E. coli* strain DH5α (BRL, USA) was grown to an optical density (λ_{600}) of 0.6 and the flask chilled on ice for 15 to 30min. The culture was transferred to 200ml pots and the cells pelleted in a GSA rotor at 3,000rpm for 15min at 4°C. The supernatant was discarded and the cells gently resuspended in 0.5ml of ice-cold 10% glycerol solution. The cells were then pelleted as above, the supernatant discarded, and resuspended in 20ml of ice-cold 10% glycerol solution. Cells were transferred to 30ml tubes, pelleted in a HB4 rotor at 4,000rpm for 15min at 4°C, and

resuspended in 2.0ml of ice-cold, 10% glycerol solution. The electrocompetent cells were transferred to 1.5ml Eppendorf tubes in aliquots of 140 μ l, snap frozen in liquid nitrogen, and stored at -80 $^{\circ}$ C until use.

Transformation of electrocompetent cells with plasmids was performed according to the recommendations supplied with the Gene-Pulser. Electrocompetent cells (40 μ l) were combined with 1.0 μ l milliQ H₂O containing 5.0ng plasmid DNA or 60ng of DNA from a ligation reaction. The mixture was transferred to an ice-cold, disposable electroporation cell (0.1cm electrode gap, supplied with the Gene-Pulser), and subject to electroporation using a Gene-Pulser (Bio-Rad), set at 1.8kV, 125 μ FD and 200 Ω . Immediately following electroporation, the cells were mixed with 1.0ml LB broth without antibiotic, and grown at 37 $^{\circ}$ C in a 1.5ml Eppendorf tube for one hr on a shaker. Two-hundred μ l of culture were then plated onto each plate of solid media containing antibiotic, and grown at 37 $^{\circ}$ C overnight.

2.2.4 Mini-preparation of plasmid DNA

The protocol used for plasmid DNA isolation was essentially the procedure described by Sambrook *et al.* (1989) for the small-scale isolation of plasmid DNA by alkaline lysis. All steps were performed at room temperature unless otherwise stated, and centrifugations were performed in an Eppendorf 5415C bench centrifuge. Liquid culture of plasmid-containing bacteria was used to fill a 1.5ml Eppendorf tube and the cells pelleted by centrifuging at 14,000rpm for 30sec. The supernatant was discarded and the cells resuspended in 100 μ l ice-cold GET buffer (50mM glucose, 25mM Tris-HCl, 10mM Na₂EDTA, pH 8.0) by vortexing for 1min. Two-hundred μ l of freshly made, ice-cold 0.2M NaOH, 1.0% SDS was added and mixed in by gentle inversion of the tube. One-hundred and fifty μ l of 3.0M KOAc, 11.5% glacial acetic acid was added to the lysate and the tube vortexed gently before placing the tube on ice for 3 to 5min. The tube was then centrifuged for 5min at 14,000rpm and 350 μ l of the supernatant transferred to a new tube. Nucleic acid was precipitated by combining the supernatant with 35 μ l of 3.0M NaOAc, pH 5.2, and 400 μ l ice-cold iso-propyl alcohol, and pelleted by centrifugation at 14,000rpm at 4 $^{\circ}$ C.

Pellets were washed in 1.0ml ice-cold 70% ethanol, dried completely, and resuspended in 20 μ l of 10mM Tris-HCl, 0.1mM Na₂EDTA, pH 8.0, containing 40mg/ml pancreatic RNase A.

2.2.5 Large scale preparations of plasmid DNA

The following method was used to purify large amounts ($\geq 150\mu$ g) of plasmid DNA. A plasmid-containing bacterial culture was grown overnight to stationary phase (~16h) in 400ml of 2YT containing appropriate antibiotic(s), in a baffled 2 litre flask at 37°C. Cells were sedimented by centrifugation (5,000rpm, 15min, 4°C, Sorvall GSA rotor) and washed in STE buffer (50mM NaCl, 1mM EDTA, 20mM Tris-HCl pH 8.0). The cells were pelleted by centrifugation as before then resuspended in 4ml of GET buffer containing 1 mg/ml lysozyme. The resuspended cells were incubated on ice for 10min, before the addition of 8ml of freshly prepared 0.2M NaOH, 1% SDS, followed by gentle mixing. After incubation on ice for 10min the mixture was centrifuged as previously except that centrifugation was at 10,000rpm for 10min. The supernatant was removed and strained through four layers of cheesecloth, before precipitation of nucleic acids by the addition of 12ml of ice-cold 2-propanol. The pellet was washed with ice-cold 70% ethanol, after which it was dried in air at room temperature (RT) before resuspension in 3ml TE. LiCl (10M) was added to a final concentration of 2.5M to precipitate RNA, and the solution placed on ice for 10min. RNA was pelleted by centrifugation in a Sorvall HB4 rotor at 10,000 rpm for 15min at 4°C. The supernatant was removed and DNA precipitated by addition of an equal volume of ice-cold 2-propanol, followed by centrifugation as previously to pellet DNA. The pellet was washed in ice-cold 70% ethanol and dried in air at RT. DNA was resuspended in 400 μ l TE and transferred to a micro-centrifuge tube. RNase A was added to 20 μ g/ml and the mixture incubated at 37°C for 1h. The solution was extracted twice with phenol:chloroform (Section 2.2.6), and once with chloroform to remove proteins. Plasmid DNA was precipitated from solution by the addition of an equal volume of 13% PEG 8000, 1.6M NaCl, followed by incubation at RT for 5min. DNA was recovered by centrifugation at full speed in a bench micro-centrifuge for 5min at RT, and the pellet resuspended in 400 μ l TE. DNA was again precipitated from solution by addition of 3M sodium acetate pH 4.6 to a concentration of 0.3M, and 2.5 volumes of ice-cold ethanol. The DNA was pelleted by centrifugation as previously, and the

pellet washed in 400µl ice-cold 70% ethanol before re-centrifugation. The pellet was dried and resuspended in 400µl TE.

2.2.6 Phenol:chloroform extraction and ethanol precipitation of DNA

DNA solutions were vortexed thoroughly with one volume of phenol:chloroform (containing one volume of redistilled phenol (BDH, Australia) equilibrated in 50mM Tris-HCl pH 8.0, and one volume of chloroform) and centrifuged for 10min at room temperature (full speed in an Eppendorf micro-centrifuge for small quantities, or 10,000rpm in a Sorvall HB4 rotor for larger solutions). The aqueous phase was recovered and the extraction repeated as necessary.

DNA was routinely precipitated from solutions with ethanol. Briefly, $1/10$ th volume of 3M sodium acetate (pH 4.6) was added followed by 2.5 volumes of ice-cold ethanol. The solutions were incubated on ice for 15min, followed by centrifugation at high speed at RT for 15min in an Eppendorf micro-centrifuge for small volumes, or at 10,000rpm at 4°C for 15min in a Sorvall HB4 rotor for larger volumes. Pellets were washed in 70% ethanol prior to drying *in vacuo* or on the bench at RT.

2.2.7 Agarose gel electrophoresis

Large-scale agarose gels were cast from 100ml of 0.9 to 1.3% (W/V) molten agarose solution containing 1×TAE buffer (0.04M Tris-acetate, 1.0mM Na₂EDTA, pH 8.0), using a 15 × 20cm mould and a comb for making wells of 15µl volume (for Southern analysis) or 30 to 50µl volume (preparative electrophoresis). DNA samples were mixed with 0.2 volume 6 × FLB loading buffer (15% (W/V) ficoll 400, 0.25% (W/V) bromophenol blue, 0.25% (W/V) xylene cyanol) and electrophoresed overnight at 40 to 50V and 18 to 50mA in 1×TAE buffer. DNA size markers made from *Hind*III cut λ DNA, *Eco*R1 cut SPP-1 DNA, or *Hpa*II cut pUC19 DNA (0.2 to 0.5µg) were run alongside the sample DNAs when needed. Gels were soaked in 0.5mg/l solution of ethidium bromide for 10min, destained by rinsing in water for 10min and photographed for future reference using U.V. light of wavelength 302nm (preparative gels) or 260nm (gels for Southern

analysis).

Mini agarose gels were cast by pouring 15ml of 0.9 to 1.5% (W/V) molten agarose solution containing 1×TBE buffer (0.045M Tris-borate, 1.0mM Na₂EDTA, pH 8.0) onto a 6.0 × 7.0cm glass plate with the appropriate comb set above it. DNA samples were mixed with 0.2 volume 6 × ULB loading buffer (40% (W/V) sucrose, 4.0M urea, 0.25% (W/V) bromophenol blue, 0.25% (W/V) xylene cyanol, 10mM Na₂EDTA) prior to electrophoresis at 80 to 180mA in 1×TBE buffer. Mini agarose gels were stained and photographed as described for large scale agarose gels.

2.2.8. Polyacrylamide (sequencing) gel electrophoresis

Denaturing polyacrylamide gels were prepared from 50ml solutions containing 6% (W/V) acrylamide, 0.3% (W/V) bisacrylamide, 7M urea, 1×TBE. Polymerization was initiated by the addition of 400µl freshly prepared 10% (W/V) ammonium persulfate and 40µl of TEMED. The polymerizing solution was poured into gels of 20 x 40 x 0.04cm, with well formation by shark's tooth combs. Gels were allowed to set for at least 60min, then pre-electrophoresed at 50 W until gel temperature was approximately 50°C. Gels were electrophoresed at 50°C at constant power after loading and denaturing of samples in formamide loading solution (95% (v/v) formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 10 mM EDTA).

2.2.9 Cereal genomic DNA preparation

The method for genomic plant DNA preparation was devised by Peter Langridge, and was based on the one used by Guidet *et al.* (1991). All centrifugations were performed at 14,000rpm in an Eppendorf bench centrifuge at room temperature. For each DNA preparation, approximately 0.4g leaf tissue was harvested from one or more seedlings that were two to four weeks old. Tissue was placed inside a 2.0ml snap-top Eppendorf tube, frozen by dropping the tube into liquid nitrogen, and pulverized with a spatula to make 0.8ml of powder. The powder was stirred

with 0.75ml extraction buffer (0.1M NaCl, 0.1M Tris-HCl, 10mM Na₂EDTA, 1.0% (w/v) sarkosyl, pH 8.5), 0.75ml phenol:chloroform:iso-amyl alcohol (25:24:1; phenol was equilibrated with 0.1M Tris-HCl, pH 8.0 prior to use) added, and the tube shaken vigorously for 1min prior to leaving it on a rotator for 15min. Tubes were centrifuged for 10min and the aqueous phase transferred to a new tube. The aqueous phase was then extracted with 0.8ml chloroform as above, and nucleic acid precipitated by combining the aqueous phase with 0.1 volume 3.0M NaOAc, pH 5.2 and 1.0 volume isopropyl alcohol. Nucleic acid was pelleted by centrifugation for 5min, and the pellet washed in 1.0ml 70% ethanol for 15min on an rotator, recovered by centrifugation, dried completely, and resuspended in 45µl of 5.0mM Tris-HCl, 0.05mM Na₂EDTA, pH 8.0, containing 40mg/mL pancreatic RNase A.

2.2.10 DNA restriction, electrophoresis and Southern transfer

For RFLP analysis, 7.0µl of genomic DNA preparation (approximately 3.0µg DNA) was digested at 37°C for three to five hours in 11µl reactions containing 1.0mg/ml BSA, 1.3mM spermidine and 1 × concentration of the buffer supplied with the enzyme. Genomic DNA digests were electrophoresed on large-scale agarose gels (Section 2.2.7), until the bromophenol blue from the loading dye had run ³/₄ of the length of the gel. DNA transfer procedures used were essentially those supplied by Amersham with the Hybond N⁺ membrane. Each gel was shaken gently in 300ml denaturing solution (1.5M NaCl, 0.5M NaOH) for 30min, rinsed briefly in water, and shaken gently in 300ml neutralizing solution (1.5M NaCl, 0.5M Tris-HCl, 1.0 mM Na₂EDTA, pH 7.2) for a further 30min. DNA was transferred from the gels to Hybond N⁺ membrane (Amersham) by Southern transfer (Southern, 1975) overnight, using 20×SSC (3.0M NaCl, 0.3M trisodium citrate, pH 7.0) as the transfer buffer. DNA was fixed to the membrane by placing the membrane DNA side up on a pad made from three sheets of Watmann 3MM paper, soaked in 0.5M NaOH. After 20min, membranes were shaken in a solution of 2×SSC for at least 5min.

2.2.11 Preparation of ^{32}P -labelled RFLP probes

Radioactively labelled probes were synthesised by random priming, essentially as described by Feinberg and Vogelstein (1983). Purified cloned insert DNA (20ng) was combined with 6.0 μl random sequence 9-mer oligonucleotide (0.1mg/ml), and the mixture incubated at 95°C for 3min to denature the DNA. The mixture was cooled on ice for 5min and combined with 10 μl probe labelling buffer (0.5 M HEPES, 0.125M Tris-HCl, 12.5mM DTT, 12.5mM MgCl₂, 1.0mg/ml BSA)¹, 2.5 μl dNTP mixture (0.2mM dATP, 0.2mM dTTP, 0.2mM dGTP), 1.0 U Klenow fragment, 3.0 μl α - ^{32}P -dCTP (10 $\mu\text{Ci}/\mu\text{l}$), and enough milliQ H₂O to make the total volume 25 μl . Probe labelling reactions were incubated at RT overnight. To make spin columns, 1.0ml syringes with their plungers removed were plugged at the bottom using glass wool, filled to the top with Biogel P-10 (Pharmacia) hydrated in TEN solution (0.1M NaCl, 1.0mM Na₂EDTA, 10mM Tris-HCl, pH 7.2) and centrifuged at 3,000 $\times g$ for 4min to remove excess TEN. Probe reactions were combined with 120 μl TEN solution and passed through the column by centrifugation at 3,000 $\times g$ for 4min to remove unincorporated dNTPs.

2.2.12 Hybridisation and autoradiography

The prehybridisation and hybridisation procedures used were based on protocols supplied with the Hybond N⁺ membrane (Amersham). Each 10ml of prehybridisation/hybridisation solution was made by combining 3.0mL of 5 \times HSB solution (3.0M NaCl, 0.1M PIPES, 25mM Na₂EDTA, pH 6.8), 2.0mL of 50 \times Denhardt's solution (2.0% (W/V) BSA, 2.0% (W/V) ficoll 400, 2.0% (W/V) PVP), 3.0ml 25% (W/V) dextran sulphate, 2.0ml milliQ H₂O, and 200 μl of 10mg/ml salmon sperm DNA which had been denatured at 95°C for 10min prior to its addition. Membranes were placed in hybridisation bottles containing 5.0 to 20ml of prehybridisation/hybridisation solution (up to 10 membranes per bottle) and prehybridised for 3 to 5hr at 65°C. Following prehybridisation, probes was denatured by heating for 10min at 95°C, cooled on ice for 5min,

¹The Tris-HCl and HEPES stock solutions used to make the probe labelling buffer were 1M, pH 8.0 and 0.8 M, pH 6.6, respectively.

added to the hybridisation mixture, and hybridisation performed at 65°C for 12 to 20hrs. Membranes were then washed (i) three times for 5min in 2.0×SSC, 0.1% SDS at room temperature, and then (ii) two times for 10min in 0.2×SSC, 0.1% SDS at 65°C. Two final washes of 10min in 1.0×SSC, 0.1% SDS at 55°C were used instead of step (ii) when probes derived from non-barley species were used. Autoradiography was performed for one to five days at -80°C with Konica X-ray film and an intensifying screen.

Following autoradiography, the probes were stripped from the membranes by placing the membranes in a lunch box with at least 100ml of boiling 0.1% SDS solution. The lunchbox was left on a shaker at room temperature for at least 10min before replacing the solution with fresh 0.1% SDS at room temperature. Membranes were stored in this solution at 4°C until their reuse.

2.2.13 Total Plant RNA Isolation

The method used was adapted from Chomczynski and Sacchi (1987). One gram of leaf or root material was frozen in liquid nitrogen. It was subsequently ground to a fine powder in a 50ml sterile plastic centrifuge tube using ball bearings. Ball bearings were removed using a magnet without the need to handle the material. Ten mls of Solution D (4M guanidinium thiocyanate, 25mM sodium citrate, pH 7; 0.5% sarcosyl, 0.1M 2-mercaptoethanol) was subsequently added to the mixture, and vortexed for 10sec. Sequentially 1ml of 2M Sodium acetate, pH 4, 10ml of phenol (water saturated), and 2ml of chloroform:isoamyl alcohol mixture (49:1) were added to the homogenate, with thorough vortexing after the addition of each reagent. The final suspension was shaken vigorously for 10sec and cooled on ice for 15min. Samples were centrifuged at 10,000×g for 20min at 4°C. After centrifugation the aqueous phase was transferred to a fresh tube, mixed with 2.5 volumes of ice cold absolute ethanol, and then placed at -20°C overnight to precipitate RNA. The RNA was sedimented at 10,000×g for 20min and the resulting RNA pellet was dissolved in 3ml of solution D, transferred to a new corex tube and placed on ice for 15min and then precipitated with 2.5 volumes of absolute ethanol overnight. The RNA was sedimented at 10,000×g for 20min and the resulting RNA pellet was washed in 5ml of 70% ethanol and then sedimented, air dried and dissolved in 200µl of 1×TE buffer. Quality and concentration was

determined spectrophotometer and gel electrophoresis.

2.2.14 Northern Blot hybridisation

Gel preparation: 2.16g of agarose was boiled in 156.6ml of water and cooled to 60°C. 5.4ml of 37% formaldehyde (BDH) and 18ml of 10×MOPS buffer [0.23M MOPS(3-N-Morpholino) propanesulfonic acid; Sigma) (pH7.0), 0.01M EDTA and 0.05M NaAC] were then added before the gel was poured into a gel box (14×21cm). The running buffer was 1×MOPS.

Sample preparation: 5µg of RNA, 2.5µl 10×MOPS, 4.4µl formaldehyde and 12.5µl formamide(BHD) were incubated at 65°C for 15min. 5µl of formamide loading buffer (FLB: 95% formamide, 20mM EDTA, 0.5% bromophenol blue, 0.05% xylene cyanol) was then added before loading. The gel was run at 6V/cm until the first bromophenol blue reached about 3/4 of the gel.

Northern Blotting and hybridisation: Before the transfer was set up the gel was soaked in 10×SSC for 15min. The RNA transfer sandwich consisted of the following layers (from bottom to top): 4 sheets of Waterman 3MM prewetted with 10×SSC, the gel (upside down), the nylon membrane (Hybond⁺, Amersham), 2 sheets of Waterman 3MM prewetted with 10×SSC and dry paper towels. After overnight transfer, the sandwich was disassembled. RNA on the membrane was fixed by U.V. light (GS Genelinker, BIORAD) and photographed under short-wave length U.V. light. Prehybridisation was performed at 45°C for a minimum of 8hrs in a bottle containing 20ml of solution consisting of 3ml of 50 × Denhardt's Reagent, 5ml of 20×SSC, 1ml of 10%SDS, 2ml of Carrier DNA (10mg/ml) and 9ml of deionised formamide (BDH). The hybridisation was performed at 45°C in a bottle containing 20ml of hybridisation solution consisting of 2ml of 50 × Denhardt's Reagent, 5ml of 20×SSC, 1ml of 10%SDS, 1ml of Carrier DNA (10mg/ml), 9ml of deionised formamide (BDH) and 2ml of Dextran Sulphate (25%). Membranes were then washed, subjected to autoradiography and stripped and stored as described for Southern analysis in Section 2.2.12.

2.2.15 Purification of DNA clone inserts

To excise insert DNA from DNA clones, 10 μ l of plasmid miniprep was digested for three hr at 37 $^{\circ}$ C, in 20 μ l reactions containing 20 U of the appropriate restriction enzyme, 1.0 mg/ml BSA, 1.3mM spermidine and 1 \times concentration of the buffer supplied with the enzyme. Alternatively, cloned DNA inserts were PCR amplified from the plasmid DNA, using the oligonucleotide primers M13-40P (5'- CAG GGT TTT CCC AGT CAC GAC -3') and M13 RSP (5'- ACA GGA AAC AGC TAT GAC CAT G -3') for clones in the plasmid vectors pBluescriptSK-, pUC18, pUC19 and pUC119, or the primers SP6 (5'- GAT TTA GGT GAC ACT ATA G -3') and T7 (5'- TAA TAC GAC TCA CTA TAG GG -3') for clones in pGEM-4. PCR reactions were 50 μ l in volume, and contained 0.5 U *Taq* DNA polymerase, 0.25 μ M of each primer, 0.2mM (each) dTTP, dATP, dCTP and dGTP, 0.8 to 3.0mM MgCl₂, 1.0 μ l of 1/500 dilution of plasmid DNA miniprep, and 1 \times reaction buffer supplied with the *Taq* enzyme (67mM Tris-HCl, 16.6mM (NH₄)₂SO₄, 0.45% Triton X-100, 200 μ g/ml gelatin, pH 8.8). Reactions were overlaid with mineral oil and subject to 25 cycles of (95 $^{\circ}$ C for 1min, 50 $^{\circ}$ C for 1min, 72 $^{\circ}$ C for 2min) and a final incubation at 72 $^{\circ}$ C for 10min, in a PTC-150 Mini Cycler (MJ Research, USA). A MgCl₂ concentration of 1.3mM was effective for the amplification of most inserts, although inserts larger than 1.5kb generally required the MgCl₂ concentration to be optimized.

Plasmid digests and PCR reactions were electrophoresed in large scale agarose gels (Section 2.2.7), the bands corresponding to the insert DNA excised, and the DNA purified from the gel slices using the Bresa-Clean kit (Bresatec, Australia), according to the manufacturer's instructions. Samples of the purified RFLP clone insert DNA preparations were electrophoresed on mini agarose gels (Section 2.2.7), alongside known amounts of DNA markers in order to verify the sizes of the inserts and to estimate their concentration.

CHAPTER 3

**DEVELOPMENT OF A VIRULIFEROUS APHID MANAGEMENT
STRATEGY AND A QUANTITATIVE RESISTANCE ASSAY**

3.1 Introduction

The unreliability and inconvenience of the biological assay for Yd_2 -elicited resistance has always limited the extent to which Yd_2 has been used in barley breeding programs. If breeders are to conduct assays, they must first obtain and maintain cultures of the appropriate aphid vector, carrying the appropriate isolate of the virus. A system for assessing the severity of BYD symptom expression must be established, a process easily confounded by environmental conditions, stresses such as frost, water logging and nitrogen deficiency (Conti *et al.*, 1990). Moreover, distinguishing between Yd_2 -containing individuals from non- Yd_2 individuals is often hampered by variation in the effectiveness of the gene in different genetic backgrounds and growth conditions, and by the fact that Yd_2 -mediated resistance is often expressed in an incompletely dominant or recessive manner (Rasmusson and Schaller, 1959; Jones and Catherall, 1970; Catherall *et al.*, 1970).

Essential to our effort to clone Yd_2 was the development of a reliable resistance assay system. Previous resistance assays and aphid rearing were carried out in the glasshouse or field during the months of May and September where temperature means in Adelaide were 15 to 20°C, but temperature ranges were between -1 to 35°C (Bureau of Meteorology at www.bom.gov.au/climate/averages). Symptoms were then scored visually or by measuring shoot fresh weight four weeks after infection. These conditions did not suit aphid fecundity or symptom expression (Sections 1.2.2 & 1.5.2) therefore, high levels of viral titre in plants did not ensue. Hence, aphids used for resistance assays were not highly viruliferous and able to transmit BYDV.

The aims of the work described in this chapter were firstly to develop an aphid management system which would produce copious quantities of highly viruliferous aphids for our infection studies and for use in field trials associated with the barley breeding program. Secondly, determine whether a molecular assay based on viral titre levels could distinguish between resistant and susceptible barley genotypes and therefore, be used to determine the resistance status of individual plants.

3.2 Materials and Methods

3.2.1 Plant Lines

Seed of the following barley lines and cultivars used in these experiments were provided by Mandy Jenkin, formerly of the University of Adelaide (CM67), Wayne Vertigan of the Tasmanian Department of Agriculture (Shannon and Proctor), the Australian Winter Cereal Collection at Tamworth, NSW (Sutter, UC337, UC566, Venus and Vixen), or were obtained from the University of Adelaide barley seed collection. F₂ seed from our Atlas × Atlas68 population were derived from crosses made by Nick Collins.

3.2.2 Viruliferous aphid management strategy

Rhopalosiphum padi L. aphids for the transfer of BYDV were collected from field-grown wheat in Glen Osmond, South Australia, and their species identity verified using the guide by Blackman *et al.* (1990). Virus-free aphid nymphs were taken as they were born from the field-collected aphids and placed on virus-free plants to establish aphid stocks. The BYDV-PAV isolate used was kindly donated by Monique Henry (formerly of the University of Adelaide). Because this BYDV isolate was originally collected from the field near Adelaide it will be referred to here as BYDV-PAV_{adel}.

Aphid and BYDV-PAV_{adel} cultures were maintained on the oat (*Avena sativa* L.) cultivar Stout in an environmental chamber (Phoenix research environmental simulator) at 22°C days and 18°C nights with a 14hr photoperiod (20-30μE m⁻²s⁻¹ at plant height). Stout plants were sown in 13cm pots (4 seeds per pot) and grown in aphid proof cages made from fine nylon mesh. Three weeks after germination oat plants were then transferred to one of five viruliferous aphid cages depending on position vacancy. These plants were then used for aphid and virus multiplication.

Once in the cage leaves with viruliferous aphids from a previous occupant were detached from the infected plants and placed on the newly introduced plant. Newly introduced plants were kept

in viruliferous aphid cages for a maximum period of 24 days, after which they were removed, sprayed with the insecticide Rogor (Hortico, Australia) (3ml/L) and put in a separate death cage for 2 days prior to being taken outside the environmental growth chamber and discarded.

Viruliferous aphids were only used from plants between 14 and 24 days after introduction into viruliferous aphid cages when viral peaks were highest (Hammond *et al.*, 1983). Viruliferous aphid cages housed plants at a variety of stages ensuring that at any point in time a number of plants would have viruliferous aphids available for inoculations. Aphids were harvested by shaking the oat plants over a large plastic tray dusted lightly with talcum powder to prevent the aphids sticking together. The harvested aphids were either used immediately for resistance assays or stored for up to two days in lunch boxes at 4°C. BYDV dot-blot hybridisation analysis (Section 3.2.5) was used periodically to confirm the virus was present in aphid cultures and these cultures were not cross contaminated with BYDV-RPV strains.

3.2.3 Barley BYDV resistance assays

Barley plants which were to be assayed for BYDV resistance were germinated on damp filter paper in a petri dish for two days before being sown into individual 13cm pots of Waite RC (Recycled) soil mix. When seedlings were 7 days old, viruliferous aphids were applied with a fine camel hair paint brush to the base of the plant so as to allow a minimum of 20 aphids to walk onto each seedling. Aphids were allowed to feed for a minimum of 48hrs before being killed by spraying with the insecticide Rogor. Infected plants were grown in an environmental growth chamber at 22°C days and 18°C nights with a 14hr photoperiod (20-30 μ E m⁻²s⁻¹ at plant height). Dot-blot hybridisation analysis (Section 3.2.5) was used to confirm and quantify BYDV infection. For dry weight analysis, shoot tops were harvested and dried at 80°C for 72hrs.

3.2.4 BYDV resistance assay field trial

A field trial designed by Steve Jefferies (Senior Research Fellow in the Barley Improvement Breeding Program at the Waite Institute) was conducted at a site in Strathalbyn, South Australia. The aim of the trial was to analyse resistance to BYDV of a number of barley backcross 2 lines

(BC₂) which had been developed using the *YLM* marker, a marker closely linked to *Yd₂* (Paltridge *et al.*, 1998). This was done in order to try and incorporate the *Yd₂* gene into a more suitable agronomic background. The BC₂ lines were derived from the recurrent parent Sloop (*Yd₂*-minus) and donor parent Franklin (*Yd₂*-plus). To scale aphid production up for use in the field trial, aphids were multiplied on 100 oat plants in the same manner described in Section 3.2.2. Leaves and stems of the oat plants were harvested 18 days after initial aphid colonisation and aphids were then spread over the field trial as a green trash. Aphids were then allowed to multiply, spread and colonise the field trial over a period of 18 days before being sprayed with a systemic insecticide Rogor. Non-infected controls were treated with both contact and systemic insecticides. Plots were machine harvested and grain yield calculated from the harvested samples.

3.2.5 BYDV dot blot hybridisation analysis

Dot blot hybridisation analysis was used to determine whether individual plants were infected with BYDV. A 707 bp DNA probe derived from nucleotides 1326 to 2032 of the RNA genome of an Australian BYDV-PAV isolate (Miller *et al.*, 1988; Young *et al.*, 1991) and a BYDV-RPV DNA probe derived from nucleotides 2025 through to 3209 of the RNA genome of an Australian BYDV-RPV as defined by Vincent *et al.* (1991) were used to generate ³²P labelled radioactive probes by *in vitro* transcription. The plasmids containing these probes were linearised by digestion with the appropriate restriction endonuclease at the terminus of the sequence of interest. Linearised DNAs were purified by ethanol precipitation (Section 2.2.6). Transcription mixtures were set up as follows: 1-2µg of linearised DNA was transcribed in a mixture containing 40mM Tris-HCl pH7.5, 6mM MgCl₂, 2mM spermidine, 10mM NaCl, 10mM DTT, 0.5U/µl RNasin (Promega), 0.5mM each of dATP, dCTP, and dGTP, 12µM UTP, 50 uCi alpha-³²P-UTP, and 20-40 units of T3 or T7 RNA polymerase (Promega) as appropriate. Transcription reactions were incubated at 37°C for 90min, transcripts were purified by phenol:chloroform extraction (Section 2.2.6) followed by a minimum of 3 precipitations in 2.5M ammonium acetate and 2.5 volumes of ethanol to remove unincorporated radioactive label. Probes were stored in 10mM Tris-HCl, 1mM Na₂EDTA (TE- buffer pH 8.0) containing 5mM β-mercaptoethanol.

To prepare cereal nucleic acid samples for dot blot hybridisation analysis, the same procedure described for cereal genomic DNA isolation (Section 2.2.9) was used, but the chloroform extraction step was omitted, and RNase A was not added to the nucleic acid re-suspension buffer. Total nucleic acid concentration was determined by spectrophotometric adsorption (260nm/280nm) and samples were adjusted to equivalent concentrations for quantitative analysis. Ten μl samples of nucleic acid were dotted 1.0cm apart onto Hybond N⁺ membrane (Amersham, USA). The spots were allowed to dry and the nucleic acid fixed to the membrane by fixation with 60mJ of U.V energy. The membrane was rinsed in 2 \times SSC for at least 5min. Prehybridisation and hybridisation conditions used were the same as those described for Northern membrane hybridisation (Section 2.2.14), except that a temperature of 68 $^{\circ}\text{C}$ was used. Following hybridisation, membranes were washed three times for 5min in 2 \times SSC, 0.1% SDS at room temperature, and twice for 10min in 0.1 \times SSC, 0.1% SDS at 68 $^{\circ}\text{C}$. Autoradiography was performed for 1 to 48hrs at -80 $^{\circ}\text{C}$ with Konica X-ray film and an intensifying screen. For quantitative dot-blot analysis a phosphorimager (Molecular Dynamics Storm 860) and phosphorimaging screen were used. Screens were exposed for 1 to 48hrs at room temperature.

3.2.6 PCR amplification of the cleaved amplified polymorphic sequence (CAPS) for the *Ylp* locus and the co-dominant *YLM* marker, both closely linked to the *Yd₂* gene.

Two PCR markers, the *Ylp*-CAPS and *YLM* markers, both closely linked to *Yd₂* were used to help determine the *Yd₂* genotype for F₂ individuals used in this work (Ford *et al.*, 1998 and Paltridge *et al.*, 1988). Template for PCR amplification comprised approximately 100ng of DNA prepared as described in Section 2.2.9 or, alternatively, was prepared using the simple NaOH extraction method of Wang *et al.* (1993). In the later procedure, a tissue homogenate was prepared by grinding 5mg of leaf tissue in 50 μl of 0.5M NaOH. One microlitre of the homogenate diluted tenfold in 0.1M Tris-HCl pH 8.0 was then used in PCR reactions.

Reactions for both PCR amplifications were performed in a 20 μl volume using either MJ Minicycler or PTC-100 Thermal cyclers (MJ Research, USA). PCR amplification conditions were 0.63 μM each DNA primer (YlpPCRMF, YlpPCRMR for the *Ylp* locus-Ylp-CAPS PCR

marker) (YLMF, YLMR for the *YLM* locus) 1.5mM MgCl₂, 0.2mM each dNTP, 1×*Taq* DNA polymerase activity buffer [67mM Tris-HCl (pH8.8 at 25°C), 16.6mM (NH₄)₂SO₄, 0.45% Triton X-100, 0.2mg/ml gelatin), 1µl of DNA sample and one unit of *Taq* DNA polymerase (Biotech International, Perth, Western Australia). Reactions, were overlaid with oil, and subjected to thermal cycling using 40 cycles of 30sec at 94°C, 30sec at 58°C and 1min at 74°C for the *Ylp* amplification and 40 cycles of 30sec at 94°C, 30sec at 58°C and 1min at 72°C for the amplification of the *YLM* locus. Post-amplification restriction digests for the *Ylp* amplified locus were performed by adding 1.2µl of the manufacturer's 10×reaction buffer, 1.2µl of 10mg/ml BSA and 2 units of *Nla*III (New England Biolabs) to 10µl of amplification mix following thermal cycling. Digestions were performed at 37°C for 60min. Electrophoretic analyses of the PCR products were performed using 0.75% agarose/2.25% NuSieve GTG agarose (FMC Bioproducts) gels and 1×TBE electrophoresis buffer.

3.2.7 Total nucleic acid Maxiprep of Oat material infected with BYDV-PAV_{adel}

The Maxiprep method for total nucleic acid extraction was scaled up from the genomic plant DNA preparation described in Section 2.2.9. However, all centrifugations were performed at 4°C in a Sorvall centrifuge at 14,000rpm. Ten grams of material was removed from a 4 week old Oat (*Avena sativa* L.) cultivar, Stout, 14 days after infection with viruliferous aphids. Tissue was cut into manageable pieces (approximately 4-5cm in length) and placed inside a mortar before being frozen in liquid nitrogen and ground to a fine powder using a pestle. The powder was transferred to a centrifuge pot and 55ml of extraction buffer (0.1M NaCl, 0.1M Tris-HCl, 10mM Na₂EDTA, 1.0% (W/V) sarkosyl, pH 8.5) added and the homogenate mixed. Fifty-five ml of phenol:chloroform:iso-amyl alcohol (25:24:1) equilibrated prior to use with 0.1M Tris-HCl pH 8.0, was added and the centrifuge pot shaken vigorously for 1min prior to leaving it on a rotor for 15min. Pots were centrifuged at 4°C for 10min at 14,000rpm and the aqueous phase transferred to a new tube. The aqueous phase was then extracted with 50ml of chloroform as described above, the nucleic acids were precipitated by the addition of 0.1 volume of 3.0M NaOAc, pH 5.2 and 1 volume of isopropanol, and centrifuged at 4°C for 10min at 14,000rpm. The pellet was washed in 10ml of 70% ethanol for 15min on a rotor, recovered by centrifugation, air dried, and

re-suspended in 7ml of 5.0 mM Tris-HCl, 0.05 mM Na₂EDTA, pH 8.0 and aliquoted into 400µl. Total nucleic acid concentration was determined by spectrophotometric adsorption (260nm/280nm). This nucleic acid was then used as the standard between experiments so that radioactive counts measured by the phosphorimager could be compared between hybridisations regardless of differences in the specific activity of probes and exposure times.

3.3 Results

3.3.1 Production of viruliferous aphids

High aphid density was achieved on oat plants 14 days after their introduction into the aphid cages. Aphid numbers peaked between 14-22 days and dot-blot analysis of shoot material showed high levels of viral infection (Figure 3.1). Twenty-four days after the introduction of oat plants into viruliferous aphid cages, viral loads were sufficiently high that strong BYDV symptoms such as leaf discolouration and wilting were observed. Aphid numbers subsequently decreased as plants rapidly died.

3.3.2 BYDV resistance assays on *Yd₂*-plus/*Yd₂*-minus cultivars

Individual barley cultivars with and without *Yd₂* were assayed to test the resistance assay system and the viruliferous nature of the aphids produced. Dot-blot analysis of nucleic acid extracted from shoot material of 12 barley cultivars 2 weeks after infection with BYDV-PAV_{adel} showed 100% infection of all individuals (Figure 3.2).

Four weeks after infection with BYDV-PAV_{adel} the level of resistance of these individuals was compared by measuring the level of stunting and necrosis obtained in infected plants to noninfected control plants (Table 3.1). All individuals without *Yd₂* showed high levels of stunting and yellowing. While Proctor(*Yd₂*-minus) showed a moderate level of susceptibility, the cultivars Atlas(*Yd₂*-minus) and CM(*Yd₂*-minus) showed an extreme degree of sensitivity to BYDV-PAV_{adel} infection resulting in complete death after 4 weeks. Individuals containing *Yd₂* exhibited milder symptoms to BYDV-PAV_{adel}, such as leaf brittleness, mild dwarfing and some

Figure 3.1 Copious quantities of a highly infectious aphids for the BYDV-PAV_{adel} culture

(a) Aphids of the genus *Rhopalosiphum padi* L, which were grown on the Oat (*Avena sativa* L.) cultivar Stout in an environmental chamber at 22°C days and 18°C nights with a 14hr photoperiod, reached high population densities covering the 3-4week old oat plant within 14 days after their introduction into an aphid cage. (b) Radioactively labelled RNA probes derived from the genomes of an Australian PAV (Miller *et al.*, 1988; Young *et al.*, 1991) and RPV isolate (Vincent *et al.*, 1991) were used to probe duplicate membranes containing total nucleic acid samples (Section 3.2.5) from these oat plants infected with PAV-BYDV_{adel}. Both healthy and infected controls with both viral isolates were included on these membranes. Dot-blot taken between 14 and 24 days after the plants initial introduction into the cages showed high levels of virus present and the absence of any contaminating RPV-Like strains. Five cages containing in total 30 of these plants housed at a variety of introduction dates, enabled the production of copious quantities of highly viruliferous aphids of the correct BYDV-isolate throughout the year, ensuring that during any date a number of plants would have viruliferous aphids available for inoculations.

(a)



(b)

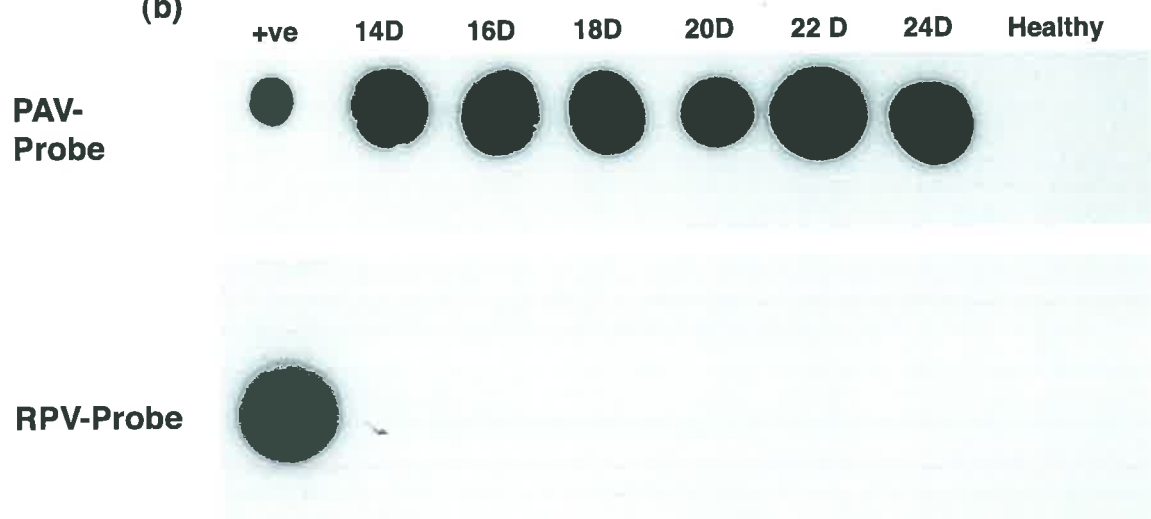
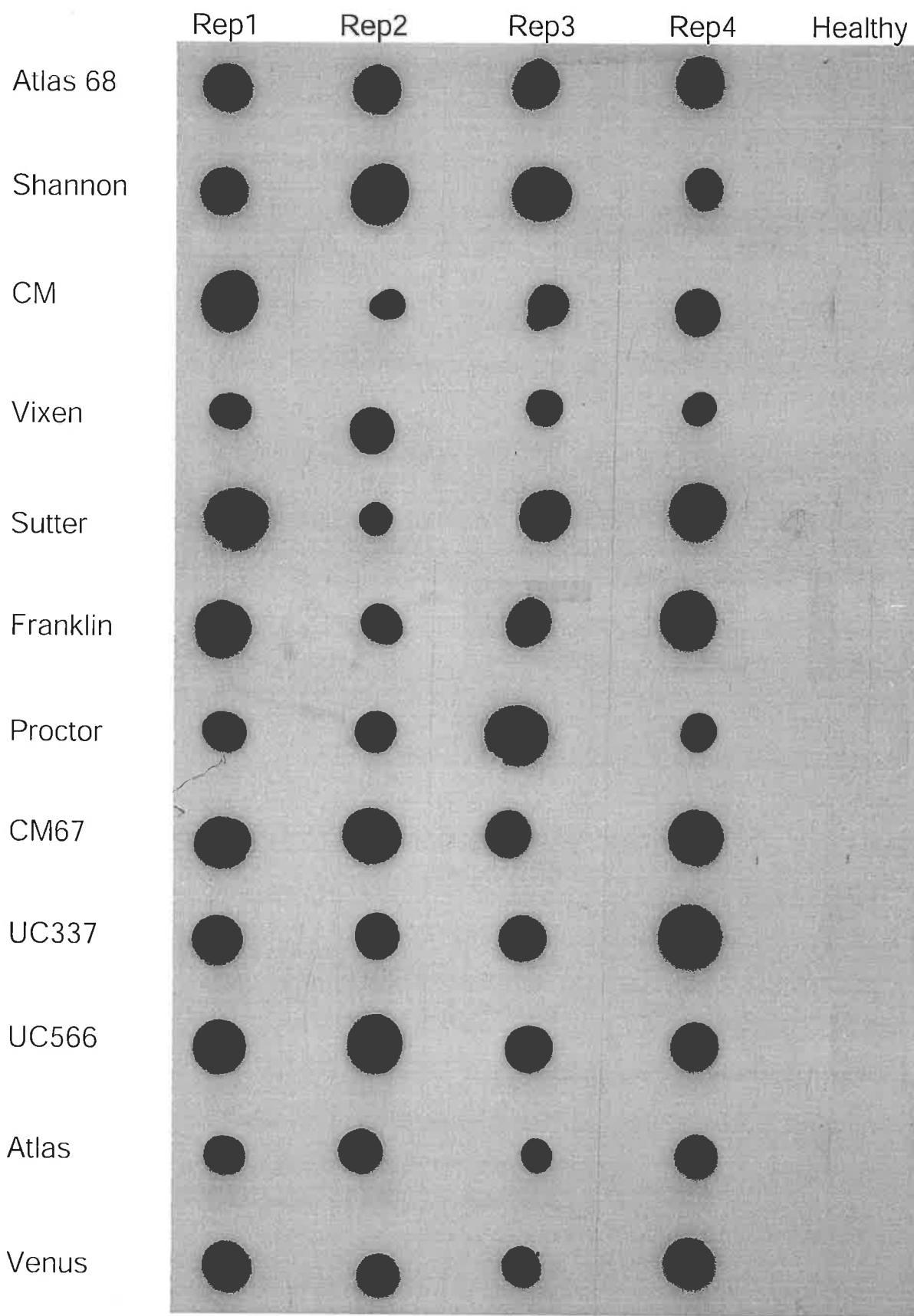


Figure 3.2 Dot-blot hybridisation analysis for the detection of BYDV-PAV_{adel} in *Yd*₂-plus and *Yd*₂-minus barley cultivars

A radioactively-labelled RNA probe derived from the genome of an Australian isolate of BYDV-PAV (Miller *et al.*, 1988 Young *et al.*, 1991) was used to probe a membrane containing total nucleic acid samples from shoot material of a range of barley cultivars positive for *Yd*₂ (Atlas 68, CM67, Franklin, Shannon, Sutter, UC337, UC566, Venus and Vixen) and negative for *Yd*₂ (Atlas, CM and Proctor). These cultivars had been infected using aphids produced by the Viruliferous Aphid Management System (Sections 3.2.2; 3.2.3). The probe showed that all cultivars and replicates were infected with BYDV-PAV_{adel}. Therefore, aphids produced by this system were highly viruliferous resulting in 100% infection for all plants assayed.



**Table 3.1 Visual rating of Yd_2 -minus and Yd_2 -plus cultivars infected with BYDV-
PAV_{adel}**

Cultivar	†Ethiopian accession	Visual rating
Atlas	-	1
Atlas68	CI3920-1	4
CM	-	1
CM67	CI2376	4
Proctor	-	2
Shannon	CI3208-1	3
Franklin	CI3208-1	3
Sutter	CI1237	3
UC337	CI1237	4
UC566	CI2376	4
Venus	CI3920-1	3
Vixen	CI3906-1	3

Visual Rating

- 1 Total dwarfing, complete yellowing and necrosis of leaves, resulting in death 4 weeks after infection.
- 2 Severe dwarfing, high degree of yellowing and leaf necrosis, unable to set fertile seed, no resistance.
- 3 Dwarfing present, mild leaf yellowing and some necrosis, fertile seed set, mild resistance.
- 4 Some dwarfing, little yellowing or necrosis, some leaf brittleness, highly resistant.
- 5 Healthy.

†The Ethiopian accession from which the Yd_2 gene was derived is included in the opposite column to the cultivar.

necrosis however, the differences in tolerance to BYDV-PAV_{adel} between *Yd*₂-minus cultivars and *Yd*₂-plus cultivars were startling (Figure 3.3). The faster growing *Yd*₂-plus genotypes (Atlas68, CM67, UC337 and UC566) showed milder symptoms than those exhibited by the slower growing genotypes (Franklin, Shannon, Sutter, Venus and Vixen).

The dry weight of noninfected plants was measured and compared to that of infected plants (Figure 3.4). The dry weights between the different Near Isogenic Lines (NILS) [Atlas(*Yd*₂-minus) and Atlas68(*Yd*₂-plus); CM(*Yd*₂-minus) and CM67(*Yd*₂-plus); and Proctor(*Yd*₂-minus) and Shannon(*Yd*₂-plus)] were examined, and showed no significant difference (according to a t-test: $P > 0.05$). However, the dry weight differences between these lines differed dramatically after infection with BYDV-PAV_{adel} (according to a t-test: $P > 0.01$). There was no significant difference between infected and noninfected dry weights of those cultivars which were *Yd*₂-plus.

3.3.3 Field trial results

Field data was collected and analysed by Steve Jefferies. Viruliferous aphids for the trial were reared and supplied by the author. The BC₂ lines positive for the *YLM* associated resistant allele showed only a 13% reduction in yield, while those carrying the susceptible associated allele incurred a 71% yield penalty. On average, lines with the *YLM* resistant associated alleles were 347% higher yielding than those with the susceptible associated allele (Figure 3.5).

3.3.4 BYDV resistance assays on the F₂ progeny from the cross of Atlas × Atlas 68

Sixty F₂ individuals from the cross of Atlas × Atlas 68 were typed using the *YLM-1* and *Ylp*-CAPS markers to help denote the *Yd*₂ genotype (Section 3.2.6) in comparison to the phenotypic response. Eleven individuals were homozygous for the *YLM-1* and *Ylp*-CAPS resistant associated alleles, 14 individuals were homozygous for the susceptible associated alleles and 35 individuals were heterozygous for both resistant and susceptible associated alleles. Resistant assays conducted on these F₂ progeny were based on a visual estimate of resistance. While the 11 individuals showing the resistant associated alleles for both the *YLM-1* and *Ylp*-CAPS

Figure 3.3 Symptoms of BYDV-PAV_{adel} infection in the barley cultivars CM and CM67

(a) The barley cultivar CM (*Yd₂-minus*) when infected with BYDV-PAV_{adel} shows no resistance to the pathogen and is completely overcome by the viral infection. The symptoms displayed in this figure occurred only 5 weeks post infection. The onset of symptoms are visible as early as 7 days post-infection. Symptoms include stunting, leaf yellowing, leaf necrosis, as well as brittleness of the leaves. Plant death usually occurs during the 4th or 5th week after infection.

(b) The barley cultivar CM67 (*Yd₂-plus*) when infected with BYDV-PAV_{adel} shows high level of tolerance to the viral pathogen. Typical symptoms such as stunting, leaf necrosis and brittleness are present but are not nearly as severe as those exhibited in its near isogenic counterpart CM. Heading occurs much earlier due the viral stress imposed on the plant.

(a)



(b)



Figure 3.4 The effect of the Yd_2 gene on the dry weight in BYDV-PAV_{adel} infected barley plants

Five seedlings of each of the barley cultivars Atlas68, CM67, Franklin, Shannon, Sutter, UC337, UC566, Venus and Vixen (Yd_2 plus) and Atlas, CM and Proctor (Yd_2 minus) were infected with viruliferous aphids 7 days post germination. Sixty days after infection, shoot tops were harvested, dried at 80°C for two days and the dry weights measured. For comparison, the dry weight of 5 virus free plants of each cultivar were measured at the same growth stage. The graph shows the mean and standard error of the dry weights. The dry weights of those cultivars with the Yd_2 gene infected with BYDV-PAV_{adel} did not differ significantly with their noninfected controls (t-test; $P>0.05$). However those not containing Yd_2 showed a dramatic difference (t-test; $P>0.01$).

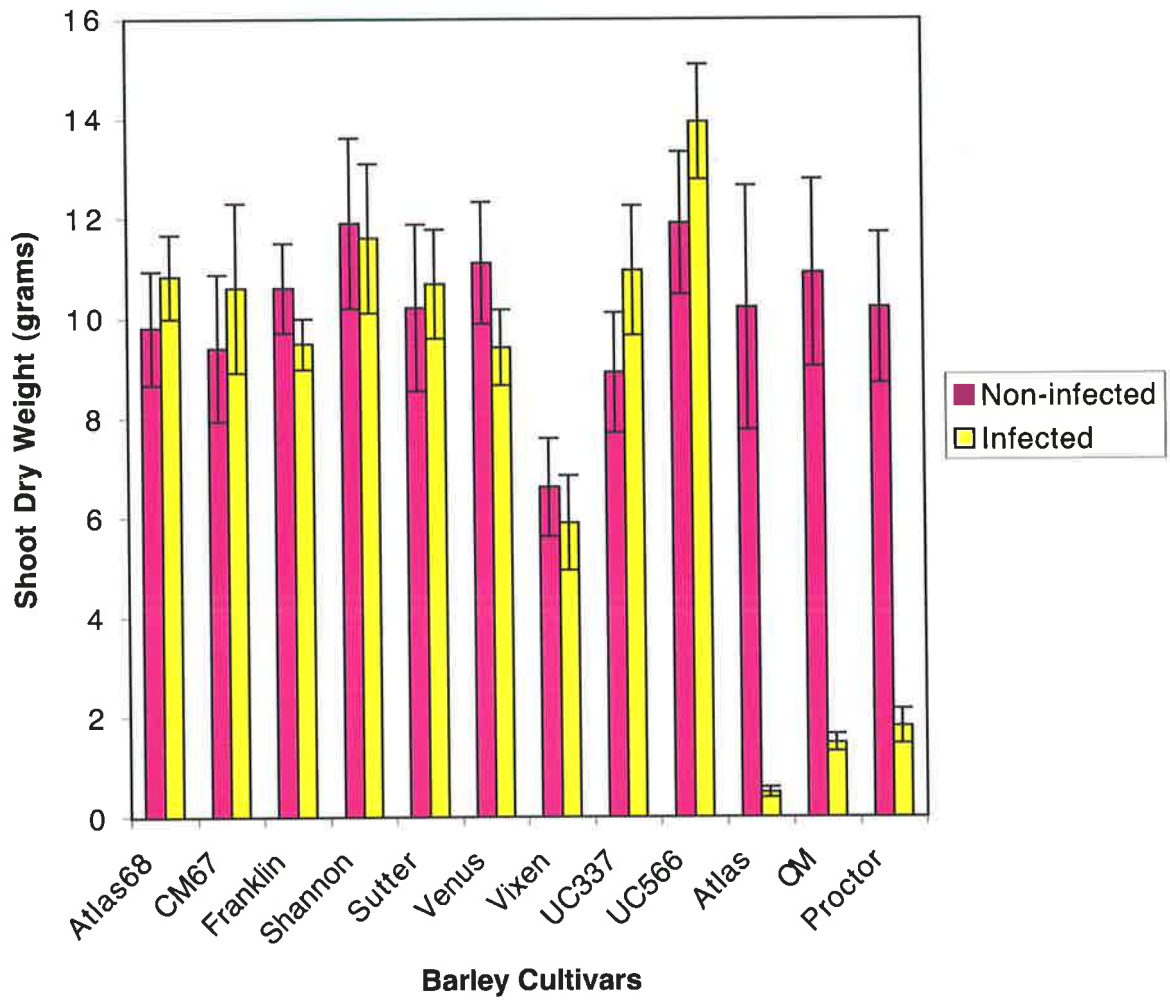


Figure 3.5 BYDV field trial resistance assay on Backcross 2 lines derived from the new malting variety Sloop (recurrent parent) and the Yd_2 positive parent Franklin (donor parent)

A split-plot field trial designed by Steve Jefferies (Senior Research Fellow in barley improvement breeding program at the Waite Institute) was used to examine the resistance status of 10 *YLM* (++; Yd_2 resistance associated allele) and 10 *YLM* (--; Yd_2 susceptible associated allele) F₂ derived BC₂ lines. The donor parent Franklin (Yd_2 -plus) and the recurrent parent Sloop (Yd_2 -minus) were also included in the trial. Aphids produced from the viruliferous aphid management system set up by this author were used to inoculate plants in the field. An epidemic of BYDV was achieved in the field experiment at Strathalbyn, South Australia in 1998. Severe stunting and chlorosis was observed in all viruliferous aphid inoculated Sloop check plots with only a trace of symptoms observed in Franklin plots. Viruliferous aphid inoculated F₂ derived BC₂ lines carrying the Sloop *YLM* variant incurred a mean grain yield reduction from uninoculated lines of 71%. In contrast, lines carrying the Franklin *YLM* variant were on average, 347% higher yielding than the Sloop *YLM* variant in plots inoculated with viruliferous aphids. The yellow plot in the foreground is a *YLM*(--) BC₂ line inoculated with viruliferous aphids, and the one immediately behind is a *YLM*(++) BC₂ line inoculated with aphids. A number of other yellow plots are visible in the background, all are *YLM*(--). Therefore, aphids produced from the viruliferous aphid management system proved to be useful for field trial assays, and allowed Yd_2 -plus and Yd_2 -minus genotypes to be easily distinguished.



markers were resistant, all other progeny (49) were highly susceptible (Figure 3.6). Individuals heterozygous for Yd_2 were unable to be differentiated from those homozygous susceptible.

3.3.5 The relationship between increased BYDV viral load and radioactive counts as measured by a phosphorimager

In order to develop a quantitative dot-blot assay using a radioactively labelled RNA probe to measure differences in viral titre between resistant and susceptible plants, a linear relationship between increasing viral load and radioactive counts as measured by a phosphorimager had to be established. A set of serial dilutions performed on total nucleic acid extracted from the shoot material of a 4 week old Oat (*Avena sativa* L.) cultivar, Stout, 14 days after it had been infected with viruliferous aphids (Section 3.2.7) showed a linear response between nucleic acid concentration and radioactive counts as measured by a phosphorimager (Figure 3.7). This result demonstrated that viral concentration increased as sample load increased and therefore, differences in viral titre can be quantified using this technique.

3.3.6 Quantitative difference in BYDV viral titre between Shannon and Proctor in root and shoot tissue

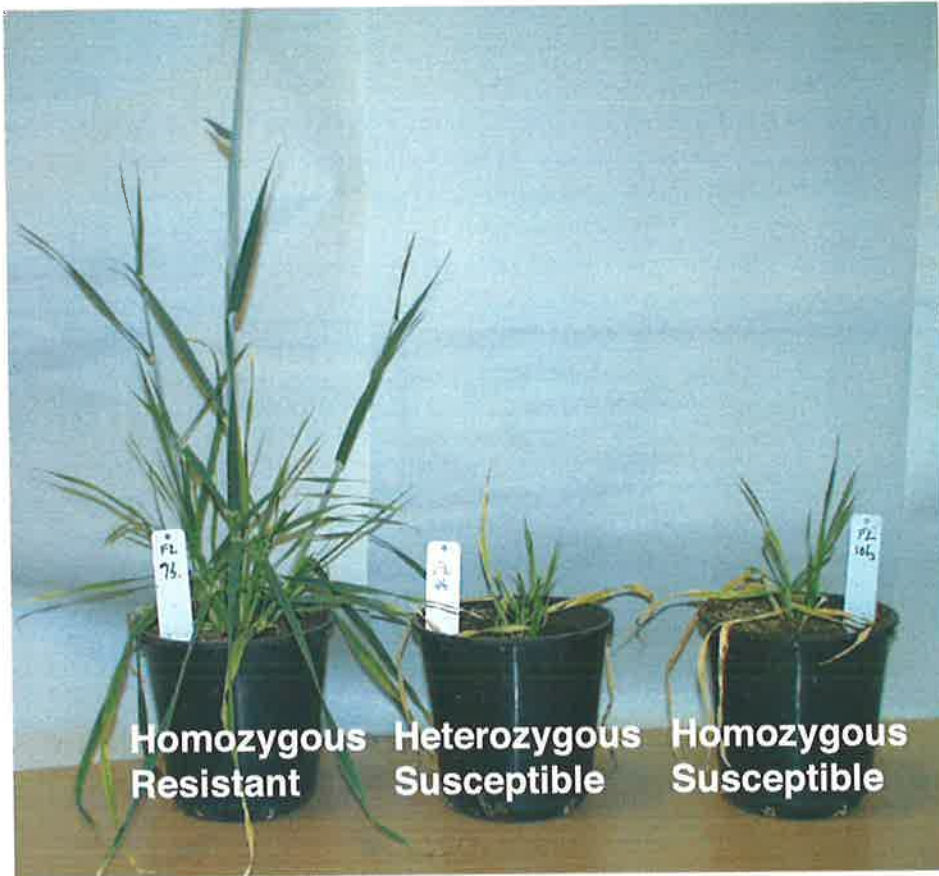
Nucleic acid extractions from root and shoot material of Shannon (Yd_2 -plus) and Proctor (Yd_2 -minus) barley seedlings extracted 2, 5 and 8 days after infection with viruliferous aphids were dotted on a membrane and hybridised to a radioactively labeled RNA probe. Differences in BYDV viral titre between Shannon and Proctor root material 5 days after infection were highly significant (Figure 3.8). While differences in viral titre were also evident in the shoot material between Shannon and Proctor, the difference was substantially less than that seen in root tissue. Differences in viral titre were significant but less pronounced at 8 days in both root and shoot tissue (data not shown). Differences in viral titre could not be detected two days after the end of the inoculation period (data not shown).

Figure 3.6 F₂ progeny analysis of the cross of Atlas (*Yd₂-minus*) and Atlas 68 (*Yd₂-plus*)

(a) Resistance assays (Section 3.2.3) performed on 60 F₂ progeny of the cross of Atlas and Atlas 68 were able to identify homozygous resistant progeny (**9b**). However resistance assays were unable to distinguish between those progeny which were heterozygous susceptible (**8b**) and homozygous susceptible (**10b**). Progeny were identified for their *Yd₂* genotype based on a cleaved amplified polymorphic sequence PCR marker for the *Ylp* locus (*Ylp*-CAPS analysis) and the co-dominant *YLM* PCR marker, two markers closely linked with *Yd₂* (Ford *et al.*, 1998; Paltridge *et al.*, 1998).

(b) The *Yd₂* genotype analysis of progeny using *Ylp*-CAPS PCR analysis is shown. Resistant [Atlas68 (**68**)], susceptible [Atlas (**At**)], heterozygous (**H**) and no template (**-ve**) controls are shown. The F₂ progeny **8b**, **9b**, and **10b** in the above pallet (described in **a**) are also analysed. Post-amplification restriction digests with *Nla*III were performed on amplified DNAs prior to electrophoresis. The 311 base-pair fragment is the allele linked with resistance, with the *Nla*III restriction enzyme site absent. The susceptible associated allele contains the *Nla*III site and when restricted is 253 base-pairs. (The other 58 base-pair restriction fragments are not visible in figure 3.5b). The marker lane (**M**) contains pUC19 DNA digested with *Hpa*II.

(a)



(b)

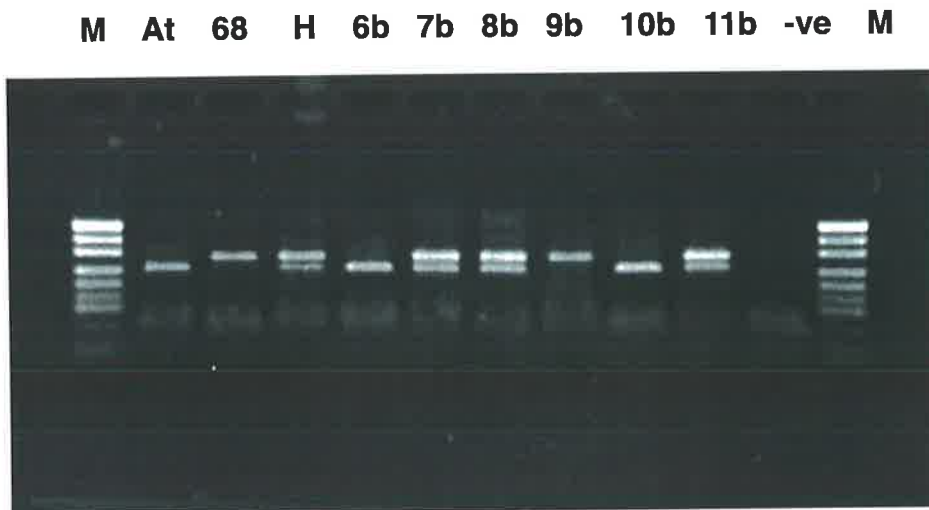


Figure 3.7 A linear response of increasing nucleic acid concentration from Oat material infected with BYDV-PAV_{adel} to radioactive counts measured by a phosphorimager

Total nucleic acid was extracted from the shoot material of a 4 week old Oat (*Avena sativa* L.) cultivar, Stout, 14 days after it had been infected with viruliferous aphids (Section 3.2.7). Nucleic acid concentration was measured using a spectrophotometer and serial dilutions were performed and spotted on a nylon membrane which was then probed with a radioactive-RNA probe derived from the genome of an Australian PAV-isolate (Miller *et al.*, 1988 Young *et al.*, 1991). After hybridisation and washing (Section 3.2.5) the membrane was exposed to a phosphorimager screen for 2 hours. Radioactive counts were then quantified using a phosphorimager. The linear response between nucleic acid concentration and radioactive counts is shown and demonstrates that viral concentration increases with increased sample load and therefore, differences in viral titre can be quantified using this technique.

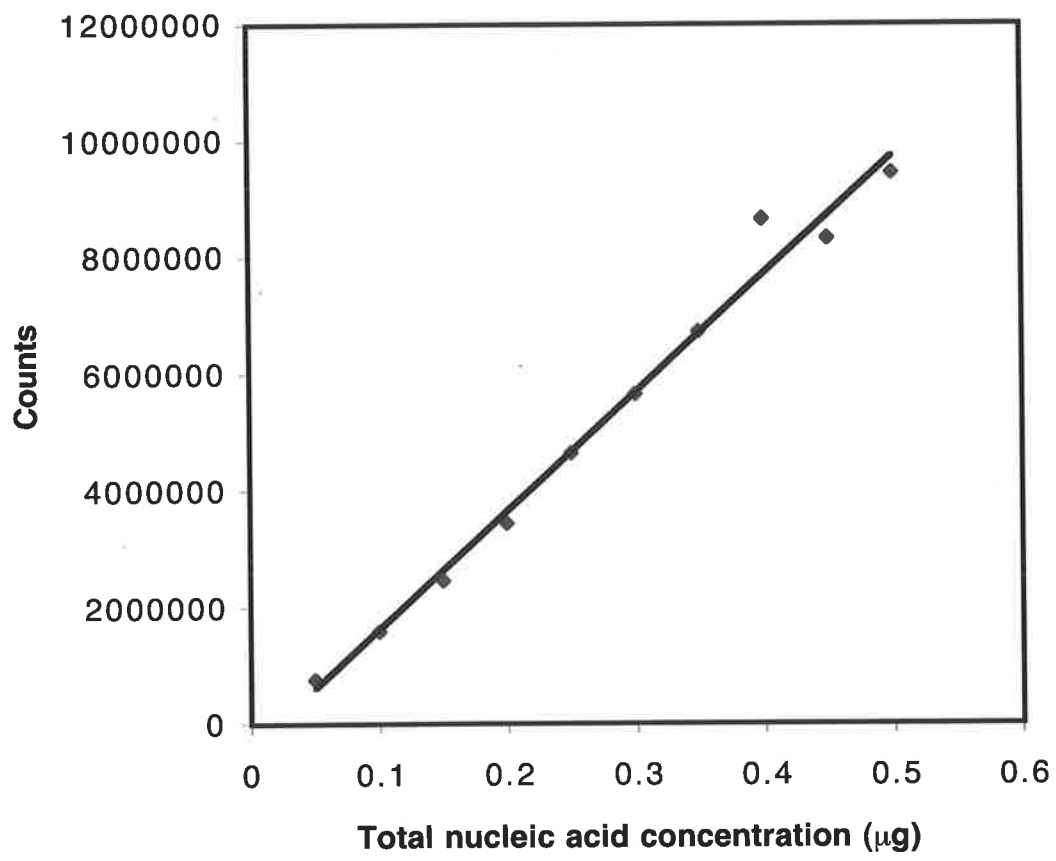
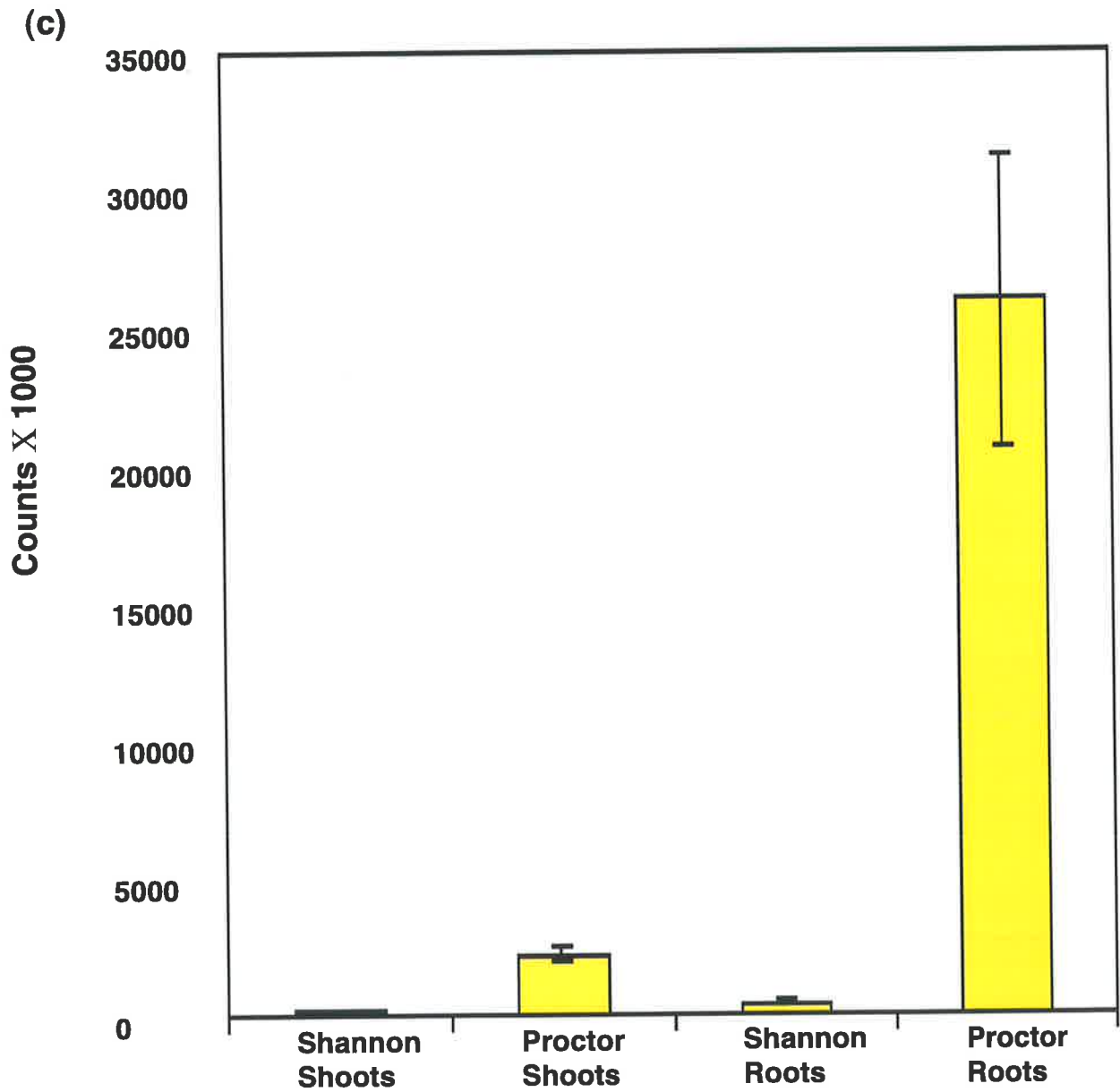
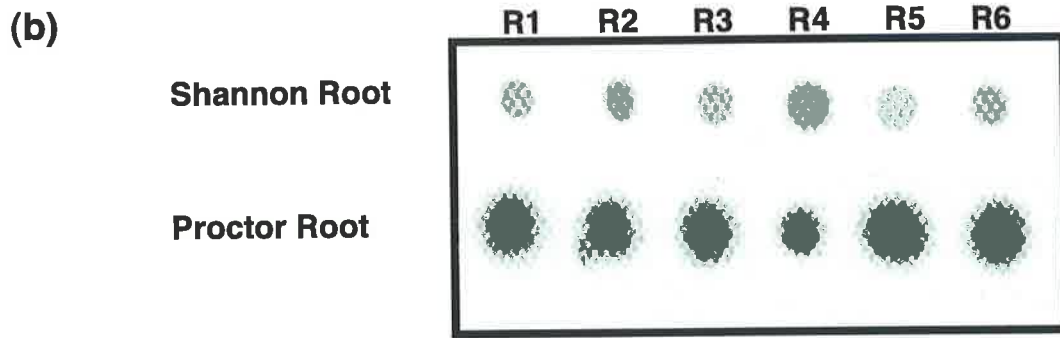
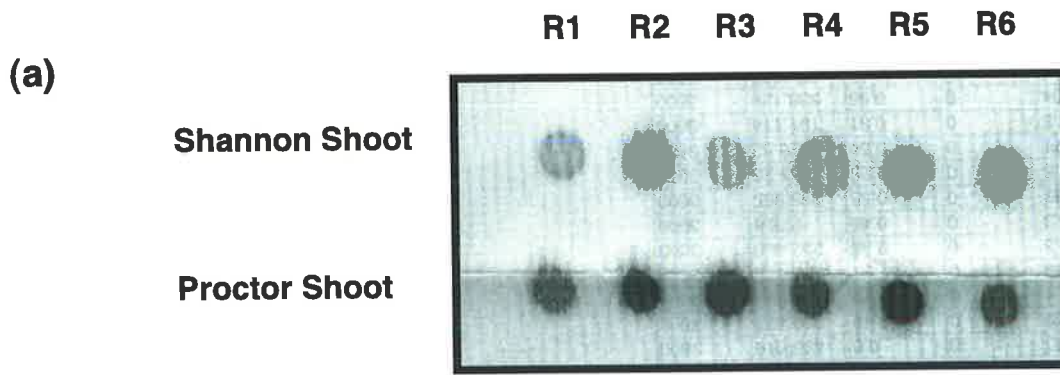


Figure 3.8 Quantitative dot-blot measuring viral titre of BYDV-PAV_{adel} in resistant and susceptible shoot and root material

Total nucleic acid samples from six barley seedlings of Shannon (*Yd₂-plus*) and Proctor (*Yd₂-minus*) were extracted from (a) shoot and (b) root material 5 days after infection with BYDV-PAV_{adel}. Dot-blot analysis was performed with a radioactive-RNA probe derived from the genome of an Australian PAV-isolate (Miller *et al.*, 1988; Young *et al.*, 1991)(Section 3.2.5). Nucleic acid concentration was quantified using a spectrophotometer and equal quantities of total nucleic acid were applied for each sample. After radioactive hybridisation and washing, the membrane was exposed to a phosphorimager screen for two hours before radioactive counts were measured using a phosphorimager (Section 3.2.5).(c) The graph shows the mean and standard error of the radioactive counts measured by the phosphorimager. Shannon (*Yd₂-plus*) shoot material showed significantly lower level of counts (according to a t-test; $P > 0.01$) compared to Proctor (*Yd₂-minus*), indicating a lower viral titre in the resistant cultivar. However, in the root material the difference between the resistant and susceptible cultivars was much greater (t-test; $P > 0.01$). Therefore, this assay allows the difference in viral titre between the two cultivars which differ in *Yd₂* status, to be quantified well before symptom expression is evident.



3.3.7 Quantitative differences in BYDV viral titre between a range of Yd_2 and non- Yd_2 cultivars in root tissue

Five days after infection with BYDV-PAV_{adel} total nucleic acid samples from a number of barley cultivars with and without Yd_2 were extracted from root material and quantitative dot-blot analysis performed. Barley cultivars containing Yd_2 consistently showed a lower viral titre than those without Yd_2 (Figure 3.9). Therefore, this assay enables the difference in viral titre of Yd_2 -minus and Yd_2 -plus cultivars to be quantified well before the expression of symptoms is evident and should prove a useful tool to determine the resistance status of barley cultivars and their progeny.

3.3.8 Quantitative differences in BYDV viral titre between F₂ individuals of the cross Atlas × Atlas68

Thirty F₂ progeny of the cross Atlas × Atlas68 were screened using both the *YLM* and *Ylp*-CAPS markers to denote the Yd_2 genotype. Five days after infection with BYDV-PAV_{adel}, total nucleic acid samples were extracted from root material and quantitative dot-blot analysis performed. Progeny homozygous for the resistant associated allele for both markers showed low viral titre. However, progeny heterozygous for both alleles and progeny homozygous susceptible for both alleles for the two markers had a high viral titre and could not be distinguished based on quantitative dot-blot analysis.

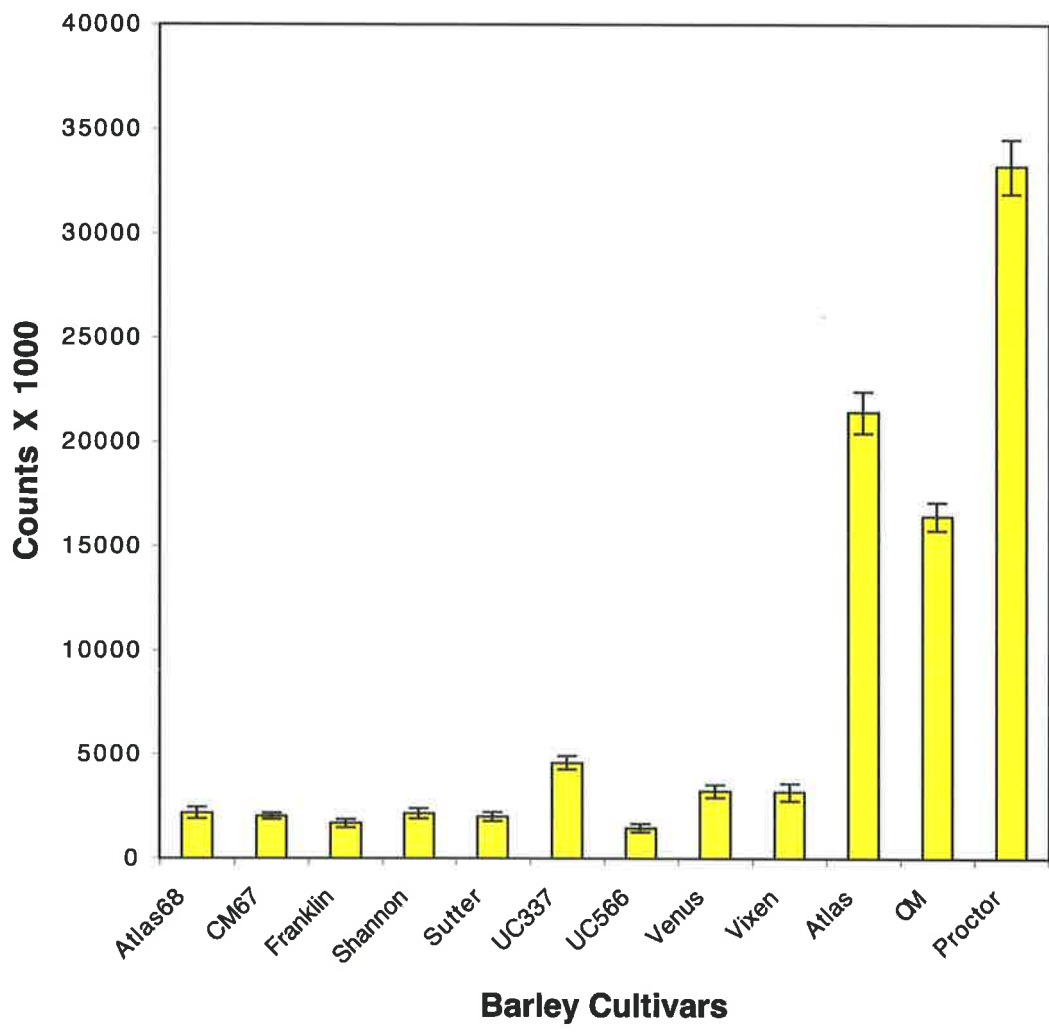
3.4 Discussion

3.4.1 The Design of a Viruliferous Aphid Management Strategy

One of the main aims of the work presented in this chapter, was the production of copious quantities of viruliferous aphids available all year round for infection studies. As previously mentioned (Section 3.1) aphid production strategies in the glasshouse reared inconsistent quantities of aphids with varying infectivity. Resistant assays carried out in the field or

Figure 3.9 Quantitative dot-blot measuring viral titre of BYDV-PAV_{adel} in root material from a range of *Yd*₂-plus and *Yd*₂-minus barley cultivars

Total nucleic acid samples from 13 barley cultivars Atlas 68, CM67, Franklin, Shannon, Sutter, UC337, UC566, Venus and Vixen (*Yd*₂ plus) and Atlas, CM and Proctor (*Yd*₂ minus) were extracted from root material 5 days after infection with BYDV-PAV_{adel}. Dot-blot analysis was performed with a radioactive-RNA probe derived from the genome of an Australian PAV-isolate (Miller *et al.*, 1988; Young *et al.*, 1991) (Section 3.2.5). Nucleic acid concentration was quantified using a spectrophotometer and equal quantities of total nucleic acid were applied for each sample. After radioactive hybridisation and washing, the membrane was exposed to a phosphorimager screen for 48 hours before radioactive counts were measured using a phosphorimager (Section 3.2.5). The graph shows the mean and standard error of the radioactive counts measured by the phosphorimager. All *Yd*₂-plus cultivars showed significantly lower level of counts (according to a t-test; $P > .01$) than the *Yd*₂-minus cultivars, indicating a lower viral titre in the resistant cultivars. Therefore, this assay allows the difference in viral titre between *Yd*₂-plus and *Yd*₂-minus cultivars to be quantified well before symptom expression is evident.



glasshouse did not suit symptom expression (Section 1.5.2), so characterisation of Yd_2 genotype was difficult. Therefore, an environmental growth chamber was secured and conditions established in the literature suiting both aphid fecundity, aphid infectivity (Section 1.2.2) and symptom expression (Section 1.5.2) for BYDV were used to design a reliable Viruliferous Aphid Management Strategy and Resistance Assay Screen.

Essential to transmission of BYDV is use of the correct vector-isolate combination (Johnson and Rochow, 1972). Because the main goal of the work presented in this thesis is the cloning of Yd_2 , then it was essential to use an isolate which does not overcome Yd_2 resistance. BYDV-PAV does not overcome Yd_2 resistance and therefore, was chosen to be used for this study. While BYDV-PAV is transmitted by both *Rhopalosiphum padi* L. and *Stiobion avenae* Fabr, *R. padi* is the most efficient vector of PAV (Johnson and Rochow, 1972). Therefore, in this study *R. padi* aphids were used for the transfer of BYDV-PAV_{adel} from infected oat plants to barley progeny.

Aphids produced by the viruliferous aphid management system were only available for resistance assays from 3 week old oat plants which had then been infested with aphids for more than 14 days and less than 24 days, grown at 22°C days and 18°C nights, with a 14 hr photoperiod. Dot-blot analysis determined these conditions consistently produced high levels of viral titre in all oat plants used for rearing aphids (Figure 3.1). This is consistent with results described by Hammond *et al.* (1983) whom reported when BYDV was purified from oats, the viral yield peaked in the shoots about 2 or 3 weeks after infestation. The growth conditions stated as being optimum for BYDV yield in oats by Hammond *et al.* (1983) are identical to those used in the Viruliferous Aphid Management System.

Aphids which were reared off oat plants 4 days after the introduction of the oat plants into the aphid cage had an extended acquisition access period (AAP) of greater than 24hrs considering that viral titre levels can be first detected in shoots 3 days after infection with BYDV (Ranieri *et al.*, 1993). Considering that large increases in aphid numbers has already occurred by day 14 in our system, the majority of aphids used by or after this date had an acquisition access period (AAP) of greater than 24 hours. Gray *et al.* (1991) reported that for an AAP greater than 24 hours the efficiency of transmission for each individual aphid was 95%. However, there was no

increase in transmission efficiency for *R.padi* for BYDV-PAV for AAPs longer than 24 hours. Therefore, the majority of aphids used for our resistance assays should have individual transmission efficiencies greater than 95%. Based on this assumption if a minimum of 20 aphids are used for infection of an individual plant, the probability of infection is almost 100%.

This is calculated using the following formula;

The probability of infection $p = 1 - q^x$;

where $q = 0.05$, and is the probability of the failure of a single aphid to infect;

where $x = 20$ which is the minimum number of aphids used.

Therefore, $p = 1 - (0.05)^{20}$; $p = 1 - 9.53^{-27}$

While highly infectious aphids were of paramount importance for our resistance assays, large populations were also needed to ensure that aphid numbers were never limiting for resistance assays. Aphids were raised on oats instead of barley since Comeau (1983) reported that for the rearing of *R.padi*, best results come from oats. While reproduction of *R.padi* is good on barley seedlings, by flowering time it is an undesirable species as aphid production is low (Leather and Dixon, 1981) and the awns of barley can interfere with aphid collection. Temperature is one of the most critical factors concerning growth of *R.padi*, which suffers damage above 32°C and death at 37°C (Richter and Balde, 1993). A problem frequently encountered in our study with South Australian summers reaching temperatures as high as 44°C (www.bom.gov.au/climate/averages). Using 22°C days and 18°C nights in a environmental growth chamber aphid densities were at their greatest between 14 and 24 days after introduction into aphid cages. This is consistent with the optima for growth of *R.padi* to be between 18-24°C as reported by Richter and Balde (1993). Therefore, optimal temperatures for both viral titre and aphid growth were the same, thereby maximising the viral titre level in plants to ensure highly viruliferous aphids as well as maximising numbers of these viruliferous aphids for infection studies.

A minimum number of 20 aphids were used to inoculate individual plants in our resistance assay studies in order to overcome any possible dosage effect reported as reported by Boulton and Catherall (1980). Boulton and Catherall (1980) inoculated spring barleys with BYDV using 5,10, 20 and 50 aphids. Infection with all aphid numbers had significantly adverse effects. Progressive effects with increasing aphid numbers, indicative of dosage response, occurred in some varieties. These effects included delay in heading, and increased stunting, but not less yield. Significant difference between infection with 5 aphids and 50 aphids for some characters such as days to heading were evident, but not between 20 aphids and 50 aphids, indicating a plateau of the dosage curve effecting these yield components. The use of a minimum of 20 aphids for each individual, in our infection studies therefore, ensured that a dosage effect was avoided.

By using the most efficient vector-isolate combination, optimal conditions ensuing high viral titre levels in host plants, a long AAP and the correct number of aphids for infecting each individual barley seedling, 100% infection was achieved in the resistance assay study (Section 3.3.2). The Viruliferous Aphid Management Strategy designed here has since been used for typing Yd_2 genotypes in our mapping population (Paltridge, 1998) and studies into the host-pathogen interaction of Yd_2 -BYDV (Shams-Bakhsh, 1997). As yet there have been no escapes from BYDV infection as determined by dot-blot analysis. Aphids produced by this system have also been successfully used in field trials in the Waite Institute barley breeding program (Jefferies *Pers.comm.*) to test the incorporation of the Yd_2 genotype into superior agronomic backgrounds. Finally this aphid system is currently being used to perform resistance assays for a number of breeders within Australia and New Zealand (Ata Rehman of NSW Agriculture, Wagga Wagga; Dr Richard Pickering of New Zealand Crop and Food Research, Mana Kai Rangahau)

3.4.2 Resistance assay screening

While aphids may be highly viruliferous and able to transfer BYDV, distinguishing between Yd_2 -containing individuals and non- Yd_2 individuals is often hampered by variation in growth conditions and the effectiveness of the gene in different genetic backgrounds (Rasmusson and Schaller, 1959; Catherall and Hayes 1966; Jones and Catherall, 1970; Catherall *et al.*, 1970).

Catherall and Hayes (1966) noted that the amount of leaf yellowing on barley plants of the same inbred varieties infected with BYDV varied with different environmental conditions. They also noted that some varieties which were tolerant under glasshouse conditions were not under field conditions. They concluded that under glasshouse conditions, which increase growth rate of the host by reducing the length of the growing period, multiplication of the virus may be less able to keep pace with the development of the plant, thereby apparently increasing varietal tolerance (Jones and Catherall, 1970). Therefore, the resistance assays described in this study were conducted under growth conditions conducive to symptom expression (Section 3.2.3) so Yd_2 and non Yd_2 genotypes could be easily distinguished.

Visual Symptoms were scored, and dry weights measured for noninfected and infected Yd_2 and non- Yd_2 cultivars. Dramatic differences were apparent between Yd_2 and non- Yd_2 cultivars, with the BYDV resistance from the Yd_2 containing cultivars effective in limiting the degree of stunting caused by the PAV_{adel} isolate of BYDV (Figures 3.4). This is consistent with previous observations which have shown Yd_2 to be effective against PAV isolates of BYDV (Skaria *et al.*, 1985; Herrera and Plumb, 1989). While dry weight showed highly significant differences between noninfected and infected non Yd_2 containing genotypes, significant differences in dry weight were not found between noninfected and infected Yd_2 containing cultivars (Figures 3.4). However, visual scoring recorded stunting and yellowing of leaves of infected treatments in comparison to noninfected healthy controls for Yd_2 containing cultivars, though symptoms were much milder than those exhibited by the non- Yd_2 cultivars. Jenson, (1968a) found that while total fresh weight is reduced, there is a dramatic increase in percentage dry weight in oats infected with BYDV. Therefore, visual estimation of resistance status appeared to be a more sensitive indicator of resistance than dry weight. A more suitable quantitative indicator for BYDV may be fresh weight (Chalhoub *et al.*, 1995; Collins, 1996). Other indicators used to study the effects of BYDV on the host include days to heading, height at an intermediate growth stage, height at maturity and grain yield (Boulton and Catherall, 1980).

Of nine Yd_2 containing cultivars, the four faster growing genotypes showed milder symptoms, than those exhibited by the slower growing genotypes. This is consistent with previous observations which have shown that the effectiveness of Yd_2 -mediated BYDV resistance is

strongly influenced by growth rate and can be seen when the Yd_2 gene is introduced into genetic backgrounds of barley lines which differ in their rate of maturity (Jones and Catherall, 1970). Jones and Catherall (1970) demonstrated that in homozygous resistance lines selected from a segregating population, the level of BYDV resistance was significantly correlated with the rate at which these lines grew under virus-free conditions. Of three non- Yd_2 containing cultivars, both Atlas and CM showed extreme sensitivity to BYDV in comparison to Proctor. Both cultivars are faster growing genotypes than Proctor. Therefore, the hypothesis that faster growing Yd_2 -containing genotypes show increased resistance cannot be extrapolated to faster growing non- Yd_2 genotypes showing increased resistance.

Catherall *et al.*, (1970) reasoned that the variation in effectiveness and dominance shown by Yd_2 may also be due to differences between the Yd_2 alleles present in the Ethiopian barleys. Therefore, it is possible that these susceptible cultivars may contain an allele homologous to Yd_2 conferring extreme susceptibility. To confirm this suspicion, crosses between two susceptible cultivars, one of extreme sensitivity (Atlas) the other of milder sensitivity (Proctor) would need to be performed and F₂ progeny analysed for segregation for differences in their sensitivity to BYDV infection. If such a gene mapped orthologous to Yd_2 this would raise the interesting possibility that a number of alleles for the Yd_2 locus may exist from both resistance and susceptible cultivars and different products may be encoded by these alleles, conferring either high or intermediate levels of resistance, while others products may confer low or extreme levels of susceptibility to BYDV.

Resistance assays were performed on F₂ progeny from the cross Atlas (Yd_2 -minus) × Atlas68 (Yd_2 -plus). While progeny homozygous resistant for Yd_2 were easily distinguishable from progeny homozygous susceptible, F₂ progeny heterozygous for Yd_2 (identified by PCR markers linked to Yd_2) were unable to be distinguished from those which were homozygous susceptible. Therefore, the Yd_2 allele appears to be completely recessive in the background of the donor parent Atlas. Catherall *et al.* (1970), observed either complete recessiveness or dominance of BYDV in Yd_2 heterozygotes and suggested the level of dominance was related to the level of resistance in the Ethiopian parent used as a source of Yd_2 . To definitively characterise the Yd_2 genotype of F₂ individuals derived from the parents Atlas and Atlas68, F₂ individuals would need to selfed to

obtain F₃ individuals which could then be tested for segregation of resistance, in order to identify heterozygous susceptible from homozygous susceptible F₂ individuals.

3.4.3 A quantitative resistance assay

While the resistance assay screen was able to easily distinguish between individuals which were homozygous resistant and homozygous susceptible, the assay took a minimum of 4 weeks before *Yd*₂ genotypes could be reliably scored. Therefore, a quick and quantitative resistance assay was sought which could be used to uniformly distinguish between resistant and susceptible genotypes of different *Yd*₂ sources. Using dot blot analysis and a phosphorimager to measure radioactive counts relating to viral titre, our results showed significant differences between viral titre in both shoot and root material between non-*Yd*₂ and *Yd*₂ genotypes, with the differences being even more pronounced in the root material. The dot-blot assay used by this author was able to effectively distinguish between homozygous resistance and homozygous susceptible individuals as early as 5 days after the end of the infection period from a range of cultivars with different sources. These results are consistent with previous findings which have used the enzyme-linked immunosorbent assay (ELISA) to measure BYDV-PAV titre in both root and shoot material (Skaria *et al.*, 1985; Ranieri *et al.*, 1993; Chalhoub *et al.*, 1994; Chalhoub *et al.*, 1995). Arundel *et al.*, (1988) using probes prepared from cDNA clones measured viral titre in barley and was able to distinguish a BYDV susceptible cultivar from a BYDV resistant cultivar if the BYDV content of leaves was measured 7-14 days after inoculation. Therefore, the technique developed by this author is an improvement in sensitivity for the detection of viral titre differences between *Yd*₂-minus and *Yd*₂-plus lines.

While the quantitative dot-blot assay was able distinguish between individuals which were homozygous resistant and homozygous susceptible, it was unable to distinguish between progeny which were homozygous susceptible and heterozygous susceptible for *Yd*₂. Therefore, one copy of the *Yd*₂ allele does not appear to slow replication of the virus in the Atlas68 donor background under the growth conditions trialed here. This is consistent with previous observations which have shown that the effectiveness of *Yd*₂-mediated BYDV resistance is strongly influenced by the

genetic background into which the *Yd₂* gene is introduced and that *Yd₂* is often expressed in an incompletely dominant or recessive manner (Jones and Catherall, 1970).

Due to the greater magnitude of reduction in viral titre in the root tissue in comparison to the shoot tissue of *Yd₂* containing genotypes, it is tempting to speculate that a *Yd₂* gene product may be differentially expressed between these tissues. Therefore, strategies aimed at identifying *Yd₂* candidate genes such as cDNA library screening and Northern analysis (Chapters 6 and 8) may consider using mRNA derived from root tissue as well as shoot-tissue.

Finally the quantitative assay developed by the author in combination with resistance screening has been used to help type individuals in our mapping populations (Chapter 4).

CHAPTER 4

**GENETIC MAPPING OF THE Yd_2 REGION OF BARLEY
CHROMOSOME 3**

4.1 Introduction

Genes for which no product is identifiable can be isolated using a map-based approach (for reviews, see Schulze-Lefert 1995, and Tanksley *et al.*, 1995). Using this approach, a genetic map is constructed of sufficient resolution to enable the identification of one or more DNA markers at a physical distance from the target gene, which is less than the average insert size of the genomic library being used. Thus, the markers are used to 'land' on a clone containing the gene (Tanksley *et al.*, 1995).

At the time that this study commenced, three plant disease resistance genes had been cloned using a map-based approach; namely, the *Pto* gene for resistance against *Pseudomonas syringae* in tomato (Martin *et al.*, 1993) and the *RPS2* and the *RPM1* genes for resistance against *Pseudomonas syringae* in *Arabidopsis thaliana* (Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Grant *et al.*, 1995). Each of these studies commenced with the construction of a high resolution genetic map around the gene of interest. Two key developments in this strategy have been the realisation that saturation of the target region with markers and the development of very high resolution maps (with less than 1cM between the target locus and linked marker) can greatly expedite gene cloning (Tanksley *et al.*, 1995). It is relatively easy in plants to analyse large segregating populations, and the development of high throughput DNA markers has expedited high resolution mapping of many loci. As a result, map-based cloning, although still not routine, has been successful in plants ranging from *Arabidopsis* (genome size 145Mb), to rice, sugar beet, tomato and barley (400Mb, 760Mb, 950Mb, and 4800 Mb, respectively) (Buschges *et al.*, 1997; Cao *et al.*, 1997; Cai *et al.*, 1997; Ori *et al.*, 1997).

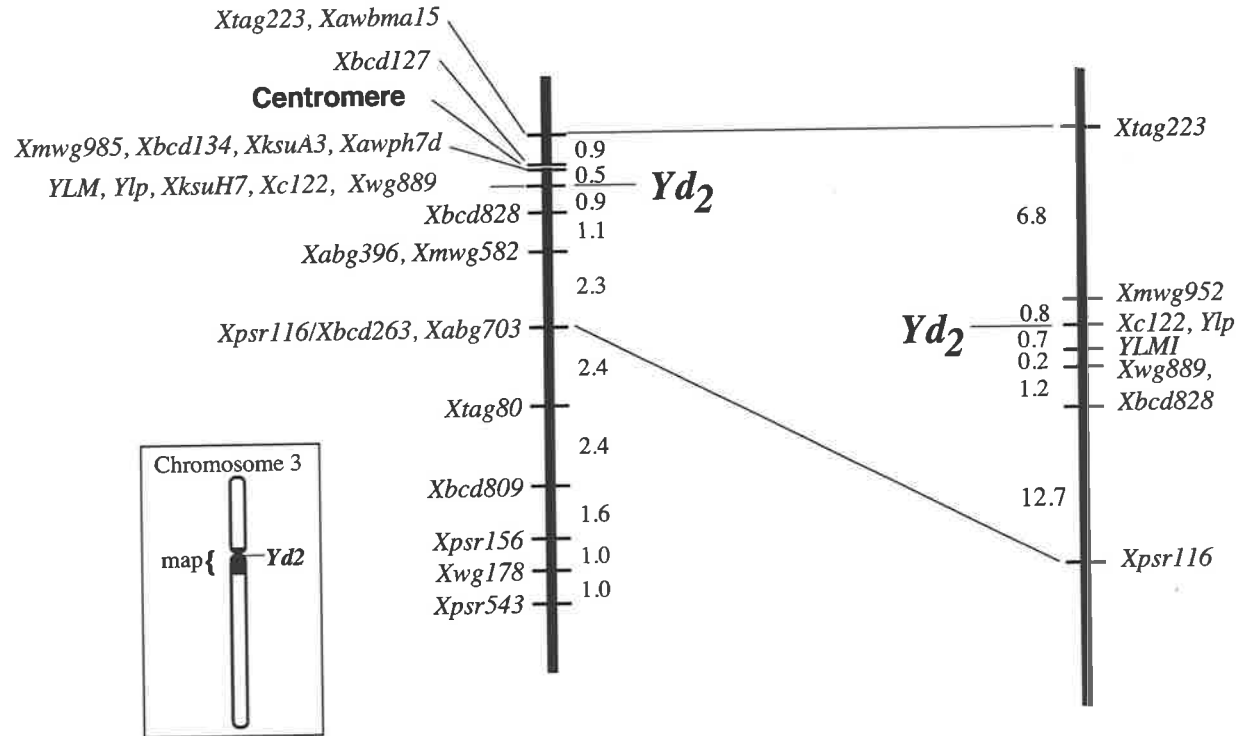
A genetic map of the *Yd₂* region had previously been constructed by Nick Collins and Nick Paltridge in this laboratory using 106 F₂ individuals of a cross between Proctor (*Yd₂*-minus) × Shannon (*Yd₂*-plus). The map contained 23 molecular markers, the *Yd₂* gene and the centromere. The map identified 10 markers within 1cM of *Yd₂* (Collins *et al.*, 1996) (Figure 4.1). In order to provide more accurate information on the genetic distances between the closest flanking markers a high resolution map of the *Yd₂* region was then constructed by Nick Paltridge in the cross Atlas

Figure 4.1 Linkage maps of the Yd_2 region of barley Chromosome 3 developed in the two mapping populations Proctor \times Shannon and Atlas \times Atlas 68

RFLP markers have the "X" prefix. Loci that perfectly co-segregated are separated by commas, and the PCR marker *YLM* was generated in this laboratory. The Proctor \times Shannon population was constructed by Nick Collins and Nick Paltridge using 106 F_2 individuals (Collins *et al.*, 1996) while the Atlas \times Atlas68 cross was constructed by Nick Paltridge using 572 F_2 individuals (Paltridge, 1998). The maps comprise 24 molecular markers and the Yd_2 gene. Distances are in centiMorgans. The inset indicates the position and size of the map relative to total genetic map of Chromosome 3.

Proctor x Shannon map

Atlas x Atlas 68 map



(Yd_2 -minus) \times Atlas 68 (Yd_2 -plus). This commenced with the analysis of 572 F₂ individuals with the pair of Yd_2 flanking markers Xtag223 and Xpsr116 and was further analysed for recombination between Xtag223 and Xbcd828. Individuals carrying events of genetic recombination in the Yd_2 region were identified, and used for fine scale mapping of those markers shown on the Proctor map to be within 2cM proximal and distal of the Yd_2 gene (Figure 4.1).

One of the main benefits to arise from the construction of linkage maps of cereal chromosomes has been the identification of molecular markers linked to agronomically desirable traits. Such linkage enables the indirect selection of desired genes in breeding programs by assaying for marker genotype rather than the trait of interest (reviewed in Section 1.6). This is especially useful in cases where the trait of interest is recessive or difficult to assay (Melchinger, 1990). Comeau and Jedlinski (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). Therefore, the molecular markers identified in this study could be used to help break these linkages.

This chapter describes RFLP mapping studies which complete the second stage of this map-based project, the development of a high resolution map around the target locus. It also provides more molecular markers which may be useful in barley breeding programs. Thirdly it describes the construction of a group of individuals homozygous for their critical recombination event between the two closest flanking markers of the Yd_2 gene in the Atlas \times Atlas 68 population. This work was undertaken using DNA probes from other mapping populations (Liu and Tsunewaki 1991; Heun *et al.*, 1991; Gill *et al.*, 1991; Devos and Gale, 1993b; Kleinhofs *et al.*, 1993; Graner *et al.*, 1994; Kurata *et al.*, 1994a; Nelson *et al.*, 1995b) close to the Yd_2 region and mapping these DNA probes as RFLPs using the genetic resources developed in this laboratory.

4.2 Materials and Methods

4.2.1 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. Finally, the author would also like to acknowledge Nick Collins and Nick Paltridge for the initial construction, development and characterisation of the Proctor × Shannon and Atlas × Atlas68 mapping populations.

4.2.2 Plant lines and genetic materials

Seed of the barley lines and cultivars used in this study were provided by Mandy Jenkin, formerly of the University of Adelaide (CM67), Wayne Vertigan of the Tasmanian Department of Agriculture (Proctor, Shannon), the Australian Winter Cereal Collection at Tamworth, NSW (Atlas 68), Rafiq Islam of the University of Adelaide (Betzes), or were obtained from the barley seed collection at the University of Adelaide (Atlas, California 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). F₂ and F₃ seed and DNA from the Proctor × Shannon and Atlas × Atlas68 populations were supplied by Nick Collins and Nick Paltridge.

4.2.3 RFLP probes

Information regarding the type and source of the DNA clones mapped as RFLPs to Yd_2 in the linkage maps (Figure 4.1) are presented in Table 4.1. Clones were obtained by Nick Collins and Nick Paltridge formerly from this laboratory, from the Australian Triticeae Mapping Initiative

Table 4.1 Legend for DNA clones mapped to the linkage map of *Yd₂*

Clone Prefix	Clone Type	Source
ABG	Barley genomic DNA	A. Kleinhofs, Washington State Univ.
AWBMA	Barley root cDNA	P. Langridge, University of Adelaide
AWPh	<i>Phalaris coerulescens</i> Desf. cDNA	P. Langridge, University of Adelaide
BCD	Barley cDNA	M. Sorrells, Cornell University
CDO	Oat cDNA	M. Sorrells, Cornell University
KSU	<i>T. tauschii</i> genomic DNA	B. Gill, Kansas State University
MWG	Barley genomic DNA	A. Graner, Institute for resistance genetics
PSR	Wheat cDNA	M. Gale, John Innes Institute, UK
RGC	Rice cDNA derived from callus	Y. Nagamura, Rice Genome Research Program
TAG	Wheat genomic DNA	K. Tsunewaki, Kyoto University
WG	Wheat genomic DNA	M. Sorrells, Cornell University
YLM	PCR-marker (AFLP-derived)	N. Paltridge, University of Adelaide
Ylp	Barley cDNA	C. Ford, University of Adelaide

RFLP clone collection at the University of Sydney. With the exception of the AW and RG probes all of the DNA clones 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, 1993; Kleinhofs *et al.*, 1993; Graner *et al.*, 1994; Nelson *et al.*, 1995b), or chromosome addition line analysis (Peter Sharp and Peter Langridge, personal communication). The RG probes had been previously mapped in rice, to the central portion of rice Chromosome 1, by Kurata *et al.* (1994a) (<http://www.staff.or.jp/>). This region had been identified as the region of the rice chromosome which corresponds to the barley *Yd₂* region (Chapter 6.1). AW probes had been localised to the long arm of Triticeae chromosome group 3 (Peter Langridge, personal communication). RFLP clones were received as stab cultures, as bacterial colonies on solid media, or as plasmid DNA. Plasmids were transformed into the *E. coli* strain DH5 α (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 2.2.5) and 15% glycerol stocks (Sambrook *et al.*, 1989) for storage of the bacteria at -80°C.

4.2.4 Using Near Isogenic Lines (NILs) to identify RFLPs linked to *Yd₂*

The BYDV resistant barley cultivars CM67 (Schaller and Chim, 1969b), Atlas68 (Schaller and Chim, 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 (CM), Atlas and Proctor, respectively, and represent three pairs of NILs for *Yd₂*. Genes for aleurone colour, awn morphology and resistance to other pathogens were also introgressed into the CM and Atlas backgrounds to produce CM67 and Atlas68. Hence, these two cultivar pairs are nearly-isogenic for these genes as well as for *Yd₂*. Since the Barley Improvement Program aimed to incorporate *Yd₂* from Franklin (*Yd₂*-plus) into the WI2875 (*Yd₂*-minus) background these two other lines were also included for RFLP analysis. RFLPs between these lines, which are linked to *Yd₂*, may prove useful as molecular markers to assist in the incorporation of *Yd₂* into the WI2875 background.

All barley cultivars were grown in the glasshouse as described in Section 2.2.1 and DNA extractions performed as described in Section 2.2.9. Barley DNA was digested with the following restriction enzymes *AluI*, *BamHI*, *BanII*, *BclII*, *BstNI*, *ClaI*, *DraI*, *EcoRI*, *EcoRV*, *HaeIII*, *HindIII*, *HinfI*, *HpaII*, *KpnI*, *NcoI*, *NdeI*, *RsaI*, *Sau3AI* *TaqI* and *XbaI*. Restricted DNA was analysed with a range of DNA probes, using the procedures for RFLP analysis outlined in Sections 2.2.10-2.2.12. Those RFLPs showing associations with Yd_2 were likely to be closely linked to Yd_2 and were chosen for segregation analysis.

4.2.5 Segregation analysis

Polymorphisms identified for DNA probes between Proctor and Shannon were mapped in the Proctor × Shannon F₂ population to determine if they were Yd_2 linked. DNA probes showing RFLPs between Atlas and Atlas68 were mapped in a group of 203 F₂ individuals showing a recombination event between *Xgk223* and *Xpsr116*, to determine if they were closely linked to Yd_2 . In the Proctor × Shannon mapping population linkage analysis was performed using the program MapMAKER (Lander *et al.*, 1987). The Kosambi Mapping Function (Kosambi, 1944) was used to convert recombination fractions to genetic distances in centiMorgans. In the Atlas × Atlas68 mapping population estimates of genetic distances within the *Xbcd828-Xgk223* interval were based on the relative frequency with which recombination occurred in genetic intervals within the *Xpsr116-Xgk223* interval.

4.2.6 BYDV resistance assays

Resistance assays to determine the Yd_2 genotype of F₂ individuals were conducted by Collins (1996) and Paltridge (1998) using viruliferous aphids produced by the author as described in Chapter 3.

4.2.7 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 an RFLP loci was located on the Chromosome 3, and to locate that RFLP to the long or the short arm of barley Chromosome 3.

4.2.8 The identification of F₃ progeny homozygous for a recombination event between the two loci *YLM* and *Xmwig952*

A minimum of 15 F₃ seedlings from each F₂ individual shown to be recombinant between the genetic interval *YLM* and *Xmwig952* were grown as described in Section 2.2.1. DNA extractions from all seedlings were performed as described in Section 2.2.9. Both the *Ylp*-derived (CAPS) PCR marker and *YLM* PCR markers were used for analysis of critical recombinant individuals with a recombination event between *YLM* and *Xmwig952*. The protocols for both PCR markers are described in Section 3.2.6. Southern analysis for *Xmwig952* on F₃ individuals was performed as described in Sections 2.2.10-2.2.12.

Results 4.3

4.3.1 The detection of *Yd₂*-associated polymorphisms using three sets of nearly isogenic lines

Sixteen DNA probes were analysed for RFLPs linked to *Yd₂* (Table 4.2). As 14 of these probes had either been mapped to the proximal region of the long arm of Triticeae chromosome group 3, or to the long arm of barley Chromosome 3 (Section 4.2.3), they were expected to map close to *Yd₂* and therefore, selected for further analysis. Although the other 2 rice probes had not been mapped in the Triticeae, they were selected for this study based on their map position in rice (Kurata *et al.*, 1994a).

Table 4.2 Results of RFLP analysis between 3 sets of NILs and WI/Franklin

DNA Probe	Loci detected	RFLPs detected between Atlas/At68	RFLPs detected between Proctor/Shannon	RFLPs detected between CM/CM67	RFLPs detected between WI/Franklin
BCD134	Single Locus	No polymorphism	P.M	N.T	N.T
BCD366	Single Locus	<i>BamHI, BanII, EcoRI, EcoRV, NcoI, NdeI,,</i>	<i>BamH, BanII, EcoRI, EcoRV, KpnI NcoI, Nde Sau3 AI, XbaI</i>	N.T	N.T
BCD1145	Single Locus	<i>BstI, KpnI, XbaI</i>	<i>BstI, KpnI, XbaI</i>	N.T	N.T
BCD1380	Multi Loci	<i>BstI, DraI</i>	<i>BstI, DraI, EcoRI, EcoRV, KpnI, NcoI, NdeI, TaqI, XhoI</i>	<i>BstI, DraI, NcoI, NdeI</i>	<i>EcoRI, EcoRV, NcoI, NdeI, Xho</i>
CDO54	Multi Loci	No polymorphism	<i>XbaI,</i>	No polymorphism	<i>EcoRI, NdeI</i>
CD0328	Single Locus	<i>XhoI, KpnI,</i>	<i>EcoRI</i>	No polymorphism	No polymorphism
CD0345	Single Locus	No polymorphism	No polymorphism	No polymorphism	No polymorphism
CD01406	Single Locus	No polymorphism	No polymorphism	<i>NdeI</i>	No polymorphism
RGC808	Multi Loci	No polymorphism	<i>EcoRI, Hae,III, NcoI, RsaI</i>	<i>EcoRI, KpnI</i>	No polymorphism
RGC270	Multi Loci	<i>KpnI</i>	No polymorphism	No polymorphism	No polymorphism
MWG802	Single Locus	<i>KpnI</i>	<i>KpnI</i>	No polymorphism	<i>KpnI</i>
MWG844b	Multi Loci	No polymorphism	<i>BamHI, BstI, XbaI</i>	<i>BstI, XbaI</i>	<i>BstI, XbaI</i>
MWG852	Single Locus	No polymorphism	No polymorphism	No polymorphism	No polymorphism
MWG2065	Multi Loci	No polymorphism	<i>EcoRI, BamHI, KpnI, NcoI</i>	No polymorphism	<i>BamHI, KpnI, EcoRI, NcoI</i>
MWG2105	Single Locus	<i>BamHI, EcoRI, KpnI</i>	No polymorphism	<i>BamH, TaqI</i>	No polymorphism
MWG2138	Multi Loci	<i>SauI, NdeI</i>	<i>KpnI, NcoI</i>	No polymorphism	<i>KpnI, NcoI</i>

P.M previously mapped by Collins *et al.*, 1996

N.T. not tested

All 16 DNA probes produced hybridisation patterns which were divided into three classes (Figure 4.2). The first class of hybridisation pattern comprised of specific restriction fragments detecting polymorphisms between NILs which were unlinked to Yd_2 ; *ie.*, the restriction fragment unique to the Yd_2 -donor did not accompany Yd_2 during introgression into the resistant cultivar. In the second class of hybridisation pattern, the RFLP did appear to be associated with Yd_2 ; *ie.*, the restriction fragment unique to the Yd_2 -donor was introgressed, along with Yd_2 , during the breeding. While the third class of hybridisation pattern detected specific restriction fragments which were monomorphic between a Yd_2 -donor barley and the susceptible parent cultivars of a given near-isogenic set.

In the Atlas68 and Shannon NIL sets, 13 of the 16 DNA probes tested with 20 different restriction enzymes detected RFLPs (Table 4.2). Two probes CDO345 and MWG852 showed no polymorphisms and were unable to be mapped. CDO1406 showed no polymorphsim between the two sets of NILs Atlas/Atlas 68 and Proctor/Shannon for which mapping populations had been developed and could not be mapped in these populations. However, as an RFLP was present between the CM/CM67 NIL set therefore, CDO1406 could be mapped if a mapping population between these two crosses was constructed. The genetic loci *Xbcd134*, had been previously mapped in the Proctor × Shannon population (Collins, 1996) and was chosen to be mapped in the larger Atlas × At68 population for increased genetic resolution. However, it could not be mapped in the expanded Atlas × Atlas68 population since all restriction fragments were monomorphic between the parents for all 20 restriction enzymes tested.

4.3.2 Addition line analysis

Using Chinese Spring wheat-Betzes barley disomic and ditelosomic addition lines (Islam *et al.*, 1981; Islam, 1983), the centromere of Chromosome 3 had been previously localised to a position between *Xbcd127* and *Xbcd134* in the Proctor × Shannon mapping population (Collins *et al.*, 1996). In the present study, these addition lines were used to analyse the chromosomal location of the loci *Xmwig952*, previously mapped within 1cM of Yd_2 in the Atlas × Atlas68 mapping population (Paltridge, 1998). However it had not yet been determined if it was on the long or short arm of Chromosome 3.

Figure 4.2 Southern hybridisation analysis of Near Isogenic Lines (NILs) for Yd_2

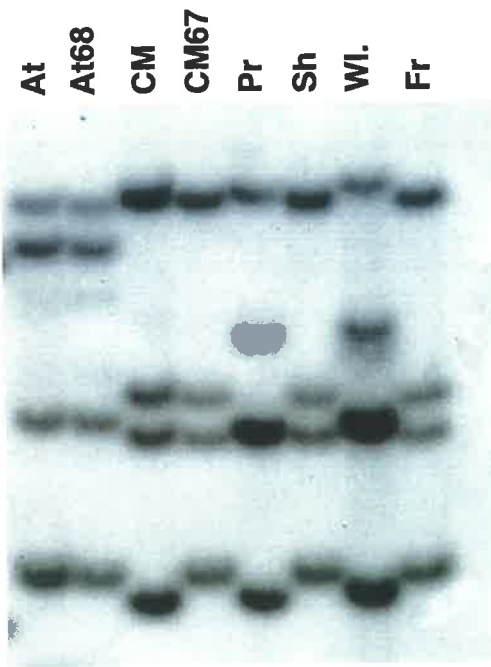
Genomic DNA extracted from the barley NILs Atlas (**At**) and Atlas68 (**At68**), **CM** and **CM67**, Proctor (**Pr**) and Shannon (**Sh**), and the non- Yd_2 and Yd_2 containing lines WI2733 (**WI**) and Franklin (**Fr**) was subjected to restriction enzyme digestion before electrophoresis on a 1×TAE 1% agarose gel. After Southern transfer, membranes were subjected to Southern hybridisation with the DNA probes BCD1380, MWG2105, BCD134 and visualised by autoradiography.

(a) The hybridisation of BCD1380 to the NIL lines of Atlas and Atlas68, CM and CM67, Proctor and Shannon, and WI2733 and Franklin is an example of the 1st type of hybridisation pattern where RFLPs were not associated with Yd_2 . The restriction enzyme used to cut the genomic DNA was *XhoI*.

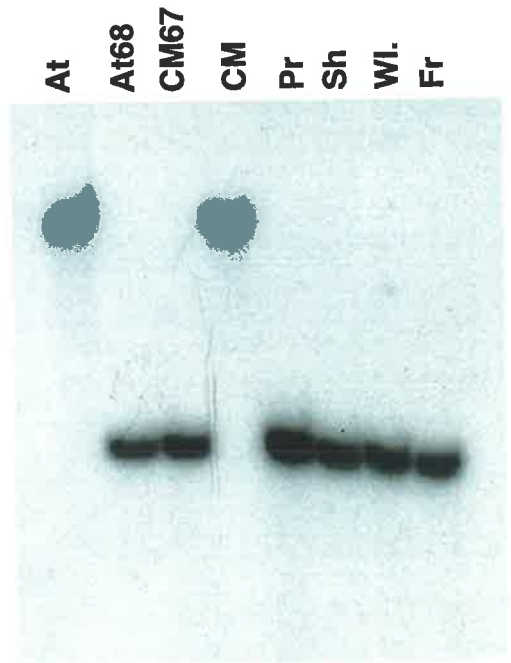
(b) The hybridisation of MWG2105 to the NIL lines of Atlas and Atlas68, CM and CM67, Proctor and Shannon, and the non- Yd_2 and Yd_2 containing lines WI2733 and Franklin is an example of the 2nd class of hybridisation pattern found where a specific restriction fragment was polymorphic between the Yd_2 -donor barley and the susceptible parent cultivars of a given near isogenic set (Atlas/Atlas68) and found to be associated with Yd_2 . The genomic DNA was cut with the restriction enzyme *BamHI*.

(c) The hybridisation of BCD134 to the NIL lines Atlas and Atlas 68 is an example of the 3rd class of hybridisation pattern found where specific restriction fragments were monomorphic between the Yd_2 -donor barley and the susceptible parent cultivars of a given near isogenic set when tried with all available restriction enzymes. Genomic DNA was cut using the following restriction enzymes *BamHI*, *DraI*, *EcoRI*, *EcoRV*, *HindIII*, *NdeI*, *NcoI* and *XhoI*.

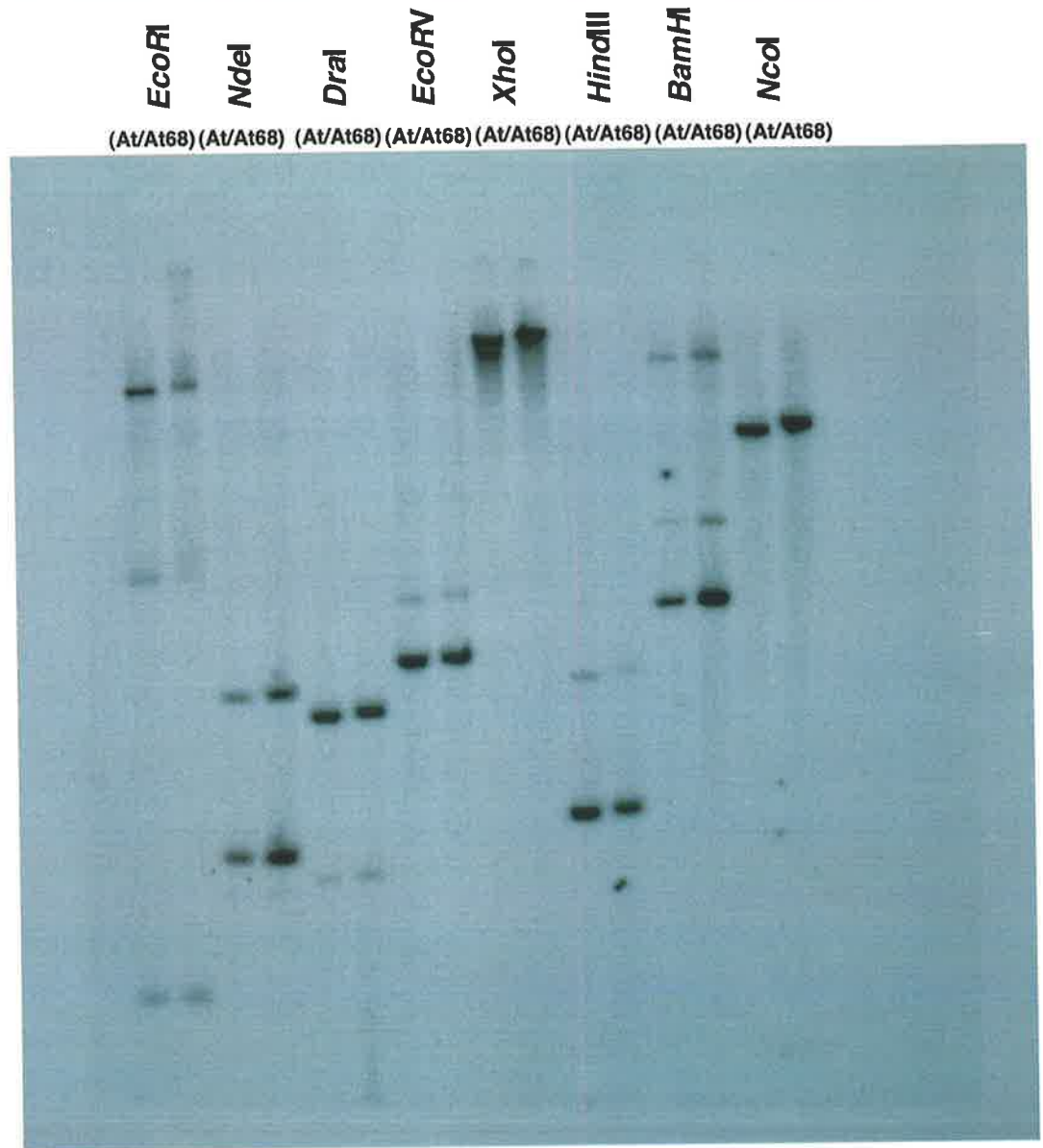
(a)



(b)



(c)



The Proctor allele of the mapped restriction fragment was present in the barley cultivar Betzes and the 3H disomic addition line, but absent from the wheat cultivar Chinese Spring. This confirmed *Xmwg952* to be located on Chromosome 3H. The presence of the same band in the 3HL but not 3HS ditelosomic addition line further localised *Xmwg952* to the long rather than short arm of Chromosome 3H. The addition line analysis of the RFLP marker *Xmwg952* is shown in Figure 4.3. Therefore, the centromere is placed above *Xmwg952* on the revised map of the Atlas × Atlas68 mapping population.

4.3.3 Segregation analysis

Twelve DNA probes and their associated RFLPs were mapped in either the Atlas × Atlas68 or Proctor × Shannon mapping populations (Table 4.3). Where possible, probes were mapped in the larger Atlas × Atlas68 population for increased genetic resolution. However, if no polymorphism existed in this cross they were then mapped in the Proctor × Shannon population. Five of the 12 probes mapped to *Yd₂* (Table 4.4) and were placed on the revised linkage map (Figure 4.4).

DNA probes which detected single loci were mapped using one restriction enzyme. Since mapping in the Atlas × Atlas68 population was carried out only within the genetic interval between *Xtag223* and *Xpsr116*, RFLPs which did not map to *Yd₂* were considered to be either unlinked or not closely linked with *Yd₂*. Probes resulting in multiloci hybridisation patterns were further analysed using more than one restriction enzyme for mapping if the first RFLP mapped was found to unlinked to *Yd₂* (Table 4.3), because other loci detected using this probe may be linked to *Yd₂*.

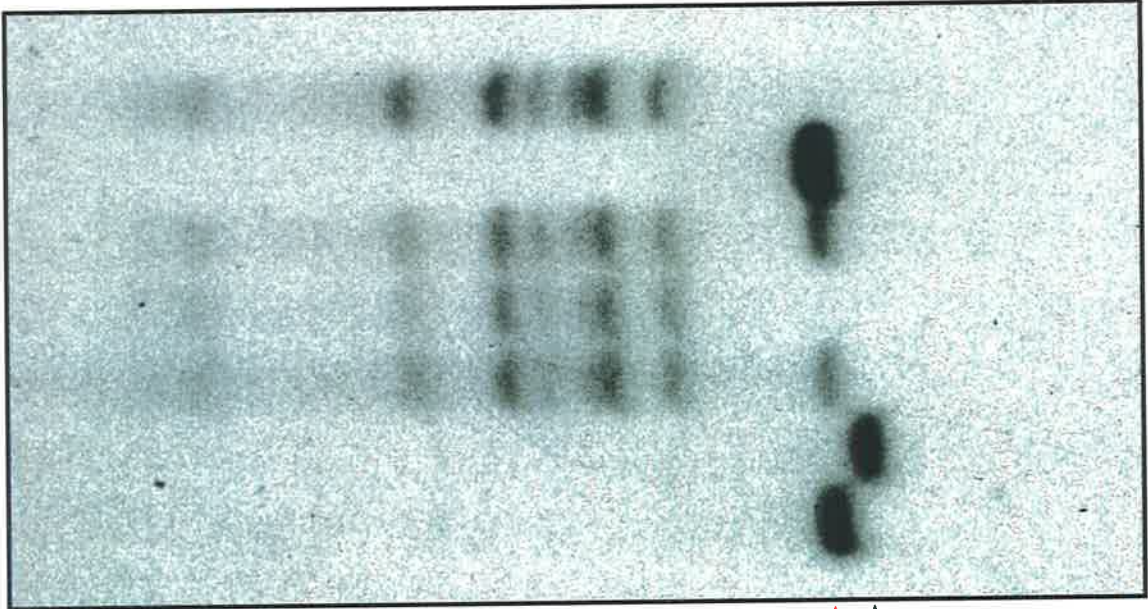
4.3.4 Detection of *Yd₂*-associated polymorphisms between WI2875 (*Yd₂*-minus) and Franklin (*Yd₂*-plus)

RFLPs linked to *Yd₂* may be useful as molecular markers to incorporate *Yd₂* into the WI2875 background. The DNA probe MWG802 when hybridised to DNA restricted with *KpnI* produced a hybridisation pattern resulting in a single loci with the same RFLP for the NIL pair Atlas/Atlas68 as with WI2875/Franklin (Table 4.2). This restriction enzyme was used to map

Figure 4.3 Addition line analysis of the physical location of the marker *Xmwg952*

Genomic DNA was extracted from Chinese Spring (CS), Betzes barley, the disomic addition line of Chinese Spring carrying Betzes barley Chromosome 3H (CS+3H), the ditelosomic addition line carrying the long arm of Chromosome 3H (CS+3HL), the addition line carrying the short arm of Chromosome 3H (CS+3HS) and the barley NILs Proctor and Shannon. DNA was restricted with the restriction enzyme *DraI* before electrophoresis on a 1% agarose gel in a 1×TAE running buffer. After Southern transfer, membranes were subjected to Southern hybridisation with the DNA probes MWG952 and visualised by autoradiography.

The Proctor and Shannon alleles of the RFLP marker *Xmwg952* are marked with the red and black arrows, respectively. The Proctor allele of the locus is clearly present in Betzes barley but not Chinese Spring (CS) wheat. The disomic addition line carrying Betzes barley Chromosome 3H in the Chinese Spring wheat genetic background confirmed the RFLP to be derived from Chromosome 3H. The presence of the same band in the ditelosomic addition line carrying the long arm of Chromosome 3H (CS+3HL), and the absence of the band from the addition line carrying the short arm of Chromosome 3H (CS+3HS), further localised the marker to the long arm of Chromosome 3H.



CS

Betzes

CS + 3H

CS + 3HS

CS + 3HL

Shannon

Proctor



Table 4.3 DNA probes and the restriction enzymes used to detect RFLPs which were mapped in either the Atlas × Atlas68 or Proctor × Shannon crosses

DNA probe	Restriction enzymes used for mapping in the NIL pairs At/At68 and P/Sh
BCD366	<i>Nco</i> I (At/At68)
BCD1145	<i>Kpn</i> I (At/At68)
BCD1380	<i>Bst</i> I, <i>Dra</i> I, (At/At68)
CDO54	<i>Xba</i> I (P/Sh)
CDO328	<i>Kpn</i> I (At/At68)
RGC808	<i>Dra</i> I, <i>Hind</i> III, <i>Xba</i> I (P/Sh)
RGC270	<i>Kpn</i> I (At/At68)
MWG802	<i>Kpn</i> I (At/At68)
MWG844B	<i>Bam</i> HI (At/At68)
MWG2065	<i>Bam</i> HI (P/Sh)
MWG2105	<i>Bam</i> HI(At/At68)
MWG2138	<i>Sau</i> I, <i>Nde</i> I (P/Sh)

Table 4.4 DNA probes and the restriction enzymes detecting RFLPs associated with Yd_2 in the NIL pairs At/At68 and P/Sh

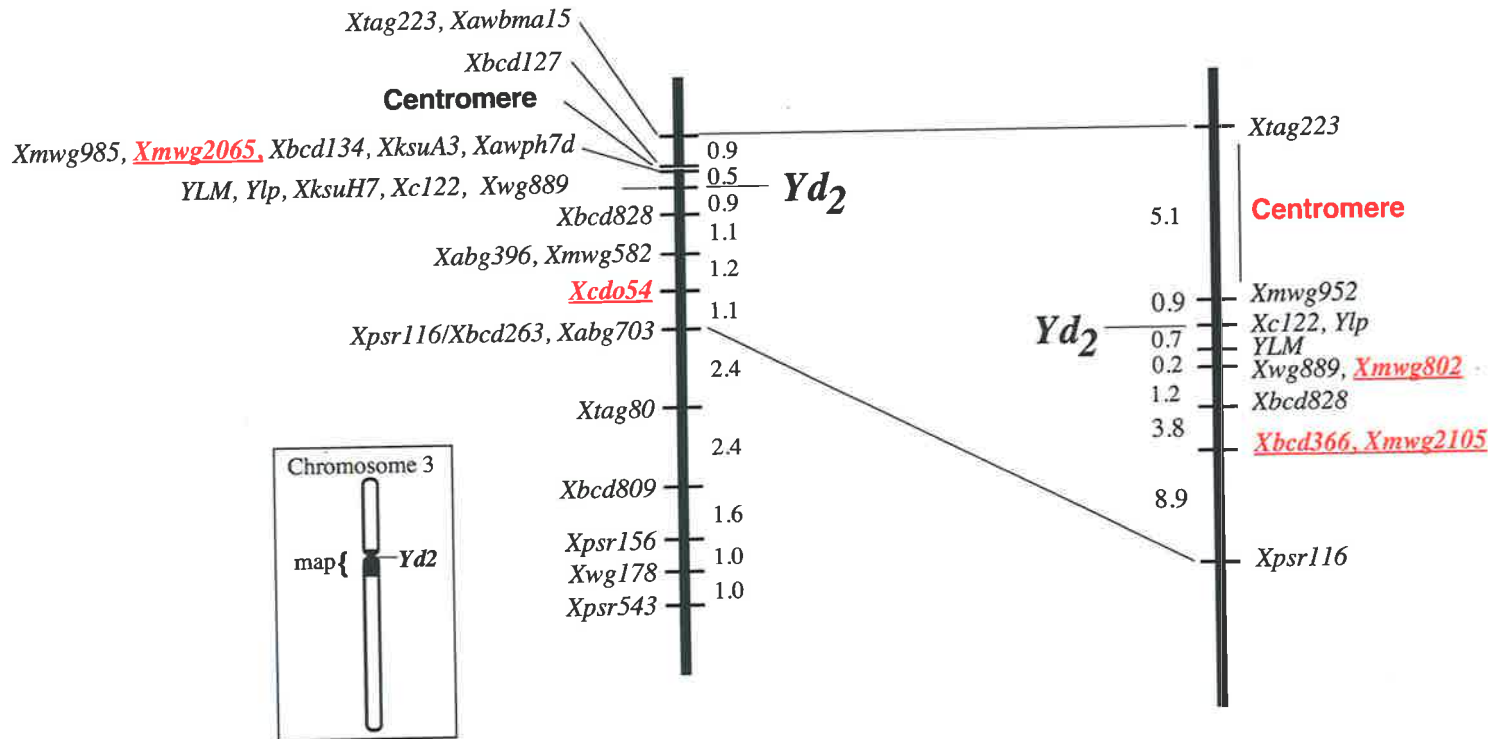
DNA probe	Restriction enzymes used to map a Yd_2 linked RFLP
BCD366	<i>Nco</i> I (At/At68)
CD054	<i>Xba</i> I (P/Sh)
MWG802	<i>Kpn</i> I (At/At68)
MWG2065	<i>Bam</i> HI (At/At68)
MWG2105	<i>Bam</i> HI (P/Sh)

Figure 4.4 Revised linkage maps of the Yd_2 region of barley Chromosome 3 developed in the two mapping populations Proctor \times Shannon and Atlas \times Atlas 68

Revised linkage maps of the Yd_2 region of barley Chromosome 3 are presented with 5 new markers highlighted and underlined in red. The centromere was also localised to a position between the markers *Xglk223* and *Xmwg952* on the Atlas \times Atlas 68 map. The other markers had been mapped previously in this laboratory by Collins *et al.*, (1996) and Paltridge (1998)(Distances are in centiMorgans).

Proctor x Shannon map

Atlas x Atlas 68 map



Xmwig802 less than 1.0cM from *Yd₂* (Figure 4.4). Therefore, this RFLP could provide a useful marker for the incorporation of *Yd₂* from Franklin into WI2875 background.

4.3.5 The construction of a group of individuals homozygous for a recombinant event between the loci *YLM* and *Xmwig952*

F₃ progeny of 18 individuals originally identified by Paltridge (1988) showing recombination between the two flanking markers *Xmwig952* and *YLM* (Figure 4.5) were chosen for analysis using the PCR marker *YLM*, the *Ylp*-derived-CAPS PCR marker and the RFLP marker *Xmwig952* in order to identify individuals homozygous for the recombination event within this interval. Any F₃ individuals identified as being homozygous for the recombination event within the interval were seen as a potentially valuable genetic resource for the future isolation of genetic markers close to *Yd₂* and the possible generation of new markers by their inclusion in resistant and susceptible bulks. A minimum of 15 F₃ progeny for each recombinant were analysed using three molecular markers, *YLM*, *Ylp* and *Xmwig952*, in order to identify individuals homozygous for the recombination event within the interval defined by the markers *Xmwig952* and *YLM*, (Figure 4.6). A number of individuals were identified as being homozygous for their recombinant chromosome. Four different classes of recombinant individuals were obtained which were homozygous for the recombinant chromosome (Figure 4.7). These individuals were selfed to the F₄, and seed and plant material stored as a genetic resource. This resource was subsequently used for the development of resistant and susceptible bulks to generate more markers linked to *Yd₂* (Chapter 8).

4.4 Discussion

4.4.1 The value of *Yd₂* linked RFLP markers in barley breeding

In this study 5 new RFLP markers were identified as being closely linked to *Yd₂*, 2 of which were within 1cM of *Yd₂*. Importantly, one of these markers *Xmwig802* is polymorphic between the line WI2875 (*Yd₂*-plus) and the cultivar Franklin (*Yd₂*-minus), both lines the Waite Barley

Figure 4.5 Critical recombinants identified between the two closest flanking markers amongst F₂ individuals of the mapping population of Atlas × Atlas68

In the analysis of 572 F₂ individuals, 18 F₂ individuals were previously identified by Nick Paltridge in this laboratory for recombination events between the closest flanking markers and *Yd₂*. Three RFLP markers *Xrgc122*, *Ylp* and *Xmwg952* and a PCR marker *YLM* were used to type recombination events close to *Yd₂*. F₃ seed was collected from each of these individuals and resistant assays performed to identify *Yd₂* genotype. No recombination events were observed between *Yd₂* and the two RFLP markers *Xrgc122* and *Ylp*. Schematic representation of chromosome types are presented. Red represents resistant associated DNA, while blue represents susceptible associated DNA. The area shaded yellow-orange is representative of the region of recombination. Numbers in brackets indicate the number of individuals identified for each type of recombination event described. These recombinants were seen as a potentially valuable source of genetic material for the resolution of genetic markers close to *Yd₂* and their potential use for the construction of resistant and susceptible bulks for bulked segregant analysis. Therefore, F₃ progeny which were homozygous for the recombinant chromosome needed to be identified (Figure 4.6 & 4.7).

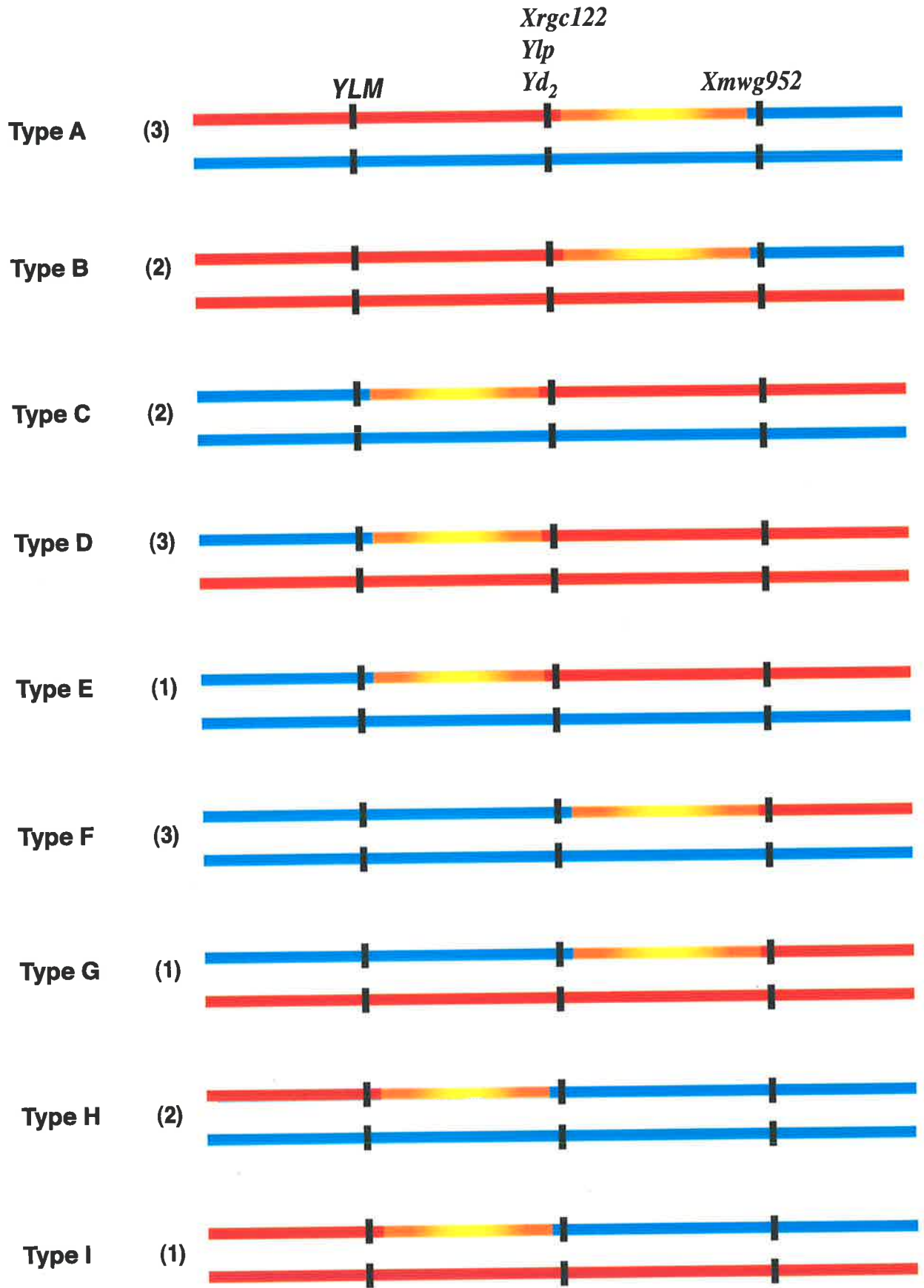


Figure 4.6 The identification of F₃ progeny homozygous for a recombination event between the two closest flanking markers *YLM* and *Xmwig952*

Genomic DNA was extracted from a minimum of 15 F₃ seedlings derived from each F₂ individual shown to be heterozygous for a recombination event between the genetic interval *YLM* and *Xmwig952* (Figure 4.5). The RFLP marker (a) *Xmwig952* and the PCR markers (b) *Ylp*-CAPs marker and (c) *YLM* were used to identify F₃ progeny homozygous for a recombination event between the two closest flanking markers *YLM* and *Xmwig952*. PCR products of the *Ylp*-derived (CAPS) PCR marker and *YLM* PCR markers were run on a 0.75% agarose/2.25% Nusieve GTC agarose gel in 1×TBE electrophoresis buffer. PCR products were visualised after staining with ethidium bromide. For analysis with the marker *Xmwig952*, DNA was restricted with the restriction enzyme *NcoI* before electrophoresis on a 1% agarose gel in 1×TAE running buffer. After Southern transfer, membranes were subjected to Southern hybridisation with the DNA probe MWG952 and visualised by autoradiography.

(a) The Black arrows indicate those individuals homozygous for the critical recombination event of interest at the *Xmwig952* locus which are progeny of Type B and Type F F₂ recombinants (Figure 4.5). The position of the resistant and susceptible *Yd₂*-associated alleles is denoted at the side of the palate.

(b) The white arrows indicate those individual homozygous for the critical recombination event of interest at the *Ylp* locus which are progeny of Type I recombinants (Figure 4.5). The position of the resistant and susceptible *Yd₂*-associated alleles is denoted at the side of the palate. The 311 base-pair fragment is the allele linked with resistance, with the *NlaIII* restriction enzyme site absent. The susceptible associated allele contains the *NlaIII* site and when restricted is 253 base-pairs. (The other 58 base-pair restriction fragments are not visible). The marker lane (M) contains pUC19 DNA digested with *HpaII*.

(c) The white arrows indicate those individual homozygous for the critical recombination event of interest at the *YLM* locus which are progeny of Type D recombinants (Figure 4.5). The position of the resistant and susceptible *Yd₂*-associated alleles is denoted at the side of the palate. The 101 base-pair fragment is the allele linked with susceptibility. The 90bp fragment is linked with resistance. The marker lane (M) contains pUC19 DNA digested with *HpaII*.

Those individuals found to be homozygous for the recombinant chromosome were selfed to the F₄ and seed and plant material stored as a genetic resource.

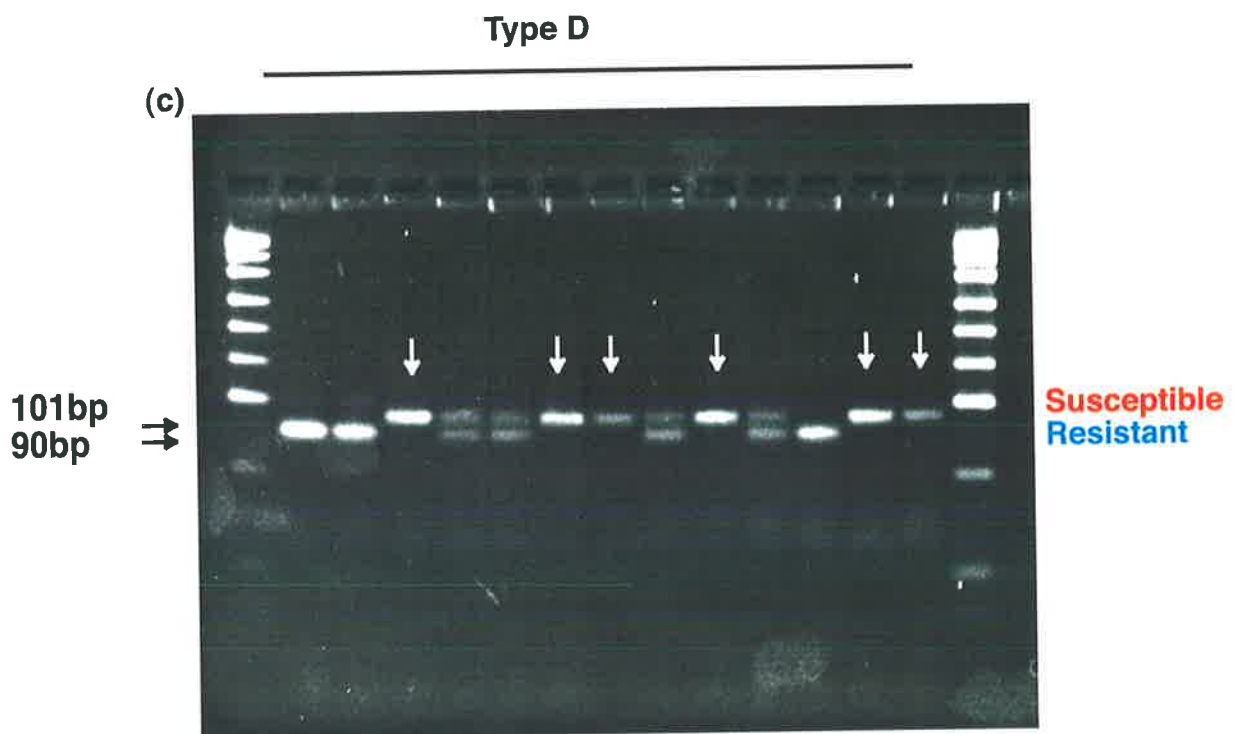
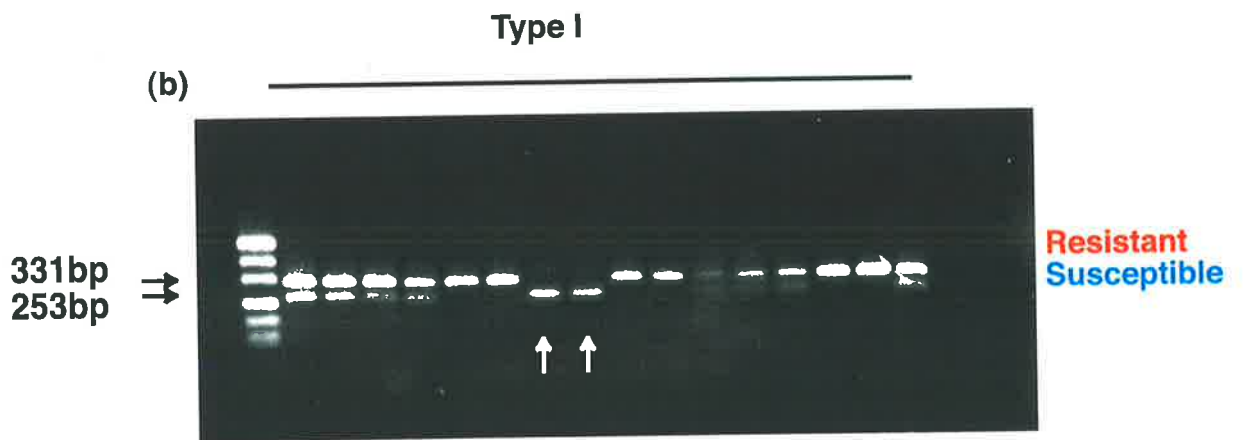
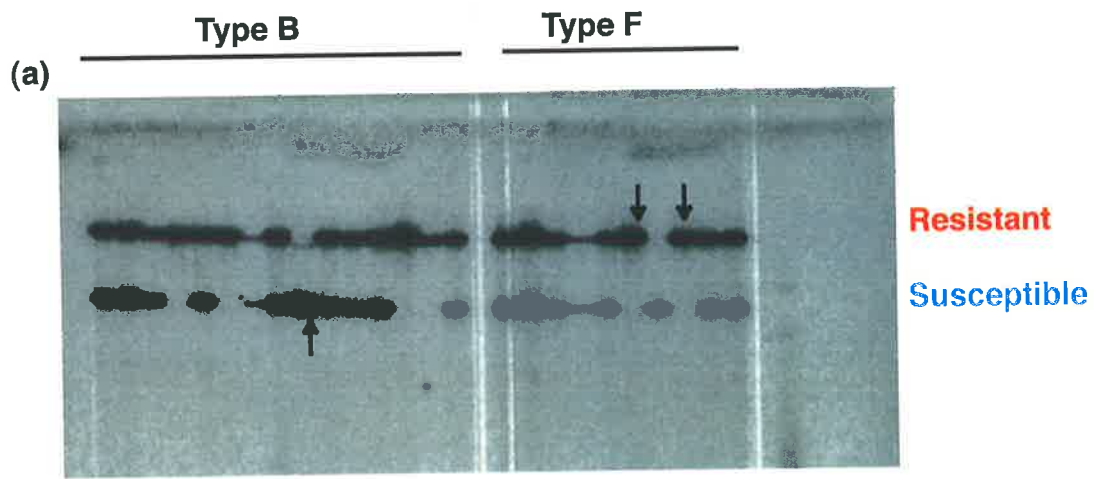
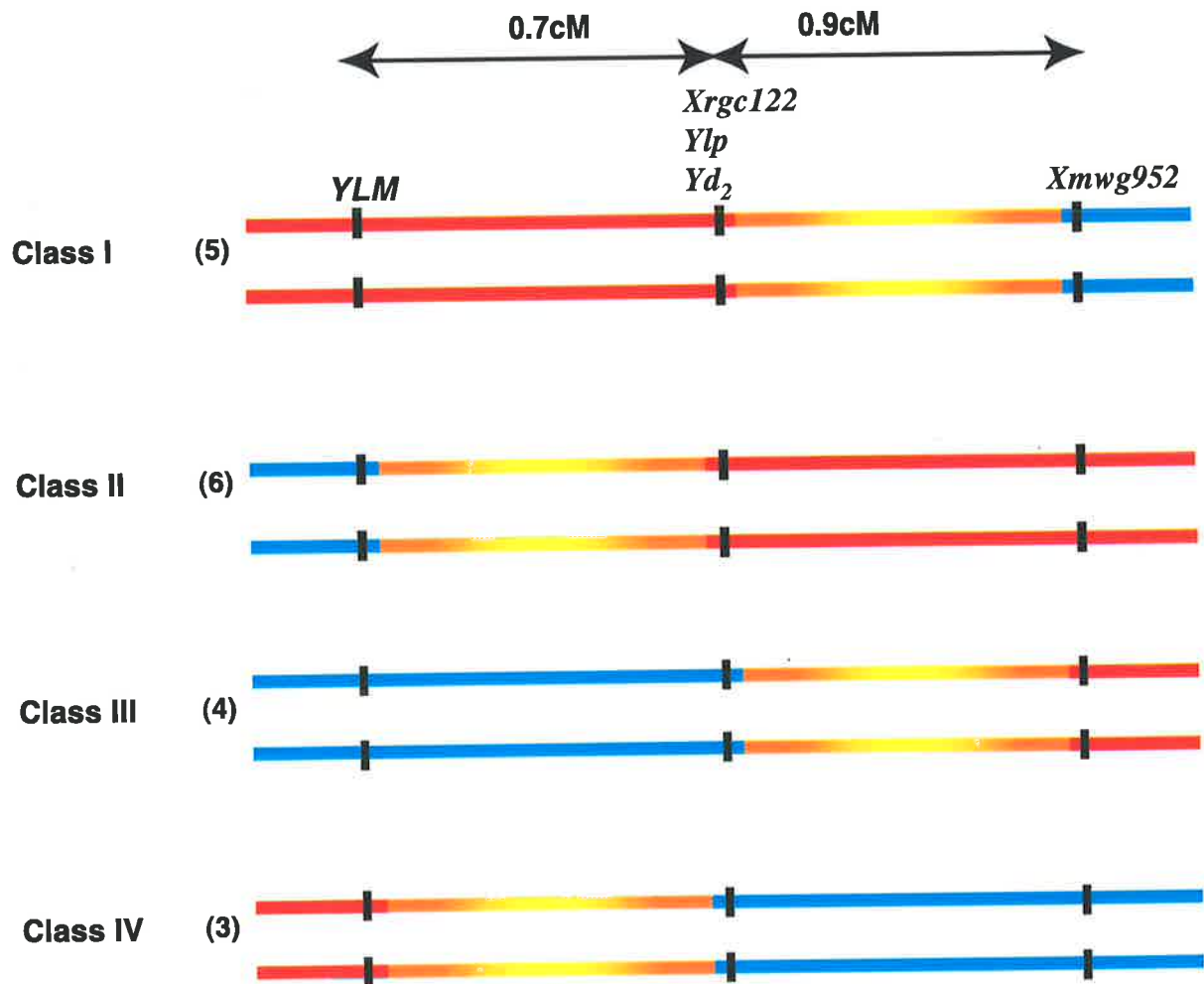


Figure 4.7 Four different classes of recombinant individuals homozygous for the recombinant chromosome

A genetic resource of individuals homozygous for the recombinant chromosome between the two closest flanking markers *YLM* and *Xmwig952* was established from screening F₃ progeny of F₂ individuals shown to be heterozygous for recombination event between the genetic interval *YLM* and *Xmwig952* with the RFLP marker *Xmwig952* and the *Ylp* and *YLM* PCR markers (Figures 4.5 & 4.6). A schematic representation of chromosome types are presented. Red represents resistant associated DNA, while blue represents susceptible associated DNA. The area shaded yellow-orange is representative of the region of recombination. Numbers in brackets indicate the number of individuals identified for each type of recombination event described. Classes I&II consisted of individuals resistant for *Yd₂* while Classes III&IV consisted of individuals susceptible for *Yd₂*. Classes I&III had individuals with a recombination event defined between the molecular markers *Ylp* and *Xmwig952* while Classes II&IV contained individuals with recombination events as defined by the markers *Ylp* and *YLM*.



Improvement Program aims to use for the incorporation of Yd_2 into a more suitable agronomic background. Typically, up to seven backcrosses have been used during the breeding of BYD-resistant barley cultivars (Schaller and Chim, 1969a,b; Schaller *et al.*, 1973, 1977, 1979; Vertigan, 1979). To have a greater than 95% chance of retaining the Yd_2 gene during seven backcrosses, whilst avoiding BYD-resistance bioassays, any single marker used to predict Yd_2 status should be within 0.7cM of the gene. (This figure was obtained by assuming that the probability of recombination occurring at each opportunity equals the genetic distance between loci expressed as a recombination fraction; *i.e.*, 0.007. Because of the small genetic distances involved, recombination percentage can be regarded as equivalent to genetic distance in cM.) Therefore, if *Xmwg802* is used as an RFLP marker to incorporate the Yd_2 gene into WI2875 during seven backcrosses, whilst avoiding BYD-resistance bioassays, this would result in a 94% confidence level of keeping the Yd_2 gene. Therefore, this marker should prove useful for the incorporation of the Yd_2 gene into WI2875.

At the completion of these RFLP mapping studies a total of 11 molecular markers including two PCR markers are within 1cM of Yd_2 on the Proctor \times Shannon map, (Figure 4.4) and therefore, sufficiently close to Yd_2 to be used in the marker-assisted selection of Yd_2 -carrying lines in breeding programs involving up to seven backcrosses to a susceptible recurrent parent. While in the Atlas \times Atlas68 map only 6 markers are genetically close enough (Figure 4.4). However, due to the higher rates for recombination in this cross resulting in an increased genetic resolution, these markers maybe physically closer and therefore, more relevant. Often in plant molecular breeding, a polymorphism detected between two lines for a marker linked to the trait of interest may not exist between the donor and the recurrent parent of interest to the plant breeder. The number of other RFLP markers closely linked to Yd_2 from both genetic maps presented in this thesis provides a comprehensive resource of genetically close markers for which polymorphisms should be able to be found between most combinations of recurrent and donor parents of interest to barley breeders. Thereby, maximising the chance of being able to use molecular markers for the incorporation of Yd_2 into breeding material of interest to the plant breeder.

4.4.2 Completion of RFLP mapping of the Yd_2 region of Chromosome 3 of barley and implications for future mapping and isolation of the Yd_2 resistance gene

The work in this chapter aimed to finalise the RFLP mapping studies around the Yd_2 region of barley Chromosome 3. Therefore, 16 DNA probes shown from other genetic linkage maps to be from the long arm of Triticeae chromosome group 3 (Liu and Tsunewaki 1991; Heun *et al.*, 1991; Gill *et al.*, 1991; Devos and Gale, 1993b; Kleinhofs *et al.*, 1993; Graner *et al.*, 1994; Kurata *et al.*, 1994a; Nelson *et al.*, 1995b) were chosen to be scanned for polymorphism between the parents of both mapping populations. This work was undertaken and polymorphisms between one or both pairs of NILs used as parents in our mapping populations were identified for 12 of the 16 DNA probes. These DNA probes were mapped relative to Yd_2 , and 5 were found to be linked. Importantly two of these linked polymorphisms were within 1cM of the Yd_2 gene. Therefore, at the finalisation of these RFLP mapping studies a total of 29 molecular markers had been placed on the map, 12 of which had been mapped within 1cM of Yd_2 (Figure 4.4).

Since the isolation of the Yd_2 gene is the main goal of this project, it is essential to consider how useful the present genetic maps may be in this endeavour. Assuming the barley genome to measure approximately 4800Mb (Arumuganathan and Earl, 1991), and that the genetic map covers about 1800cM (Becker *et al.*, 1995), the average relationship between genetic and physical distance can be calculated to be about 2.7 Mb per centiMorgan. The map resolution required for map-based cloning in barley has been estimated previously to be about 0.1cM (Simons *et al.*, 1997). This is based on the calculation that loci 0.1cM apart may be close enough to identify a single genomic clone such as a yeast artificial chromosome (YAC), or series of overlapping Bacterial Artificial chromosomes BACs in barley that span a distance of approximately 300kb.

In order to have a 95% chance of resolving loci 0.1cM apart, a mapping population carrying approximately 3000 meiotic products (for example, 1500 F₂ individuals) is required. This is calculated by assuming the probability of no recombination between such a marker and Yd_2 is 0.999 in each of the 3000 meioses; therefore the probability of no recombination events in the entire mapping population = $0.999^{3000} = 0.05$, and the probability of recombination = $1 - 0.05$

= 0.95. For the Proctor × Shannon map there were only 106 F₂ individuals (212 meiotic products) used for construction of the map. The probability that a mapping population of 106 F₂ individuals (212 meiotic products) could resolve markers within 0.1cM of *Yd₂* is approximately 19%. However, the Atlas × Atlas68 mapping population consisted of 572 F₂ individuals (1144 meioses). This population offered a 95% chance of resolving markers 0.3cM apart, but only a 60% chance of resolving markers 0.1cM apart. Two markers were unable to be resolved from *Yd₂* in both our mapping populations, and based upon such assumptions we can be confident that the markers are within 0.3cM of the gene. Based on this information and assuming an average relationship of genetic and physical distance of 2.7Mb/cM and an average insert size of a large insert genomic BAC library to be approximately 110kb, our co-segregating markers may be close enough for us to start a chromosome walk. However, the chances of obtaining a large genomic clone with one of our co-segregating markers and encompassing the *Yd₂* gene is not high, and the chance of identifying a large genomic clone with two markers flanking our gene are very poor based on the assumptions made. If such assumptions are correct then an increase in genetic resolution, and increased marker saturation of the target region may be required.

While RFLP markers are always likely to become available from other researchers, the number of potentially *Yd₂* linked markers that could be obtained is limited by the extent of their mapping populations, as well as being mappable in our populations. While 14 of the 16 DNA probes tested in this study showed mappable polymorphisms, only 5 were found to be closely linked to *Yd₂*. Therefore, giving the author a success rate of less than 30%. As previously mentioned all 16 DNA probes were chosen for mapping based on their predicted proximity in other cereal linkage maps to the *Yd₂* area (Section 4.2.3). Of the 9 DNA probes which did not map to *Yd₂*, many of these were mapped outside the area of interest, as mapping in the Atlas × Atlas68 population was carried out within the genetic interval denoted by the markers *Xtag223* and *Xpsr116*. What must also be considered is that a number of markers produced multiloci hybridisation patterns and therefore, polymorphisms mapped may have been of loci unlinked to *Yd₂*.

If a higher marker density in our target area is necessary, then other marker systems may be more applicable. Additional DNA marker systems have been developed in recent years which have also been used to construct linkage maps of the cereal genomes. These new marker systems use the polymerase chain reaction (PCR) to amplify polymorphic DNA sequences. The main marker types developed for linkage analyses are Simple Sequence Repeat polymorphisms or microsatellites (SSRs; Tautz, 1989), Random Amplified Polymorphic DNAs (RAPDs; Williams *et al.*, 1990) and Amplified Fragment Length Polymorphism (AFLPs; Zabeau and Vos, 1993; Vos *et al.*, 1995) (reviewed in Section 1.6.1). More recently, degenerate primers targeted to amplify resistant gene analogues have been used to amplify products which are run on a polyacrylamide gel and scanned for size polymorphisms or presence and absence polymorphisms, and mapped as a PCR marker (Chen *et al.*, 1998).

Essential for any further saturation of the region was the development of a genetic resource of recombinants homozygous between the two closest flanking markers in our Atlas × Atlas68 mapping population. The identification and characterisation of these recombinants allows them to be put into populations of susceptible and resistant DNA bulks which can be scanned for polymorphisms using a PCR-derived marker system, this is designated bulked segregant analysis (Michelmore *et al.*, 1991). Bulked segregant analysis has been used by a number of researchers for high resolution genetic mapping of a target locus using a variety of PCR-derived marker systems (Michelmore *et al.*, 1991; Ballvora *et al.*, 1995; Meksem *et al.*, 1995) (reviewed in Section 1.6.2). Importantly, the genetic interval between the two markers *Xmwg952* and *YLM* is 1.5cM. Therefore, polymorphisms identified between bulks may be expected to map within this interval, thereby closely mapping this region. Further marker saturation at the *Yd₂* region using this resource was undertaken later in this study and is discussed in Chapter 8.

While calculations of required marker density and genetic resolution indicate that our high resolution map around *Yd₂* may not be sufficient for the identification of a large insert genomic clone physically close to *Yd₂*. It is essential to note that these calculations are based on a genome-wide estimate of genetic and physical distances, with the assumption that recombination frequency is evenly distributed throughout the genome. However, extreme

deviation from estimates have been frequently reported in the literature. For example, a correlation of 43kb/cM has been found in the tomato genome in the vicinity of the resistance locus I_2 (Segal *et al.*, 1992), whereas, in the region surrounding the *Tm-2a* gene, 1cM corresponds to 4000kb (Ganal *et al.*, 1989). Importantly, Buschges *et al.* (1997) have showed that the relationship between genetic and physical distance can deviate by one or two orders of magnitude in different regions of the barley genome. Specifically, Buschges *et al.* (1997) found that within an 820 base-pair segment of the *Mlo* gene (which controls powdery mildew resistance in barley) the recombination frequency measured just 25kb/cM; within a 30kb interval spanning *Mlo*, the recombination frequency measured 100kb/cM. According to Buschges *et al.* (1997), exceptionally high rates of recombination have been observed in coding rather than non-coding regions of the *Arabidopsis thaliana* Chromosome 4. Buschges *et al.* (1997) argue that a similar trend may apply in barley. It is important to note that the two markers which co-segregate with Yd_2 are both detected with cDNA probes. Therefore, there is the possibility that the Yd_2 region could be gene rich with higher rates for recombination than the genome average.

In contrast, the Proctor \times Shannon mapping population placed the Yd_2 gene less than 1cM from the centromere of Chromosome 3H. In a study by Linde-Laursen (1982) and more recently Kunzel *et al.* (2000), this region of the chromosome was shown to be one of low recombination frequency, relative to that observed in the more distal regions of the chromosome. Indeed, a suppression of recombination and the clustering of markers in the centromeric region seems to be a general feature of a number of Triticeae chromosomes (Devos and Gale, 1997). This information suggests that the map-based cloning of Yd_2 may be very difficult. In contrast, Paltridge (1998) identified a 5 fold increase in recombination rates for this area in the Atlas \times Atlas68 mapping population and importantly identified 18 individuals having a recombination event between the two closest flanking markers which are only separated by a genetic distance of 1.5cM. Therefore, it is possible that Yd_2 is not as genetically close to the centromere as previously thought (Collins, 1996). Before trying to decide whether we need another round of marker saturation and a possible increase in the genetic resolution of the mapping population (number of meiotic products) it appears essential that the relationship between genetic and

physical distances in this region is accurately measured. The following chapter describes the use of pulsed field gel electrophoresis (PFGE) to measure this relationship.

CHAPTER 5

**PHYSICAL MAPPING OF THE Yd_2 REGION OF BARLEY
CHROMOSOME 3**

5.1 Introduction

Subsequent to the successful construction of a high resolution genetic map of the *Yd₂* region, the next step crucial to the success of a map-based cloning effort is the establishment of the relationship between genetic and physical distance by physical mapping of the most closely linked markers. This is critical to the success of map based cloning because the relationship between genetic and physical distance can vary over 100-fold in different regions of a genome (Ganal *et al.*, 1989; Segal *et al.*, 1992).

The variation in this relationship between genetic and physical distance can depend on the composition of surrounding DNA sequences (CpG islands or heterochromatin) and the physical location on the chromosome (DeScenzo and Wise, 1996). Over the last decade, a combination of cytogenetics and molecular genetics have been used to develop physical maps and determine the ratio of physical to genetic distance in a number of plant species (Gustafson *et al.*, 1990; Gill *et al.*, 1993; Jiang and Gill, 1993; Kota *et al.*, 1993; Leitch and Heslop-Harrison, 1993; Schmidt *et al.*, 1994; Pederson and Linde-Laursen 1995, Künzel *et al.*, 2000). Generally, low recombination rates per kilobase (kb) have been observed in regions near the centromeres and very high recombination rates per kb towards the distal end of the chromosome. In addition cytogenetic analyses demonstrated that some chromosomal regions that are physically small are active sites for recombination.

Although results from molecular cytogenetic analysis indicate variation between genetic and physical distance throughout the genome, a more accurate calculation of the ratio of genetic to physical distance can be obtained via electrophoretic analysis of high molecular weight (HMW) DNA (DeScenzo and Wise, 1996). In regions of the genome which are saturated with molecular markers, it may be possible to locate two tightly linked markers on a single high molecular weight DNA fragment. If two genetically distinct markers hybridise to the same fragment, a maximum physical distance spanned by a particular genetic interval can be calculated. A number of techniques have been developed to separate high molecular weight DNA fragments electrophoretically. Pulsed field gel electrophoresis (PFGE; Schwartz and Cantor, 1984) and a variant known as contour clamped homogenous field electrophoresis (CHEF; Chu *et al.*, 1986),

have been widely used to resolve large DNA fragments. CHEF analysis has been utilised to investigate the relationship between physical and genetic distance for complex loci in plants such as *Tm-2a* (Ganal *et al.*, 1989), *Pto* (Martin *et al.*, 1993) and *Cf-2* (Dixon *et al.*, 1995) in tomato, *Xa21* (Ronald *et al.*, 1992) in rice, *Hor 1* (Sieder and Graner, 1991) and *Hor 2* (Sorensen, 1989) in barley, and the *alpha-Amy-1 loci* (Cheung *et al.*, 1991) in wheat.

An essential element of physical mapping is the ability to isolate and manipulate relatively intact and unsheared chromosomal DNA. The most commonly used method is to embed intact cells, protoplasts or nuclei in agarose plugs followed by cell lysis, proteinase-K digestion, thorough washing and restriction enzyme digestion (Schwartz and Cantor, 1984). The agarose support matrix not only prevents DNA shearing during handling but also provides enough porosity for the diffusion of molecular reagents such as the reaction buffers and enzymes. DNA prepared in agarose plugs has been used successfully for physical mapping and YAC cloning in many systems (eg. Carle and Olson, 1985; Poustka *et al.*, 1987; Smith and Cantor, 1987; Anand *et al.*, 1989; Ganal *et al.*, 1989; Albertson *et al.*, 1990; Ward and Jen, 1990; Cheung *et al.*, 1991; Martin *et al.*, 1992).

Suppression of recombination has been observed in regions physically close to the centromere, as evidenced by gene clusters lacking physical linkage. This was observed at the *Mi* (Van Daelen *et al.*, 1993) and *Tm-2a* (Ganal *et al.*, 1989) loci in tomato. Since *Yd₂* is genetically close to the centromere (Figure 4.1) it might be expected that the ratio of physical distance to genetic distance could be high. Therefore, it was imperative if we were to proceed with a map-based strategy to clone *Yd₂*, that we obtain an accurate estimate of the relationship between physical and genetic distance. The average relationship between genetic and physical distance in barley based on a genome size of 4800Mb (Arumunganathan and Earl, 1991) and a genetic map length of 1800cM is 2.7Mb/cM (Becker *et al.*, 1995). However, a repression in recombination around the centromere may result in an unfavourable increase in this relationship and make map-based cloning of *Yd₂* a difficult task. This chapter describes work carried out to obtain an estimate of the relationship between genetic and physical distance around the *Yd₂* region.

5.2 Materials and Methods

5.2.1 Acknowledgments

The author is sincerely grateful to both Peter Langridge of Adelaide University and Peter Anderson from Flinders University for the use of the DRII CHEF apparatuses in their laboratories.

5.2.2 Plant materials

Leaf material from the BYDV resistant barley cultivar Atlas 68 (Yd_2 -plus) was used for all high molecular weight DNA preparations. Plant material was grown in the glasshouse as described in Section 2.2.1

5.2.3 DNA probes

The DNA clones chosen for physically mapping were all within 1cM genetic distance from Yd_2 . For information regarding the source of DNA clones used refer to Section 4.2.3

5.2.4 Isolation of high molecular weight DNA

5.2.4.1 Leaf material (adapted from Guidet et al., 1990)

High molecular weight (HMW) DNA was isolated from 0.2g of leaf material of 14 day old seedlings. Leaf material was ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was then transferred to a crucible preheated to 50°C, mixed with 1ml of 0.7% Low Melting Point (LMP) agarose (in 10mM Tris-HCl pH 7.8, 15mM NaCl, 200mM Na₂EDTA) and gently stirred with a spatula. The mixture was poured directly into an opened mould (Biorad's CHEF-DR II mould) cast immediately and allowed to set at 4°C for 15min. The resulting plugs were incubated for 48hrs at 50°C in two changes of lysis buffer (0.5M EDTA pH 8.0, 10mM Tris

pH8.0, 1% sarkosyl, 5mg/ml proteinase K) followed by 6 washes of 0.5M EDTA at 50°C. DNA plugs were stored at 4°C in 0.5M EDTA prior to further manipulations.

5.2.4.2 Protoplasts (adapted from Cheung and Gale 1990)

HMW DNA was isolated from protoplasts obtained from leaf material of 14 day old seedlings. The leaves were removed and surface sterilised for 15min in 10% domestic bleach (Domestos), followed by 4 washes in sterile distilled water. Leaves were then transferred to a petri dish containing 4ml of protoplast buffer (10mM CaCl₂, 1µM CuSO₄, 0.2mM KH₂PO₄, 10µM KI, 1mM KNO₃, 0.7M Mannitol, 2mM MES, 1mM MgSO₄, pH5.8 with KOH), and 2ml of enzyme solution. The enzyme solution contained 0.8% Cellulase Onokzuka R-10 (Yakult, Tokyo, Japan), 0.4% Macerocyme Onokzuka R-10 (Yakult, Tokyo, Japan), and 0.04% Pectoylase Y23 (Seishin, Tokyo, Japan) made to volume in protoplast buffer. Leaf tissue was sliced longitudinally into very fine pieces with a scalpel blade and placed in a vacuum oven for 20min at 700mbar. Petri dishes containing leaf material were then removed and incubated at normal pressure at 25°C in the dark for 4hrs for cell wall digestion. Petri dishes were then transferred to an orbital shaker with gentle shaking (25rpm) for protoplast release, which was monitored by microscopic observation. Tissue was then flushed with 4ml of protoplast buffer for further protoplast release, before being sequentially filtered through 40µm and 20µm nylon sieves and then pelleted at 26×g for 10min. Protoplasts were then washed in protoplast buffer before being pelleted again at 26×g for 10min. Protoplasts were resuspended at a final concentration of 4.5×10⁶ protoplasts ml⁻¹, mixed with an equal volume of low melt agarose solution containing 2% agarose, 0.17M EDTA, in protoplast buffer, at 45°C. The molten mixture was aliquoted into plastic moulds (10mm height × 5mm width × 1.5mm thickness) and allowed to solidify at 4°C for 30min. The blocks were incubated for 48hrs at 50°C in two changes of lysis buffer (0.5M EDTA pH 8.0, 10mM Tris pH8.0, 1% sarkosyl, 2mg/ml proteinase K) followed by 4 washes of 0.5M EDTA at 50°C. The resulting DNA plugs were then stored at 4°C in 0.5M EDTA.

5.2.4.3 Nuclei agarose plugs and microbeads (adapted from Hong-Bin Zhang et al., 1995)

Reagents

(i) Homogenisation buffer (HB) (10×) Stock: 0.1M trizma base, 0.8M KCl, 0.1M EDTA, 10mM spermidine, 10mM spermine, final pH 9.4-9.5 adjusted with NaOH. This stock solution was stored at 4°C.

(ii) HB (1×): 1×HB plus 0.5M sucrose. The resultant 1×HB was stored at 4°C. Immediately prior to use β-mercaptoethanol was added to 0.15%.

(iii) Wash buffer (1×HB plus 0.5% TritonX-200). Stored at 4°C, immediately prior to use β-mercaptoethanol was added to 0.15%.

HMW DNA was isolated from 25g of leaf material up to 4 weeks of age. Leaf material was cut into suitable sized pieces placed in a mortar and pestle and ground to a fine powder in liquid nitrogen. The powder was immediately transferred to a 500ml beaker containing 200ml of ice-cold 1×Homogenisation buffer(1×HB); 0.15% β-mercaptoethanol and 0.5% TritonX-100. The contents were gently swirled using a magnetic stir bar for 10min on ice. The homogenate was filtered through two layers of cheesecloth and one layer of miracloth by squeezing with gloved hands. The homogenate was then pelleted by centrifugation in a swinging bucket centrifuge at 1800×g at 4°C for 20min. The supernatant fluid was discarded and the pellet washed twice in 5ml of ice-cold wash buffer and repelleted at 1800×g at 4°C for 20min. The pelleted nuclei were resuspended in 1ml of 1×HB without β-mercaptoethanol and stored on ice. To determine whether the nuclei were intact, an aliquot was stained with DAPI and the sample scored under an epi-fluorescence microscope. Nuclei were embedded in agarose plugs and lysed using the protocol described for protoplasts (Section 5.2.4.2).

Nuclei to be embedded in agarose microbeads were prewarmed to 45°C for 5min in a oven and mixed with an equal volume of 1% LMP agarose in 1×HB without β-mercaptoethanol. This molten mixture was kept in water bath at 45°C before being poured into a prewarmed 500ml flask. Twenty ml of prewarmed light mineral oil at 45°C was added and the contents of the flask were shaken vigorously for 3sec and poured into an ice-cold 500ml beaker containing 150ml of

ice-cold 1×HB without β-mercaptoethanol, which was then vigorously stirred for 10min on ice as agarose microbeads formed. The beads were harvested by centrifugation at 900×g at 4°C for 20min in a swinging bucket centrifuge. The supernatant was discarded and the pelleted microbeads resuspended in 5-10 volumes of lysis buffer. Microbeads were incubated for 48hrs at 50°C in two changes of lysis buffer (0.5M EDTA pH 8.0, 10mM Tris pH8.0, 1% sarkosyl, 2mg/ml proteinase K) followed by 4 washes of 0.5M EDTA at 50°C. Microbeads were then stored at 4°C in 0.5M EDTA.

5.2.5 Restriction enzyme digestion of HMW DNA

Before restriction enzyme digestion agarose plugs and beads containing HMW DNA were washed 6 times in 10-20 volumes of ice-cold TE (10mM Tris-HCl, pH8.0, 1mM EDTA, pH8.0). The first wash for 2hrs, the second wash overnight, the following 4 washes each for a duration of 15min. The plugs or beads were then ready for restriction enzyme digestion. Fifty microliters of beads were used in each reaction. Agarose plugs and beads were incubated in 500µl of restriction digestion buffer at 4°C, in the presence of 8mM spermidine and 2mg/ml of BSA for 2hrs. Restriction enzyme digestions were carried out in 500µl of the same buffer with fresh reaction mix and 20 units of enzyme at temperatures recommended by the manufacturers, between 4 to 12hrs. Restriction enzymes used for restriction of HMW DNA were *AatII*, *BamHI*, *BssHIII*, *ClaI*, *DraI*, *EagI*, *EcoRI*, *EcoRV*, *FspI*, *HindIII*, *NdeI*, *NcoI*, *NotI*, *NruI*, *PvuI*, *PvuII*, *SacI*, *SacII*, *SalI*, *SmaI*, *SnaBI*, *XhoI*. After digestion, the activity of the restriction endonuclease in each sample was stopped by incubation in 0.5M EDTA. The agarose blocks and microbeads were then ready for electrophoresis and were stored at 4°C.

5.2.6 Pulsed field gel electrophoresis (PFGE) separation, transfer and hybridisation

The digested HMW DNA was loaded onto a 0.5×TBE 1% agarose gel and sealed in position using the same agarose as the gel. Pulsed-field gel electrophoresis was performed using a CHEF DRII (Bio-Rad, USA) run at 14°C and 200 volts. Pulse and run times used to separate HMW DNA are presented in Table 5.1. The DNA was stained with ethidium bromide, photographed,

Table 5.1 Electrophoretic parameters used to resolve restricted HMW DNA

Switch Interval	Run Time	Gel	Voltage	Resolution window
30min	72hrs	0.6%, 0.5×TBE	50V	2.2Mb - 4.6Mb
60-90sec	24hrs 22hrs	1.0%, 0.5×TBE	200V	220Kb - 2.2Mb
15-50sec	24hrs	1.0%, 0.5×TBE	200V	24kb - 610kb
1-12sec	14hrs	1.0%, 0.5×TBE	200V	4.36kb - 145kb

and nicked using 60 mJoules of U.V. light in the GS gene linker (Bio-Rad, USA). The DNA was blotted onto Hybond N⁺ membrane (Amersham, USA) in 0.4M NaOH instead of 20×SSC as described in Section 2.2.10. ³²P labelled probe preparation and hybridisation conditions were as described in Sections 2.2.11 and 2.2.12 respectively. Kodak double emulsion BIOMAX MS film and a BIOMAX MS intensifying screen were used for PFGE autoradiography. Filters were exposed from 24-72hrs at -80°C.

5.3 Results

5.3.1 Isolation and restriction enzyme digestion of high molecular weight DNA

Four different methods for the preparation of HMW DNA were tested to analyse the quantity and quality of HMW DNA prepared so as to determine which method was most suitable for physical mapping. The methods chosen were the embedding of leaf material, protoplasts and nuclei in agarose plugs and nuclei embedded in agarose microbeads.

To determine the intactness of nuclei isolated, an aliquot of nuclei from the homogenate was stained with DAPI, observed and counted under an epi-fluorescence microscope. A suitable amount of intact nuclei (> 95%) were present in the sample. This technique was then deemed suitable for the production of intact nuclei, which could then be embedded in agarose for HMW DNA preparation and subsequent pulsed field gel electrophoresis to test the quantity and quality of the DNA. Protoplast release and integrity was also monitored by microscopic observation using an Olympus compound inverted microscope (CK2). A 10µl aliquot was taken after each hour of enzymatic digestion for 12 hours after initial digestion, and protoplast release and integrity checked by microscopic observation. Enzymatic digestion of cell walls for greater than 4 hours resulted in a decrease in the percentage of intact protoplasts. Therefore, to increase protoplast release without protoplast lysis, tissue subjected to enzymatic treatment was flushed with 4ml of protoplast buffer for increased protoplast release and increased yield.

Uncut HMW DNA produced by all 4 methods was analysed by PFGE using *Saccharomyces cerevisiae* and *Scizosaccharomyces pombe* chromosomes to resolve DNA fragments ranging from 220 to 2.2Mb and 2.2Mb to 4.6 Mb in size. After electrophoresis, the gel was stained with ethidium bromide and de-stained in water overnight. DNA prepared by nuclei and protoplasts embedded in agarose plugs or beads showed a light smear of DNA in the region of the gel between 220kb and 2Mb (Figure 5.1). However, the majority of the DNA was retained in the wells or compression zone. These results indicated that for protoplasts and nuclei the majority of the DNA prepared was larger than 4.6Mb in size. While a greater quantity of DNA was produced by embedding leaf material in agarose plugs, a large amount DNA of lower molecular weight was present in the region of the gel from 220kb to 2.2Mb (Figure 5.1). Therefore, a high proportion of the DNA produced by this method was not a suitable size for physical mapping purposes (Figure 5.1). While DNA quality (size) for nuclei and protoplasts was similar, the quantity of DNA produced by nuclei embedded in agarose plugs was greater than that of either protoplasts embedded in agarose plugs or nuclei embedded in microbeads. Importantly, a substantial proportion of microbeads prepared were lost during the lysis and wash steps thereby, reducing the efficiency of this method.

In order to determine the quality of the HMW DNA in terms of restriction enzyme digestion for physical mapping, barley DNA embedded in agarose plugs prepared by the 3 different methods (leaf, protoplasts and nuclei) and barley nuclei embedded in microbeads were digested with eight different restriction enzymes, *EcoRI*, *EcoRV*, *NcoI*, *NdeI*, *DraI*, *HindIII*, *BamHI*, *XbaI*. The restricted DNA was run on a pulsed field gel, a Southern transfer performed and the membrane hybridised sequentially with 2 probes, WG889 and BCD1380. The DNA probe WG889, a mutli loci probe had been mapped as an RFLP within 1cM of *Yd₂* in this laboratory (Paltridge, 1998). The DNA probe BCD1380 a strongly hybridising multiloci probe had also been mapped as an RFLP in the vicinity of *Yd₂* by Nelson *et al.* 1995b. DNA produced from both nuclei (Figure 5.2) and protoplasts was sensitive to restriction enzyme digestion. Hybridisation with the two DNA probes showed that DNA had been restricted into discrete bands and was not degraded by the restriction digestion conditions. In contrast, while DNA produced from embedding leaf material was sensitive to restriction enzyme digestion, subsequent hybridisation to the DNA probes by Southern analysis showed the DNA had been degraded under restriction enzyme conditions.

Figure 5.1 Analysis of high molecular weight (HMW) DNA prepared from nuclei, protoplasts and intact leaf material

Pulsed field gel electrophoresis (PFGE) was used to analyze HMW DNA produced from nuclei, protoplasts and leaf material. *Saccharomyces cerevisiae* (M1) and *Schizosaccharomyces pombe* (M2) chromosomes were used as high molecular weight DNA size markers. Two PFGE conditions were used to resolve DNA fragments ranging from 220kb to 2.2Mb and from 2.2Mb to 4.6Mb. After electrophoresis, gels were stained with ethidium bromide and destained in water overnight and photographed.

(a) & (b) Uncut DNA prepared from nuclei (N1), protoplasts (P1) and leaf material (L1, L2, L3) embedded in agarose plugs were run on a 1% agarose gel for 24 hours at 200 volts in 0.5×TBE. A linear ramp from 60-90 seconds was used to resolve DNA between 240kb and 2.2Mb in size. The majority of DNA was of HMW and did not migrate out of either the wells (w) or the compression zone (cz). A greater quantity of DNA was present in both nuclei and leaf tissue preparations, then in the protoplast sample. A light smear of sheared lower molecular weight DNA is visible in both DNA samples prepared from protoplasts and nuclei. Large amounts of sheared lower molecular weight DNA is visible in the leaf material preparations, indicating that the HMW DNA prepared from nuclei and protoplasts is of better quality than that prepared from leaf material.

(c) Uncut DNA prepared from nuclei (N1) and protoplasts (P1) as well as DNA prepared from nuclei digested with *PvuI* (ND1) and *NotI* (ND2) were run on a 0.6% agarose pulsed field gel (PFG) for 72 hours at 50 volts in 0.5×TBE. The switch time was 30 minutes. The majority of uncut DNA prepared from both nuclei and protoplasts was of high molecular weight and above 4.6Mb in size (in the wells and compression zone). A lighter smear of sheared DNA of smaller molecular weight is also visible. All DNA prepared from nuclei digested with the 6 base cutter *PvuI* (ND1) was less than 2.2Mb in size. As expected, a slight smear of DNA greater than 2.2Mb in size was generated by digestion with *NotI* (ND2) an 8-base cutter. While all three methods produced DNA of high molecular weight, nuclei were found to give consistently sufficient quantity and better quality HMW DNA than that prepared from protoplasts and leaf material.

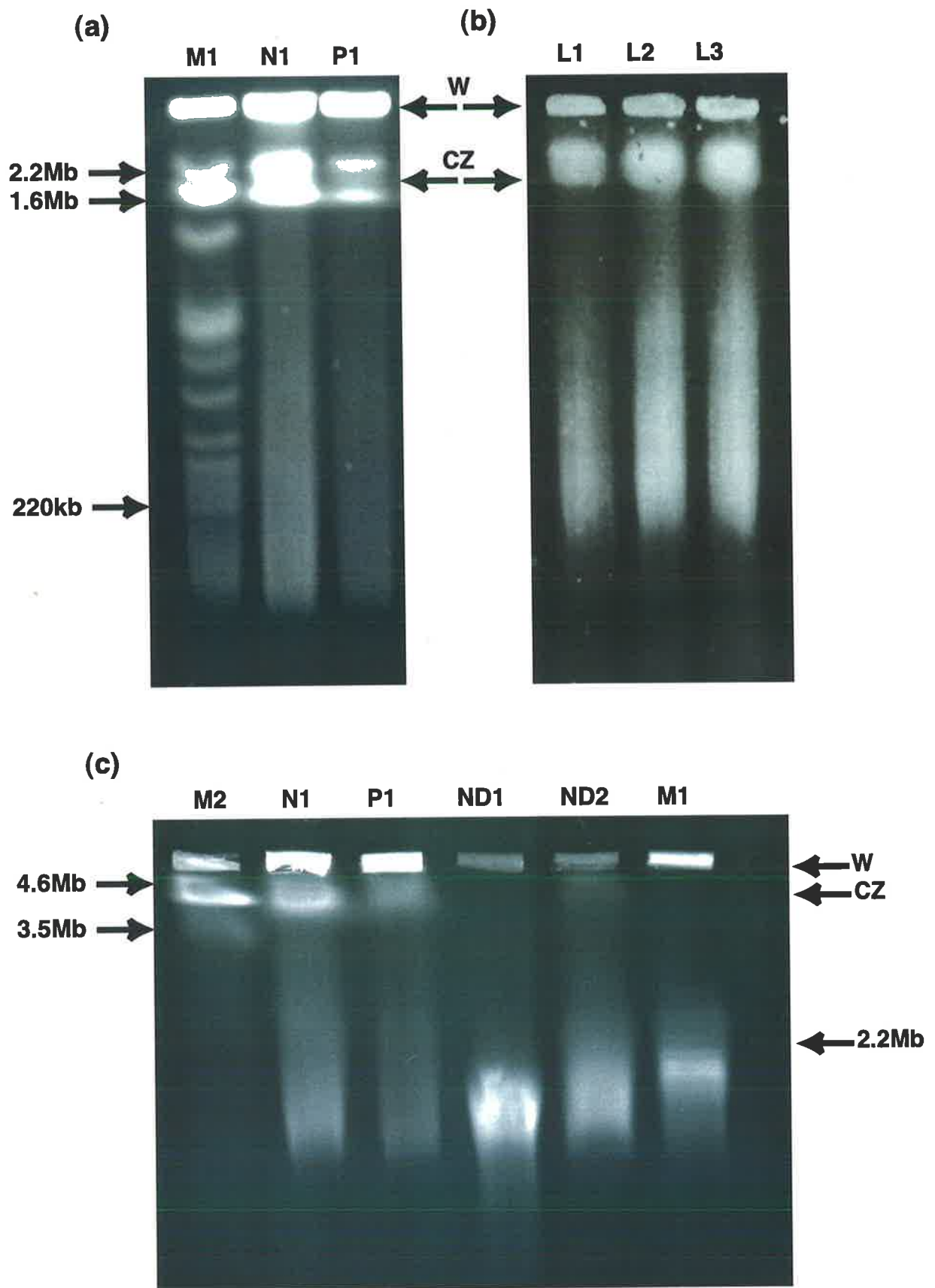
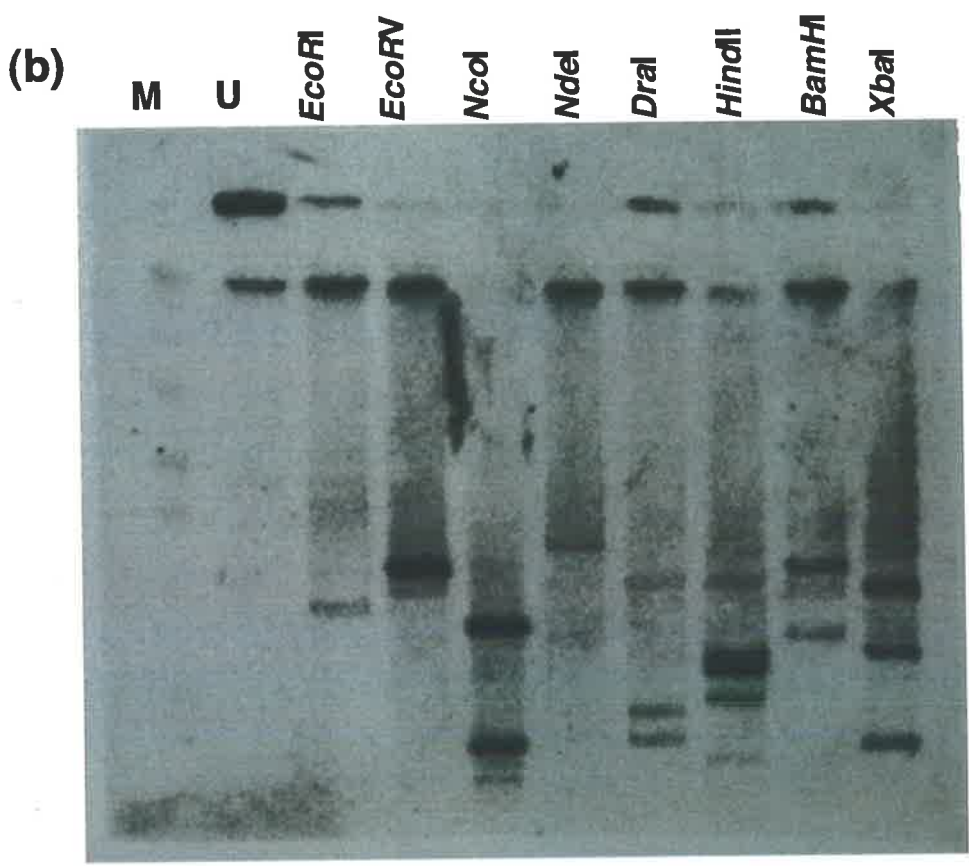
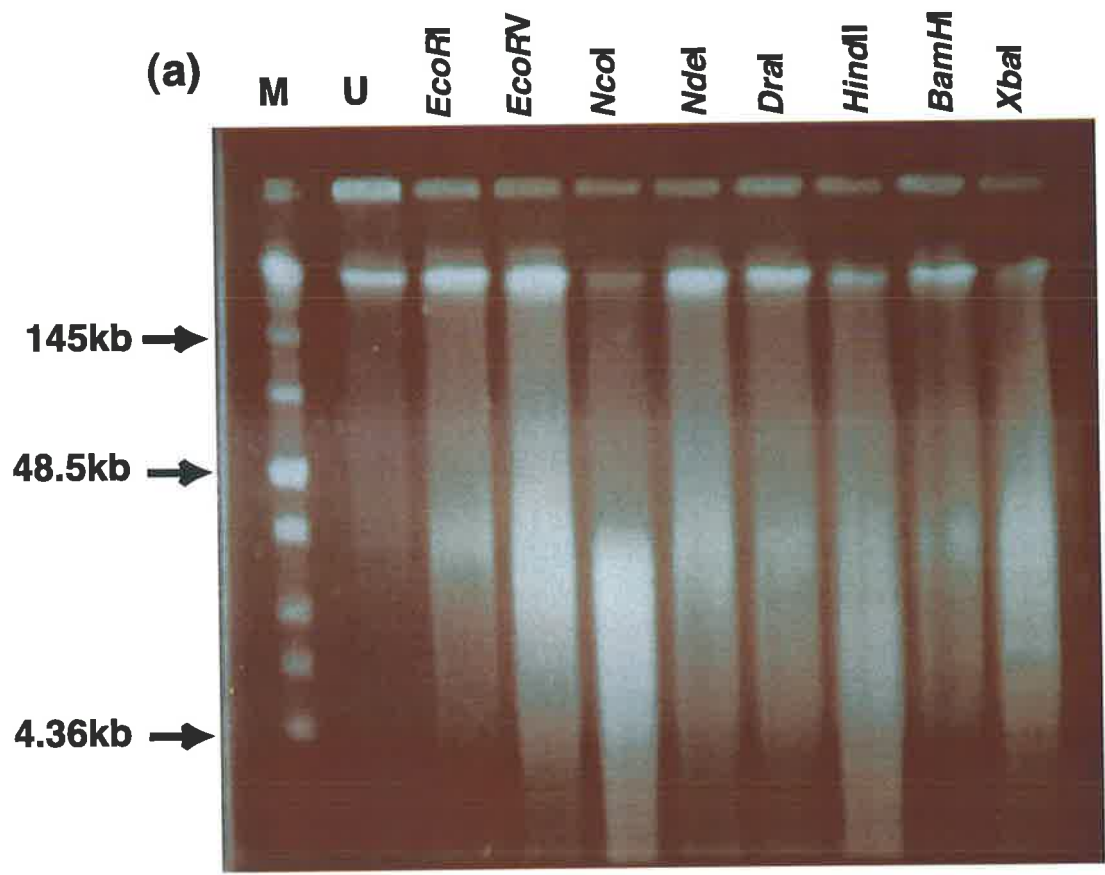


Figure 5.2 Restriction enzyme digestion and Southern hybridisation of HMW DNA

(a) To determine the quality and the suitability of the HMW DNA for physical mapping eight restriction enzyme digestions (*EcoRI*, *EcoRV*, *NcoI*, *NdeI*, *DraI*, *HindIII*, *BamHI*, *XbaI*) were performed on HMW DNA prepared from barley nuclei. Samples were run in a 1% agarose gel for 14 hours at 200 volts. A linear ramp of 1-12 seconds was used to resolve DNA between 4kb and 145kb in size. All DNA was found to be sensitive to restriction enzyme digestion. An undigested control (U) and low range lambda concatemer ladder (M) were included on the gel.

(b) Alkaline transfer was performed on the PFG and the resulting membrane hybridised with WG889, a DNA probe closely linked *Yd₂*. The DNA was restricted into discrete bands and not degraded by the restriction digestion conditions used. Therefore, it was concluded that DNA produced from nuclei was suitable for physical mapping.



Although HMW DNA prepared from protoplasts embedded in agarose plugs and nuclei embedded in microbeads produced DNA of adequate quality and quantity, nuclei embedded in agarose plugs consistently gave higher quantities and better quality in comparison to the other techniques. Therefore, embedding of nuclei in agarose plugs was chosen for the preparation of HMW DNA for physical mapping of the *Yd₂* region.

5.3.2 Identification of probes suitable for physical mapping of the *Yd₂* region

Eleven DNA probes which mapped within 1cM of *Yd₂* were tested for their suitability for use in physical mapping studies of the *Yd₂* region (Table 5.2). Seven rare cutting restriction enzymes (*BssHII*, *ClaI*, *NotI*, *NruI*, *PvuI*, *SalI*, *SmaI*) were initially tested to determine if HMW restriction fragments could easily be detected by hybridisation to these DNA probes. If so, these probes would be useful for physical mapping studies. Of the 11 probes tested only 6 gave signals easily detected by Southern hybridisation of pulsed field gels. The other 5 DNA probes produced hybridisation signals with heavy background and no discrete restriction fragments were detected. Therefore, these 5 probes were excluded from the study and physical mapping studies were undertaken using the 6 DNA probes which produced good hybridisation signals.

5.3.3 PFGE DNA restriction fragment sizes generated by different restriction enzymes as detected by DNA probes genetically close to the *Yd₂* locus

Twenty two restriction enzymes were tested for physical mapping of the *Yd₂* region. Of these, 8 (*EcoRI*, *EcoRV*, *NcoI*, *NdeI*, *DraI*, *HindIII*, *BamHI*, *XbaI*) generated DNA fragments of less than 20kb in size which were detected by Southern hybridisation to the 6 probes shown to be suitable for physical mapping (Section 5.3.2). Four restriction enzymes *AatII*, *EagI*, *FspI*, and *SacI* produced poor hybridisation signals with all 6 probes. The remaining ten restriction enzymes generated DNA fragments greater than 100kb as detected by Southern analysis. The actual sizes of DNA fragments detected by the six probes closely linked to *Yd₂* are summarised in Table 5.3. With the single copy probes, most restricted enzyme digests produced a single major band. However, some single copy probes recognized multiple bands of equal intensities. Multiple copy

Table 5.2 DNA probes suitable for physical mapping studies

DNA Probe	Suitability for Physical Mapping
AWPH7D	Unsuitable
BCD134	Suitable
KSUA3	Unsuitable
KSUH7	Unsuitable
MWG802	Suitable
MWG952	Suitable
MWG2065	Unsuitable
YLM	Suitable
Ylp	Suitable
RGC122	Unsuitable
WG889	Suitable

Table 5.3 Summary of fragment sizes (kb) detected by DNA probes which map close to Yd_2 after restriction digestion and PFGE

Restriction enzyme	BCD134	MWG802	MWG952	YLM	Ylp	WG889
<i>Bss</i> III	N.B.	610	450, 300, 190, 180	48	450	560,432, 360, 288
<i>Cla</i> I	N.B.	N.B.	120	155	450	144,CZ.
<i>Not</i> I	C.Z.	N.B.	N.B.	C.Z.	C.Z.	810, 560,360
<i>Nru</i> I	360	N.B.	165, 100, 90	150	N.B.	500, 360
<i>Pvu</i> I	180*	440	200	180*	180*	450
<i>Pvu</i> II	N.B.	N.B.	N.B.	140	N.B.	N.B.
<i>Sac</i> II	N.B.	N.B.	N.B.	180	500	N.B.
<i>Sal</i> I	N.B.	N.B.	210	100	450	360
<i>Sma</i> I	300	N.B.	460	700	C.Z.	48
<i>Snab</i> I	C.Z.	N.B.	120	155	C.Z.	290,300

C.Z: A very strong signal in the compression zone was evident, with little background in the lane, suggesting that a HMW fragment too large to be resolved by the experimental conditions used remained in the compression zone.

N.B: No discrete band was detected but a smear throughout the entire lane was visible.

* Fragments of the same size which co-migrated on the PFG indicating a putative physical linkage are highlighted in bold.

probes such as WG889 and MWG952 produced a variation in band number and intensity with different digests. These differences were thought to be due to partial cleavages caused by methylation at CpG nucleotides.

NotI is a methylation-sensitive enzyme containing CpGs and CpXpGs in its eight base pair recognition site. This enzyme usually creates large restriction fragments in plant species (Ganal *et al.*, 1989; Ganal *et al.*, 1991). This was also found to be the case in the region genetically close to *Yd₂* where the largest resolvable fragment was 810kb and the smallest 360kb. However, three *NotI* restriction fragments did not migrate out of the compression zone of the gel and were unresolvable by the PFG conditions used for this study. Therefore, they were assumed to be greater than 2Mb in size. The other nine restriction enzymes digested the HMW DNA into a variety of sizes that were either resolved using PFGE or did not migrate out of the compression zone or well. A number of restriction enzyme × probe combinations resulted in poor hybridisation patterns with background smearing throughout the entire gel lane (Table 5.3). This was probably due to variation in the methylation pattern in a particular region of the genome, resulting in multiple restriction fragment sizes and a smeared appearance of the hybridisation signal.

5.3.4 The physical linkage of genetically close markers

Restriction digestion analysis of HMW barley DNA revealed a putative physical linkage between genetically close molecular markers from the *Yd₂* region. Three DNA probes YLM, Ylp and BCD134 were shown to hybridise to the same 180kb *PvuI* restriction fragment (Table 5.3). However, such co-migration may be the result of two restriction fragments of similar size migrating to the same point on the gel, 'a chance co-migration', resulting in the appearance that both DNA markers were hybridising to the same DNA fragment and physically linked. Thereby, a physical linkage may be inferred with a particular restriction enzyme when it does not exist, a real danger to physical mapping exercises using PFGE. These putative physical linkages were further investigated using a series of different pulsed field gel electrophoresis conditions in an attempt to separate HMW DNA which may be of a similar size but not physically linked (Table 5.4).

Table 5.4 Switch and run times used to resolve the 180kb *PvuI* restriction digest fragment identified by hybridisation of the DNA probes BCD134 and YLP

Switch Interval	Run Time
1-12sec	15hrs
2-50sec	20hrs
5-60sec	20hrs
15-50sec	23hrs 24hrs
60-90sec	20hrs 24hrs 26hrs

To check for co-migration, Southern membranes made from PFGs were hybridised sequentially with DNA probes thought to be physically linked. Between each hybridisation, membranes were stripped and subjected to autoradiography to ensure the previous probe had been completely removed. Autoradiographs were overlaid and aligned using the radioactive marker, radioactive dye dots on the side of the membrane and membrane background. Restriction fragments could then be checked for co-migration (Figure 5.3). Furthermore, markers were hybridised simultaneously to look for close doublet or triplet bands, since overlaying autoradiographs may not pick up very slight differences in fragment sizes and chance co-migration could be misinterpreted as physical linkage (Figure 5.4). Importantly, the two genetically distinct molecular markers BCD134 and Ylp were not resolved from each other, therefore, indicating physical linkage. However, the DNA marker YLM, was shown to be of slightly lower molecular weight and not physically linked to the same 180kb *PvuI* restriction fragment as BCD134 and Ylp, and was a chance co-migrator.

A series of restriction digests (*EcoRI*, *EcoRV*, *MluI*, *NotI*, *SalI*, *SmaI*, *SnaBI*) were performed on HMW DNA already restricted with *PvuI* to develop a restriction map of the 180kb PFG restriction fragment identified by the hybridisation of the two DNA probes Ylp and BCD134 (Table 5.5). With the exception of *NotI* and *SnaBI* all restriction enzymes used cut the HMW fragment. *EcoRI* and *EcoRV* restricted the fragment multiple times, *MluI*, *SalI* and *SmaI* only cut the fragment once. Hybridisation of Ylp and BCD134, to the restriction fragments generated by the double digestions showed their cumulative molecular weights were never greater than 180kb (Table 5.5). Single restriction enzyme digests of HMW DNA with *NotI* or *SnaBI* generated a HMW restriction fragment which did not move out of the compression zone as identified by hybridisation of both Ylp and BCD134. Therefore, the double digestions with *NotI* and *SnaBI* (Figure 5.5) after an initial *PvuI* digest provide two other restriction enzymes physically linking these DNA probes. This data, with restriction enzyme mapping of this 180kb fragment, provides strong evidence that a single 180kb *PvuI* restriction fragment encompasses both the *Ylp* and *Xbcd134* loci, and is not merely a 'chance co-migration'. The two loci *Ylp* and *Xbcd134* were separated on the Proctor × Shannon Map by 0.5cM and here are shown to be a maximum distance of 180kb apart. Therefore, a relationship of 1cM=360kb can be estimated for this region of the genome.

Figure 5.3 The co-hybridisation of two genetically distinct markers closely linked with Yd_2 , to a single restriction enzyme fragment on a pulsed field gel

(a) HMW DNA prepared from nuclei was digested with the restriction enzyme *PvuI* and subjected to PFGE. Restricted DNA was run at 200 volts for 24 hours. A linear ramp of 15-50 seconds was used to resolve DNA from 6.55kb to 533.5kb in size. Low range (M1) and long range (M2) lambda concatemer ladders as well chromosomes of a rice yeast artificial chromosome (YAC) clone (Y) positive for one of the markers were used to help size the DNA. Undigested HMW DNA (U) was also included as a control. An alkaline transfer was performed on the PFG and the resulting membrane used for Southern blot hybridisation analysis.

(b) A Southern hybridisation of the membrane described above with the probe BCD134 was performed and the membrane exposed to highly sensitive film at -80°C for 48 hours. The genetic loci *Xbcd134* identified by the probe BCD134 is separated from Yd_2 by 0.5cM in genetic distance. Radiolabelled lambda DNA was used to visualise the concatemer ladder. BCD134 hybridised strongly to a restriction fragment of approximately 180Kb in size, which was not present in the undigested control. BCD134 did not hybridise to the rice YAC. The membrane was washed for 10 minutes in 0.4M NaOH and then stripped 3 times with boiling stripping solution (2mMEDTA, 0.1% SDS) and re-exposed to highly sensitive film for 72 hours to check the probe had been successfully removed. The membrane was then used for re-hybridisation.

(c) A subsequent hybridisation to the same pulsed field membrane was made with the DNA probe *Ylp*, which maps to the *Ylp* loci that co-segregates with Yd_2 . The probe hybridised strongly to the rice YAC as well as to a restriction fragment of approximately 180Kb. The autoradiographs were overlaid using the concatemer ladders and autorad background to correctly align them, both restriction fragments were found to hybridise to the same point. Therefore suggesting physical linkage of both genetically distinct markers *Xbcd134* and *Ylp* to the single *PvuI* restriction fragment.

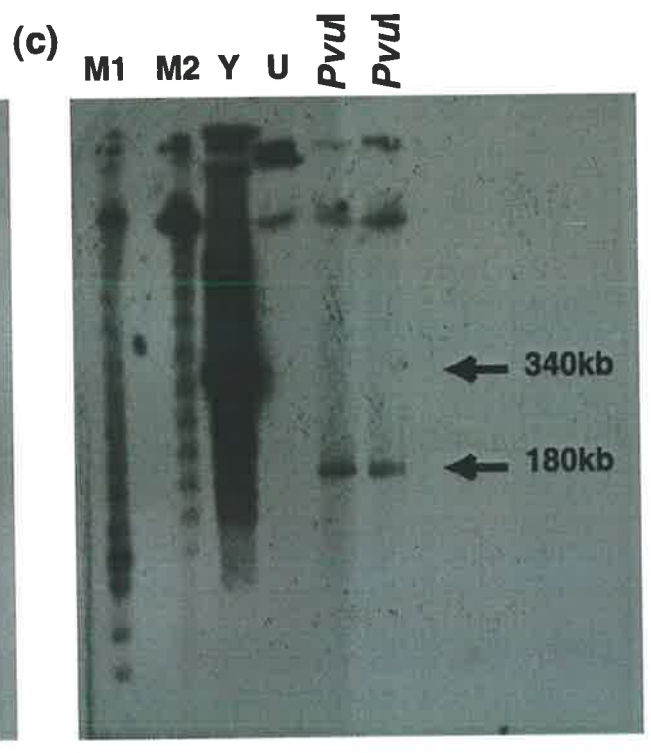
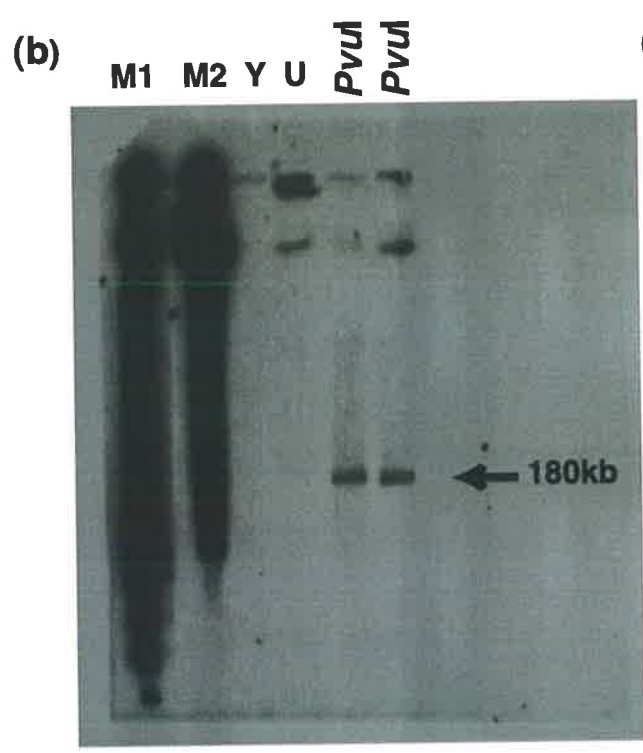
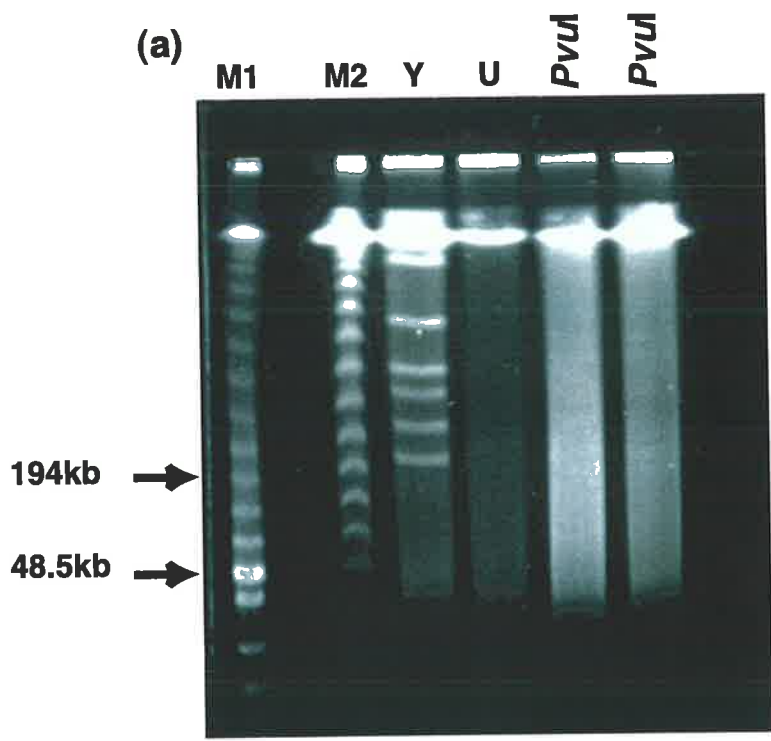


Figure 5.4 The physical linkage of the genetically distinct markers *Xbcd134* and *Ylp*, two markers closely linked to *Yd₂*

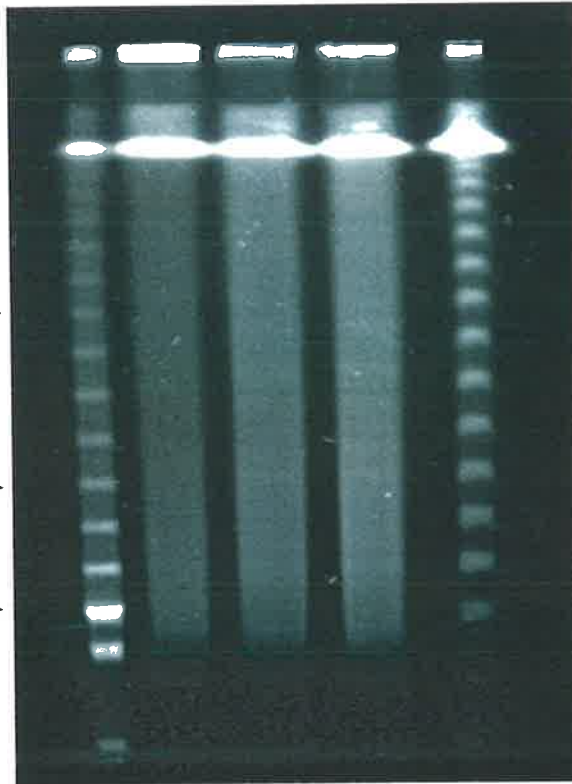
(a) High molecular weight DNA prepared from nuclei was digested with the restriction enzyme *PvuI* and subjected to PFGE. Restricted DNA was run at 200 volts for 23 hours. A linear ramp of 15-50 seconds was used to resolve DNA from 6.55kb to 533.5kb in size. Low range (M1) and long range (M2) lambda concatemer ladders were included to size the restricted DNA. A HMW undigested control (U) was also included. After alkaline transfer the membrane was then subjected to Southern hybridisation and exposed to highly sensitive film at -80°C for 48 hours. Oligolabelled lambda DNA (Section 2.2.11) was hybridised to identify the concatemer ladder.

(b) DNA probes BCD134 and *Ylp* were hybridised to the same membrane simultaneously to exclude the possibility that both probes co-migrated to different restriction fragments of similar size(chance co-migration). If two fragments did co-migrate then a doublet band would be expected. However, an intense single hybridisation band was detected providing strong evidence that *Xbcd134* and *Ylp* are a maximum physical distance of 180kb apart. These two loci are separated by 0.5cM in genetic distance therefore, giving us an estimate of the ratio of physical to genetic distance of 1cM being equivalent to 360kb for this region of the genome.

(a)

M1 U *PvuI* *PvuI* M2

388kb →
194kb →
48.5kb →



(b)

M1 U *PvuI* *PvuI* M2

← 194kb
← 48.5kb

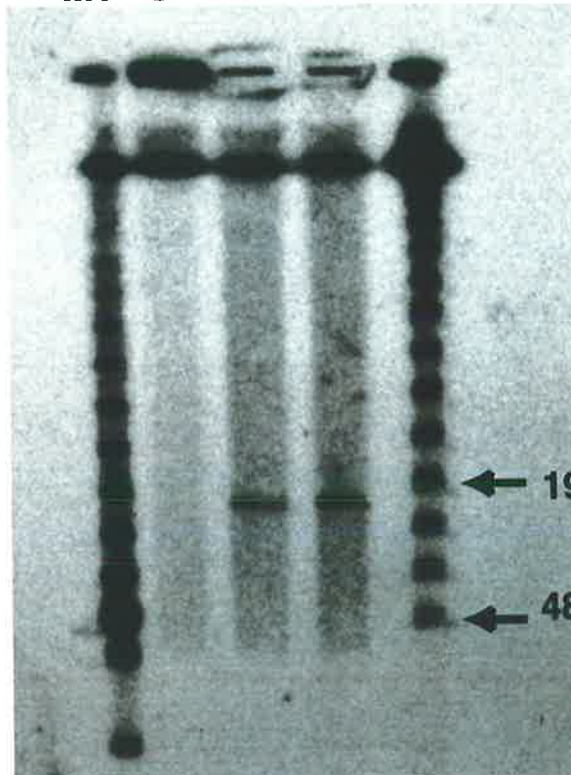


Table 5.5 Restriction enzyme mapping of the 180Kb *PvuI* restriction fragment identified by hybridisation of the two DNA probes BCD134 and YLP

Restriction enzyme	Fragment size identified by BCD134	Fragment size identified by YLP	Cumulative fragment size
<i>EcoRI</i>	6.5kb	9kb, 23kb	38kb
<i>EcoRV</i>	4kb	9kb	13kb
<i>MluI</i>	23kb	157kb	180kb
<i>NotI</i>	180kb#	180kb#	180kb
<i>SalI</i>	130kb	50kb	180kb
<i>SmaI</i>	10kb	170kb	180kb
<i>SnabI</i>	180kb#	180kb#	180kb

* All restriction digests were performed on HMW DNA already digested with *PvuI*.

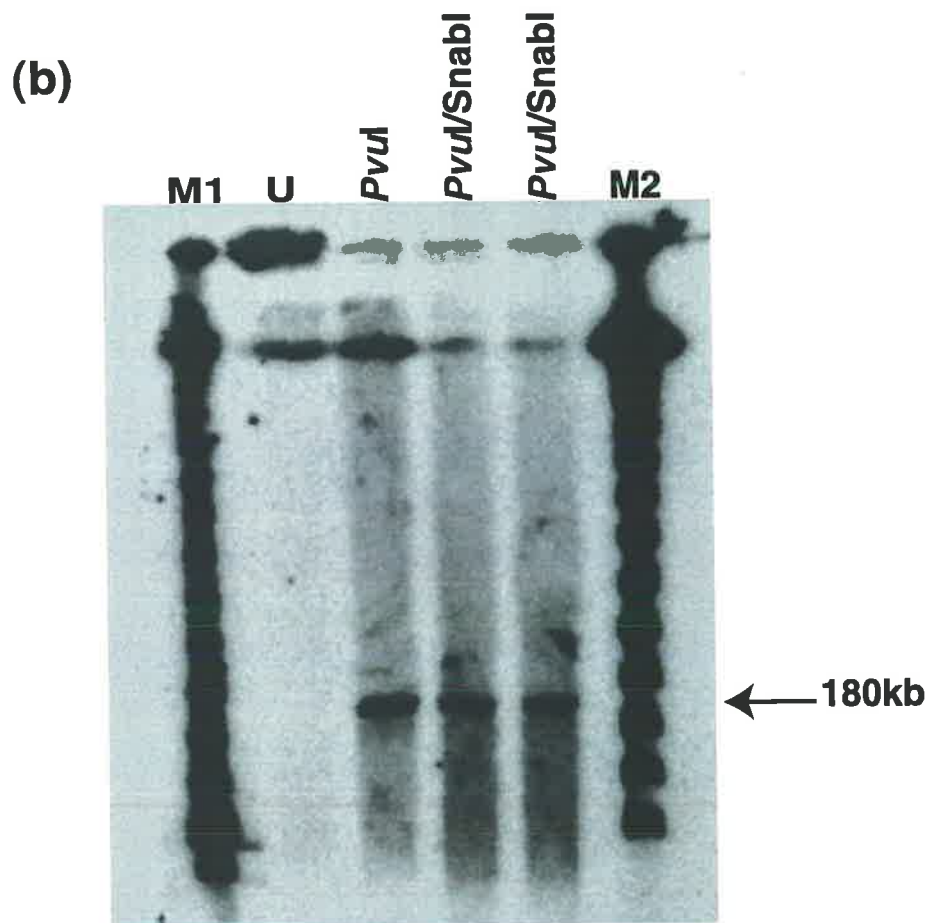
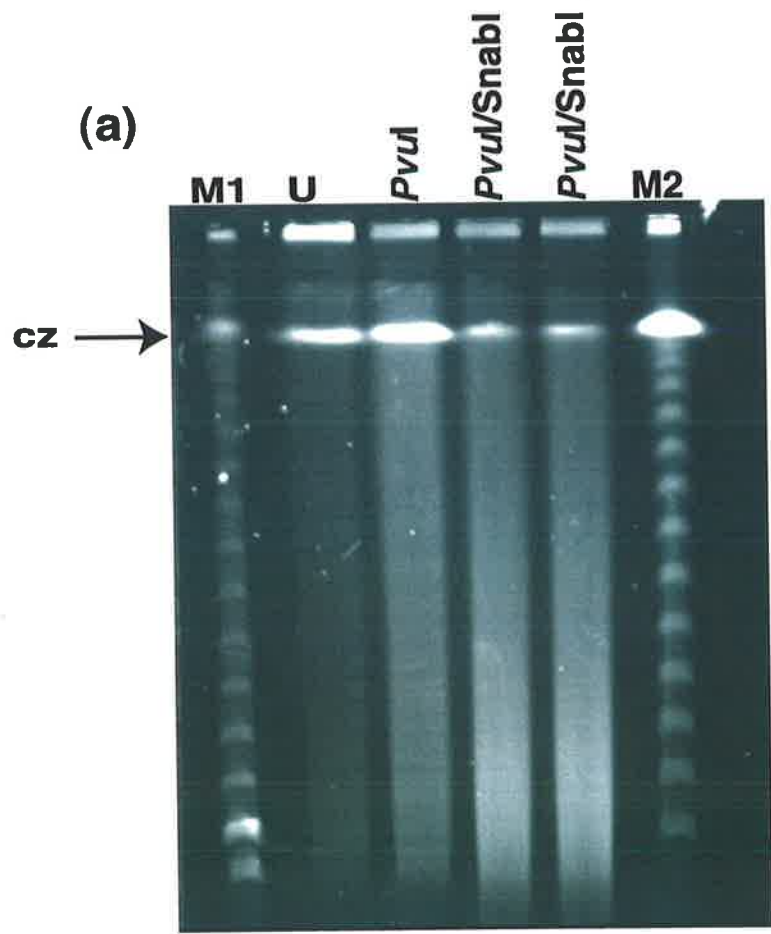
** Two sets of switch and run times were used to resolve the restricted DNA (a) 1-12sec for 15hrs; (b) 15-50sec for 24 hrs.

These restriction enzymes did not appear to restrict the 180kb *PvuI* restriction fragment.

Figure 5.5 The physical linkage of two genetically distinct markers *Xbcd134* and *Ylp* as shown with a second restriction enzyme *SnaBI*

(a) A PFG was run to resolve HMW DNA digested firstly with *PvuI* and then with *SnaBI*. DNA was run at 200 volts for 24 hours. A linear ramp from 15-50 seconds was used to resolve DNA between 24kb and 436.5kb in size. Low range (M1) and long range (M2) lambda concatemer ladders were included to size DNA. Undigested (U) and *PvuI* single digest controls were included in the gel. Double digests used both the restriction enzymes *PvuI* and *SnaBI*. Considerably more DNA was digested in the double digests as evident by less DNA in the compression zone (cz) and also more DNA at lower molecular weight than in the single *PvuI* digest. This indicated the DNA was sensitive to restriction digestion with *SnaBI* after *PvuI* digestion.

(b) After alkaline transfer the membrane was then subjected to Southern hybridisation and exposed to highly sensitive film at -80°C for 24 hours. Both BCD134 and *Ylp* were probed simultaneously. Oligolabelled lambda DNA was also hybridised to identify the concatemer ladder. No difference in band migration between the double digests were observed in comparison to the single *PvuI* digest. Therefore, *SnaBI* provides a second restriction enzyme physically linking *Xbcd134* and *Ylp*. This provides strong evidence that genetic loci *Xbcd134* and *Ylp* are a maximum physical distance of 180kb apart and are not two different fragments of similar size co-migrating to the same spot on the gel.



While a favourable physical linkage between *Xbcd134* and *Ylp* was identified, no physical linkages were found between any of the other loci identified with DNA probes determined to be suitable for physical mapping. Importantly, no physical linkage was detected between the two loci flanking *Yd₂* in the Atlas × Atlas68 mapping population, *YLM* and *Xmwg952*. However, from restriction enzyme data produced by the single restriction digest *SmaI* (Table 5.3) and the double digest data of *PvuI* with *SmaI* and *SalI* (Table 5.5), a minimum distance can be calculated between both these flanking markers and the *Ylp* loci (Figure 5.6). *YLM* can be calculated to be a minimum of 1860kb from *Ylp* based on the assumption that the *SmaI* fragment detected in the compression zone by the *Ylp* probe is a minimum size of 2Mb and the *Ylp* locus is a maximum of 140kb from the end of this restriction fragment flanked by the locus *Xbcd134* as determined by the double digestion data (Table 5.5) (Figure 5.6).

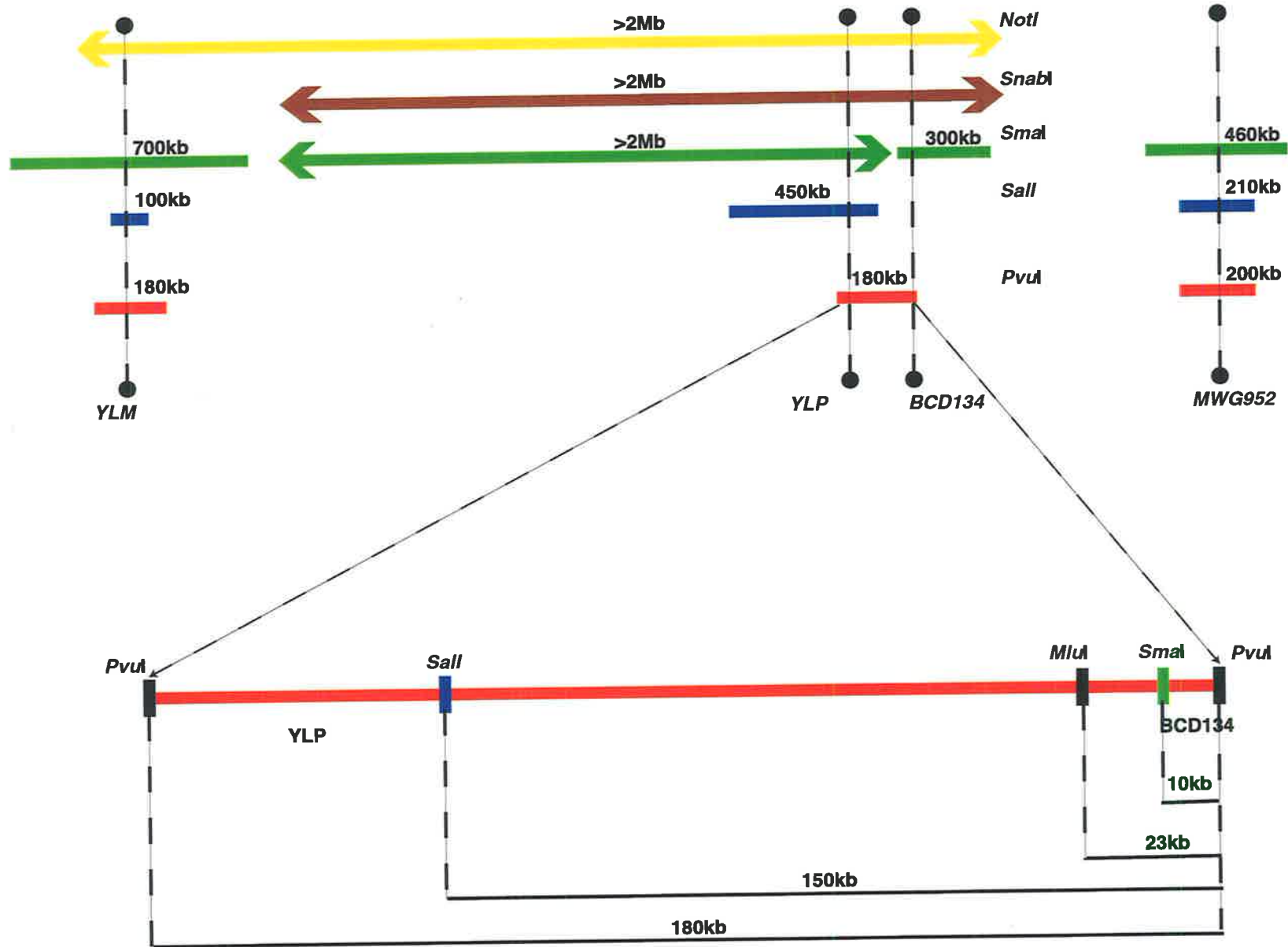
Based on the identification of a 300kb *SmaI* restriction fragment identified by BCD134 which is not physically linked to either the 460kb *SmaI* fragment detected by MWG952 or the *SmaI* fragment in the compression zone detected by *Ylp*, and because *Ylp* was shown to be a maximum distance of 140kb from the end of the *SmaI* fragment in the compression zone identified by *Ylp* and flanked by *Xbcd134* as determined by the double digestion of HMW barley DNA using *PvuI* and *SmaI* (Table 5.5) (Figure 5.6). *Xmwg952* can be calculated to be a minimum of 460kb from *Ylp*.

5.4 Discussion

Map-based cloning in complex plant genomes generally involves genetic fine mapping and DNA marker identification in the target interval to delimit the physical size of the chromosome segment in which the target gene resides (Lahaye *et al.*, 1998). Therefore, construction of a high resolution genetic map at the respective locus is a prerequisite for the isolation of a gene based only on its map position (Tanksley *et al.*, 1995). Chapter 4 described RFLP mapping studies of the *Yd₂* locus, placing a total of 29 molecular markers on two genetic maps, with 12 of these markers within 1cM of *Yd₂*. Calculations based on the total genetic map and genome size of barley, together with the number of meiotic products in our mapping populations suggested that the likelihood of identifying a large genomic clone with one of our co-segregating markers,

Figure 5.6 A long range restriction enzyme map of the Yd_2 region of barley Chromosome 3

Pulsed field gel electrophoresis restriction mapping data of the Yd_2 region was used to construct a preliminary physical map (opposite) surrounding the immediate area of the Yd_2 gene and flanking markers. Fragments cut by the restriction enzymes *PvuI*, *NotI*, *SalI*, *SmaI*, and *SnaBI* are represented by red, yellow, blue, green, and brown solid horizontal lines respectively. Solid lines without arrows represent defined physical sizes, with their size denoted above each solid line, while those with arrows represent undefined physical sizes with a minimum size limit denoted directly above each solid line. The approximate position of molecular markers on these restriction fragments is denoted by dashed lines proceeding from each individual marker.



which also encompassed Yd_2 , was poor (Section 4.4.2). However, these calculations are based on genome-wide estimates of genetic and physical relationships, and locus-dependant deviations from these estimates have frequently been reported (Section 5.1). Therefore, it was essential to obtain an accurate measurement of the relationship between genetic and physical distance for the region of Yd_2 before proceeding with a map-based cloning strategy.

5.4.1 Requirements for physical mapping

The technique of PFGE was used to establish the relationship between genetic and physical distance for the Yd_2 region. There are three basic requirements for the analysis of plant genomes by PFGE:

- (1) a convenient method to prepare HMW DNA;
- (2) restriction enzymes which are able to generate large fragments;
- (3) saturation of the target region with a number of DNA markers able to be physically mapped by PFGE (Wing *et al.*, 1994).

Four methods for the production of high molecular weight barley DNA were tested. Barley DNA produced from leaf material, protoplasts and nuclei were embedded in agarose plugs. Barley nuclei were also embedded in agarose microbeads. HMW DNA produced from leaf material was found to be unsatisfactory for restriction enzyme digestion. The HMW DNA prepared by this method is mixed with unlysed intact cells and tissue debris. Therefore, access of restriction enzymes to the DNA may be affected, resulting in partially restricted DNA which is unsuitable for physical mapping.

DNA prepared from both protoplasts and nuclei was sensitive to restriction digestion. However, preparations of nuclei consistently produced more DNA and were less labour intensive. The use of protoplasts for HMW DNA preparation has been used successfully in previous physical mapping studies (Wu and Tanksley 1993; Wing *et al.*, 1994). However, it requires different conditions for isolating protoplasts between species (Ganal and Tanksley, 1989; Cheung and Gale, 1990). In contrast, the conditions for nuclei, however, have been shown to be universal over a wide range of species (Zhang *et al.*, 1995).

Wing *et al.* (1993) reported that DNA prepared from nuclei embedded in agarose microbeads was more readily digestible than the DNA prepared from nuclei embedded in agarose plugs. Microbeads in addition, offer a greater degree of control over restriction digestion, which is essential for the partial digestions needed for cloning of HMW DNA into YAC or BAC vectors. However, for the work described in this study complete restriction enzyme digestions of HMW DNA using agarose plugs were more amendable, due to ease of handling, and a greater quantity of DNA recovered, with minimal loss of nuclei. In contrast to the significant loss of microbeads incurred during wash steps, resulting in a lower yield of restricted HMW DNA for our mapping studies.

The current limit for PFGE separation of DNA fragments is approximately 10Mb. Although this size range is sufficient for separating intact chromosomes from many microorganisms, plant chromosomes are too large to be resolved by PFGE. Therefore, restriction enzymes are needed to reduce the chromosomes to DNA fragments of a suitable size for PFGE resolution (Birren and Lai, 1993). To apply PFGE to genome analysis, one needs rare cutting restriction enzymes which are able to generate these required large DNA fragments. Rare-cutting restriction enzymes usually have octameric recognition sites (eg. *NotI*) or are hexameric enzymes which contain the nucleotide sequence CpG and CpXpG. These DNA motifs are often under-represented in the genomes of higher plants and are frequently methylated at the cytosine residue (Gruenbaum *et al.*, 1981). The inability of many restriction enzymes to cut at the methylated sites can generate larger than expected DNA restriction fragments, suitable for physical mapping studies.

Physical mapping studies in barley by Sielder and Graner (1991), Kilian *et al.*, 1997 and Lahaye *et al.* 1998 found that the average sizes of restriction fragments generated by restriction digestion in their regions of interest were 100-300kb, 230kb and 95kb, respectively. In this study, ten restriction enzymes were shown to generate large DNA fragments detected by probes linked to *Yd₂* with the average fragment size resolvable by PFGE approximately 300kb (Table 5.3). Therefore, the sizes of the restriction fragments generated by these enzymes were large enough to increase the chance of identifying two tightly linked markers on a single HMW restriction fragment thereby, giving an estimate of the relationship of genetic to physical distance in this region of the genome.

Eleven DNA probes identifying loci within 1cM of Yd_2 were chosen to be tested for their suitability for physical mapping using PFGE. However, only 6 of these probes identified restriction fragments resolvable using the PFGE conditions tested. All but one of these 6 DNA probes was a barley source probe (barley genomic or barley cDNA). With the exception of one DNA probe, the other probes which gave poor hybridisation signals were all derived from non-barley DNA. Importantly, the use of these other 5 probes under conventional Southern analysis resulted in poorer hybridisation patterns when compared to those probes which detected bands under PFGE conditions. Previous studies have found that the detection sensitivity of PFG blots is usually lower than that of filters obtained from conventional gel electrophoresis (Birren and Lai, 1993). Therefore, the non-barley probes which detect RFLPs linked to Yd_2 and represent expressed sequences could potentially be used to identify homologues from a barley cDNA library which may then be used for physical mapping studies.

A number of smeared autoradiographs were obtained with certain enzyme \times probe combinations. This may be the result of a variation in the methylation of certain regions of the plant genome where these target restriction sites are located (Birren and Lai, 1993). Such non-uniform methylation will result in partial restriction digestion products which are unsuitable for probe detection on Southern blots.

In this study it was clearly demonstrated that:

- (1) DNA probes for physical mapping are more specific if derived from the species being investigated;
- (2) a large number of markers are needed for saturation of the target region, especially if DNA probes from other species have been used in the construction of the linkage map, as some of these may be unsuitable for physical mapping studies.

5.4.2 The relationship of genetic to physical distance in the Yd_2 region and implications for the isolation of Yd_2

Physical mapping of the Yd_2 region revealed a physical linkage between the two loci *Xbcd134* and *Ylp*. These loci are separated by 0.5cM in genetic distance on the Proctor \times Shannon genetic

map and the loci *Ylp* was found to co-segregate with *Yd₂* in both mapping populations (Section 4.1). Therefore, a relationship of physical to genetic distance around this immediate area can be calculated as 1cM being less than 360kb. This relationship is a ten-fold reduction from the genome average calculated in Chapter 4. As *Yd₂* was placed genetically close to the centromere in the Proctor × Shannon mapping population, it was expected that the relationship between genetic and physical distance would be extremely unfavourable for map-based cloning strategy. However, as discussed in Section 5.1, the relationship between physical and genetic distance can vary throughout the genome.

This relationship between genetic and physical distance suggests that the *Yd₂* gene may be within 108kb of the *Ylp* loci, assuming that *Ylp* is closer than 0.3cM in genetic distance from *Yd₂* (95% confidence level; Section 4.4.2). If we assume that the *Yd₂* gene is within 0.1cM of the *Ylp* loci (60% confidence level; Section 4.4.2) then it may be as close as 36kb to *Ylp*. Such a relationship greatly improves our chances of identifying a single genomic clone with our co-segregating marker *Ylp* which may also contain the *Yd₂* gene. These results provide the foundation for the next step towards the isolation of the *Yd₂* gene by chromosome walking or jumping. Based on this physical data and the average insert size of large insert genomic libraries, we should be able to chromosome walk to *Yd₂* in two steps.

Additional common restriction fragments with other rare-cutting restriction enzymes encompassing the *Xbcd134* and *Ylp* loci may of been expected to be found. However, no additional restriction enzymes were found which physically linked these loci together on restriction fragments which were resolvable using the PFGE conditions used in this study (Section 5.3.4). However, the distribution of rare-cutter recognition sites has been reported to be clustered rather than randomly distributed (Bickmore and Bird, 1992; Larsen *et al.*, 1992). This complicates the interpretation of PFGE Southern analysis. Results in this study indicated that a clustering of restriction sites may also occur around the *Yd₂* locus (Table 5.5, Figure 5.6).

While a favourable physical linkage between *Xbcd134* and *Ylp* was identified, no physical linkages were found between any of the other loci identified with DNA probes determined to be suitable for physical mapping (Section 5.3.4). Importantly, no physical linkage was detected

between the two loci flanking Yd_2 in the Atlas \times Atlas68 mapping population, *YLM* and *Xmwg952*. Restriction enzyme mapping suggested that both these loci may be physically distant to Yd_2 (Section 5.3.4; Figure 5.6). Importantly, this demonstrates that while *Xbcd134* and *Ylp* are physically close, there may be a lot of variation in the relationship between physical and genetic distance within the genetic interval defined by our closest genetic markers.

Since chromosome walking (Section 1.7.1) can be complicated by the occurrence of chimeric or rearranged HMW clones and, in plants with large and complex genomes, by the amount of DNA, the frequency of repetitive DNA and the difficulty in establishing useful endclones, chromosome landing (Tanksley *et al.*, 1995) (Section 1.7.1) is the most promising strategy to establish the physical coverage of the region of interest. In order to do this, one or more markers are required within a physical distance from the target gene that is less than the average insert size of the YAC or BAC library being used for the screening of the relevant markers. Based on our physical mapping data *YLM* may be too far way from *Ylp*, and most probably Yd_2 to start a chromosome walk in this direction, since this would involve more than 15 walking steps with BAC vectors with an average insert size of 110kb, or 6 steps with YAC vectors with a 300kb average insert size. It is also possible that *Xmwg952* may be too far away to start a walk from the opposite direction. Based on the physical data described in this chapter, a chromosome walk is most likely to be successful using the DNA markers *Ylp* and *BCD134* to identify large insert genomic clones closely linked to the Yd_2 gene. End clones could then be derived from these large genomic clones to identify other overlapping clones linked to the Yd_2 region, eventually constructing a complete contig over the Yd_2 region encompassing the Yd_2 gene. Importantly large insert genomic clones identified would provide an additional source of DNA template from which to derive markers linked to Yd_2 to help improve our genetic and physical maps.

The next step in our map-based strategy was to use the DNA probes *BCD134* and *Ylp* to identify genomic clones linked to Yd_2 . At the time that these physical mapping studies were carried out no large insert genomic barley library was available for our use. However, rice had been proposed to be a model plant cereal genome due to its small genome size and the low amount of repetitive DNA it contains (Moore *et al.*, 1993; Kurata *et al.* 1994b). These features of the rice genome, combined with the conservation of gene order among grass species (synteny) could

provide a basis for the isolation of genes from cereals with larger genomes. The work in Chapter 6 describes the use of a well characterised rice YAC library to identify clones from the area of rice syntenous to the *Yd₂* region of barley Chromosome 3 and determine whether they may be useful for identifying a *Yd₂* orthologue in rice to help with the eventual isolation of the barley *Yd₂* gene.

CHAPTER 6

**THE IDENTIFICATION AND CHARACTERISATION OF LARGE
INSERT GENOMIC CLONES FROM THE REGION OF RICE
SYNTENOUS WITH THE Yd_2 REGION OF BARLEY
CHROMOSOME 3**

6.1 Introduction

Based on the fossil record, the grass (Gramineae) family arose relatively recently, approximately 50 to 60 million years ago (Crepet and Feldman, 1991). Despite the recent origin of the Gramineae from a common ancestral species, there has been considerable expansion and contraction in genome size. The genomes of barley and wheat are among the largest at 4.9×10^9 and 1.6×10^{10} bp per haploid nucleus, respectively, while the genome of rice is one of the smallest at 4.0×10^8 bp per haploid nucleus (Arumuganathan and Earle, 1991). These changes in genome size have been accompanied by very little change in the content of genes and other low copy sequences. Much of the additional DNA in the larger cereal genomes consists of repetitive sequences which constitute 83% and 76% of the genomes of barley and wheat, respectively, but only 50% of the genome of rice (Flavell *et al.*, 1974; Deshpande and Ranjekar, 1980). Repetitive DNA evolves rapidly in the grasses, and shows little sequence homology between species (Flavell *et al.*, 1986).

In contrast to repetitive sequences, cereal genes appear to have been highly conserved during evolution. Among a number of economically important grasses, 44 to 100% of cDNA fragments cloned from any one species will hybridise at moderate stringency to homologous sequences present in the genome of any one of the other species (Hulbert *et al.*, 1990; Wang *et al.*, 1992; Kurata *et al.*, 1994a; Causse *et al.*, 1994; Van Deynze *et al.*, 1995a). Sequences identified in two grass species by a single cDNA probe are likely to represent genes which have descended from a single ancestral gene (orthologous) (Hulbert *et al.*, 1990). Therefore, the hybridisation of cDNA clones across the Gramineae implies that there has been little sequence divergence within genes and only limited gene deletion or gain during the evolutionary divergence of the grasses from a common ancestral species.

Rice possesses a number of qualities which make it an ideal cereal species from which to isolate genes by positional cloning methods. The rice genetic map is 1575cM distributed over 12 linkage groups (Kurata *et al.*, 1994a), with the rice haploid genome size approximately 400Mb (Arumuganathan and Earl, 1991) therefore, yielding an average of 250kb/cM. By comparison,

the barley genetic map is 1245cM (Kleinhofs, 1994) distributed over seven linkage groups with the barley haploid genome 4900Mb (Arumuganathan and Earl, 1991), yielding an average of 4Mb/cM. These size comparisons clearly indicate the advantage of using the rice genome for map-based cloning rice orthologues of genes from barley and other large genomic cereals. Gene order colinearity among cereals has been well established at the macro level (Ahn and Tanksley, 1993; Bennetzen and Freeling, 1993; Devos *et al.*, 1993; Kurata *et al.*, 1994b; Dunford *et al.*, 1995; Kilian *et al.*, 1995; Dubcovsky *et al.*, 1996). 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). However, in order to apply the synteny relationships between the small genome of rice and a large genome such as barley for map-based cloning of genes, the conservation of gene order at the sub-centiMorgan and sub-Mbase level needs to be established.

Comparative mapping studies have shown that a central portion of rice Chromosome 1 is the region of the rice genome containing genes orthologous to those close to Yd_2 on barley Chromosome 3 (Figures 6.1, 6.2)(Causse *et al.*, 1994; Kurata *et al.*, 1994a; Collins, 1996). Collins *et al.* (1996) identified a number of RFLP markers that showed conserved order in barley and rice, including markers which were located to within 1.0cM proximal and distal of Yd_2 . This suggests that no translocations, large deletions or other major chromosomal rearrangements have occurred close to the point corresponding to the Yd_2 locus during the evolution of barley and rice from a common ancestor. The conserved RFLP marker linkage shown by Collins (1996) is therefore, likely to extend to regions immediately surrounding the Yd_2 locus. Overall, there appears to be adequate conservation in RFLP marker colinearity between the Yd_2 region of barley and the corresponding region of the rice genome to allow rice to be used to generate additional markers close to Yd_2 and possibly to identify a gene orthologous to Yd_2 .

PFGE described in from Chapter 5 indicated that the relationship of physical and genetic distance around the Yd_2 region is favourable to a map-based cloning strategy. However, at the completion of the PFGE physical mapping studies, no comprehensive barley large insert clone libraries were available for use. In contrast, a well characterised rice Yeast Artificial Chromosome (YAC)

Figure 6.1. The location of RFLP markers mapped in rice using DNA probes which map close to Yd_2 (Reproduced from Collins, 1996)

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 by Collins (1996) to construct the map of the Yd_2 region of barley Chromosome 3. All of these loci in rice 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)

*Yd*₂ region of barley
Chromosome 3

rice Chromosome 1
(Causse *et al.*, 1994)

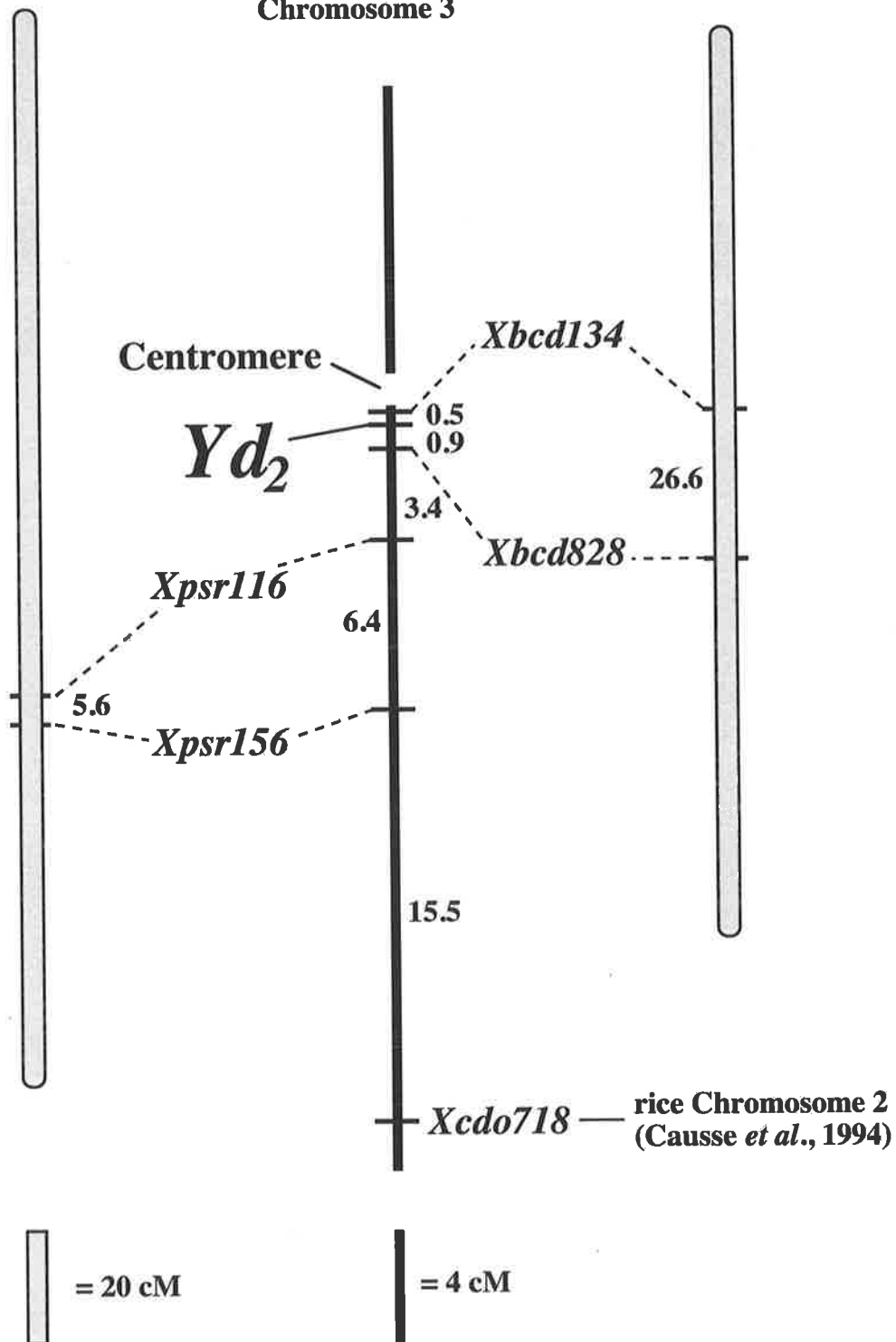
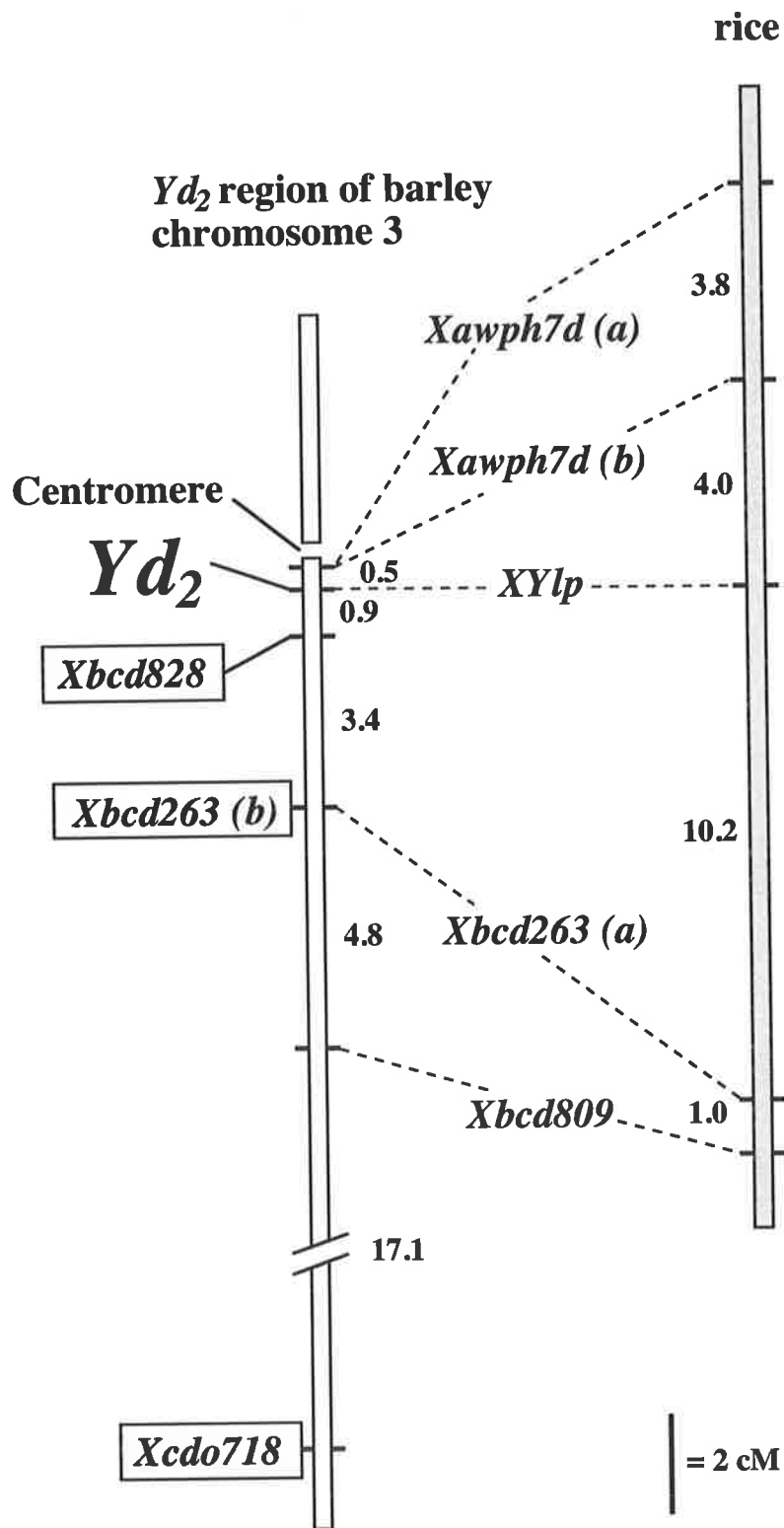


Figure 6.2. Positions of RFLP loci mapped in rice using the Doongara × Hungarian F₂ population, using probes mapped close to *Yd₂* on barley Chromosome 3 (Reproduced from Collins, 1996)

The locations of RFLPs mapped in rice by Collins (1996) using the Doongara × Hungarian F₂ population, and the locations of RFLPs mapped close to *Yd₂* on barley Chromosome 3 using the same probes are shown. 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 AWP7d was used to map separate loci using the restriction enzymes *Hpa*II {*Xawph7d(a)*} and *Rsa*I {*Xawph7d(b)*}. BCD263 was used to map two loci (*Xbcd263a* and *Xbcd263b*) using *Hind*III. Distances are in cM. Mapping data presented in Figure 6.1 and Figure 6.2 show a high level of synteny and colinearity within this portion of rice Chromosome 1 and barley Chromosome 3.



library had been established by the Japanese Rice Genome Project (<http://www.staff.or.jp/>). Therefore, this resource was obtained and the use of rice as a tool for cloning the *Yd₂* gene was explored.

It was reasoned that if a *Yd₂* orthologue could be identified in this syntenous region of rice Chromosome 1, it could be isolated from rice using a map-based approach. Such a gene could then be used as a probe to identify clones representing the *Yd₂* gene from a barley cDNA library. If a *Yd₂* orthologue did not exist, then it may still be possible to use these large genomic clones as a source for markers to further saturate the *Yd₂* locus. Therefore, the aims of the work described in this chapter were to identify and characterise large insert genomic clone(s) from the area of rice syntenous with *Yd₂* which may then prove useful in cloning a rice *Yd₂* orthologue or further marker saturation of the *Yd₂* region in barley.

6.2 Materials and Methods

6.2.1 Acknowledgments

The rice YAC filters and YAC clones supplied from the Rice Curator Takuji Sasaki, and his staff at the MAFF DNA Bank of the National Institute of Agrobiological Resources, Kannondai, Tsukuba, Ibaraki, Japan is gratefully acknowledged. The yeast clone ABG1380 and the vector plasmid pBR322 was generously supplied by Ursula Langridge. The random 10mer primers were a generous gift Graham Collins.

6.2.2 YAC filter screening

Filters of a 5 genome equivalent rice YAC library were imported from the MAFF DNA Bank. Protocols for prehybridisation, hybridisation and autoradiography are described in Section 2.2.12. Between consecutive hybridisations the membrane was washed in 0.4M NaOH for 10min and then stripped 3 times at 85°C stripping solution before being re-exposed for autoradiography to ensure probe removal was complete.

6.2.3 Growth of YAC clones

Yeast clones received in stab culture were plated onto Single Selection Media (SSM){1.7g/l yeast nitrogen base, without amino acids or ammonium sulphate, 5.3g/l ammonium sulphate, 20g/l of glucose, 56mg/l of adenine, 56mg/l of tyrosine, 100mg/l of tryptophan, 70ml of 20% caesin (acid hydrolysed, pH 6.5-7.0) and 30g/l of bacto-agar; autoclaved} grown for 2-3 days at 30°C before single colonies were picked and grown in liquid SSM until stationary phase was reached, OD₆₀₀ 0.5-0.8.

6.2.4 Extraction of total genomic DNA from YAC clones for dot-blot analysis

Yeast cells from 10ml of stationary phase culture were pelleted in a bench top centrifuge for 5min at 1500rpm. Cells were transferred to a 2ml Eppendorf tube and washed twice with 1.5ml of TE buffer (10mM Tris-HCl, 1mM EDTA). The cell pellet was resuspended in 300µl of zymolyase buffer (SCEM buffer)(1M sorbitol, 100mM tri-sodium citrate, 10mM EDTA, 10mM β-mercaptoethanol, zymolyase 10mg/ml) and incubated at 37°C for 30min. Six hundred µl of lysis buffer (100mM Tris-HCl pH 8.6, 1.5M NaCl, 50mM EDTA, 8% Dodecyltrimethylammonium bromide) was gently mixed with the cells and the suspension incubated at 68°C for 5min. One volume of chloroform (900µl) was added, the tubes inverted to mix phases, and the phases separated by centrifugation at 14,000rpm for 5min. The upper aqueous phase was transferred to a new 2ml Eppendorf tube. Nine-hundred and Ninety µl of MQH₂O and 5µl of RNase A (10mg/ml) were added and incubated at 68°C for 30min. One-Hundred and fifty µl of CTAB solution (5% Hexadecyltrimethylammonium bromide, 0.4M NaCl) was added and gently mixed by inversion. Eppendorfs were centrifuged for 2min at 10,000rpm and the supernatant discarded. The DNA-CTAB pellet was resuspended in 300µl of 1.2M NaCl, before the addition of 600µl of absolute ethanol and centrifugation for 10min at 14,000rpm. The DNA pellet was washed in 70% ethanol, air-dried and resuspended in 300µl of TE buffer.

Dot-blot analysis was performed on total genomic DNA for the identification of YAC clones. Ten µl samples of total genomic DNA were incubated at 94°C for 5min before and then put on

ice. Five μl samples were spotted on Hybond N⁺ membrane and air dried for 10min. DNA was cross-linked to the nylon membrane using 125 mJ of U.V. light in a Biorad GS gene linker and the membranes washed 3 times in 2 \times SSC. ³²P labelled probe preparation, hybridisation, and autoradiography are described in Sections 2.2.11-2.2.12.

6.2.5 High molecular weight DNA preparation from YAC clones

YAC clones were grown in 2ml of SSM in 30ml conical flasks at 30°C for 2 days. Media and cells were transferred into 2ml Eppendorf tubes, centrifuged for 5min at 1500rpm and washed twice with 1.5ml of TE buffer (10mM Tris-HCl, 1mM EDTA). After the final TE wash the cell pellet was vortexed in the TE buffer remaining after the majority of the TE had been removed by aspiration. Twenty μl of 10mg/ml zymolyase was added to the resuspended pellet and the mixture vortexed. Two-hundred μl of 2% low melt Seaplaque agarose in SCEM buffer at 65°C was mixed with the cell mixture using a wide bore pipette tip and aliquoted into Biorad plastic moulds (10mm \times height \times 5mm width \times 1.5mm thickness) and set on ice.

Plugs were transferred to 10ml round bottom plastic centrifuge tubes and 5ml of SCEM buffer and 40 μl of zymolyase (10mg/ml) were added. Plugs were incubated at 37°C for 3hrs to spheroplast the yeast cells. SCEM buffer was exchanged with 2 changes (5ml each) of lysis buffer (0.5MEDTA pH8.0, 10mM Tris-HCl pH8.0, 1% sarkosyl, 0.5mg/ml of proteinase K) at 50°C for 48hrs, followed by 4 washes of 0.5M EDTA at 50°C.

6.2.6 Restriction enzyme digestion of HMW YAC clone DNA

Agarose plugs containing total HMW DNA of YAC clones were treated to restriction enzyme digestion analysis as described in Section 5.2.5. The restriction enzymes used for digestion were; *AatII*, *BssHII*, *ClaI*, *EcoRI*, *EcoRV*, *FspI*, *MluI*, *NcoI*, *NotI*, *NruI*, *PvuI*, *PvuII*, *Sall*, *SmaI*, *SnaBI*.

6.2.7 PFGE separation, transfer and hybridisation of YAC clones and restricted HMW DNA

Undigested and digested HMW YAC DNA was separated using PFGE as described in Section 5.2.6. Pulse intervals (switch times) and run times used to separate HMW DNA are presented in Table 5.1 of the previous chapter, any variations in these conditions are noted in individual figures. ^{32}P labelled probe preparation and hybridisation are described in Sections 2.2.11 and 2.2.12 respectively. Between hybridisations the membrane was washed in 0.4M NaOH for 10min and stripped 3 times with boiling stripping solution before being re-exposed for autoradiography to ensure complete probe removal before subsequent hybridisations. For PFGE autoradiography, Kodak double emulsion BIOMAX MS film with a BIOMAX MS intensifying screen was used and hybridisations exposed from 24-72hrs at -80°C .

6.2.8 Purification of Y2733 template

To purify Y2733 template from yeast chromosomal DNA, YAC clone Y2733 was run on a $0.5\times\text{TBE}$ 1% agarose stacking gel. The PFG was set by pouring 1% Seakem LE agarose in the PFG mould and allowing it to set. The area of the gel where the YAC was expected to migrate under the PFGE conditions used in the experiment was excised (approximately 2cm in width), and replaced with 1% SeaPlaque low melt agarose. This increased the resolution of the YAC from the yeast genomic chromosomal DNA. PFGE was carried out at 200 volts and a linear ramp of 15-40sec for 15hrs. Y2733 was excised from the gel, and the DNA nicked using 60mJ of U.V. in a Biorad GS gene linker. Y2733 DNA was purified using a Bresaspin gel extraction kit (Geneworks, Australia). Purified DNA was precipitated by the addition of 3M sodium acetate pH4.6 to a final concentration of 0.3M, and 2.5 volumes of ice-cold ethanol. The DNA was pelleted by centrifugation in a benchtop centrifuge at 14,000rpm, the pellet washed in 70% ethanol, dried and resuspended in 20 μl of TE buffer.

6.2.9 PCR amplification of YAC ends

PCR amplifications were performed in a 20 μ l volume using either MJ Minicycler or PTC-100 Thermal cyclers (MJ Research, USA). A YAC-left arm specific primer (5'-CGCGATCATGGCGACCACAC-3') and a YAC-right arm specific primer (5'-ATATAGGCGCCAGCAACC-3') were used in combination with one of 9 random 10mers (Table 6.1). PCR amplification conditions were 0.63 μ M for each primer 1.5mM MgCl₂, 0.2mM each dNTP, 1 \times Taq DNA polymerase activity buffer [67mM Tris-HCl (pH8.8 at 25°C), 16.6mM (NH₄)₂SO₄, 0.45% Triton X-100, 0.2mg/ml gelatin), 20ng of purified Y2733 DNA and one unit of Taq DNA polymerase (Biotech International, Perth, Western Australia). Reactions were overlaid with oil, and subjected to thermal cycling using 15 cycles of 60sec at 94°C, 60sec at 53°C, 60sec at 72°C followed by 25 cycles of 60sec at 94°C, 60sec at 35°C and 60sec at 72°C. Electrophoretic analyses of the PCR products was performed using 1.0% agarose gels and 1 \times TBE electrophoresis buffer. Amplified products were the gel purified using a Bresaspin gel extraction kit (Geneworks, Australia). The purified DNA products were then oligolabelled and probed against a PFGE membrane of Y2733 to check that the DNA products were specific to rice Y2733 and not yeast chromosomal DNA.

6.2.10 Determination of the suitability of YAC-end probes for mapping in barley

YAC-end probes were hybridised to polymorphism membranes of barley to determine their suitability for mapping in the Atlas \times Atlas68 and Proctor \times Shannon mapping populations. DNA extractions on barley cultivars are described in Section 2.2.9. Restriction enzymes used to digest the DNA were *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Nco*I, and *Nde*I. Southern analysis was as carried out as described in Section 2.2.10-2.2.12.

Table 6.1 Random 10mer oligonucleotides used in combination with YAC right and left arm specific primers for the amplification of YAC end probes

Random 10mer oligonucleotide	5' to 3'
OPA-O1	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-04	AATCGGGCTG
OPA-05	AGGGGTCTTG
OPA-06	GGTCCCTGAC
OPA-08	GTGACGTAGG
OPA-09	GGGTAACGCC
OPA-13	CAGCACCCAC
OPA-18	AGGTGACCGT

6.3 Results

6.3.1 Identification of YACs from the region of rice syntenous with the *Yd₂* region of barley

In order to identify large insert genomic clones from the region of rice syntenous with the *Yd₂* region of barley Chromosome 3, the DNA probes Ylp and C122 which identify RFLPs that co-segregate with the *Yd₂* gene, were used to screen a 5 genome equivalent rice YAC library. Three areas with strong hybridisation signals were detected on a single filter which were common to both probes (Figure 6.3). The addresses (positions on the filter identified) of all three areas were sent to the Rice Curator at the DNA MAFF Bank in Japan. Two of the areas were identified as containing the YAC clones Y2733 and Y2763, both of which had been placed on the area of the rice physical map syntenous with *Yd₂* in barley. Importantly, both clones had already been shown to encompass the rice loci *Xrgc122* (Figure 6.4). The third area contained no clones from the region of rice syntenous region with that of *Yd₂*.

The clones Y2733 and Y2763 and another six clones flanking these clones on the rice physical map [(Y3025, Y4493, Y4848; identified by the DNA probe C667), (Y1134, Y6758, Y3742; identified by the DNA probe R886)] were obtained from the MAFF DNA Bank (Figure 6.4). Dot-blot analysis confirmed that YAC clones Y2733 and Y2763 strongly hybridised to both Ylp and C122, confirming they were the clones detected by the YAC filter screen (Figure 6.5). Dot-blot analysis of YAC clones with 13 other DNA probes that detect RFLPs close to *Yd₂* (Table 6.2) showed that the DNA probe WG889 strongly hybridised to Y4848 (Figure 6.5). PFGE analysis of HMW DNA was used to confirm that the three DNA probes hybridised to the YAC DNA and not yeast genomic chromosomal DNA (Figure 6.6).

6.3.2 PFGE size analysis of rice YAC clones

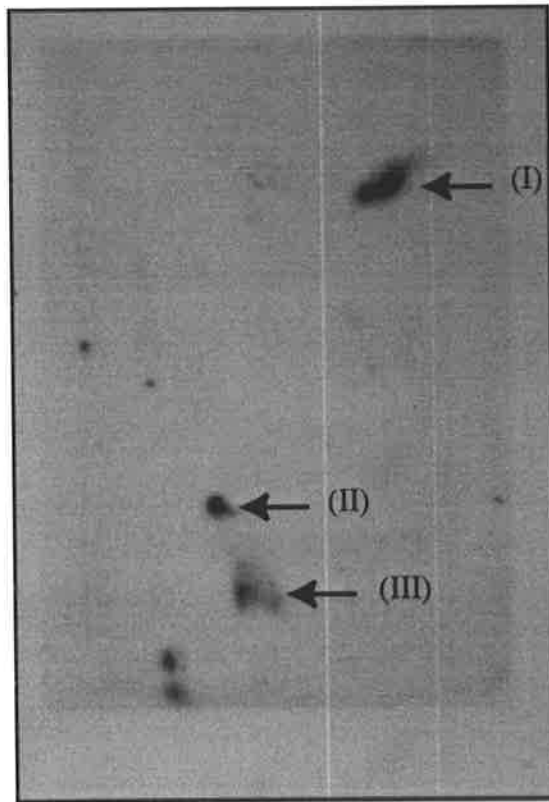
YAC clones obtained from the MAFF Bank were sized by PFGE. YACs were identified using the DNA probe pBR322, specific for the vector sequence of the YACs (Figure 6.7). The probe pBR322 identified a hybridisation signal for 7 of the 8 clones. The absence of a signal from the

Figure 6.3 Screening a yeast artificial chromosome (YAC) library with two DNA probes Ylp and C122 which identify RFLPs closely linked to *Yd₂*

Five high density YAC filters representing 5 genome equivalents of the rice genome were screened consecutively with the DNA probes Ylp (a) and C122 (b). To identify areas of common hybridisation autoradiographs were overlaid using radioactive dye and hybridisation background to correctly align them. Three strong hybridisation signals marked I, II & III are common to both probes. These areas were addressed and the Rice Curator at the MAFF DNA Bank notified. Two of these areas (I and II) contained clones identified as being from the region syntenous to the *Yd₂* in rice (Figure 6.4).

(a)

YLP Hybridisation



(b)

C122 Hybridisation

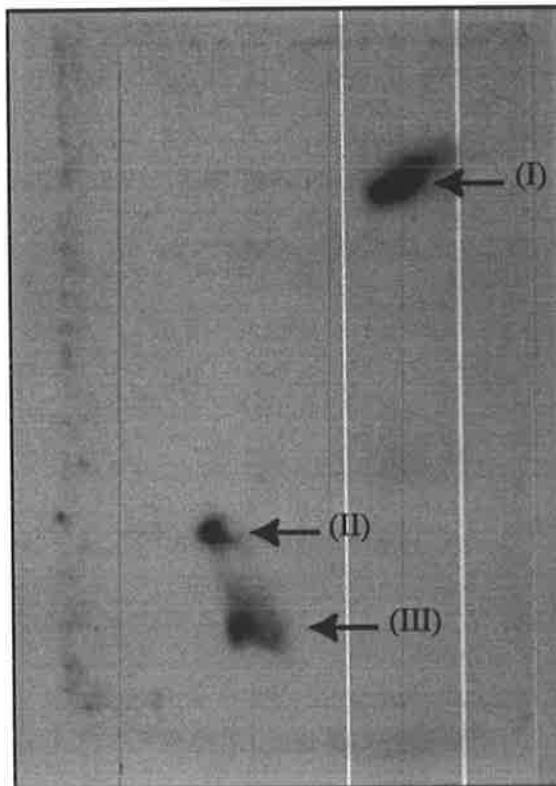


Figure 6.4 A partial rice YAC contig of rice Chromosome 1, the rice region syntenous with the *Yd₂* region of barley Chromosome 3 (Adapted from the Rice Genome Project physical data page at [http://www.staff.or.jp/rpg/publicdata/physical map/physicalmap.html](http://www.staff.or.jp/rpg/publicdata/physical%20map/physicalmap.html))

The position of YAC clones Y2733 and Y2763, identified by hybridisation to the DNA probes C122 and Y1p, on rice Chromosome 1 region 3, the area syntenous with *Yd₂* in barley Chromosome 3 is indicated. The thick horizontal bar at the top of the diagram represents the region of Chromosome 1 on the high density rice genetic map on which these two rice YACs have been positioned. Distances are in centiMorgans. The solid vertical bars denote positions of molecular markers with respect to their genetic distance along this region of rice Chromosome 1. The solid lines with circles represent the YACs forming the physical map of this region which have been identified by the respective molecular markers shown immediately above. Two YACs Y2733 and Y2763 identified by the YAC library screen, along with another 6 YAC clones from both sides of these clones were obtained from the rice curator of the MAFF DNA Bank in Japan. The clones obtained are shown in red or blue. However, the area that these YACs cover on this part of the rice physical map is not continuous, with the gaps between the solid lines representing a break in the YAC contig

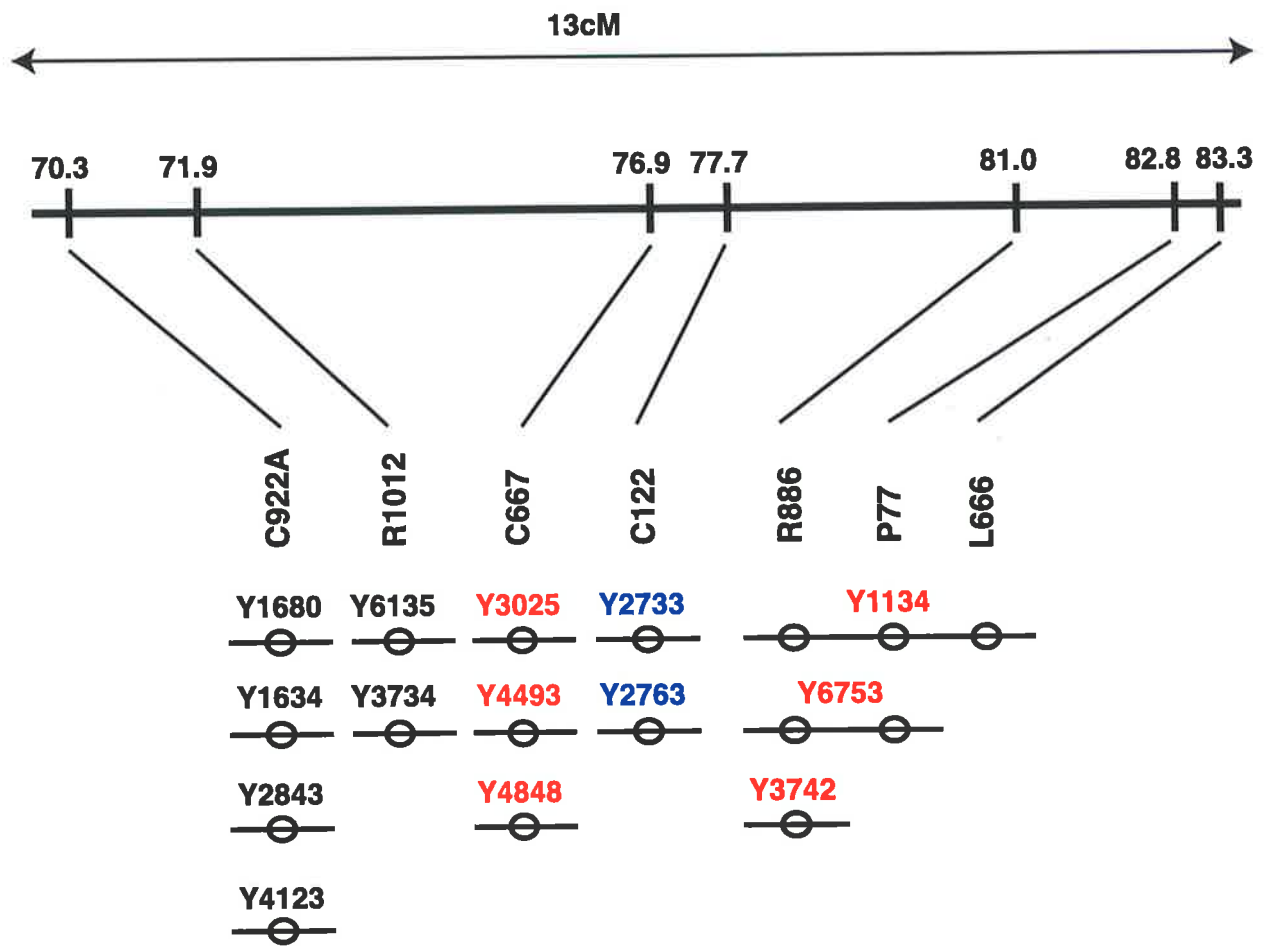
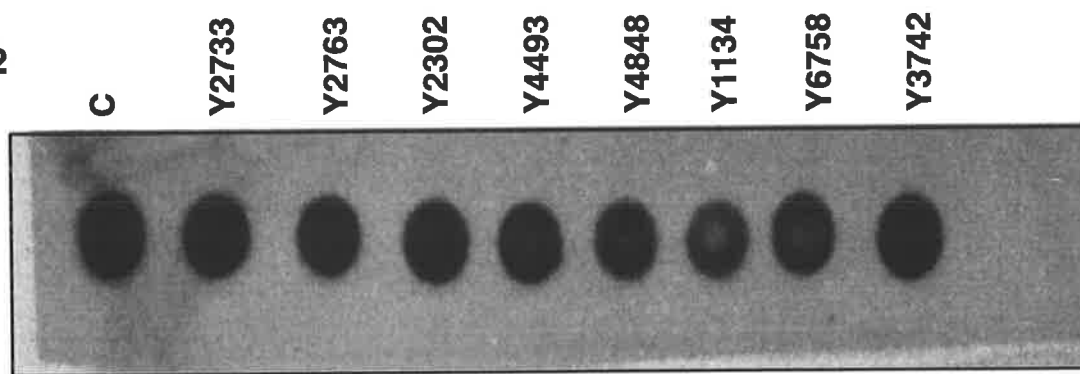


Figure 6.5 Dot-blot-analysis of YAC clones using to DNA probes closely linked to Yd_2

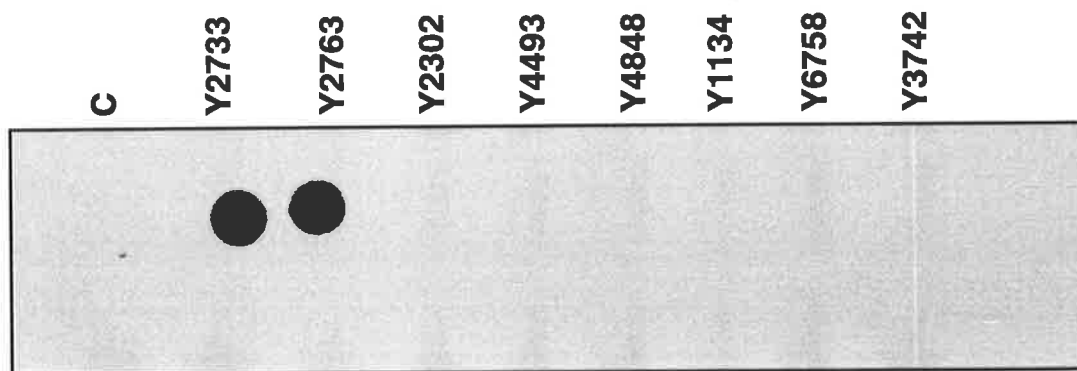
DNA probes which detect RFLPs closely linked to Yd_2 (Table 6.2) were hybridised to DNA from YAC clones from the region of rice syntenous with Yd_2 region of barley Chromosome 3. DNA from the YAC clones Y2733, Y2763, Y3025, Y4493, Y4848, Y1134, Y6758, Y3742 was spotted onto multiple H⁺ bond membranes and hybridised. Total DNA from the yeast clone ABG1380 (C), containing the yeast vector without a YAC insert was used as a control on all membranes.

(a) The plasmid vector PBR322 from which the YAC vector was derived was oligolabelled and used as a control DNA probe, as expected it hybridised to all clones. Three DNA probes (b) Ylp, (c) C122 and (d) WG889 which identify RFLPs linked to Yd_2 were found to hybridise to three YACs. Ylp and C122 both strongly hybridised to YAC clones Y2733 and Y2763, while WG889 hybridised strongly to Y4848.

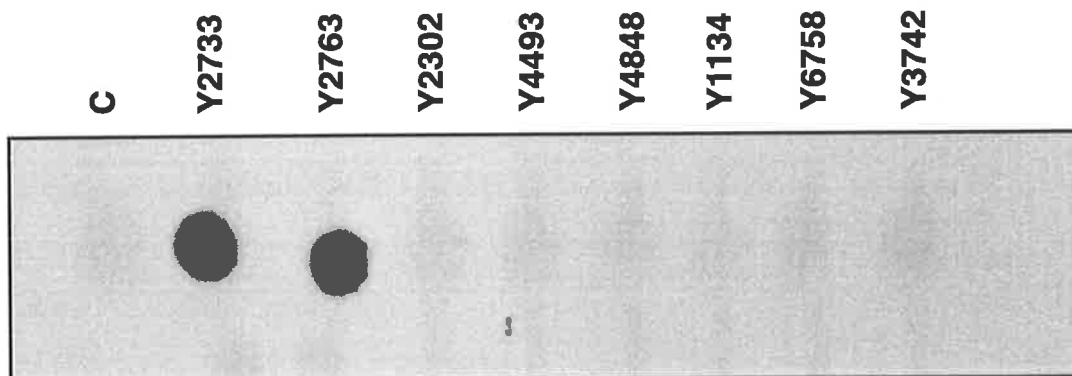
(a) pBR322



(b) Ylp



(c) C122



(d) WG889

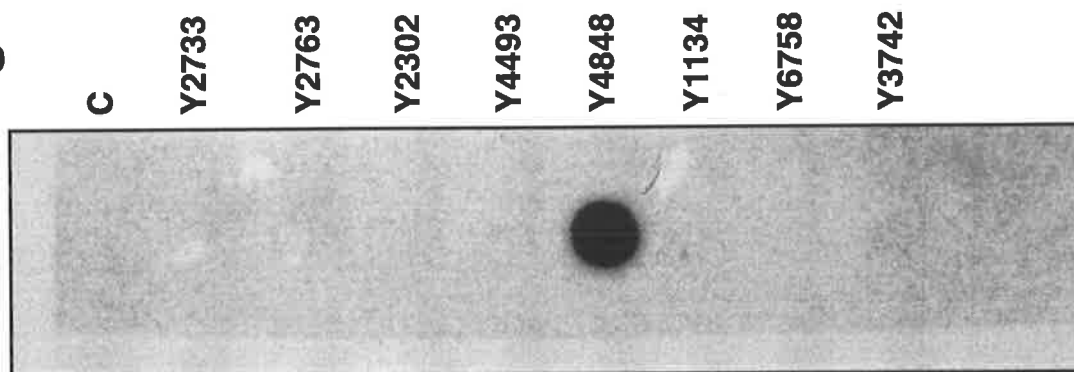


Table 6.2 DNA probes linked to Yd_2 that were used to screen the rice genomic YAC clones obtained from the MAFF Bank of the Rice Genome Project

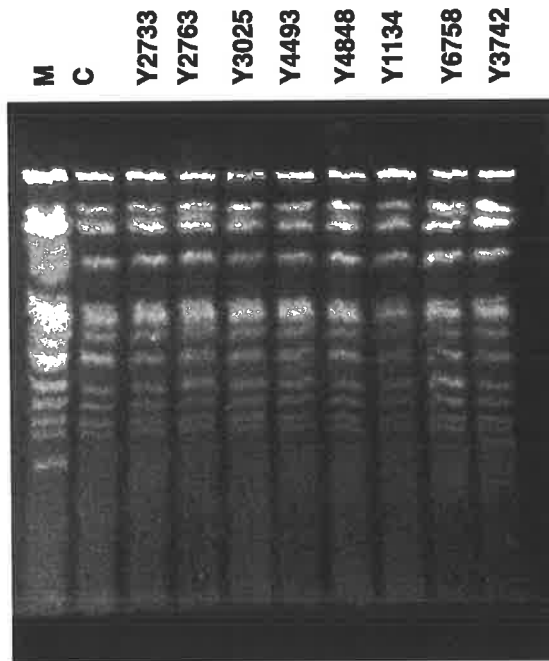
AWph7d
BCD134
BCD366
BCD828
BCD1380
KsuA3
MWG802
MWG952
MWG985
MWG2105
MWG2065
WG889
YLM

Figure 6.6 PFGE analysis of YAC clones with the DNA probes Ylp and WG889

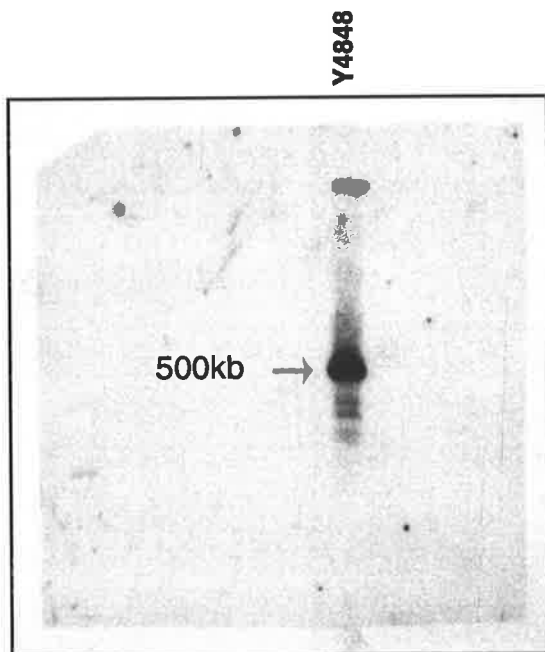
(a) High molecular weight DNA prepared from YAC clones was run on a 0.5×TBE 1% agarose gel for 24 hours at 200 volts. A linear ramp of 60-90 seconds was used to separate yeast chromosomes and YAC DNA. *Saccharomyces cerevisiae* (M) was used to size YAC DNA. High molecular weight DNA from the yeast clone ABG1380 (C), containing the yeast YAC vector without insert was used as a control. YAC clones were loaded in the following order; Y2733, Y2763, Y3025, Y4493, Y4848, Y1134, Y6758, Y3742. After ethidium bromide staining, alkaline transfer was performed and the membrane was then used for hybridisation.

The DNA probe WG889 (a) identified the clone Y4848, while the probe Ylp (b) identified both Y2733 and Y2763. Showing both probes are specific for the YAC DNA.

(a)



(b) WG889 Hybridisation



(c) Yip Hybridisation

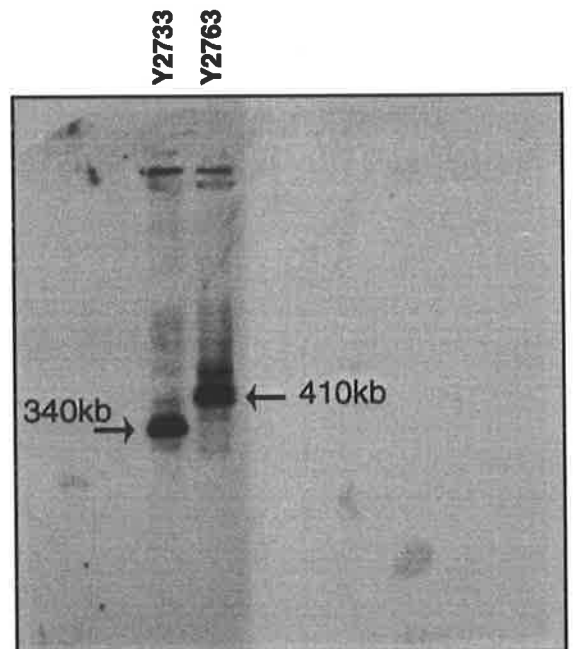
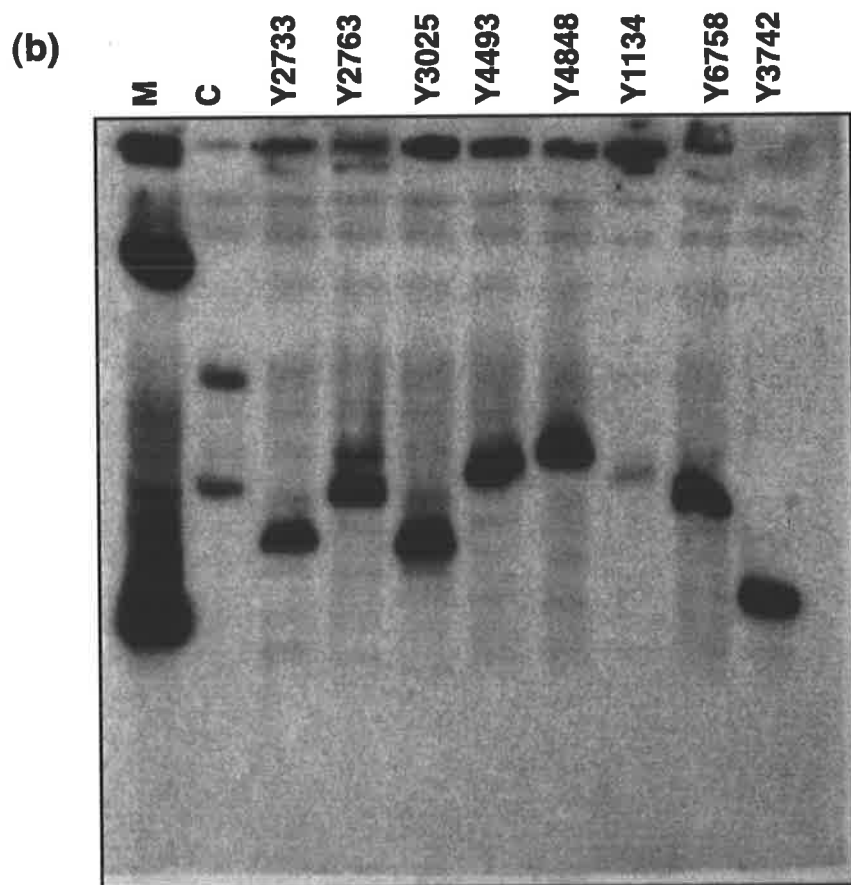
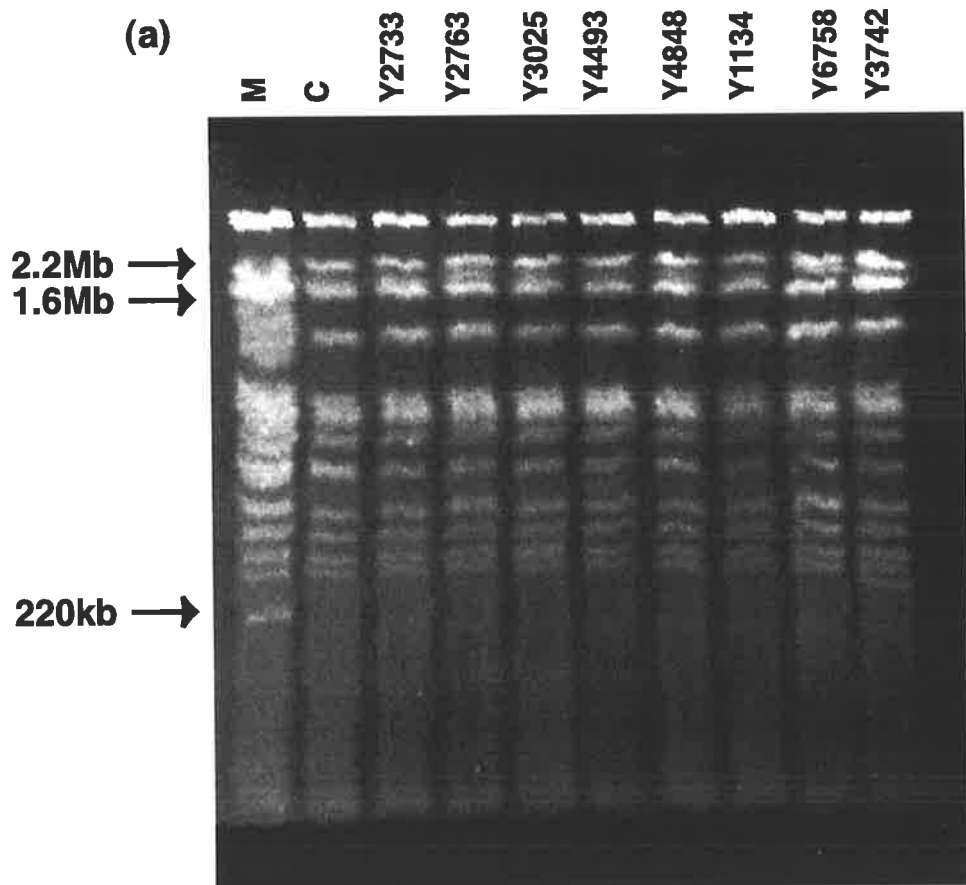


Figure 6.7 PFGE of YAC clones from the region of rice syntenous with the *Yd₂* region of barley

(a) High molecular weight DNA prepared from YAC clones was run on a 0.5×TBE 1% agarose gel for 24 hours at 200 volts. A linear ramp of 60-90 seconds was used to separate yeast chromosomes and YAC DNA. *Saccharomyces cerevisiae* (M) was used to size YAC DNA. High molecular weight DNA from the yeast clone ABG1380 (C), containing the yeast vector without YAC insert was used as a control. YAC clones were loaded in the following order Y2733 (1), Y2763 (2), Y3025 (3), Y4493 (4), Y4848 (5), Y1134 (6), Y6758 (7), Y3742 (8). After ethidium staining an alkaline transfer was performed on the PFG and the membrane was used for hybridisation.

(b) Hybridisation was performed on the PF membrane with the DNA probe pBR322 to detect and size the YAC DNA (Table 6.3). The probe pBR322 detected YAC DNA in 7 of the 8 clones. The YAC insert Y1134 appeared to have been lost from the yeast vector, and no further analysis of this clone was undertaken.



YAC clone Y1134 indicated that had lost its insert, a problem encountered in the YAC cloning system (Dunford *et al.*, 1993) and further analysis of this clone could not be undertaken.

The average insert size of the rice YAC library is 350kb (Umehara *et al.*, 1995). The sizes of the rice YAC clones obtained from the MAFF Bank were determined using PFGE and are presented in Table 6.3. The average size of these YACs obtained from this region of rice syntenous with *Yd₂* region in barley is 388kb. However, the area they cover on the rice physical map is not a continuous contig (a series of overlapping clones) (Figure 6.4).

6.3.3 Restriction enzyme mapping of the YAC clones Y2733 and Y2763

Restriction enzyme mapping of Y2733 and Y2763 was undertaken to determine the maximum physical distance between the rice loci *XrYlp* and *Xrc122* identified by the DNA probes Ylp and C122. Initially high molecular weight DNA was restricted with 7 restriction enzymes, *BssHIII*, *NotI*, *NruI*, *PvuI*, *PvuII*, *SalI*, and *SmaI*. The restricted DNA was separated by PFGE and Southern analysis was carried out using the DNA probes Ylp and C122. However, no co-migrating restriction fragments were detected by the probes Ylp and C122 (Figure 6.8). Therefore, a physical linkage of less than 340kb between the rice loci *XrYlp* and *Xrc122* could not be inferred.

The restriction fingerprints detected by the DNA probes Ylp and C122 were identical for the clones Y2733 and Y2763. Southern analysis of these membranes with the probe pBR322 (which identifies both vector arms), detected a minimum of two sets of bands for each digest. One set of bands were found to be identical between the two clones Y2733 and Y2763, confirming that the rice genomic inserts of both Y2733 and Y2763 are identical on one side of the vector and they have the same cloning site. Y2763 extends 70kb further in one direction than Y2733, therefore, both clones should contain 340kb of common rice insert DNA. The restriction enzymes *NotI*, *NruI* and *PvuI* identified a maximum physical linkage of 9kb between *Xrc122* and one of the YAC vector arms (Figure 6.8) placing the loci *Xrc122* towards the end of the YAC clone.

Table 6.3 Sizes of rice YACs imported from the MAFF Bank of the Rice Genome Project as determined by PFGE

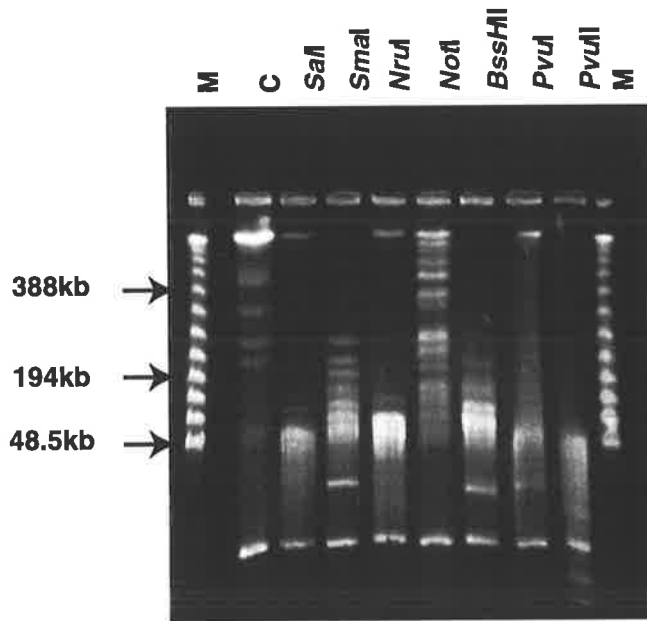
YAC clone	Size
Y1134	Absent
Y2733	340kb
Y2763	410kb
Y3025	310kb
Y3742	260kb
Y4493	490kb
Y4848	500kb
Y6758	410kb

Figure 6.8 Restriction enzyme mapping of Y2733

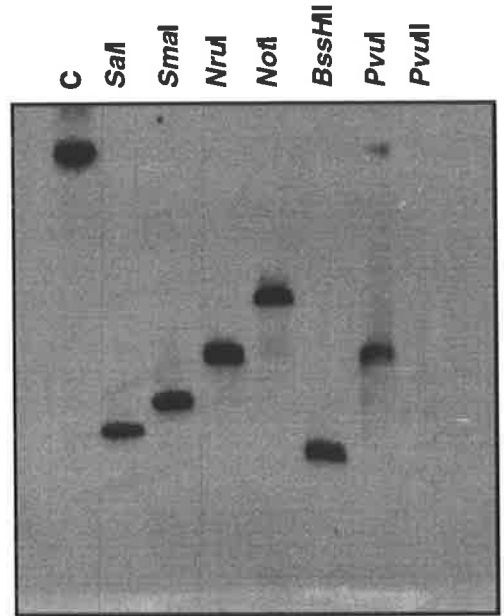
(a) High molecular weight DNA prepared from Y2733 was restricted with the restriction enzymes *SaII*, *SmaI*, *NruI*, *NotI*, *BssHII*, *PvuI*, and *PvuII*, and separated on a 0.5×TBE 1% agarose gel for 24 hours at 200volts, using a linear ramp of 15-40 seconds to resolve the restricted DNA. An undigested control (C) was included and a mid range concatemer ladder (M) to size the restricted DNA fragments. After ethidium staining alkaline transfer was carried out and the resulting membrane used for hybridisation.

The DNA probes Ylp (b), C122 (c) and pBR322 (d) were consecutively hybridised to the Southern blot membrane. Between hybridisations the membrane was stripped and re-exposed to ensure probe removal before. Autoradiographs were overlaid aligned using the radioactive dye and autorad background so restriction fragments of identical molecular weight could be identified. No restriction fragments of the same molecular weight were detected by the DNA probes Ylp (b) and C122 (c). However, restriction fragments generated by *NruI*, *NotI* and *PvuI* and detected by the DNA probes C122 (c) and pBR322 (d) were found to be identical therefore, placing the loci identified by C122, *Xrgc122*, a maximum of 9kb from one end of the vector.

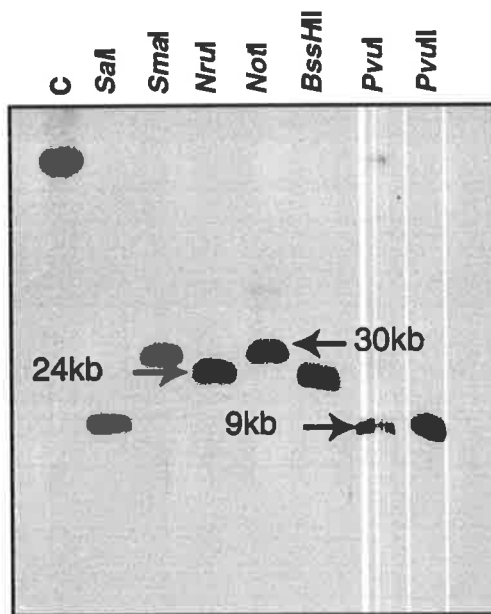
(a) PFG



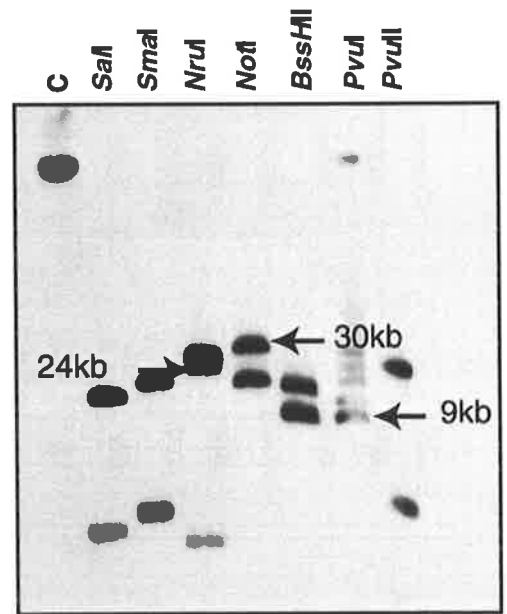
(b) Ylp Hybridisation



(c) C122 Hybridisation



(d) pBR322 Hybridisation



Additional restriction enzyme mapping of Y2733 was undertaken to identify a physical linkage between the two rice loci *XrYlp* and *Xrc122* (Table 6.4). Using the restriction enzymes *MluI*, *NcoI*, and *SnaBI* a maximum physical distance between the loci of 9kb was established. This data indicated that the two loci which co-segregate with *Yd₂* in barley, are physically located adjacent to each other in rice genome.

6.3.4 Amplification of end probes from rice YAC clones for mapping in barley

To determine whether the rice YAC clone Y2733 completely encompassed the region in rice syntenous with *Yd₂* in barley, end probes from Y2733 clone were generated. PCR products were amplified from purified Y2733 template using a combination of a primer specific to the YAC vector arm (YAC-left or right) in conjunction with a random 10mer (Table 6.1). Purified products were then oligolabelled and hybridised against a PFGE membrane of Y2733 to check that probes were specific for YAC template and not yeast chromosomal DNA (Figure 6.9). Two products amplified with the primers YAC-right/OPA-13 and YAC-left/OPA13 were shown to be specific for the Y2733 DNA and were used as hybridisation probes in barley. However, both probes produced poor hybridisation signals on barley Southern membranes and were unable to be mapped in our mapping populations.

6.4 Discussion

The identification of the loci *Xrgc122* from the rice RFLP map of Kurata *et al.* (1994a) which is orthologous to the barley loci *Xc122* which co-segregates with *Yd₂* (Paltridge, 1998), was seen to provide an opportunity to develop RFLP markers very close to *Yd₂* using a strategy developed by Kilian *et al.* (1995; 1997). At the commencement of this study excellent progress has been made in the physical mapping of the rice genome with YAC clones (Kurata *et al.*, 1997), and the locus *Xrgc122* detected by the DNA probe C122 in rice had been localised to two YAC clones (Rice Genome Research Program Homepage; <http://www.staff.or.jp/>). Therefore, YAC filters from the rice genome project were screened and both YAC clones landmarked by the DNA probe C122 were identified and obtained along with another 6 YACs flanking these clones on the rice YAC contig (Figure 6.4). These YACs were then further characterised to determine if they would be

Table 6.4 Sizes of restriction enzyme fragments of YAC2733 detected by the hybridisation probes YLP and C122 as determined by PFGE

Restriction enzyme	Ylp fragment size	C122 fragment size
<i>AatII</i>	24kb	N.B.
<i>BssHII</i>	6kb	23kb
<i>ClaI</i>	15kb	9kb
<i>EcoRI</i>	22b	9kb
<i>EcoRV</i>	18kb	N.B.
<i>FspI</i>	9kb	20kb
<i>MluI*</i>	24kb	24kb
<i>NcoI*</i>	9kb	9kb
<i>NotI</i>	120kb	30kb
<i>NruI</i>	23kb	24kb
<i>PvuI</i>	23kb	8kb
<i>PvuII</i>	N.B.	8kb
<i>SalI</i>	6.5kb	8kb
<i>SmaI</i>	9kb	30kb
<i>SnabI*</i>	9kb	9kb

* Restriction fragments shown to be of identical molecular weight therefore, indicating a physical linkage between the two DNA probes with that restriction enzyme.

N.B. No band detected

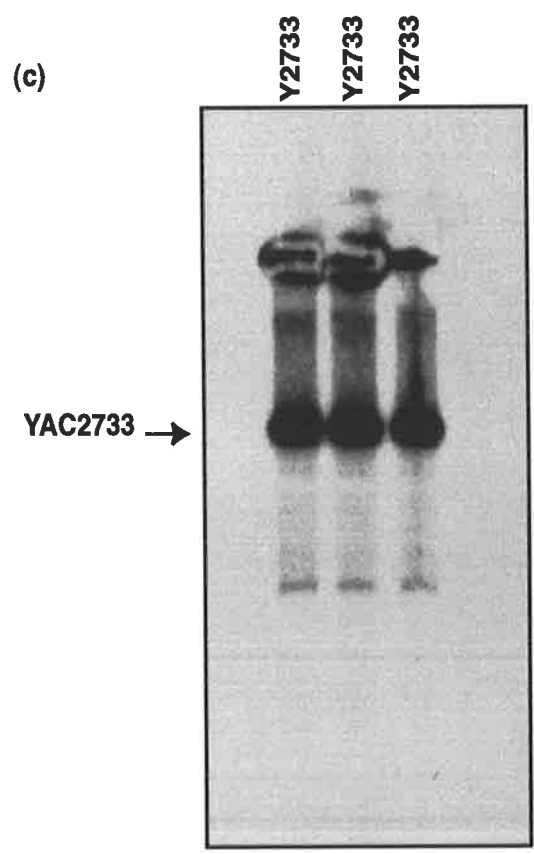
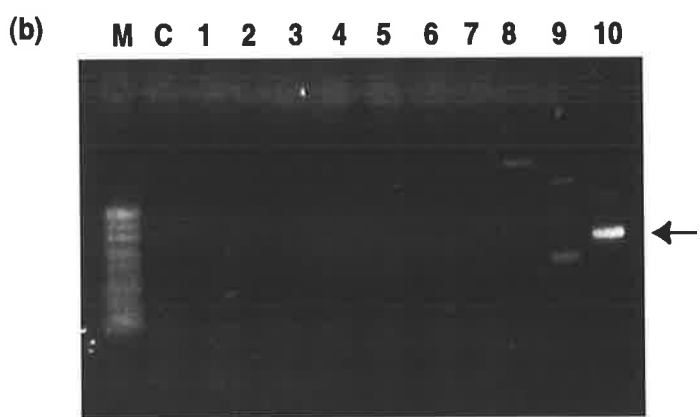
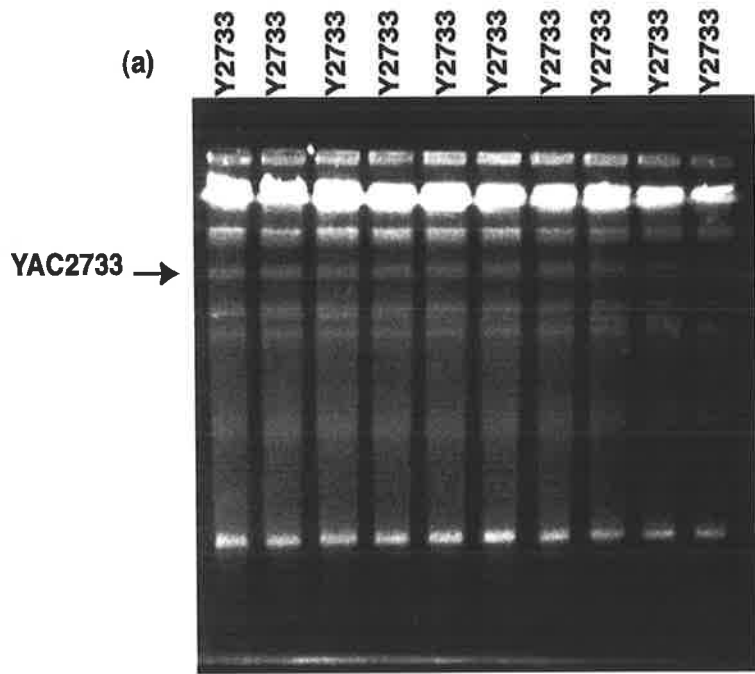
PFGE conditions were; restricted DNA was run on a 1% agarose 0.5×TBE for 24 hrs at 200volts with a linear ramp of 15-40 sec.

Figure 6.9 Development of YAC end probes from Y2733

(a) High molecular weight DNA prepared from Y2733 was separated on a 0.5×TBE 1% agarose stacking gel containing both Seakem LE agarose and low melt agarose. PFGE was carried out using a linear ramp of 15-40 seconds for 15 hours to give maximum separation of Y2733 from the host yeast chromosomes. Y2733 was then excised from the gel and purified (Section 6.2.8). Purified DNA was pooled and ready for use in PCR amplification.

(b) Purified Y2733 DNA was amplified using specific primers for both YAC left and right arm vectors with a series of random 10mers (Table 6.1). A no template control (C) was included. Amplified products were separated on a 1×TBE 1% agarose gel. Three random 10mer primers (Oligo8, Oligo9, and Oligo13) in combination with the YAC-left arm specific probe amplified products (lanes 8, 9, 10). The DNA product shown in lane 10 was excised and gel purified. The marker lane (M) contains pUC19 DNA digested *Hpa*II.

(c) Purified product of lane 10 was then oligolabelled and hybridised to a pulsed field membrane of YAC clone Y2733 which was separated on a 0.5×TBE 1% agarose gel using a linear ramp of 15-40 seconds for 15 hours. The probe strongly hybridised to the Y2733 in all three lanes and not to yeast chromosomes indicating that the product was YAC-specific and not chromosomal host yeast DNA.



useful in helping to further saturate the Yd_2 region with molecular markers and if they may possibly contain a gene orthologous to Yd_2 .

6.4.1 The level of gene order colinearity between the Yd_2 region of barley and the corresponding region of the rice genome

A number of DNA probes which detect RFLPs closely linked to Yd_2 were hybridised to YAC template from YAC clones identified as being from the region of rice syntenous with the Yd_2 region of barley Chromosome 3. However, only three DNA probes hybridised to three of the eight YAC clones. The fact that a number of these markers closely linked to Yd_2 did not hybridise to the rice YACs may imply a breakdown in marker colinearity between rice and barley at the sub-centiMorgan and sub-Mbase level. Breaks in colinearity have previously been observed between rice and barley (Kilian *et al.*, 1995; 1997). Kilian *et al.* 1997 demonstrated that while there was a high level of gene order synteny between rice and barley in the two regions they were studying, *Rpg1* and *rpg4* (Resistance loci to barley stem rust), they identified however, small DNA fragments which seemed to have moved to other regions of the rice genome, out of order. This raised the concern that their gene of interest may not be in the expected location, and after initial sequence analysis of the region, they had as yet, not identified a candidate resistance gene. Therefore, the possibility of a breakdown in synteny needs to be considered when using rice as a subgenomic cloning vehicle. This is a limitation when using a single model genome to isolate orthologous genes in grasses. However, there are alternative explanations which do not imply a breakdown in marker colinearity.

At the time this study was performed, the rice YAC contig constructed by the Japanese Rice Genome Project was not continuous over the region of rice syntenous with Yd_2 and a number of gaps of undefined size existed between the clones (Figure 6.4). Therefore, many of the DNA markers which did not hybridise to the YAC clones may be represented in the syntenous region of the rice genome, but are not represented on the rice YAC physical map. In addition, these markers may be represented in the YAC contig but hybridise to YAC clones further away from the YACs Y2733 and Y2763 and the those clones flanking them. Finally, of the 13 probes which were hybridised against the rice YACs, 7 were genomic probes from other species and therefore, most

likely contained repetitive DNA which shows little sequence homology between species (Flavell *et al.*, 1986). Such probes usually make poor hybridisation probes when cross-hybridised with other species (Devos and Gale, 1997).

Recent mapping work has revealed some exceptions to orthology and colinearity. Several disease resistance gene analogs (RGAs) and resistance genes are not well conserved among the grass genomes. Mapping of RGAs isolated from rice and barley, in rice, barley and foxtail millet showed limited orthology (Leister *et al.*, 1998). A lack of colinearity was also found between wheat and rice at the wheat-leaf rust gene locus *Lr1* on Chromosome 5DL (Gallego *et al.*, 1998). This data suggests that comparative analysis might be more difficult for genes, such as resistance genes, that evolve rapidly. However, these resistance genes completely arrest the growth of the pathogen by eliciting a hypersensitive response in the host plant, in contrast, as discussed in Section 1.5.2, the *Yd₂* gene does not prevent the virus from spreading systemically from the point of infection, rather it reduces the rate of replication of the virus and tolerates its growth. Therefore, the *Yd₂* gene may not be under the same evolutionary pressures as resistance genes which elicit a hypersensitive response and may be more amenable to cloning using rice as a model system.

6.4.2 The relationship between physical and genetic distance in the region of rice syntenous with the *Yd₂* region of barley

Recent gene density studies of repetitive genomes such as barley and wheat, have shown higher gene densities than were predicted based on genome wide averages, indicating that genes are not randomly distributed along the chromosomes but interspersed as gene islands by repetitive DNA (Rahman *et al.*, 1997; Panstruga *et al.*, 1998; Keller and Feuillet, 2000; Tarchini *et al.*, 2000). It was essential for us to try and establish an estimate of the differences that may exist between rice and barley in relation to the ratio of genetic to physical distance around this region of the genome, as this may give us an idea on the differences between gene density in the region for both species. Physical mapping data (Chapter 5) suggested that the ratio of physical to genetic distance around the immediate region of *Yd₂* in barley was considerably less than that calculated as a genome average. Also, a number of molecular markers identified as being physically and

genetically close to each other and Yd_2 , represented expressed sequences. Therefore, it is possible that the Yd_2 region in barley is gene rich and differences in gene density between this region and the syntenous region in rice may not be dramatically different.

If the relationship of genetic to physical distance in barley is far greater than that in rice, then this would confirm that the use of rice as a model subgenomic cloning vehicle may be warranted. However, if the differences in the relationship were not great, then this would suggest that with the possible difficulties of using rice as a subgenomic vehicle this avenue to cloning Yd_2 may not be worth pursuing. To obtain such a measure, information about genetic and physical distances for this region of the rice genome were needed.

The DNA probe WG889 which hybridised to Y4848 is 0.9cM distal to Yd_2 on the barley Atlas × Atlas68 map. Y4848 is landmarked by the molecular marker C667 which is 0.8cM from *Xrgc122* on the rice genetic map (Figure 6.4) (www.staff.or.jp/). Based on this information if it is assumed that the relationship between genetic distances for molecular markers are similar in this immediate region of the genome for both rice and barley, differences in the relationship between physical and genetic distances between the two species can be calculated.

Both rice YACs Y2733 and Y2763 encompassed not only the *Xrgc122* loci detected by the probe C122 but also the *XrYlp* loci detected by the probe Ylp. Physical mapping of these YACs showed that both these markers which co-segregate with Yd_2 in our mapping populations were no further than 9kb apart in rice. Based on our physical mapping data in barley, and the assumption that our co-segregating markers were within 0.3cM of Yd_2 (95% confidence level), we assumed that *Ylp* was within 108kb of Yd_2 (Section 5.4.2) and therefore, within 108kb of *Xc122* in barley. If the *Ylp* loci was within 0.1cM of the Yd_2 loci (60% confidence level) then it may be as close as 36kb to Yd_2 in barley. Therefore, a rough estimate of approximately 4-12 fold less physical distance in rice than barley was calculated for this region of the genomes between these species. The upper estimate of this calculation is still less than the 16-fold difference expected based on genome averages and a random distribution of genes in barley. The lower limit suggests that the differences in the ratio of physical and genetic distance may not be that dramatically different between barley and rice for this area of the genome. Therefore, map-based cloning of Yd_2 using

barley may be a more sensible approach considering the possible problems associated with using rice as a subgenomic cloning vehicle. However, considering the small physical distance between the two rice loci *Xrgc122* and *XrYlp* and that the probes which identify these loci in rice also identify RFLPs which co-segregate with *Yd₂* in barley, it is tempting to speculate that a rice gene orthologous to *Yd₂* may be physically close to these loci.

6.4.3 The possibility of a gene orthologous to *Yd₂* on a rice-YAC from the region of rice syntenous with the *Yd₂* region of barley Chromosome 3

For the identification of *Yd₂* using rice as a subgenomic cloning vehicle, it is essential that any large genomic clone/s identified from the region syntenous with *Yd₂* region completely span the region ensuring any possible gene orthologous to *Yd₂* is contained within the clones. If RFLP markers derived from end probes of a YAC(s) could be showed to be genetically distinct from *Yd₂* by recombination events proximal and distal of the *Yd₂* gene, this would infer that the YAC clone(s) from which the end clones were derived completely encompass the region syntenous with *Yd₂*. While the generation of end clones from Y2733 was successful, these clones however, proved to be poor hybridisation probes and were not useful for RFLP mapping in barley. Kilian *et al.* (1995) and Paltridge (1998) report similar difficulties when mapping rice cDNA and rice genomic DNA probes in barley. The problems arising from the mapping of rice probes in barley are almost certainly a consequence of the fact that rice and barley are only distantly related members of the grass family. As a result, sequence homology between rice and barley is not always sufficient for efficient hybridisation. Since non-coding sequences are less conserved amongst the grasses than coding sequences (Devos and Gale, 1997), cDNA clones generally provide better hybridisation probes than genomic clones. End probes derived from large insert genomic clones are likely to contain repetitive DNA which results in them being poor hybridisation probes, as experienced in this study.

Physical mapping of the Y2733 and Y2763 determined that *Xrgc122* was a maximum of 9kb from one end of the YAC vector. The physical proximity of both *Xrgc122* and *XrYlp* to one end of the YAC clones, dramatically reduces the chance that either of these YACs completely

encompass the region of rice syntenous with the Yd_2 region of barley. Therefore, any possible gene orthologous to Yd_2 may not be present on either of these YACs.

In validating the use of rice as a subgenomic vehicle it is assumed that a gene orthologous to the Yd_2 gene exists in rice. Since the divergence of rice and barley from a common ancestral species, the possibility that the Yd_2 resistance gene evolved independently in the wild Ethiopian Barleys from which it was first discovered must also be considered. It is possible that a gene orthologous to Yd_2 is not present in rice and using rice as a subgenomic cloning vehicle will not result in the identification of an orthologue which can be used to directly pull out the Yd_2 gene in barley. However, subclones derived from these clones may help delimit the Yd_2 region in barley.

6.4.4 Future directions for the use of rice as a subgenomic cloning vehicle

If rice is to be used as a subgenomic vehicle then we must obtain a clone(s) that completely span the region of rice syntenous with Yd_2 . While it is possible that a gene orthologous to Yd_2 is present on the two YACs which hybridise to the probes C122 and Ylp, as the loci *Xrgc122* and *XrYlp* are located towards the end of the genomic insert it is unlikely that these YACs completely span the syntenous region of Yd_2 . If a clone is identified that completely spans the syntenous region of rice it may encompass a gene orthologous to Yd_2 .

A number of Bacterial Artificial Chromosome (BAC) filters from a recently constructed rice library from Clemson University (www.genome.clemson.edu) have been obtained to screen with the DNA probes C122 and Ylp to identify BAC clone(s) that completely span the region of rice syntenous with barley Yd_2 region. BAC clones have significant advantages over YAC clones, which are often found to be chimeric and are problematic when it comes to the isolation and manipulation of YAC insert DNA (discussed in Chapter 7.1).

Once a clone or series of clones that completely span the region in rice syntenous with the Yd_2 region have been identified it would be possible to subclone that BAC(s) and use it as a source of further markers for Yd_2 . Although only a small proportion of subclones (15%) would be expected to hybridise well enough to barley DNA to make useful RFLP probes (Kilian *et al.*, 1997), this

approach should yield many closely linked markers which may further delimit the region immediate to the *Yd₂* gene. The large rice genomic insert may also be used as a hybridisation probe to identify cDNAs from libraries constructed from infected and noninfected *Yd₂* and non-*Yd₂* barleys. Changes in levels of expression of infected and noninfected *Yd₂* and non-*Yd₂* cultivars for any expressed sequences identified can then be investigated by Northern analysis. Any cDNAs identified would need to co-segregate with *Yd₂* in our mapping population to be considered as candidate genes. Any candidates could then be sequenced and BLAST searched against existing databases to identify putative function.

The development of efficient sequencing technologies means it is now possible to sequence the region syntenous with the *Yd₂* region of barley. Based on the sequence, gene prediction can be performed by appropriate computer programs (GeneMark.hmm at <http://dixie.biology.gatech.edu/GeneMark/eukhmm.cgi>). Sequences can then be BLAST searched against ever increasing databases to help in attributing putative functions to query sequences. In combination with the technique of cDNA capture and Northern analysis this can be extremely powerful. Sequence information will soon become freely available from the Rice Genome DNA Sequencing Project (<http://www.staff.or.jp>) who are currently sequencing the region of Chromosome 1 of rice, syntenous with Chromosome 3 barley. However, it may be quicker and more competitive for our research group to undertake this task immediately by subcloning any BAC/s identified as covering the syntenous region with *Yd₂* and using the Australian Genome Research Facility to sequence this entire area and assemble the data.

If a candidate clone is not identified on the rice DNA fragment, then as already discussed this may be because ;

- 1) The orthologous gene is not present in rice
- (2) The orthologous gene is not in the expected syntenic position
- (3) We may not recognize the *Yd₂* orthologue since homology searches may not recognise it due to a lack of homology with the conserved regions of resistance genes already cloned. It may be different to anything we expect and unrelated to any sequences with ascribed functions in current gene and expressed sequence databases.

Considering the difficulties of using rice as a subgenomic cloning vehicle, the favourable ratio of genetic to physical distance around the immediate area of Yd_2 in the barley inferred from our physical mapping in Chapter 5 and a newly available barley BAC library, it was decided to proceed with a map-based effort in barley and not rice. If time and resources allowed, the most favourable situation would be to undertake both map-based cloning in rice and barley simultaneously.

CHAPTER 7

**THE IDENTIFICATION AND CHARACTERISATION OF LARGE
INSERT BARLEY GENOMIC CLONES FROM THE Yd_2 REGION
OF BARLEY CHROMOSOME 3**

7.1 Introduction

Physical mapping and chromosome walking in complex genomes requires large insert DNA libraries that can be rapidly constructed, easily screened and manipulated, and are relatively free of chimeras (Martin, 1998). Large insert DNA libraries have been constructed in bacteriophage, cosmid and yeast artificial chromosome (YAC) vectors. However, the insert size of bacteriophage and cosmid libraries are limited by the phage particle size and have a maximum practical limit of 17kb (λ), 46kb (cosmid) and 100kb (P1) (Woo *et al.*, 1994).

YAC libraries, were originally introduced by Burke *et al.* (1987) and revolutionised the analysis of complex genomes. The cloning capacity of YACs appears to be limitless and primarily depends on the size and integrity of the insert DNA. In recent years, YAC libraries have been constructed from human (Burke *et al.*, 1987), mouse (Chartier *et al.*, 1992) and plant species, such as *Arabidopsis* (Grill and Somerville, 1991), tomato (Martin *et al.*, 1992), maize (Edwards *et al.*, 1992), barley (Kleine *et al.*, 1993) and japonica rice (Umehara *et al.*, 1995). These libraries have made valuable contributions towards the production of physical maps and the isolation of many important genes (Arondel *et al.*, 1992; Giraudat *et al.*, 1992; Leyser *et al.*, 1993; Martin *et al.*, 1993). The primary advantage of using YACs is their large insert size which is important for chromosome walking applications. However the large percentage of chimeric (Libert *et al.*, 1993; Umehara *et al.*, 1995) and unstable (Dunford *et al.*, 1993; Schmidt *et al.*, 1994) YAC clones, and the tedious steps in the manipulation and isolation of YAC insert DNA have limited the usefulness of these libraries.

Recently, Shizuya *et al.*, (1992) used a bacterial artificial chromosome (BAC) system to clone large DNA fragments from the human genome. This system utilises an F-factor-based-vector and is capable of maintaining human genomic fragments of > 300kb. In contrast to YAC cloning, the BAC system allows the DNA to be cloned with high efficiency, manipulated easily and is stably maintained in *Escherichia coli*. Therefore, the application of the BAC cloning system to plant species should greatly facilitate map-based cloning efforts (Martin, 1998).

Recently, a number of comprehensive BAC libraries have become available for public use (<http://www.genome.clemson.edu>; <http://hbz.tamu.edu>). Given the problematic manipulation of YAC insert DNA for the generation of end-specific clones and the potential problem of synteny breakdown between rice and barley (Section 6.4.1; Kilian *et al.*, 1995; 1997), it was decided to try to identify a genomic clone(s) from barley by screening a barley BAC library using the DNA probes BCD134, YLM and Y1p. This chapter describes the identification and characterisation of a series of overlapping barley BAC clones from the *Yd*₂ region of barley Chromosome 3.

7.2 Materials and Methods

7.2.1 Acknowledgments

The BAC filters and BAC clones supplied by Rod Wing and his staff at the Clemson University Genomics Institute (U.S.A) are gratefully acknowledged. Runzhi Li a visiting scientist from China is thanked for his contribution to the work described in this chapter including some plasmid preparations and Southern analysis.

7.2.2 Purification of DNA probe template

DNA probe template used for BAC hybridisation was extracted from *E.coli* by plasmid miniprep (Section 2.2.4) followed by restriction enzyme digestion (Section 2.2.15) to release the DNA insert which was then gel purified using a Bresaspin gel extraction kit (Geneworks, Australia). Care was taken to ensure that no vector sequence remained attached to the probe template which would result in cross-hybridisation with the vector sequence of the BAC clones.

7.2.3 Screening a barley BAC library

Filters of a 6.5 genome equivalent barley BAC library constructed from the non-*Yd*₂ cultivar Morex were obtained from the Clemson University Genomics Institute. BAC filters were prehybridised in 30ml of hybridisation buffer (160ml of 1M sodium phosphate pH 7.2 buffer,

112ml of 20% SDS, 0.6ml of 0.5M EDTA, 330 μ l of 10mg/ml of denatured salmon sperm and 27ml of MQH₂O) at 65°C overnight. The prehybridisation solution was discarded and fresh hybridisation solution containing denatured probe was added and incubated for 24hrs at 65°C. Filters were washed in the bottle twice with 2 \times SSC, 0.1% SDS at room temperature. Radioactive counts were measured to check signal strength before subsequent washing down to 0.5 \times SSC at 50°C. Subsequent washes were undertaken until membrane signal strength was no greater than 15 counts/second but not less than 10 counts/second. After washing, filters were wrapped in saran wrap and exposed to Konica X-ray film with an intensifying screen at -80°C for a minimum of 10 days to ensure that background (non-specific hybridisation) was sufficiently strong enough to help correctly address positive BAC signals. Radioactive ink was used to make alignment spots to help with the orientation and identification of the autoradiographs of the BAC membranes. BAC addresses (positions on the filter) were identified according to the instructions found at the Clemson University Genomics Institute homepage (<http://www.genome.clemson.edu>).

7.2.4 Growth of BAC clones

Clones were received as stab cultures and immediately grown in a liquid LB broth (Section 2.2.2) containing 25 μ g/ml of the antibiotic chloramphenicol.

7.2.5 Mini-preparation of BAC plasmid DNA

Individual BAC clones were grown as described in Section 7.2.4 in 10ml yellow cap tube O/N at 37°C in a rotating wheel. The culture was poured into an eppendorf tube and the cells pelleted by centrifugation for 3min at 14,000rpm. The supernatant was discarded and the cell pellet resuspended in 90 μ l of TES (25mM Tris-HCl pH8.0, 10 mM EDTA, 15% w/s sucrose) buffer by vortexing. One hundred and eighty μ l of lysis solution (0.2M NaOH, 1% SDS) was added and the mixture gently mixed by inversion with no vortexing. One hundred and thirty five μ l of 3M NaOAc, pH4.6 was added and gently mixed by inversion. The bacterial chromosome DNA was pelleted by centrifugation at 14,000rpm for 15min. The supernatant was removed to a fresh tube and 2 μ l of 10mg/ml Rnase A added and incubated at 37°C for 1hr. Four hundred μ l of Tris-

saturated phenol and 400 μ l of chloroform were added, and mixed by inversion. Samples were centrifuged at 14,000rpm for 5min and the supernatant extracted. BAC DNA was then precipitated using 2-3 volumes of -20°C absolute ethanol for 10min at room temperature. The DNA was pelleted at 14,000rpm for 15min, washed with 500 μ l of 70% ethanol, and resuspended in 20-50 μ l of TE (10mMTris-HCl, 1.0mM EDTA) buffer. DNA was stored at 4°C prior to subsequent analysis.

7.2.6 Dot-blot analysis of BAC clones

To confirm positive identification of BAC clones obtained from Clemson University, dot-blot analysis was performed on all BAC clones received. Ten μ l samples of purified BAC DNA were incubated at 94°C for 5min then put on ice. Five μ l samples were spotted on to Hybond N⁺ membrane and left to air dry for 10min. The DNA was cross-linked to the nylon membrane using 125mJ of U.V. light using a Biorad GS gene linker. The membranes were washed 3 times in $2\times\text{SSC}$ and then used for hybridisation. ^{32}P labelled probe preparation, hybridisation, and autoradiography were as described in Sections 2.2.11 and 2.2.12.

7.2.7 Confirmation that barley BAC clones are linked to the *Yd₂* locus

BAC clones were confirmed as linked to *Yd₂* by either PCR or Southern analysis. PCR amplification of the cleaved amplified polymorphic sequence (CAPS) for the *Ylp* locus and the co-dominant *YLM* marker was performed on BAC DNA (approx 0.1 μ g) and on the cereal genomic DNA Atlas, Atlas68 and Morex as described in Section 3.2.6. BAC and cereal genomic DNA to be analysed for the *Ylp* and the *Xbcd134* loci by Southern analysis was digested with the restriction enzyme *Nco*I. The cultivars Atlas, Atlas68 and Morex which were used as cereal genomic DNA controls were grown as described in Section 2.2.1. Cereal genomic DNA extractions were performed on these lines as described in Section 2.2.9. Southern Analysis was performed as described in Section 2.2.10, except that only 0.2 μ g of BAC DNA was digested and run along side of 5.0 μ g of restricted genomic cereal DNA because of the much greater proportion of target DNA in the BAC clones.

7.2.8 Screening BAC clones from the *Yd₂* region with markers closely linked to *Yd₂*

In order to screen all BAC clones identified from the *Yd₂* region with 10 DNA probes (Table 7.1) identifying RFLPs closely linked to *Yd₂*, multiple Southern membranes of BAC clones digested with *HindIII* were made. Cereal genomic DNA extractions were performed on the barley lines Atlas, Atlas68 and Morex as described in Section 2.2.9. Southern Analysis was performed as described in Sections 2.2.10-2.2.12.

7.2.9 Pulsed field gel electrophoresis

BAC clones linked to the *Yd₂* locus were sized using PFGE as described in Section 5.2.6. To size the DNA insert 15µg of purified BAC was first digested with *NotI* to release the insert and linearise the DNA. All PFGs were run for 22hrs at 200volts with a linear ramp of 5-20sec.

7.2.10 Southern fingerprint analysis of BAC clones

Ten µg of purified BAC DNA was digested with *HindIII* to release the BAC vector and digest barley DNA inserts so the BAC clones could be compared for common restriction fragment bands of the same molecular weight using Southern analysis. A common restriction fragment infers an overlap between two or more BAC clones. Southern transfers were performed as described in Section 2.2.10. Membranes were hybridised with radiolabelled BAC template (Section 7.2.11). Double restriction enzyme digests were performed on BAC clones identified by different DNA markers (e.g Ylp and BCD134) (*HindIII*, being the initial restriction digest) to confirm that any common restriction fragments were not just co-migrators of similar size but were identical. The following restriction enzymes were used for the second digest, *AluI*, *BamHI*, *EcoRI*, *EcoRV*, *HpaI*, *NcoI*, *PstI*, *PvuII*, *XhoI*.

7.2.11 Preparation of ³²P-labelled total BAC DNA probes

Total BAC template was oligolabelled using a modification of the method described by Feinberg and Vogelstein (1983) (Section 2.2.11). Purified BAC DNA (200 ng) nicked with 60mJ of U.V.

Table 7.1 DNA probes linked to Yd_2 which were hybridised to barley BAC clones that were identified as being from the Yd_2 region

AWPH7D
C122
BCD828
BCD1380
KsuA3
KsuH7
MWG802
MWG952
MWG985
MWG2065
WG889

light was combined with 9.0µl random sequence 9-mer oligonucleotide (0.3mg/ml) and the mixture incubated at 95°C for 5min to denature the DNA. The mixture was cooled on ice for 5min and combined with 14µl probe labelling buffer (0.5M HEPES, 0.125M Tris-HCl, 12.5mM DTT, 12.5mM MgCl₂, 1.0mg/ml BSA) 4µl dNTP mixture (0.2mM dATP, 0.2mM dTTP, 0.2mM dGTP), 10U Klenow fragment, 5.0µl α-³²P-dCTP (10µCi/µl), MQH₂O was added to a total volume of 35µl. To increase the specific activity of the probe for Northern analysis 5.0µl of α-³²P-dATP (10 µCi/µl) was substituted for cold dATP. Probe labelling reactions were incubated at room temperature overnight and any unincorporated dNTPs removed as described in Section 2.2.11.

7.2.12 Northern analysis of infected and noninfected resistant and susceptible barley cultivars, with BAC clones from the *Yd*₂ region as total genomic DNA radiolabelled probes

Atlas and Atlas 68 seedlings were infected at the two leaf stage as described in Section 3.2.3. Total RNA extractions (Section 2.2.13) were performed from shoot and root material 5 days after the end of the inoculation period and from noninfected control plants at the same stage of development. Northern gel electrophoresis, blotting and hybridisation was performed as described in Section 2.2.14. Total BAC genomic ³²P radiolabelled DNA of two BAC clones, one positive for the DNA probe Ylp (B22), the other positive for the probe BCD134 (B20), were used to probe the membranes. After washing, membranes were exposed to Kodak double emulsion BIOMAX MS film with a BIOMAX MS intensifying screen and exposed for 72hrs at -80°C.

7.3 Results

7.3.1 The identification of BAC clones that hybridise to the DNA probes BCD134, YLM and Ylp

Three DNA probes were used to screen seventeen BAC filters representing a 6.5 genome equivalent library from the barley cultivar Morex (*Yd*₂-minus). Filters were firstly hybridised

with the Ylp probe which detects the RFLP loci, *Ylp*, which co-segregates with *Yd₂*. Four strong positive BAC clones were identified. The BAC filters were then hybridised with the DNA probes BCD134 and YLM, simultaneously. These probes detect two loci *Xbcd134* and *YLM* which flank each side of the *Yd₂* gene (Figure 4.1). Seventeen very strong to medium hybridisation signals as well as numerous weaker signals were detected (Figure 7.1). Using two probes simultaneously increased the background of the autoradiograph making it easier to correctly address strongly hybridising signals. The twenty-one strong and medium strength hybridisation signals resulting from all three probe hybridisations were addressed (BAC positions identified) and ordered from Clemson University Genomics Institute. The clones adjacent to the BAC clones identified were also ordered where the exact position of the clones identified was uncertain. A total of 36 clones were ordered.

The 36 BAC clones were received as stab cultures and numbered according to their position in the microtitre plate (1-96). Clones were then plated, picked as single colonies, grown and BAC DNA purified from each of the 36 clones. Dot-blot analysis was performed using the three DNA probes BCD134, YLM and Ylp. Five clones were identified as strongly hybridising to BCD134, 6 clones hybridised strongly to YLM and 2 clones hybridised to Ylp (Figure 7.2). Therefore, 2 of the 4 positives were obtained from the initial BAC membrane screening with Ylp, and 11 of 17 positives were obtained from the second screening with both the DNA probes BCD134 and YLM.

7.3.2 Confirmation that the BAC clones strongly hybridising to the three DNA probes are linked to *Yd₂*

Thirteen clones hybridised strongly to the three DNA probes used to identify them from the barley BAC library screen. However, positive identification that these clones contained loci linked to *Yd₂* was needed and therefore, PCR primers developed to specifically amplify alleles linked to *Yd₂* and Southern analysis were used to confirm that these strongly hybridising BAC clones encompassed loci linked to *Yd₂*. DNA template from the susceptible and resistant cultivars, Atlas and Atlas 68, were used as genomic controls. Template from the cultivar Morex

Figure 7.1 Screening bacterial artificial chromosome (BAC) filters with DNA probes closely linked to *Yd₂*

In order to identify large insert genomic clones linked to *Yd₂*, 17 BAC filters representing 6.5 genome equivalents of the barley genome were screened with the DNA probes Ylp, BCD134 and YLM. A number of hybridisation signals in the correct duplicate pattern were identified. The filter positions (BAC address) of the strong hybridisation signals were addressed and these clones were ordered from Clemson University. Those of weaker intensity were ignored. An example of the hybridisation of the two probes BCD134 and YLM, simultaneously, to a single BAC filter is shown opposite. A single strong hybridisation signal in the correct duplicate pattern and a number of weaker signals were identified. The strong hybridisation signal is denoted by (S) and some of the weaker signals denoted by (W).

BCD134 and YLM Hybridisation

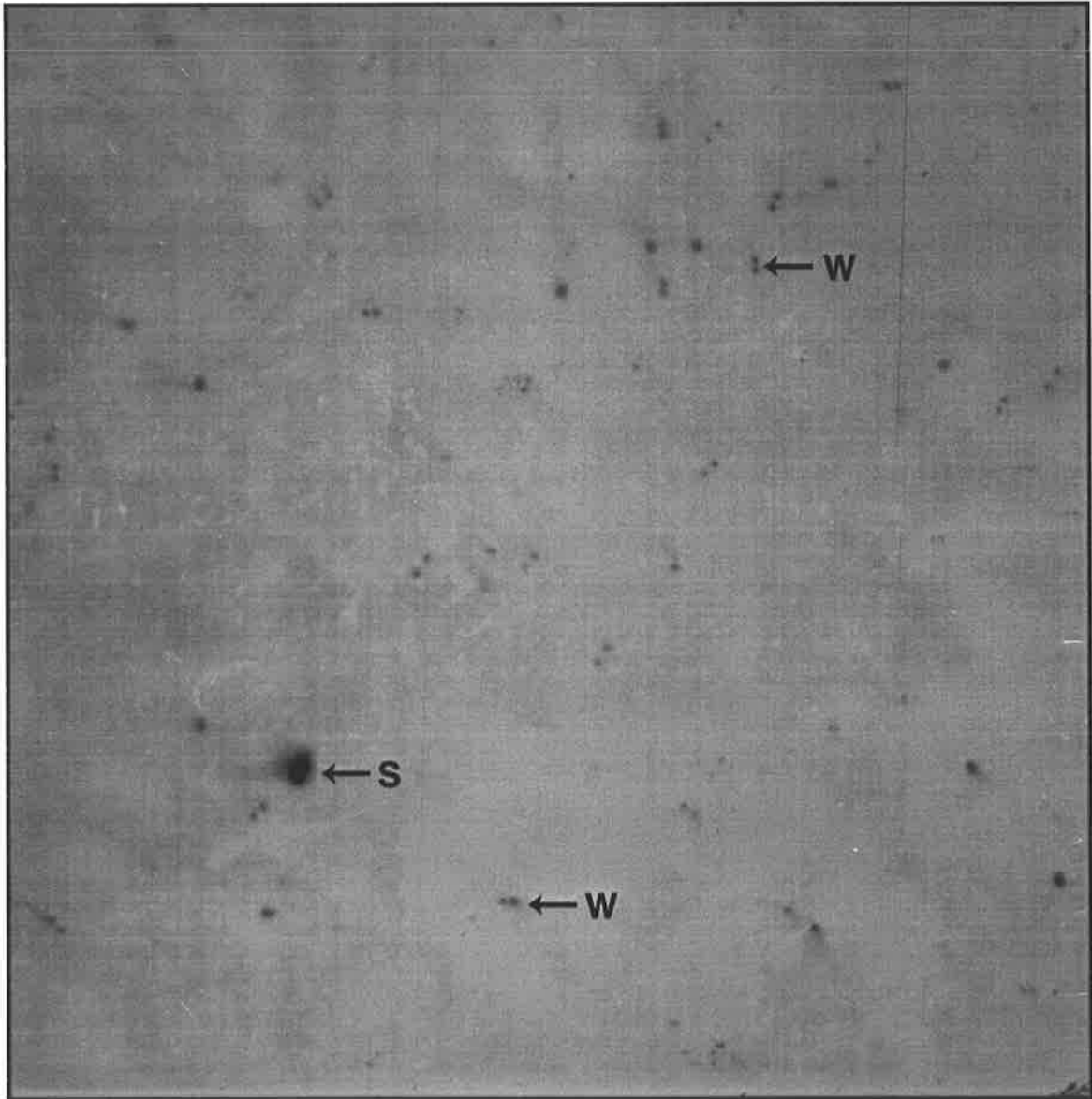
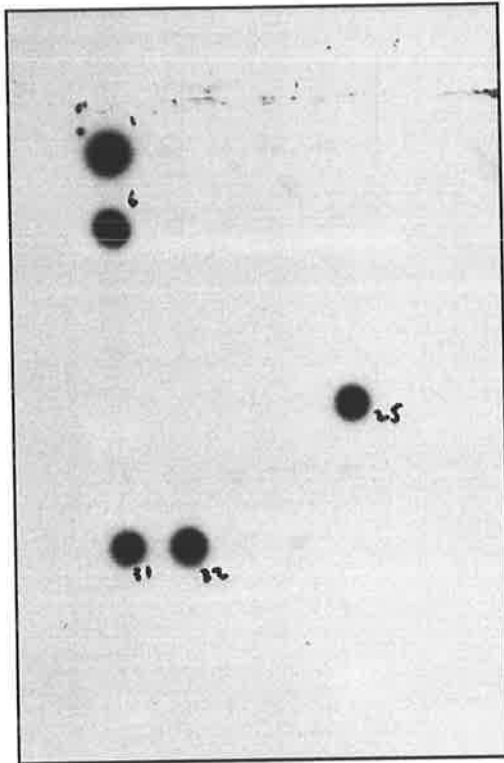


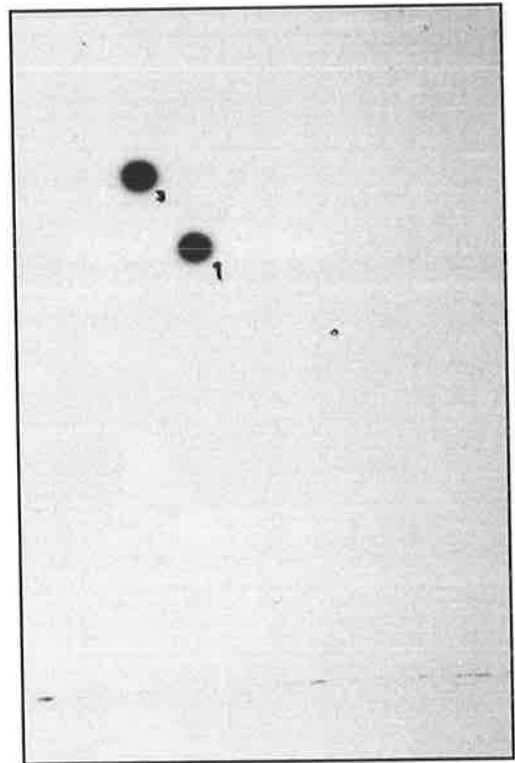
Figure 7.2 Dot-blot-analysis of BAC clones

Plasmid DNA extractions of 36 BAC clones was performed (Section 7.2.5). BAC DNA was spotted onto triplicate nylon membranes and dot-blot analysis performed (Section 7.2.6). Three separate hybridisations were performed to identify clones strongly hybridising to the DNA probes (a) BCD134, (b)Ylp and (c)YLM. After washing, membranes were subjected to autoradiography at -80°C for 16 hours. The 5 clones which strongly hybridised to BCD134, the 2 clones to Ylp and the 6 clones strongly hybridising to YLM are indicated.

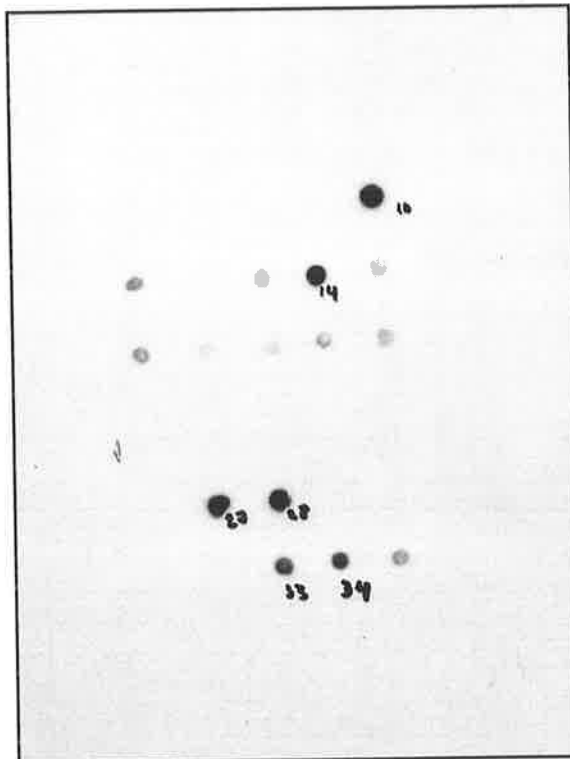
(a) BCD134 Hybridisation



(b) Ylp Hybridisation



(c) YLM Hybridisation



was also used as a genomic control as the barley BAC library had been constructed from this cultivar.

The co-dominant *YLM* PCR marker amplified the correct susceptible associated allele of the expected size from 4 of the 6 barley BAC clones (Figure 7.3-a) shown to hybridise strongly to *YLM* DNA probe (Figure 7.2). Therefore, all 4 BAC clones are linked to the *Yd₂* region of barley Chromosome 3. The genomic insert of the two BAC clones which failed to amplify a product were considered to be unlinked to the *Yd₂* locus. PCR amplification of the cleaved amplified polymorphic sequence (CAPS) for the *Ylp* locus identified the correct susceptible associated allele of the expected size from both BAC clones which had been identified by hybridisation with the *Ylp* DNA probe. In addition, Southern analysis of these BAC clones identified the same size restriction fragment with the *Ylp* DNA probe as that of the genomic controls, confirming these BAC clones encompassed the *Ylp* loci, and were linked to the *Yd₂* locus (Figure 7.3-b). Four of the 5 BAC clones identified with the DNA probe BCD134 (Figure 7.2) were shown by Southern analysis to have the same size restriction fragment as the genomic controls. Therefore, these 4 BAC clones encompass the loci *Xbcd134* which is closely linked to *Yd₂*. However, one of the five BAC clones, showed a restriction fragment of smaller molecular weight, which may be because this loci is positioned closer towards the end of the clone resulting in the loss a *HindIII* site and the generation of a smaller than expected restriction fragment. Fingerprint analysis of this BAC (Section 7.3.4) was undertaken to determine whether it was from the same region as the other 4 BAC clones which encompass the *Xbcd134* locus.

7.3.3 Size of the BAC clones from the *Yd₂* region

BAC clones identified from the *Yd₂* region were sized by PFGE (Figure 7.4). The restriction enzyme *NotI* was used to linearise the DNA and release the insert from the vector to enable the insert BAC DNA to be sized. All but one of the positive BAC clones contained an insert of 100kb or greater (Table 7.2). The restriction enzyme *NotI* did not cut the insert of any of the clones encompassing the *Ylp* or *Xbcd134* loci. However, *NotI* did cut the insert of one of the BAC clones encompassing the *YLM* loci several times. The size of this BAC was estimated by the addition of the molecular weights of the separate PFGE fragments. The average size of the

Figure 7.3 The identification of BAC clones linked to the *Yd₂* locus

Clones which strongly hybridise to the three DNA probes which detect markers closely linked to the *Yd₂* locus were shown to be from the correct locus by PCR or Southern analysis.

(a) Primers designed to specifically amplify the *YLM* locus were used for PCR with the templates of the six barley BAC clones shown to hybridise strongly to radiolabelled template of the amplified *YLM* product (Section 7.2.7). PCR products were separated on a 1×TBE 1% agarose gel. A product of the expected size (101bp) was amplified from the template of four of the six barley BAC clones (**B42**, **B53**, **B72** and **B80**) indicating they encompassed the *YLM* locus and were linked to *Yd₂*. Template from two BAC clones (**B93**, **B95**) failed to amplify a product of the correct size suggesting they were not linked to *Yd₂*. Resistant(Atlas68, **At68**) susceptible (Atlas, **At**) and no template (-ve) controls were included. The susceptible cultivar Morex (**Mx**) (*Yd₂*-minus) was also included as a control since the BAC library was constructed from this cultivar. The marker lane (**M**) contains pUC19 digested with *HpaII*.

(b) BAC DNA **B22** and **B37** and cereal genomic DNA of the cultivars Atlas68 (**At68**), Atlas (**At**), and Morex (**Mx**) were digested with the restriction enzyme *NcoI* and a Southern blot analysis performed. The marker (**M**) SPPI cut with *EcoRI* was used to size the restricted DNA. The *Ylp* DNA probe identified common restriction fragments between the BAC clones **B22** and **B37** and all three barley cultivars. Therefore, BAC clones B22 and B37 encompass the *Ylp* loci which is closely linked to the *Yd₂* gene.

(a) YLM PCR

M -ve At68 At Mx B42 B53 B72 B80 B93 B95



101bp
90bp

(b) Ylp Hybridisation

M At68 At Mx B22 B37

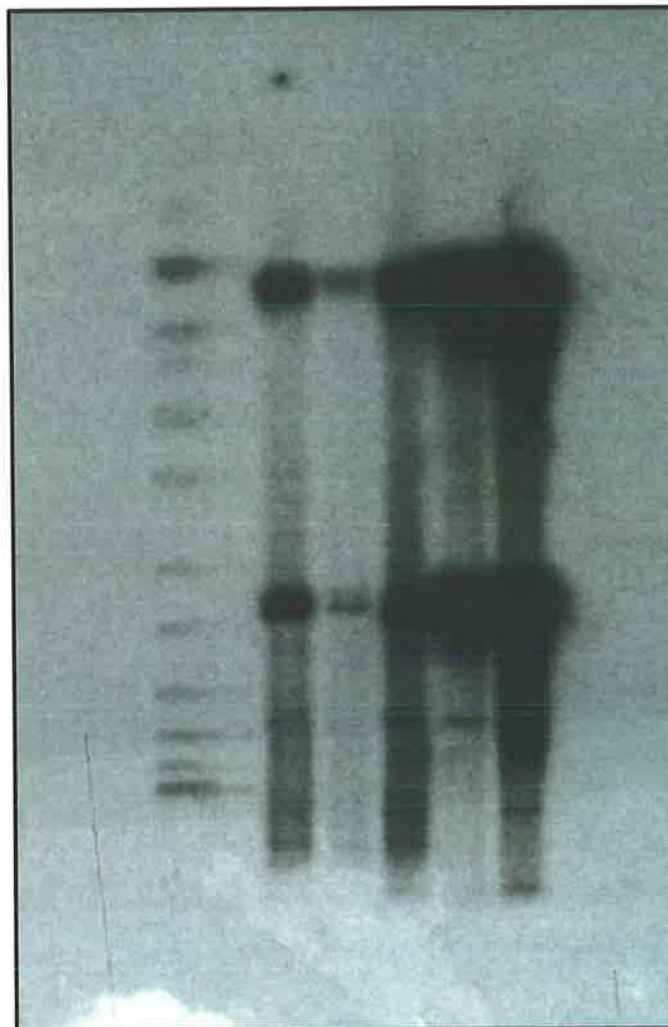


Figure 7.4 PFGE of the BAC clones positive for the *Ylp* locus

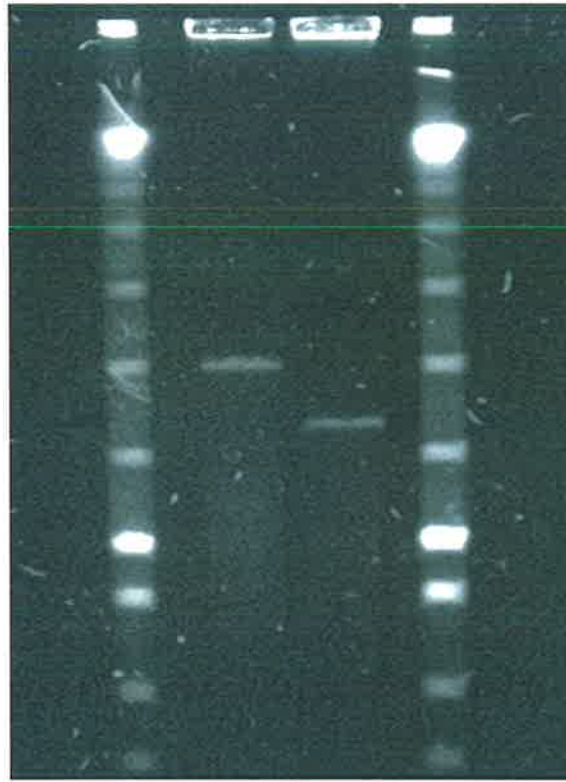
(a) An alkaline midiprep as described in Section 7.2.5 was used to purify the BAC plasmid from clones **B22** and **B37**, both encompassing the *Ylp* locus, which co-segregates with *Yd₂*. Plasmids were digested with *NotI* to release the barley genomic insert for sizing by PFGE. Digested samples were run on a 0.5×TBE 1% agarose gel for 22 hours at 200 volts. A linear ramp of 5-20 seconds was used to resolve the DNA. A low range lambda concatemer ladder (**M**) was also included to size the DNA. Alkaline transfer was performed on the PFG and the membrane used for hybridisation.

(b) The resultant membrane was hybridised with the DNA probe *Ylp*. **B22** was sized at approximately 150kb while **B37** was sized at approximately 115kb.

(a)

M BAC22 BAC37 M

194.0kb →
145.5kb →
97.0kb →
48.5kb →



(b)

BAC22 BAC37

150kb →
115kb →



Table 7.2 Size of the genomic inserts of the barley BAC clones from the *Yd₂* region

BAC clones	Loci encompassed	Insert size (kb)
BAC22	<i>Ylp</i>	150
BAC37	<i>Ylp</i>	115
BAC20	<i>Xbcd134</i>	115
BAC27	<i>Xbcd134</i>	98
BAC69	<i>Xbcd134</i>	98
BAC84	<i>Xbcd134</i>	100
BAC87	<i>Xbcd134</i>	98
BAC42	<i>YLM</i>	100
BAC53	<i>YLM</i>	60
BAC72	<i>YLM</i>	100
BAC80	<i>YLM</i>	100

barley BAC library is 110kb (<http://www.genome.clemson.edu>) and the average size of the BAC genomic inserts we obtained for 11 clones from the *Yd₂* region was 104kb.

7.3.4 Southern fingerprint analysis of BAC clones linked to *Yd₂*

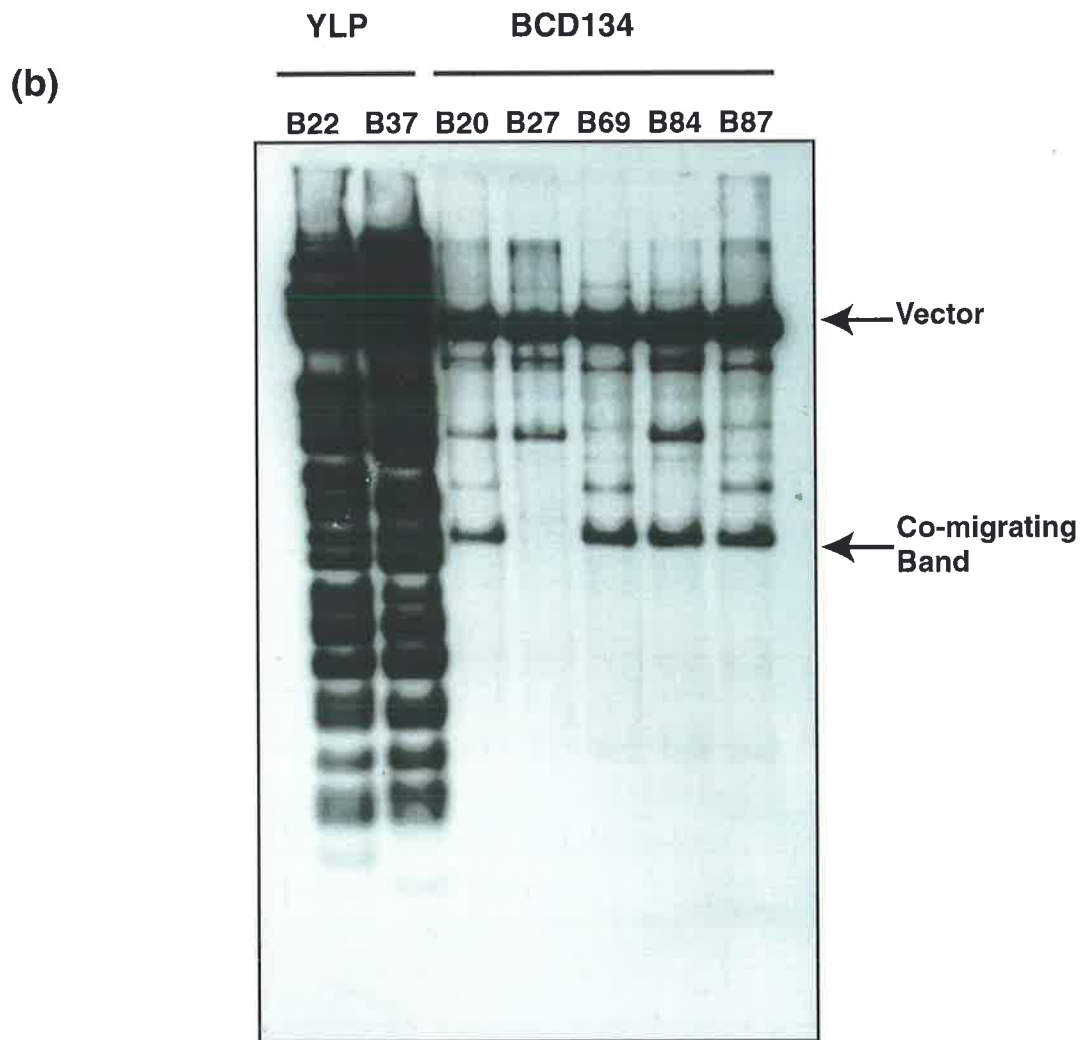
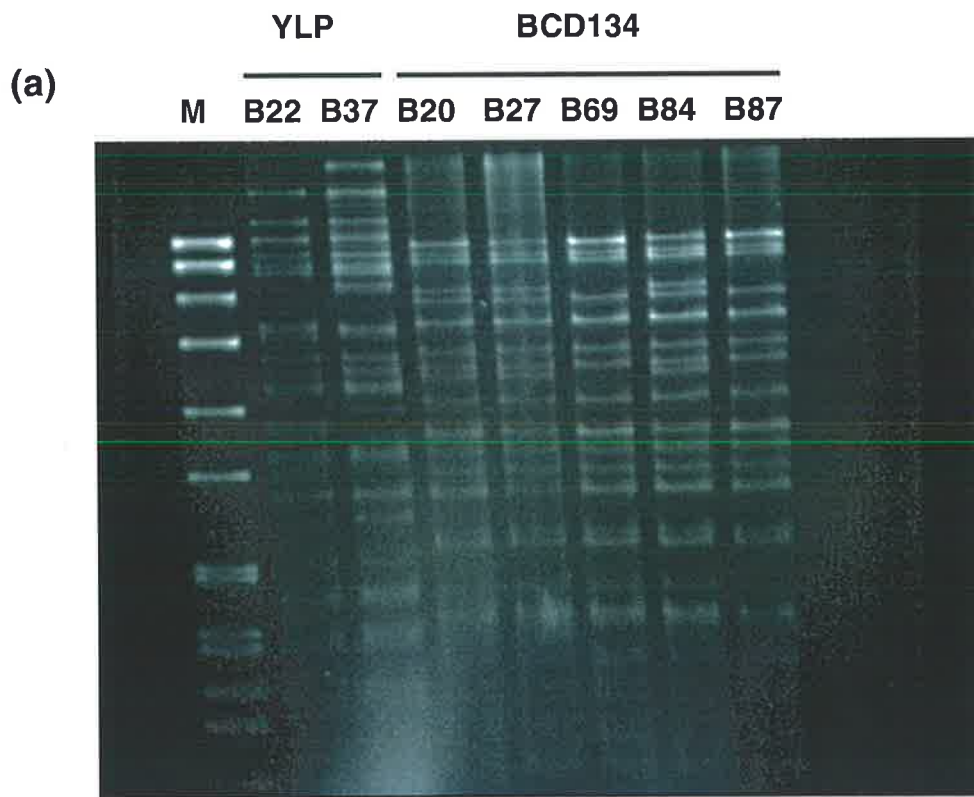
BAC clones encompassing the *Yd₂* region were digested with *Hind*III to release the BAC vector and digest the barley genomic DNA insert. The resulting restriction fragment fingerprint was used to determine the degree of overlap between those BAC clones identified that encompass the same genetic marker as well as any possible overlap between the BAC clones encompassing different genetic markers. BAC clones positive for *Xbcd134* showed almost identical restriction fragment fingerprints suggesting a high degree of overlap. BAC clones 22 and 37 which are positive for the *Ylp* loci showed a high degree of similarity, but the overlap was not as great as the BAC clones that encompass *Xbcd134* (Figure 7.5). However, this was expected as the difference in size between the two genomic inserts of the clones positive for *Ylp* is 35kb (Table 7.1).

Radiolabelled BAC DNA from the two BAC clones positive for *Ylp* was used as the hybridisation probe for a Southern membrane made from BAC DNA positive for *Xbcd134* and *Ylp* which had been digested with *Hind*III (Figure 7.5). This probe detected a common restriction fragment between 4 of the 5 BAC clones positive for *Xbcd134* and the two BAC clones positive for *Ylp*. To ensure that the restriction fragment was identical between the BAC clones and not a co-migrator of similar size a number of double restriction digests were performed. The restriction enzymes *Bam*HI, *Eco*RI, *Eco*RV, *Nco*I, *Pst*I, and *Pvu*II did not restrict this fragment in either of the BAC clones identified by BCD134 or *Ylp*. However, the restriction enzymes *Alu*I, *Hpa*I, and *Xho*I digested the fragment into the same number and sized fragments for all the BAC clones identified by these probes. Thereby, confirming the restriction fragment is identical in each of these BAC clones. This data also confirms an overlap between the BAC clones carrying genetically distinct markers and provides compelling evidence that the physical linkage between *Ylp* and *Xbcd134* is less than 200kb supporting the physical mapping data described in Chapter 5.

Figure 7.5 Southern fingerprint analysis of BAC clones encompassing the *Ylp* and *Xbcd134* loci

(a) BAC clones encompassing the *Ylp* and *Xbcd134* loci were digested with *Hind*III to release the BAC vector (pBeloBAC11) and digest the barley DNA insert. Restricted DNA was resolved on a 1×TAE 1% agarose gel. The marker (M) SPPI cut with *Eco*RI was used to size the restricted DNA. The resulting restriction digestion patterns (restriction fingerprints) provided an estimate of the similarity between those BAC clones containing the same genetic marker. BAC clones positive for *Xbcd134* showed almost identical restriction fingerprints suggesting that all five BAC clones had a high degree of overlap. While BAC clones 22 and 37 also showed a high degree of similarity, it was not as great as that between the BAC clones positive for *Xbcd134*.

(b) Alkaline transfer was performed on the PFG and the membrane hybridised with radiolabelled total BAC DNA from **B22** and **B37**. The radiolabelled BAC DNA hybridised strongly to both **B22** and **B37** restricted DNA as expected. For BAC clones positive for *Xbcd134* a number of bands including the vector band showed strong hybridisation to the probe template derived from **B22** and **B37**. Four of the five BAC clones positive for *Xbcd134* contained a restriction fragment of the same molecular weight as in the two BAC clones positive for *Ylp*. This demonstrates an overlap between these four BAC clones positive for *Xbcd134* with the two BAC clones positive for *Ylp*, and provides evidence that the physical linkage between *Ylp* and *Xbcd134* is less than 200kb as indicated by physical mapping data produced in Chapter 5.



PFGE and Southern fingerprint analysis of the BAC clones positive for *YLM* showed the degree of overlap between these clones is less than the overlap shared by the clones which encompassed the *Ylp* locus (Figure 7.6). The presence of multiple *NotI* sites in BAC53, and the lack of common restriction fragments in Southern fingerprint analysis, indicates that the overlap between B53 and the other 3 BAC clones positive for *YLM* is minimal. Oligolabelled BAC42 DNA template was used as the probe on for Southern analysis of the BAC DNA positive for *YLM* and *Ylp*, which had been digested with *HindIII*. No common bands were detected between the BAC clones with the genetically distinct markers *Ylp* and *YLM*, suggesting the physical linkage between these two genetically distinct loci is not close.

7.3.5 Southern hybridisation of *Yd₂* linked DNA probes to barley BAC clones from the *Yd₂* region for barley Chromosome 3

The 11 BAC clones shown to be from the *Yd₂* region were digested with *HindIII* and Southern analysis performed on duplicate membranes with 10 DNA probes which detect RFLPs closely linked to the *Yd₂* region (Table 7.1). However, none of the probes were found to hybridise to any of these BAC clones.

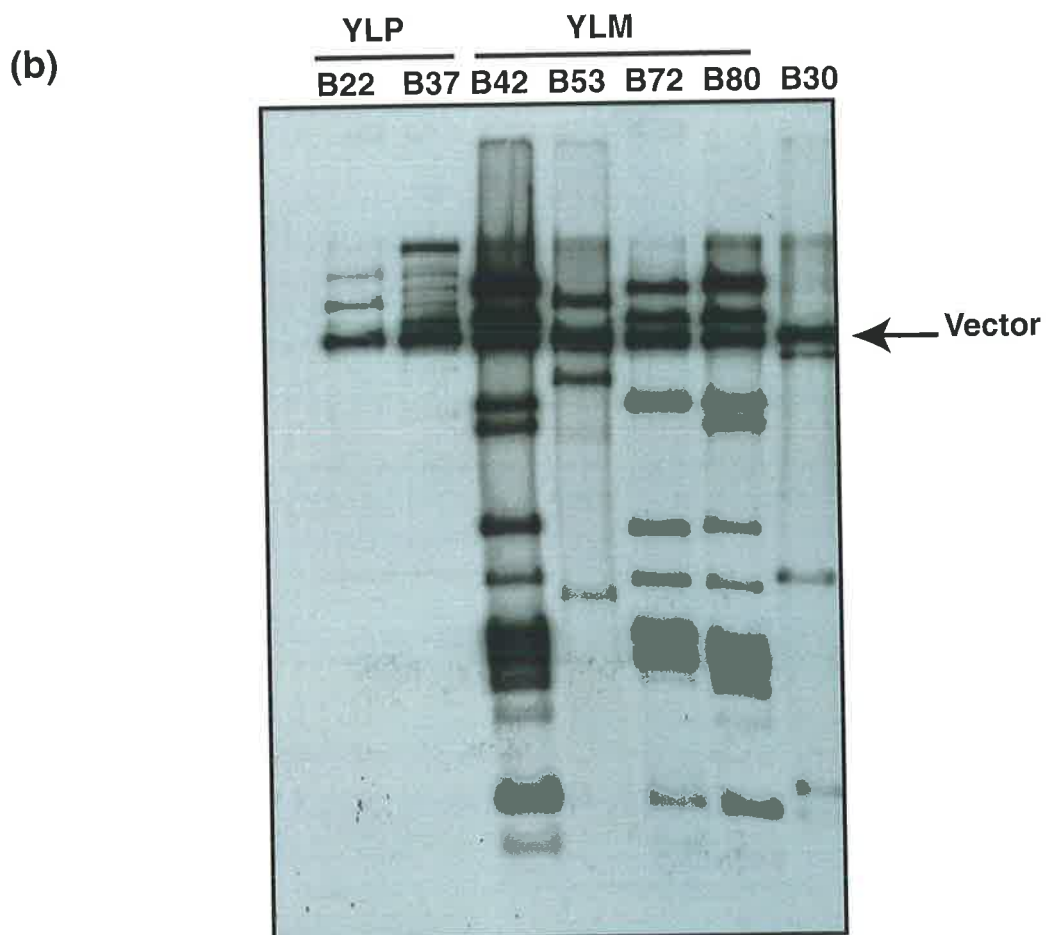
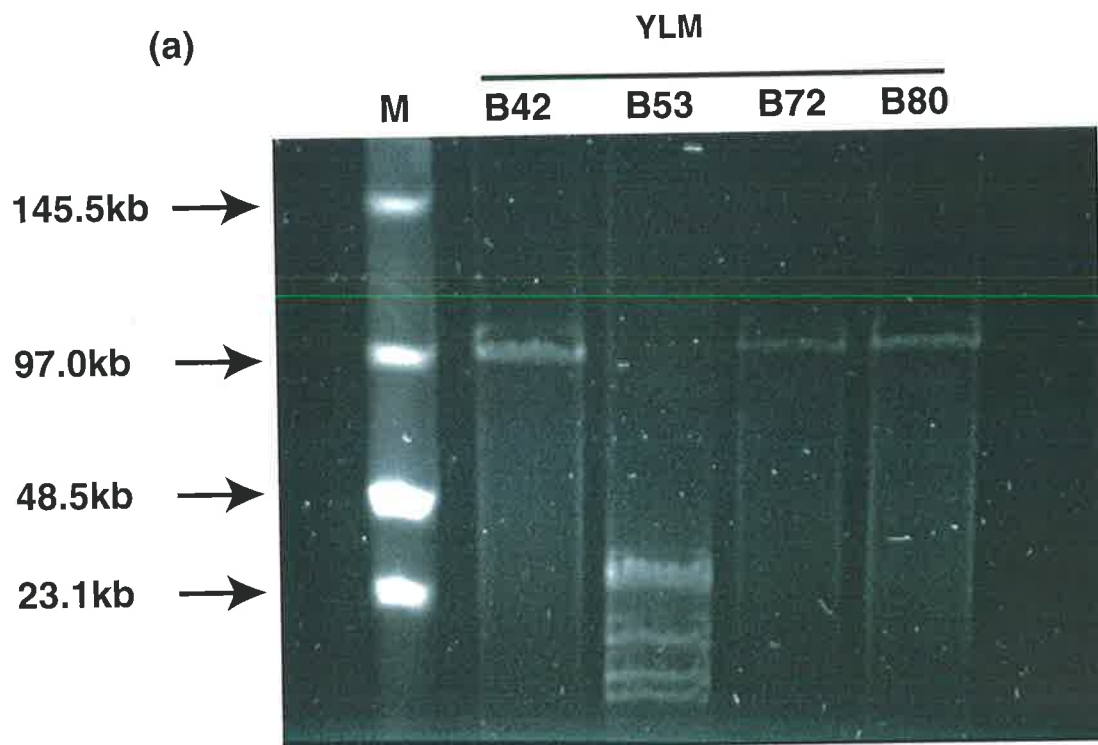
7.3.6 Northern analysis of infected and noninfected resistant and susceptible cultivars using BAC clones from the *Yd₂* region as probes

Radiolabelled BAC DNA from the clones B20 and B22 were used to probe Northern membranes of Atlas and Atlas 68 infected and noninfected root and shoot material. However, no hybridisation signals were detected.

Figure 7.6 PFGE and Southern fingerprint analysis of the four BAC clones encompassing the *YLM* locus

(a) An alkaline midiprep as described in Section 7.2.5 was used to purify the BAC plasmid from clones **B42**, **B53**, **B72** and **B80** which encompass the *YLM* locus. Plasmids were digested with *NotI* to release the barley insert for sizing by PFGE. Digested samples were run on a 0.5×TBE 1% agarose gel for 22 hours at 200 volts. A linear ramp of 5-20 seconds was used to resolve the DNA. A low range lambda concatemer ladder (**M**) was also included to size the DNA. **B42**, **B72** and **B80** were sized just under 100kb, while **B53** which contained multiple *NotI* sites within the insert, was under 50kb in size.

(b) DNA from the clones **B42**, **B53**, **B72** and **B80** was digested with *HindIII* to release the BAC vector and digest the barley insert DNA. The two BAC clones **B22** and **B37** positive for *Ylp*, were included in the fingerprint analysis to detect any common bands between the BAC clones containing genetically distinct markers which would infer a physical overlap. A non-positive BAC, **B30**, was included as negative control. Restricted DNA was resolved on a 1×TAE 1% agarose gel. Alkaline transfer was performed and the membrane hybridised with radiolabelled total BAC DNA from **B42**. The membrane was subjected to autoradiography at room temperature overnight. While **B42**, **B72** and **B80** showed similar restriction fingerprints, a number of restriction fragments present in these clones were absent in **B53**, which along with the presence of multiple *NotI* sites in **B53** {above in part (a)} indicates that the overlap between **B53** and the other 3 BAC clones positive for *YLM* is minimal. No bands appeared to be common between the BAC clones with genetically distinct markers *Ylp* and *YLM*, suggesting that physical linkage between the two loci is not close.



7.4 Discussion

7.4.1 The identification of large insert genomic clones from the *Yd₂* region of barley Chromosome 3

In order to begin a chromosome walk in the *Yd₂* region of barley with the eventual aim of constructing a series of overlapping clones forming a contig encompassing the *Yd₂* gene, three DNA probes *Ylp*, BCD134 and *YLM* which detect loci closely linked to the *Yd₂* region in barley were used as hybridisation probes to identify large insert genomic clones from the *Yd₂* region. The DNA probe *Ylp* was chosen as a hybridisation probe as it detects the *Ylp* loci shown to co-segregate with the *Yd₂* gene (Section 4.1). The DNA probe BCD134 detects the loci *Xbcd134*, which is physically linked to *Ylp* (Chapter 5), and separated by 1 recombination event proximal to *Yd₂* in the Proctor × Shannon mapping population, and is seen as physically close enough to begin a chromosome walk from the proximal side of *Yd₂*. The DNA probe *YLM* was also used to identify large insert clones from the *Yd₂* region. Although the physical information described in Chapter 5 suggested that *YLM* may be too far from *Yd₂* to begin a chromosome walk from this direction. *YLM* is the closest marker distal to *Yd₂* and remains our closest starting point distal to *Yd₂*. Secondly, there is the possibility that *YLM* may be closer than expected due to repetitive nature of the *YLM* probe and a possibility that the locus identified in physical mapping was unlinked to *Yd₂*.

While the hybridisation of the DNA probes *Ylp*, BCD134 and *YLM* to the BAC filters detected a number of positive clones, not all positive clones identified and requested were received. This problem has also been encountered by a number of other researchers who have used this library at our research facility. There are a number of possible reasons for this;

- 1) Some positive hybridisations being incorrectly addressed. However, this is unlikely considering that sufficient care was taken to orientate the autoradiographs to make the identification of positive hybridisations exact. Positive clones along with their neighbouring clones were ordered to decrease the likelihood of addressing an incorrect clone. BAC addressing (location of positive BAC clones on filters) was also done independently by two different

researchers and it is unlikely that exactly the same mistake was made by both researchers resulting in the same incorrect address.

2) Secondly, some positive hybridisations may be false positives. However, this too is unlikely, since all clones are replicated as duplicate dots in each grid in order to avoid such a problem.

3) It is more likely that some error occurred in the gridding or picking of the library. Even though the gridding of libraries are rigorously ordered and involve robotics, errors are not surprising considering the enormous size of these libraries and the enormous demand for them world-wide.

Errors in BAC gridding raise important implications for map-based cloning work. Clones which may encompass the gene of interest or complete contigs covering the area of interest may permanently be lost due to errors in gridding. This could be partially overcome by increasing the size of the libraries and thereby, increasing the chance of finding more clones of interest. However, increasing the size of the libraries would increase the likelihood of errors within libraries. An alternative to library gridding, would be the creation of pools of BAC DNA which could be analysed with PCR primers specific for the target areas. This may help reduce errors and the number of clones permanently lost from genomic libraries.

After individual growth, clones can be arrayed into primary pools which can be combined into superpools for PCR analysis (Green and Olson, 1990; Schibler *et al.*, 1998; Salimath and Bhattacharyya, 1999; Klein *et al.*, 2000). Once a positive superpool is identified, the primary pools can then be analysed to determine which pool or pools contain the sequence of interest. When these primary pool/s are identified, individual BAC clones containing the sequence of interest can be purified from a specific pool by colony hybridisation.

Salimath and Bhattacharyya (1999) constructed a soybean BAC library comprising of approximately 45,000 clones stored in 130 primary pools with approximately 350 clones per pool. For use in PCR analysis, an equal amount of DNA from 6 primary pools was combined to form a "giantpool", and only 22 giantpools need to be initially analysed using PCR. Based on this model a 6.5 genome library of barley consisting of 290,000 clones could be arranged into

approximately 828 primary pools. Six primary pools could then be combined to form 138 superpools, requiring 138 individual PCR reactions to screen for one loci. If further primary pools are included in each superpool then the initial PCR screening would be reduced. Such analysis and pooling may have the advantage over the conventional method of screening large insert genomic libraries where individuals clones are arrayed into microtiter plates and then replicated onto nylon membranes for screening and identification of positive clones. Arraying individual clones of a large-genome library containing a few to several fold genome equivalents into microtiter plates is time-consuming and a laborious procedure (Salimath and Bhattacharyya, 1999).

While pooling BAC clones for PCR analysis may partially overcome the problems involved in BAC library screening, specific primers are needed to analyse complex DNA pools to determine if clones containing the target DNA of interest are present. The more complex the BAC superpools, the less able they are to be analysed using hybridisation techniques. More importantly, pooled DNA libraries may not be as practical to send to individual researchers as are nylon filters of grid arrays replicated by robotic systems. The screening of pools by PCR analysis would undoubtedly be more labour intensive than screening membranes, especially if numerous loci need to be screened. Another important consideration with pooling BAC clones is the potential for the deletion of clones from the library when pools are replicated. Certain clones may be less vigorous under normal growth conditions and may therefore, be out competed by other clones with the end result that they may be lost from the pool after a number of successive replications.

A number of BAC clones were obtained which positively hybridised to one of three probes and were further characterised. While all 7 clones detected by either the DNA probes Y1p and BCD134 were shown by PCR, Southern analysis and BAC fingerprinting to be from the *Yd₂* region, 2 of the 6 clones which hybridised to YLM were not. This is most likely due to the repetitive nature of the YLM probe, which identified other unlinked loci, demonstrating the need to show that clones identified in an initial hybridisation screen must be shown to be from the target region, either by PCR or Southern analysis.

The size of the genomic library (number of genome equivalents) screened determines the probability of identifying a clone(s) with the target sequence. The barley BAC library screened in this work was 6.5 genome equivalents. Therefore, the theoretical probability of isolating a particular DNA sequence is 99.85%. This is calculated using the following formula;

The probability of successfully identifying a BAC clone from any genomic library = $1 - p^x$.

Where p^x = the probability of the failure to identify the target BAC.

$p = 1$ - the probability of successfully identifying a single BAC that contains the sequence of interest from a single independent cloning event. Based on the average insert size of this barley BAC library (110kb) and the haploid genome size of barley (4900Mb), the chance of successfully identifying a single BAC of interest in a single cloning event from the total haploid barley genome is $4900\text{Mb} \div 0.11\text{Mb} = 2.25 \times 10^5$. Therefore $p = 1 - 2.25 \times 10^{-5} = 0.9999775$.

x = the number of clones in the library. For a 6.5 genome equivalent library with an average insert size of 110kb and a haploid genome size of 4900Mb, the number of clones is approximately 290,000. Therefore, $1 - (0.9999775)^{290,000} = 0.9985 = 99.85\%$.

Using this formula a satisfactory genome coverage giving a 95% chance of identifying a clone of interest is 3 genome equivalents [$1 - (0.9999775)^{133635} = 0.95$] However, a 1 genome equivalent library will only give a 63% chance of identifying a clone with the sequence of interest [$1 - (0.9999775)^{44545} = 0.63$]. Screening of this barley BAC library resulted in the identification of 2 clones which encompassed *Ylp*, 5 which encompassed *Xbcd134* and 4 which encompassed *YLM*. Therefore, as expected the loci from the *Yd*₂ region are represented in this library.

7.4.2 Implications for the construction of a contig encompassing the Yd_2 region

The immediate aim of the Yd_2 work was the construction of a contig of overlapping clones over the Yd_2 region. Fingerprint analysis of clones encompassing *Xbcd134* and *Ylp* provided strong evidence of a small overlap between these clones. Therefore, important progress has been made with our first chromosome walk towards the construction of such a contig. The chromosome walk will need to be continued in the region distal to *Ylp* until we have identified a clone(s) which encompass a locus or loci which show genetic recombination from Yd_2 . If we assume recombination in this genetic interval is even, then a further chromosome walk may provide us with a complete contig over the region of interest. Currently our partial contig spans a minimum distance of 213kb. If a further chromosome walk identified a BAC of average insert size (110kb) that completes the contig spanning the gene it would result in a complete contig of approximately 323kb covering the Yd_2 region. However, Buschges *et al.* (1997) showed that the relationship between genetic and physical distance can deviate by one or two orders of magnitude in different regions of the barley genome. Specifically, they found that within an 820 base-pair segment of the *Mlo* gene (which controls powdery mildew resistance in barley) the recombination frequency measured just 25kb/cM, within a 30kb interval spanning *Mlo* the recombination frequency measured 100kb/cM. Therefore, if the relationship between physical and genetic distance distal to Yd_2 is less favourable than estimated, a number of chromosome walking steps may have to be performed.

Chromosome walking with BAC clones has been applied successfully in rice to isolate the genomic regions encompassing the *Pi-ta²* and *xa5* genes, which confer resistance against rice blast and bacterial blight, respectively (Nakamura *et al.*, 1997; Yang *et al.*, 1998). The use of BAC libraries has proven to be very efficient and, unlike YAC libraries (Burke *et al.*, 1987), no chimeric clones have been reported (Shizuya *et al.*, 1992; Wang *et al.* 1995; Zhang *et al.*, 1996). Patocchi *et al.*, 1999 began a chromosome walk starting from two molecular markers flanking the *Vf* resistance gene of apple to the fungus *Venturia inaequalis* using a BAC library. Thirteen BAC clones spanning a region of approximately 500kb between these makers were identified in 9 chromosome walking steps. Therefore, a number of walking steps should not present a problem to the construction of a complete contig over our region of interest.

To identify overlapping clones distal to *Ylp*, end probes will need to be generated from the BAC clones that encompass the *Ylp* loci. The end probes will need to be analysed by Southern hybridisation to test their suitability as hybridisation probes for BAC screening. Once suitable end probes are derived the BAC membranes can be re-screened.

The DNA probe C122 which identifies *Xc122* and co-segregates with the *Yd₂* gene, may be useful in future hybridisation screens to identify the BAC clones from the *Yd₂* region needed to complete a physical contig over the region. Because of cross-hybridisation problems with this probe and BAC vector sequence it was not included in the initial screen. However, regions of the insert of this probe are currently being tested for their suitability for BAC membrane screening, so they can be included in the next BAC library screen.

BAC clones identified using BCD134, YLP and YLM and shown to be linked to the *Yd₂* region did not encompass other loci detected by other DNA probes linked to *Yd₂* (Table 7.1). The physical mapping studies described in Chapter 5 did not identify any other physical linkages between any loci other than *Ylp* and *Xbcd134*, suggesting that these loci may be physically too distant from *Yd₂* to be used for chromosome walking steps and therefore, it is unlikely that any of these probes will be used in screening BAC membranes for future chromosome walks. However, future positive BAC clones identified in chromosome walks by end probes generated from existing BAC clones may be screened against these markers as all but C122, are genetically distinct from *Yd₂* and may help delimit *Yd₂* to a particular physical interval within a series of overlapping large insert genomic clones.

7.4.3 The possibility of a *Yd₂* homologue on the partial contig of the BAC clones encompassing the *Ylp* and *Xbcd134* loci from the *Yd₂* region of barley Chromosome 3

The barley BAC library screened in this study was constructed from the barley cultivar Morex, which is susceptible to BYDV-PAV and does not contain the *Yd₂* gene. Therefore, it was assumed that a *Yd₂* homologue and not the *Yd₂* gene would be present on any contig constructed over this region. While we do not have a complete contig over the *Yd₂* region it is possible that the partial contig of clones encompassing *Ylp* and *Xbcd134* encompass a *Yd₂* homologue. To

address this possibility, Northern analysis of infected and noninfected shoot and root material was carried out, large insert genomic clones from the partial contig were used as hybridisation probes to identify mRNA transcripts which may be candidates for a *Yd₂* homologue. However, no hybridisation signals were detected and there are several possible reasons for this;

1) The *Yd₂* homologue is distal to *Ylp* and is not contained within the partial contig of BAC clones.

2) The large insert genomic clones used as hybridisation probes have a poor relationship of probe DNA to target sequence (mRNA). Therefore, any message present on the Northern membranes may not have been detected. To address this, increased levels of total RNA were loaded into each well of the Northern gels (20µg/lane). Radiolabelled ³²P dATP as well as ³²P dCTP was used when labelling the probe template to increase the specific activity of the probe. However, if the *Yd₂* transcript is not abundant, the signal may still not be detected. The use of large insert genomic clones as total DNA probes, have been successfully used to isolate target gene(s) from cDNA libraries (Bent *et al.*, 1994; Martin, 1998). The construction of cDNA libraries from infected and noninfected resistant and susceptible root and shoot material is being considered. Once such a resource is established, and a series of BAC clones which form the contig covering the gene are identified, these clones may be used as large insert DNA hybridisation probes to isolate cDNAs of possible candidate genes.

3) As the mode of action of *Yd₂* is unknown, the possibility that the *Yd₂* gene may not produce a mRNA transcript in the tissue we are examining at the developmental stage we are using must be considered. Sequence analysis of the complete region may be more informative.

4) An important assumption in our positional cloning strategy is that a *Yd₂* homologue is present in the susceptible cultivar Morex. The possibility that the *Yd₂* gene did not evolve from an orthologous gene of a common ancestral grass species must be considered. Such a gene may have been integrated into the Ethiopian barleys from which it was discovered at a much later evolutionary date. Therefore, no such homologue may exist in conventional barley cultivars. It

may be necessary to identify BAC clones from a library constructed from a barley cultivar where Yd_2 is present. However, to our knowledge no such library yet exists.

7.4.4 Future directions for the map based cloning of Yd_2 in barley and the identification of a candidate gene/s

As previously discussed the next stage in our map-based cloning strategy is the completion of our chromosome walk to obtain a complete contig of overlapping large insert genomic clones spanning the Yd_2 region. Once this has been achieved, we will need to identify genes within this region and determine if they are candidate genes for Yd_2 . A number of different strategies are available to us to investigate the final stage of this map based cloning project. Large genomic insert clones spanning the region may be used to as a hybridisation probes to identify cDNAs from libraries constructed from infected and noninfected Yd_2 and non- Yd_2 barleys. Changes in levels of expression of infected and noninfected Yd_2 and non- Yd_2 cultivars for any expressed sequences identified can then be investigated by Northern analysis. All cDNAs identified would need to show co-segregation with Yd_2 in our mapping population to be considered as candidate genes. Any candidates could then be sequenced and BLAST searched against existing databases to identify putative function.

With the development of efficient sequencing technologies it is also now possible to completely sequence the BAC clones spanning the Yd_2 region of barley. Based on the sequence, gene prediction can be performed with adequate computer programs (GeneMark.hmmm at <http://dixie.biology.gatech.edu/GeneMark/eukhmm.cgi>). Sequences can then be BLAST searched against ever increasing databases to help to attribute putative functions to candidate gene sequences. In combination with the techniques of cDNA capture and Northern analysis this can be an extremely powerful tool. Once candidate genes are identified, putative functions will need to be confirmed through complementation of phenotype by either transformation or mutagenesis.

CHAPTER 8

A PCR APPROACH TO RESISTANT GENE ANALOGUE AMPLIFICATION FROM THE *Yd*₂ REGION OF BARLEY CHROMOSOME 3 AND THE SYNTENOUS REGION OF RICE

8.1 Introduction

Genes conferring gene-for gene disease resistance isolated from plants can be categorised into four classes based on their predicted protein products (as reviewed in Section 1.3.3). The majority of these genes belong either to a class encoding a stretch of leucine rich repeats (LRRs) with a putative nucleotide binding site (NBS) (Bent *et al.*, 1994; Mindrinos *et al.*, 1994; Whitham *et al.*, 1994; Grant *et al.*, 1995; Lawrence *et al.*, 1995; Anderson *et al.*, 1997; Ori *et al.*, 1997; Parker *et al.*, 1997; Yoshimura *et al.*, 1998), or to a class that encodes a LRR domain without a NBS (Jones *et al.*, 1994; Dixon *et al.*, 1996). The two other classes each have only one known representative and encode proteins containing LRRs and a serine-threonine protein kinase domain without a NBS (Song *et al.*, 1995), or by proteins containing a protein kinase domain without either a NBS or LRRs (Martin *et al.*, 1993).

While these genes were isolated from distantly related species (tomato, tobacco, *Arabidopsis*, flax and rice) and confer resistance to viral, bacterial or fungal isolates, their deduced protein products have structural similarities. While overall sequence similarity between resistance genes is low and insufficient to be detected by cross-hybridisation, short stretches of their peptide sequences are conserved (Staskawicz *et al.*, 1995; Hammond-Kosack and Jones 1997).

To date, the isolation of resistance genes has required the difficult and complex procedures of map-based cloning and transposon tagging. An alternative method, the polymerase chain reaction (PCR), may provide a more efficient way to isolate more resistance genes. In recent studies, PCR primers based on short stretches of amino acids conserved among NBS-LRR resistance proteins were used to amplify Resistant Gene Analogues (RGAs) from potato, soybean, *Arabidopsis thaliana*, rice, and barley (Kanazin *et al.*, 1996; Leister *et al.*, 1996, 1998; Yu *et al.*, 1996; Aarts *et al.*, 1998). Amino acid motifs targeted in these studies included the kinase-1a (P-loop) and kinase-2 motifs, which are known features of NBS (Saraste *et al.*, 1990; Traut, 1994), as well as other conserved motifs of unknown function. RFLP loci identified by some of these RGA clones were shown to be closely linked to known resistance loci, suggesting that the RGA clones may hybridise to resistance genes.

To date, all reports of the cloning of RGAs have used agarose-gel electrophoresis to resolve PCR products, and RFLP analysis to find polymorphisms among plant genotypes. Heterogeneity of DNA fragments from single bands isolated on agarose gels was observed by Kanazin *et al.* 1996; Leister *et al.* 1996; and Yu *et al.* 1996. Chen *et al.* (1998) demonstrated that these heterogenous RGA fragments could be separated and polymorphic bands directly detected using high-resolution electrophoresis.

Many RGAs have mapped to chromosomal regions known to contain resistance genes. However, studies have failed to identify candidate RGAs for all resistant gene loci and no RGAs had been mapped to the region of Yd_2 in barley (Leister *et al.*, 1998; Leister *et al.*, 1999). Concurrent to our map-based efforts in barley and rice, we investigated the cloning of the Yd_2 resistance gene using a PCR-derived approach.

The work in this chapter describes the amplification RGAs close to Yd_2 using primers from the literature previously used successfully to amplify RGAs. In the 1st approach primers were targeted at the template of the rice YAC,Y2733, syntenous with the region of Yd_2 in barley (Chapter 6) in an attempt to amplify syntenous RGAs. Secondly primers were used on the template of two different pairs of NILs (Near Isogenic Lines) for Yd_2 , using both agarose and polyacrylamide gel electrophoresis to scan for polymorphisms. Thirdly polymorphisms were sought between resistant and susceptible bulks made from critical recombinants derived from our genetic mapping population (Chapter 4).

8.2 Materials and Methods

8.2.1 Acknowledgments

The author would like to acknowledge Nick Paltridge for collaboration in part of this work, with help in cloning PCR products.

8.2.2 Purification of the YAC template Y2733

Template of the rice YAC, Y2733, was purified by PFGE as described in Section 6.2.8.

8.2.3 The use of DNA extracted from Near Isogenic Lines (NILs) to identify polymorphisms linked to Yd_2 amplified by RGA primers

DNA from two pairs of NILs for Yd_2 , Atlas (Yd_2 -minus) and Atlas68 (Yd_2 -plus), and Proctor (Yd_2 -minus) and Shannon (Yd_2 -plus) was chosen for polymorphism analysis using primers designed to amplify conserved domains of resistance genes (Table 8.1). Barley cultivars were grown in the glasshouse as described in Section 2.2.1 and DNA extractions were performed as described in Section 2.2.9.

8.2.4 The use of susceptible (Yd_2 -minus) and resistant (Yd_2 -plus) DNA bulks to identify polymorphisms linked to Yd_2 amplified by RGA primers

Eighteen individuals homozygous for a recombination event between the two flanking markers *YLM* and *Xmwig952*, described in Section 4.3.5, were used for the construction of bulk susceptible and resistant pools of DNA for polymorphism analysis using primers designed to amplify conserved regions of resistance genes. DNA extractions were performed on individual recombinants as described in Section 2.2.9, and 200ng of DNA from each individual which had been typed as Yd_2 -minus or Yd_2 -plus was mixed with DNA from other susceptible or resistant individuals for Yd_2 respectively. The susceptible DNA bulk was constructed from 7 individuals homozygous for a recombination event between the two flanking markers *YLM* and *Xmwig952* and susceptible for Yd_2 (Classes III and IV; Figure 4.7). The resistant DNA bulk was constructed from 11 individuals homozygous for a recombination event between the two flanking markers *YLM* and *Xmwig952* and resistant for Yd_2 (Classes I and II; Figure 4.7). Resistant and susceptible pools of bulk DNA were diluted for PCR amplification by RGA primers (Section 8.2.5).

Table 8.1 Primer pairs used to amplify conserved domains of resistance genes

Primer ^a	Sequence (5'-3') ^b
Pto kin 1*	GCATTGGAACAAGGTGAA
Pto kin 2	AGGGGGACCACCACGTAG
RLRR-for*	CGCAACCACTAGAGTAAC
RLRR-rev	ACACTGGTCCATGAGGTT
S2*	GGIGGIGTIGGIAAIACIAC
AS3	IAGIGCIAGIGGIAGICC
S2*	GGIGGIGTIGGIAAIACIAC
AS1	CAACGCTAGTGGCAATCC
XLRR-for*	CCGTTGGACAGGAAGGAG
XLRR-rev	CCCATAGACCGGACTGTT
RLK-for*	GAYGTNAARCCIGARAA
RLK-rev	TCYGGYGCRATRTANCCNGGITGICC
NLRR-for*	TAGGGCCTCTTGCATCGT
NLRR-rev	TATAAAAAGTGCCGGACT
NBS-F1*	GGAATGGGNGGNGTNGGNAARAC
NBS-R1	YCTAGTTGTRAYDATDAYYYTRC
CLRR-for*	TTTTCGTGTTC AACGACG
CLRR-rev	TAACGTCTATCGACTTCT

^a The primer pairs Pto kin 1 and Pto kin 2 were designed by Chen *et al.* (1998) based on the DNA sequence encoding for protein kinase in the tomato *Pto* gene conferring resistance to *Pseudomonas syringae* pv *tomato*. The primers S2, AS1, AS3 were designed by Leister *et al.* (1996) based on the resistance genes *RPS2* of *A.thaliana* and *N* of tobacco. The primer pairs RLK for and RLK rev were designed by Feuillet *et al.* (1997) to amplify serine/threonine kinase sequence II to VIII of the wheat *Lr10* gene conferring resistance to *Puccinia recondita*. The primer NBS-F1 and NBS-R1 were designed by Yu *et al.* (1996) based on the amino-acid sequences of two highly conserved motifs of the nucleotide-binding site in tobacco *N* and *Arabidopsis RPS2* genes. The primer pairs RLRR-for and RLRR-rev, XLRR-for and XLRR-rev, NLRR-for and NLRR-rev, and CLRR-for and CLRR-rev were designed based on leucine-rich repeat regions of genes *RPS2*, *Xa21* in rice against *Xanthomonas campestris* pv *oryzae*, *N*, and *Cf9* in tomato against *Cladosporium fulvum* respectively (Naweed Naqvi, IRRI).

^b Codes for mixed bases: Y=C/T, N=A/G/C/T, R=A/G, and D=A/G/T

* This primer was radiolabelled so PCR products could be visualised by autoradiography after polyacrylamide gel electrophoresis.

8.2.5 PCR amplification of RGAs from DNA template

The template for PCR amplification was approximately 30ng of DNA prepared as described in Sections 8.2.2, 8.2.3 and 8.2.4. Reactions were performed in a 25 μ l volume using either MJ Minicycler or PTC-100 Thermal cyclers (MJ Research, USA). For non-radiolabelled PCR reactions, PCR amplification conditions were 4 μ M of each DNA primer (Table 8.1), 2.0mM MgCL₂, 0.2mM each dNTP, 1 \times Taq DNA polymerase activity buffer [67mM Tris-HCl (pH8.8 at 25°C), 16.6mM (NH₄)₂SO₄, 0.45% Triton X-100, 0.2mg/ml gelatin), 1 μ l of DNA sample (30ng) and one unit of Taq DNA polymerase (Biotech International, Perth, Western Australia). Reactions were overlaid with oil subjected to thermal cycling with an initial denaturation of 5min followed by 45 cycles of 1min at 94°C, 1min at 45°C, and 2min at 72°C, followed by a final extension at 72°C for 7min.

For radiolabelled PCR reactions 8 μ M of the forward primer (Table 8.1) from each primer set was 5' labelled with 2.5 μ M of γ ³²P-ATP in 1 \times T4 kinase buffer (Geneworks) with 5units of PNK T4 kinase (Geneworks) at 30°C for 1hr. One μ M of labelled forward primer and 3 μ M of unlabelled forward primer was used in combination with 4 μ M of unlabelled reverse primer. The conditions for PCR were identical to those described above for the non-radiolabelled method. Electrophoretic analyses of the non-radiolabelled PCR products was performed using 1% agarose gels and 1 \times TBE electrophoresis buffer, while electrophoretic analyses of radiolabelled products was performed by polyacrylamide gel electrophoresis, as described in Section 2.2.8.

8.2.6 Isolation and cloning of PCR products amplified from DNA template of the rice YAC Y2733, the barley NILs, and the susceptible and resistant barley pooled DNA bulks

PCR products amplified from the DNA template of Y2733 were gel purified by agarose gel electrophoresis using a Bresaspin gel extraction kit (Geneworks, Australia). Polymorphic bands identified by polyacrylamide gel electrophoresis between DNA of barley NILs or barley bulked DNA populations were cloned as described by Brigneti *et al.* (1997) and De Jong *et al.* (1997). Firstly, exposed X-ray films were aligned to their dried polyacrylamide gels using pin

pricks made through each film/gel overlay prior to exposure. Bands of interest were then excised from the gels using a scalpel blade, taking both the gel slice containing the fragment of interest and the Whatman 3mm backing paper onto which each dried gel was stuck. Gels were then re-exposed to X-ray film to confirm that the correct bands had been excised.

The gel/paper fragment was re-hydrated in 1ml of TE (10mM Tris, 1mM EDTA) for 1 hour at room temperature, before the gel slice was carefully removed from the backing paper, crushed with a sealed micropipette tip and left to soak in 200µl of TE for a further 2hrs. Two microliters of the resulting eluate were then used as the template for PCR re-amplification using the same pair of primers and PCR conditions as had been used for the original amplification. Re-amplified product was then run in a 1% 1×TBE agarose gel and the product gel purified using a Bresaspin gel extraction kit (Geneworks, Australia).

The purified product was cloned into the pGEM®-T Vector Kit (Promega) according to the manufacturers recommendations. Transformation of E.coli with the resulting plasmids by electroporation was as described in Section 2.2.3. Colony growth and plasmid mini-preparation and large scale preparations were as described in Sections 2.2.2 , 2.2.4 and 2.2.5.

8.2.7 DNA sequencing and sequence analysis

A minimum of 2 independent clones were sequenced in both the forward and reverse directions, on an automated DNA sequencer (ABI Prism 377 DNA sequencer). The Primers used for sequencing were the T7 and SP6 sequencing primers. Sequence data was analysed using the program SeqED 1.0.3 (Applied Biosystems) and DNA Strider 2.1. If the cloned sequences were heterogenous, further clones were picked, grown and sequenced to obtain a better estimate of the heterogeneity. Database similarity searches were performed on total cloned genomic sequences using BLASTN (nucleotide sequence verse nucleotide sequence) and BLASTX (nucleotide sequence verse protein sequence) programs on databases compiled by the Australia National Genomic Information Service (ANGIS). Rice genomic sequence searches using BLASTN were run against the non-redundant (NR) nucleic acid database and the *Oryza sativa* Expressed Sequence Tags (ESTs) database. BLASTX searches were run against the NR protein database of

ANGIS. Barley genomic sequence searches using BLASTN and BLASTX were run against the NR nucleic acid and NR protein database respectively.

Cloned rice genomic sequences were translated in 6 frames using DNA Strider 2.1. Translated sequences were searched for termination and initiation codons. Database similarity searches were carried out on translated sequences which did not contain multiple termination codons using BLASTP (protein verse protein sequence) against the NR protein database of ANGIS.

8.2.8 Southern analysis

Southern membranes of restricted barley genomic DNA were probed with DNA probes amplified from the DNA template of the rice YAC Y2733, the NILs of barley, and susceptible and resistant DNA bulks. Southern analysis was performed on Atlas and Atlas68 DNA as described in Sections 2.2.9, 2.2.10 and 2.2.11. Restriction enzymes used to digest the genomic barley DNA were *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, *Nde*I, *Nco*I, and *Xho*I.

8.2.9 Pulsed field gel electrophoresis

Preparation, restriction enzyme digestion and PFGE of high molecular weight barley DNA were performed as described in Sections 5.2.3-5.2.5. Restriction enzymes used to digest the HMW DNA were *Bss*HII, *Cla*I, *Not*I, *Nru*I, *Pvu*I, *Sal*I, *Sma*I, and *Sna*BI. PFGE membranes were probed with DNA probes amplified from the DNA template of rice YAC Y2733, the NILs of barley, and resistant and susceptible DNA bulks.

8.2.10 Northern analysis of infected and noninfected resistant and susceptible barley cultivars with DNA probes amplified by RGA primers

Northern analysis was performed on total RNA extracted from Atlas and Atlas68 seedlings infected at the two leaf stage as described in Section 3.2.3. Total RNA extractions (Section 2.2.13) were performed on shoot and root material 5 days after the end of the inoculation period as well as on noninfected control plants at the same stage of growth. Northern gel

electrophoresis, blotting, hybridisation, washing and autoradiography was performed on all samples as described in Section 2.2.14. DNA probes amplified from the DNA template of rice YAC Y2733, the NILs of barley, and susceptible and resistant DNA bulks were used to probe the membranes. After washing, membranes were exposed to Kodak double emulsion BIOMAX MS film with a BIOMAX MS intensifying screen and exposed for 72hrs at -80°C.

8.3 Results

8.3.1 PCR amplification of products from the DNA template of rice YAC Y2733, using primers shown in the literature to amplify conserved domains of resistance genes

Rice YAC Y2733 DNA, was purified by PFGE and used as the DNA template for PCR in combination with the 9 primer pairs that have been shown in the literature to amplify conserved domains of resistance genes (Table 8.1). The products amplified using these primers were gel purified and oligolabelled before being used to probe PFGE membranes containing DNA from the two rice YAC clones Y2733 and Y2763. The oligolabelled template from the product amplified using the primer combination S2/A3 hybridised strongly to both rice YAC clones Y2733 and Y2763, indicating the product(s) were specific to the rice YACs and not to yeast chromosomal DNA (Figure 8.1). However, oligolabelled template from the product amplified using the primer combination NBS-F1/NBS-R1 was not specific to the rice YACs and hybridised strongly to the yeast chromosomal DNA (Figure 8.1). All of the products amplified with the other primer pairs hybridised to neither rice or yeast DNA. The product(s) amplified using the primer combination S2/A3 were cloned and sequenced.

8.3.2 Sequence analysis of products amplified using the primer combination S2/A3 from DNA template of the rice YAC Y2733

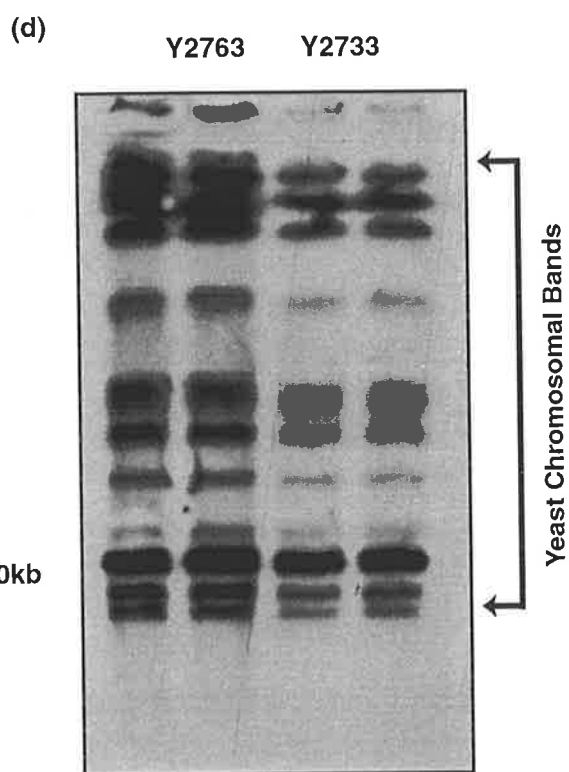
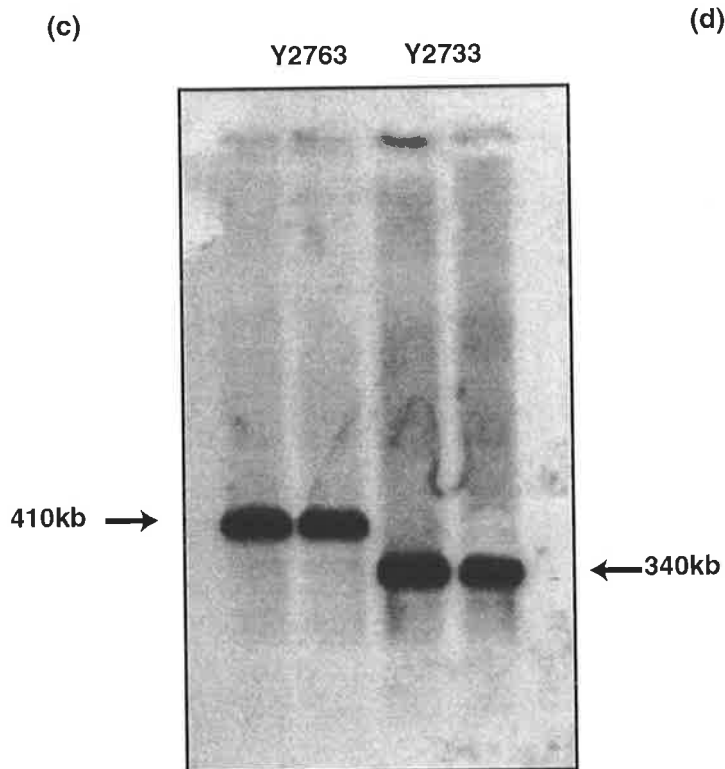
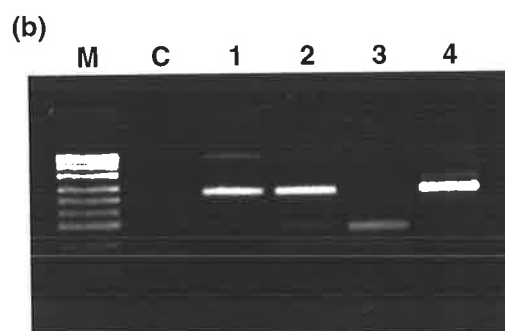
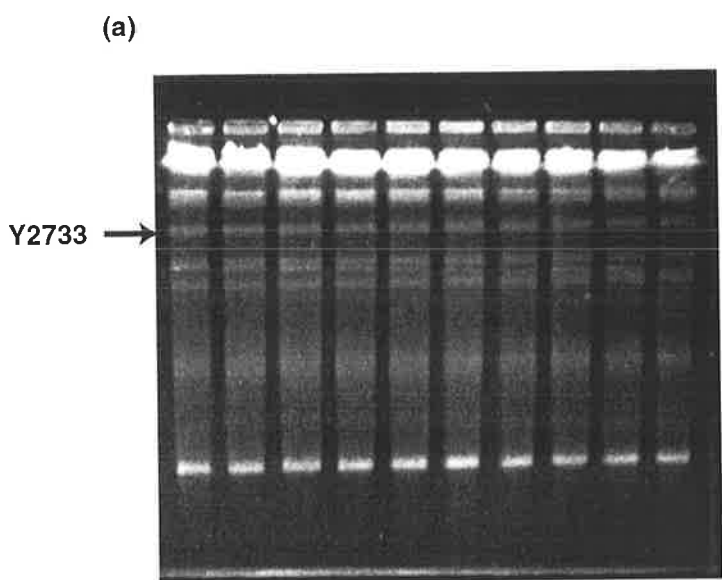
The 300bp fragment amplified from the rice YAC Y2733 using the primer combination S2/A3 was gel purified and cloned using the pGEM®-T Vector Kit. Twelve independent clones were sequenced in both directions and a number of clones were found to be identical. However, 5 distinct clones were identified. Therefore, the 300bp product amplified by the primer pair S2/A3

Figure 8.1 PCR amplification of products from the DNA template of rice YAC Y2733, using primers designed to amplify conserved domains of resistance (R) genes

(a) High molecular weight DNA prepared from Y2733 was separated on a 0.5×TBE 1% agarose stacking gel containing both Seakem LE agarose and low melt agarose. PFGE was carried out using a linear ramp of 15-40 seconds for 15 hours to give maximum separation of Y2733 from host yeast chromosomes. Y2733 was then excised from the gel and purified. Purified DNA was pooled and used for PCR amplification.

(b) Purified Y2733 was used as the template for primers designed to amplify conserved domains of resistance genes (Table 8.1). A number of products in lanes 1 through to 4 were amplified using the primer pairs (Pto kin1/Pto kin2), (S2/AS3), (NBS-F1/NBS-R1) and (XLRR-for/XLRR-rev) respectively. All products were excised from the gel and purified. No template DNA control was included (C). The marker lane (M) contains pUC19 DNA digested with *HpaII*.

Purified product from (b) lanes 2 and 3 were oligolabelled and hybridised to a pulsed field membrane of YAC clones Y2733 and Y2763. (c) The DNA probe template amplified from the primers S2/AS3, strongly hybridised to both YACs and not yeast chromosomal DNA indicating that the product was YAC-specific. (d) In contrast, DNA probe template amplified using the primers NBS/F1, NBS/R1 hybridised strongly to yeast chromosomal DNA indicating that the product was not specific to the rice YACs. All other products amplified did not hybridise to either rice or yeast DNA.



was a heterogenous mix of PCR products. The 5 clones were designated, N10, N11, N12, N13, and N14. The sequences of each clone are presented in Figure 8.2. Pair-wise comparisons of nucleotide sequences showed no significant identity between the sequences of the clones N10, N11, and N12. However, N13 and N14 had a high degree of identity at the nucleotide level except for an 85bp deletion between nucleotides 198 and 283 in the N13 clone.

Database similarity searches carried out on total cloned genomic sequences using BLASTN against the NR nucleic acid and *Oryza sativa* Expressed Sequence Tags (ESTs) databases revealed all of the genomic sequences shared sequence similarity with resistance or putative resistance genes. However, the regions of similarity were with only that of the primers used to amplify these products. BLASTX results however, showed no significant identity matches with cloned resistance genes, putative resistance genes, or resistance gene analogues. Similarity searches revealed the internal region of some clones contained a duplication of both primer sequences (Figure 8.2).

BLASTN searches on the rice ESTs database for the clones N10, N11, and N12 identified short stretches (70bp) of moderate sequence identity (>75% sequence identity) with BAC and PAC genomic clones from the rice sequencing project. BLASTN searches on the rice ESTs database for the clones N13 and N14 identified very short stretches (40bp-50bp) of higher sequence identity (>80% sequence identity) with a number of ESTs. BLASTN and BLASTX searches conducted on the genomic sequences and BLASTP searches conducted on the translated sequences however, did find a number identity matches of low probabilities with genes or transcripts which could be considered as potential candidates for *Yd₂*. A number of these identity matches along with their probability scores are summarised in Table 8.2.

8.3.3 Southern analysis of the rice clones obtained from Y2733

Southern analysis on restricted barley genomic DNA to identify RFLPs which could be mapped in either the Atlas (*Yd₂-minus*) × Atlas68 (*Yd₂-plus*) or Proctor (*Yd₂-minus*) × Shannon (*Yd₂-plus*) mapping populations was carried out using DNA insert template from all 5 rice clones as a hybridisation probes. Southern hybridisation signals for each of the 5 probes failed to identify

Figure 8.2 Sequences of rice clones derived from a 300bp PCR product amplified from the DNA template of rice YAC Y2733

The sequence of five clones, cloned from a 300bp PCR product template of rice YAC Y2733 using the primer pair S2/A3 are present represent primer sequence at the start of each clone. Lettering in duplications of primer sequence within these clones.

N10 sequence

**GAGGGCGAGGGGGAGGCCAAAGACTCGAATCCACATCGGGATTATTTGAATTCATATTCATCTA
CAATTTTCTCCGGGTCTCTTCCCCACCCCCCACACACACAGAGGCTTCGGCCAATGCGGAGA
CGTCCGGATGGGGTACCCGTCTGATCGGACCGAGGGGTCGGATGGCCGTTCTAATGGTGACGG
AGATGATTGAACTCATATGAGGGCGAGGGGGAGGCCTCCTTGGTGTGGAACCCCGAGCCGTGGG
TCTCGTCCGAGGCAGAGCGCGAGGATCTCGTCTCGCCGGCGTTCGTCCATGTAGAAGAAGCTGTG
GTCGTCTTCCCCACCCCCC**

N11 sequence

**GAGGGCGAGGGGGAGGCCCTGAATGGCTGACAGTGGGCCATGCTTAGGTCATGTCTTTGGTCCGT
ACGTAAGGCCCGAAGCCCAATATCAGCACCCCGAGTGGATGCTCCATCCTACTCCCTCCGTCCCA
CAAACCTGCCGTTTACCCGCCCTGGATTGTCCCAGAAAAAATGTCGTCGAGAGAGAAGTGGT
GGGCCCCCTCCGTATACGTGGAGGTGAACCAGAAAAAAGAAGTGCCTTTTTATTGTTGCGCTAG
TACTACTCCGATCTTATCCTCTCTCTAGTCGCCCCATCCATCGCTCTCCGTCGTCTTCCCCAC
CCCCC**

N12 sequence

**GGGGGGGTGGGGAACACGACGGACAGCGATGGATGGGGGCGACTATAGAGAGGATAAGATCGGA
GTAGTACTAGCGCAACAAATAAAAACGCACTTCTTTTTTCTGGTTCACCTCCACGTATACGGAG
GGCCCCACCACTTCTCTCTCGACGACATTTTTTCTGGGACAATCCAGGGGCGGTGAAACGGCAG
GTTTTGTGGGACGGAGGGAGTAGGATGGAGCATCCACTGGGGTGCTGATATTGGGCTTCGGGCC
TTACGTACGGACCAAAGACATGACCTAAGCATGGCCACTGTCAGCCATTCAGGCCTCCCCCTC
GCCCTC**

N13 sequence

**GGGGGGGTGGGGAAGACGACTAGACGCTCTTCTGTATGGCCTCCCCCTCGCCCTCACTATGAGT
TCAATCATCTCCGTCACCATCAGAACGGCCATCCGACCCCTCGGTCCGATCAGGACAGGTACCC
CATCCGGACGTCTCCGATTGGCCGAAGCCTCTGTGTGTGTGCAGGGGGGTGGGGAAGACGACC
TTAGGCCTCCCCCTCGCCCTC**

N14 sequence

**GGGGGGGTGGGGAAGACGACTAGTCATAGAAAGCCTCCCCCTCGCCCTCACTATGAGTTCAATC
ATCTCCGTCACCATCAGAACGGCCATCCGACCCCTCGGTCCGATCAGGACAGGTACCCCATCCG
GACGTCTCCGATTGGCCGAAGCCTCTGTGTGTGTGTGGGGGGGTGGGGAAGACGACGATGCAA
CATCACGTGGATCACGTTGGTGAGCTCGATTTTCTTCTCCATTAACGAAGATCCTCTTTGGG
AGCTCCTTACCTCCATGGCCTCCCCCTCGCCCTC**

Table 8.2 Identity matches from database similarity searches against the rice genomic clones with genes or transcripts identified as possible candidate genes orthologous to *Yd₂*

Clone	Search Program	Identity matches	Smallest Sum Probability
N10	BLASTN	<i>O. sativa</i> lipoxygenase	P=0.82
	BLASTX	Glycoprotein precursor* Chitinase*	P=0.0047 P=0.0083
	BLASTP	Serine proteinase	P=.0036
N11	BLASTX	Extensin-like protein precursor*	P=0.20
		Hydroxyproline-rich glycoprotein*	P=0.51
N12	BLASTX	Extensin*	P=0.12
		Hydroxyproline-rich glycoprotein*	P=0.67
N13	BLASTN	Phenylalanine ammonia lyase	P=0.59
	BLASTX	Extensin Proline rich protein	P=0.00061 P=0.0046
		Phenylalanine ammonia lyase**	P=0.14
N14	BLASTN	Phenylalanine ammonia lyase*	P=0.47
	BLASTX	Extensin Proline rich protein	P=0.12 P=0.67

* More than one identity match in database similarity searches with a different source.

**Numerous identity matches in database similarity searches with different sources.

any RFLPs. Therefore, these DNA probes were unmappable in our mapping populations with the restriction enzymes tested. While single major loci were identified with these probes, numerous other loci of weaker hybridisation intensity were identified. Southern hybridisation signals were also similar between probe hybridisations inferring close physical proximity to each other, and a possible repetitive element within these probes.

8.3.4 Physical mapping of rice clones on Y2733

PFG membranes of restricted Y2733 DNA were probed with the insert of the clones N10, N11, N12, N13, and N14 in order to physically map these clones on Y2733. Clones N10, N11 and N12 produced identical hybridisation signals, suggesting the loci identified by these three clones were extremely physically close or identical. The hybridisation signals produced by N13 and N14 were identical but different from the hybridisation signals produced by N10, N11 and N12. The restriction enzyme *MluI* detected a restriction fragment common to all 5 clones, physically linking both sets of clones by a maximum of 10kb (Figure 8.3). The restriction enzyme *SnaBI* detected a restriction fragment common to clones N10, N11 and N12 with the Ylp probe hybridisation, physically linking the loci identified by these rice clones with the rice loci *XrYlp* by a maximum of 10kb (Figure 8.4). Therefore, the loci identified by these rice clones are physically close to the rice loci *XrYlp* and *Xrc122* identified by the DNA probes Ylp and C122. Both DNA probes Ylp and C122 identify RFLPs in barley which co-segregate with the *Yd₂* gene (Chapter 4).

8.3.5 Northern analysis of infected and noninfected resistant and susceptible barley cultivars using DNA probes amplified by primers designed to amplify RGAs

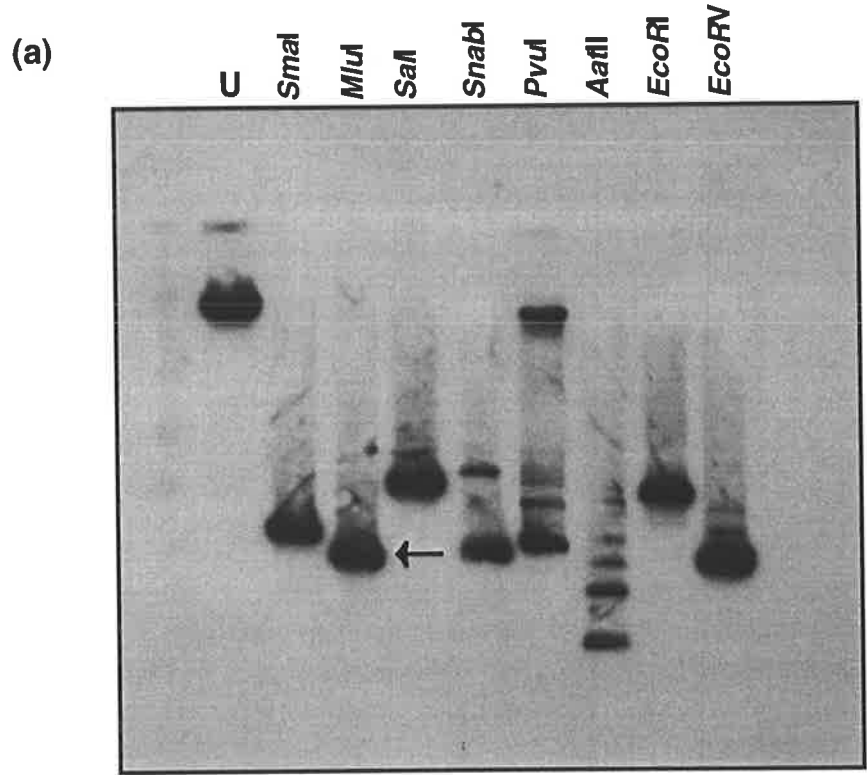
³²P radiolabelled DNA probes of the rice clones amplified from the DNA template of rice YAC, Y2733 were used to probe Northern membranes of Atlas and Atlas 68 infected and noninfected root and shoot material. No hybridisation signals were detected.

Figure 8.3 Physical mapping of the clones N10 and N14 on the rice YAC Y2733

High molecular weight DNA prepared from Y2733 was restricted with the following restriction enzymes; *SmaI*, *MluI*, *Sall*, *SnaBI*, *PvuI*, *AatII*, *EcoRI* and *EcoRV* and run on a 0.5×TBE 1% agarose gel for 15 hours at 200volts. A linear ramp of 1-12 seconds was used to resolve restricted DNA. An undigested control (U) was included and a mid range concatemer ladder (M) also included to help size restricted DNA. After ethidium staining an alkaline transfer was then performed on the PFG and the membrane used for hybridisations.

The DNA probes (a) N10 and (b) N14 were consecutively hybridised to the PF membrane. Between hybridisations the membrane was stripped and re-exposed to ensure probe removal before re-hybridisation. Autoradiographs were overlaid and aligned using radioactive dye and autorad background so restriction fragments of identical molecular weight could be identified. Radiolabelled lambda DNA was also used to help visualise the concatemer ladder (b). The restriction fragment generated by *MluI* for N10 and N14 was found to be identical placing the loci identified by these probes a maximum of 10kb from each other.

N10 Probe Hybridisation



N14 Probe Hybridisation

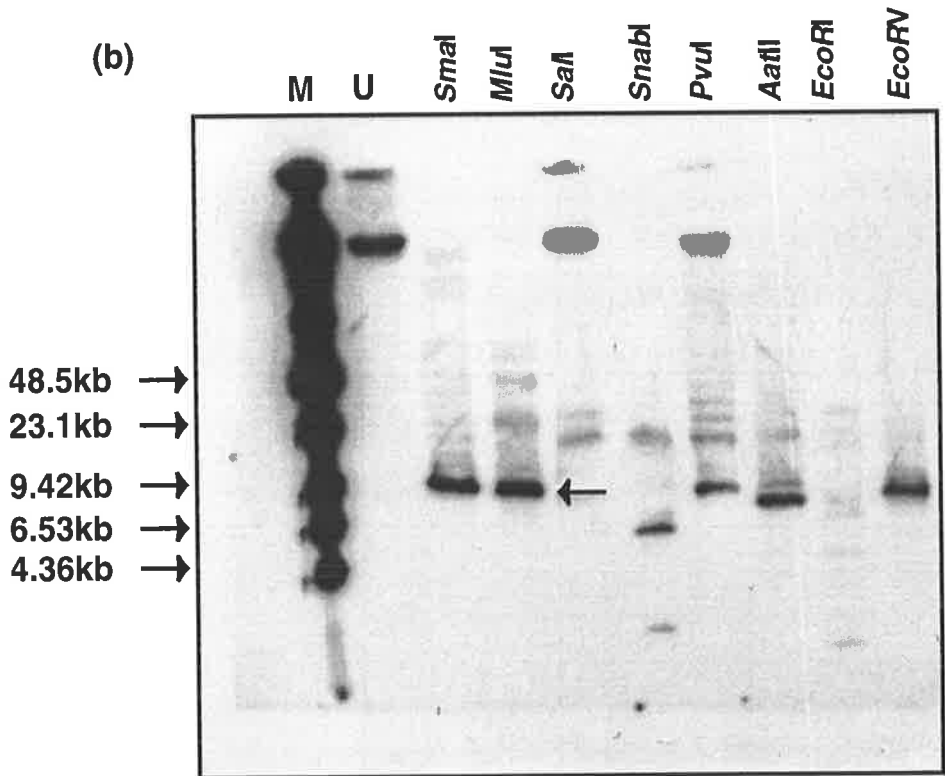
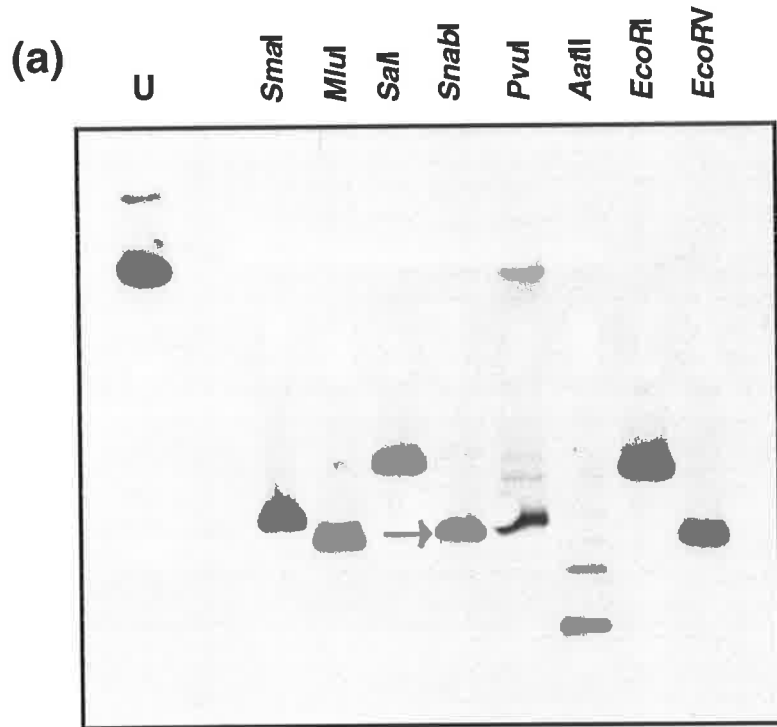


Figure 8.4 Physical mapping of product N10 on the rice YAC Y2733 with relation to the loci identified by the DNA probe Y1p

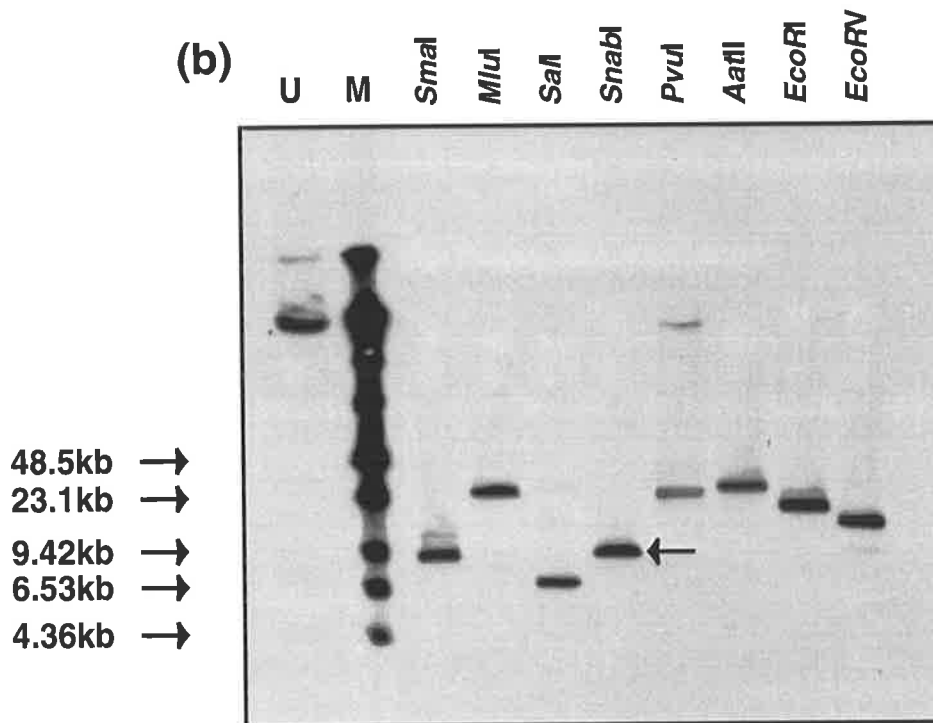
High molecular weight DNA prepared from Y2733 was restricted with the following restriction enzymes; *SmaI*, *MluI*, *SalI*, *SnaBI*, *PvuI*, *AatII*, *EcoRI* and *EcoRV* and run on a 0.5×TBE 1% agarose gel for 15 hours at 200volts. A linear ramp of 1-12 seconds was used to resolve restricted DNA. An undigested control (**U**) was included and a mid range concatemer ladder (**M**) included to help size the restricted DNA. After ethidium staining an alkaline transfer was performed on the PFG and the membrane used for hybridisations.

The DNA probes (**a**) N10 and (**b**) Y1p were consecutively hybridised to the PF membrane. Between hybridisations the membrane was stripped and re-exposed to ensure probe removal before re-hybridisation. Autoradiographs were overlaid and aligned using radioactive dye and autorad background so restriction fragments of identical molecular weight could be identified. The 10kb restriction fragment generated by *SnaBI* for N10 and Y1p was found to be identical placing the loci identified by these probes a maximum of 10kb from each other. The restriction enzyme *SnaBI* had previously linked both loci identified by C122 and Y1p (Chapter 6, Section 6.3.3). Thereby placing the loci identified by N10 physically close to the two rice loci syntenous with those loci shown to co-segregate with *Yd₂* in barley.

N10 Probe Hybridisation



Ylp Probe Hybridisation



8.3.6 The resolution of PCR products amplified from genomic barley DNA

The PCR products amplified from genomic barley DNA using the 9 primer pairs designed to amplify conserved domains of resistance genes, were resolved using agarose and polyacrylamide gel electrophoresis. Amplification of genomic barley DNA with these RGA primers resulted in one to 15 bands being detected in 1% agarose gels when stained with ethidium bromide. Previously it had been reported that cloning of a single major band from agarose gels often resulted in several different clones (Kanazin *et al.*, 1996; Leister *et al.*, 1996). Chen *et al.*, (1988), reported that a large number of bands and fragment length polymorphisms could be detected by high resolution electrophoresis. Therefore, a single primer in each primer set was radiolabelled so that the PCR products amplified could be visualised by autoradiography after polyacrylamide gel electrophoresis. This resulted in a large increase in the number of loci detected using this system (Figure 8.5). These results confirm the observations that highly heterogenous products are produced by PCR-amplification with RGA primers (Kanazin *et al.*, 1996; Leister *et al.*, 1996). The number of loci detected using polyacrylamide gel electrophoresis for all 9 primer sets is summarised in Table 8.3.

8.3.7 Polymorphisms detected between the DNA of barley NILs and bulked pools of *Yd₂-minus* and *Yd₂-plus* DNA

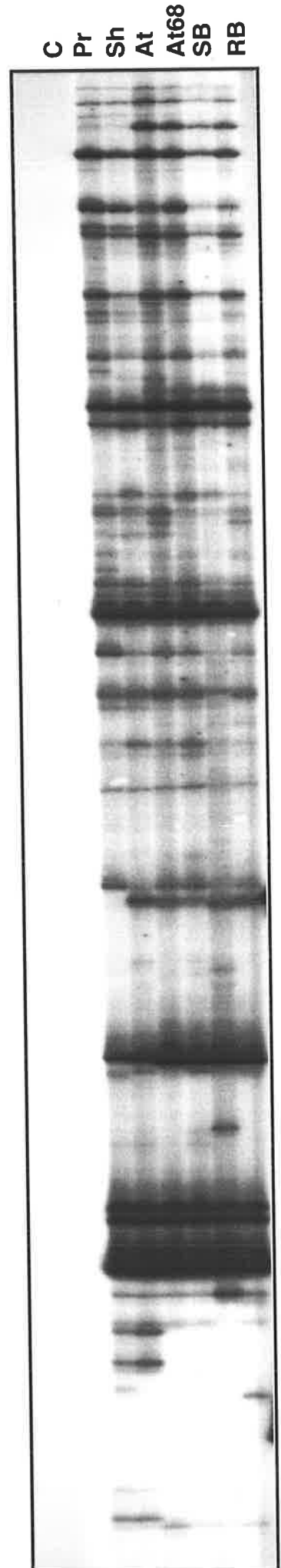
Using 9 primer pairs designed to amplify conserved domains of resistance genes, PCR products amplified from genomic barley DNA were analysed for polymorphisms between two pairs of NILs for *Yd₂* (Atlas *Yd₂-minus*; Atlas 68 *Yd₂-plus*) (Proctor *Yd₂-minus*; Shannon *Yd₂-plus*) and between bulked pools of susceptible and resistant DNA derived from the critical recombinant mapping population (Section 4.3.5). In total more than 400 loci were detected using these primers. A number of polymorphisms were found between the different sets of NILs, (Atlas and Atlas 68 verse Proctor and Shannon) (Figure 8.6). However, these polymorphisms were not considered to be *Yd₂* associated, but reflect differences between donor parent background. Six of the 9 primer sets amplified more than 40 loci each which were detected by polyacrylamide gel electrophoresis and autoradiography (Table 8.3). However, only three primer sets S2/AS3, S2/AS1 and NLRR-for/rev amplified bands polymorphic between the NILs {Atlas and Proctor

Figure 8.5 The resolution of PCR products amplified by the primer set Pto kin1/Pto kin 2 using both agarose and polyacrylamide gel electrophoresis

PCR products were amplified from the barley genomic template Proctor (**Pr**)[*Yd₂*-minus], Shannon (**Sh**)[*Yd₂*-plus], Atlas (**At**)[*Yd₂*-minus], Atlas68 (**At68**)[*Yd₂*-plus], and from bulked pools of resistant(**RB**) and susceptible(**SB**) DNA derived from the critical recombinant mapping population (Section 4.3.5). A no template control was included (**C**). The marker lane (**M**) in (a) contains pUC19 DNA digested with HpaII.

(a) The amplification of genomic DNA with the Pto primers resulted in a maximum of 7 visible bands when run in a 1% agarose gel and stained with ethidium bromide. (b) However greater than 50 bands were easily scored when PCR products were run out on a polyacrylamide sequencing gels with one primer radiolabelled so that the PCR products could be visualised by autoradiography.

(b) Polyacrylamide gel electrophoresis



(a) Agarose gel electrophoresis

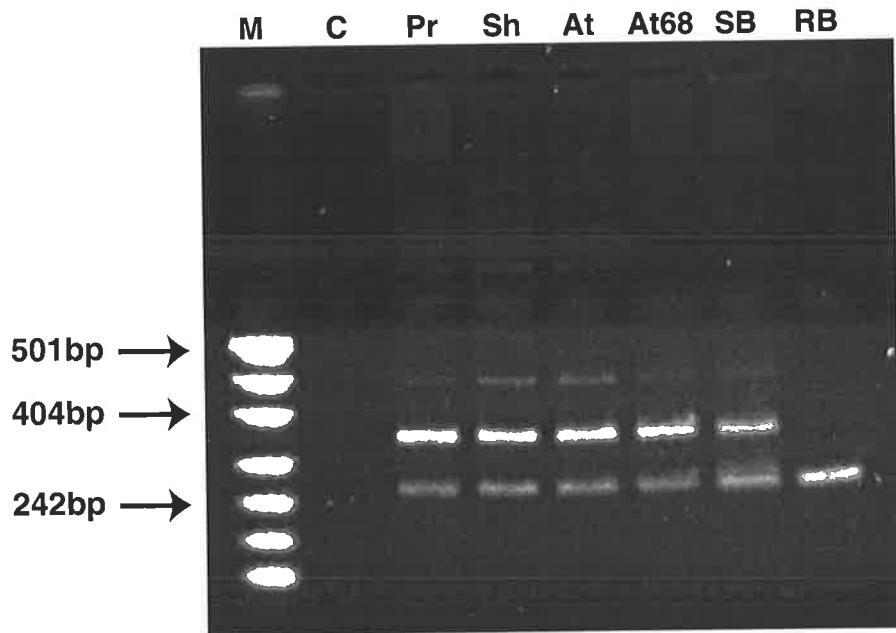


Table 8.3 Number of bands scored which were amplified from genomic barley DNA by primers derived from the conserved domains of resistance genes

Primer set	Number of loci detected using polyacrylamide gel electrophoresis	Number of polymorphisms detected between NILs	Number of polymorphisms detected between bulks
Pto kin 1 Pto kin 2	58	0	0
RLRR-for RLRR-rev	31	0	0
S2 AS3	>100	2	1*
S2 AS1	54	4	4*
XLRR-for XLRR-rev	18	0	0
RLK-for RLK-rev	8	0	0
NLRR-for NLRR-rev	48	2	0
NBS-F1 NBS-R1	66	0	0
CLRR-for CLRR-rev	44	0	0

*All polymorphisms detected between bulked pools of resistant and susceptible DNA were also present between the NILs. However, not all polymorphisms detected between NILs were present between the two bulks.

Figure 8.6 The detection of polymorphic PCR products amplified from barley genomic DNA using primers designed to amplify conserved domains of resistance genes

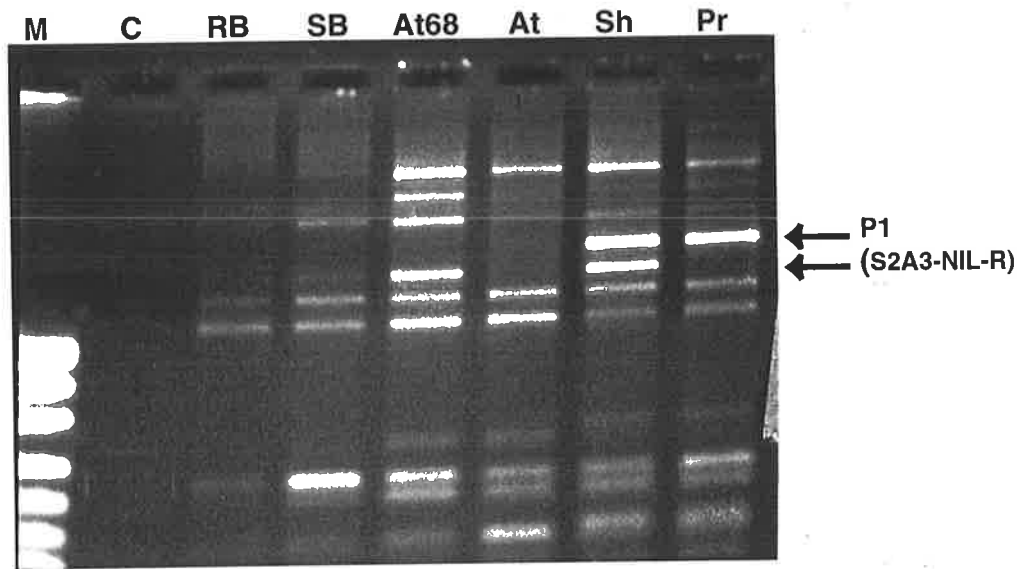
PCR products were amplified from barley genomic template using the primer pairs (a) S2/A3, (b) S2/A1 and (c) NLRR-Forward and Reverse. Polymorphisms were sought between two pairs of NILs for Yd_2 {(Atlas[At] Yd_2 -minus; Atlas 68 [At68] Yd_2 -plus) (Proctor[Pr] Yd_2 -minus; Shannon[Sh] Yd_2 -plus)} and between bulked pools of susceptible[SB] and resistant[RB] DNA derived from the critical recombinant mapping population (Section 4.3.5). A no template control was included (C). The marker lane (M) in (a) contains pUC19 DNA digested with *HpaII*. For palate (a) agarose gel electrophoresis was carried out on a 1% gel in 1×TBE running buffer as described in Section 2.2.7. For palate (b) and (c) polyacrylamide gel electrophoresis was carried as described in Section 2.2.8.

A number of polymorphic bands were detected between the different sets of NILs (P1), which, were not considered to be Yd_2 associated. Polymorphisms found between NILs (S2A3-NIL-R, NLRR-NIL-R, NLRR-NIL-S) but not present between bulked resistant and susceptible DNA pools were considered to be Yd_2 associated. These polymorphisms were mapped in the critical recombinant population and placed on the Yd_2 map (Figure 8.8). A number of polymorphisms were detected between NILs which were also present between bulked resistant and susceptible DNA pools (S2A1-BL-R, S2A1-(a&b&c)-BL-R). These polymorphisms were also mapped in the critical recombinant population to confirm their co-segregation with Yd_2 .

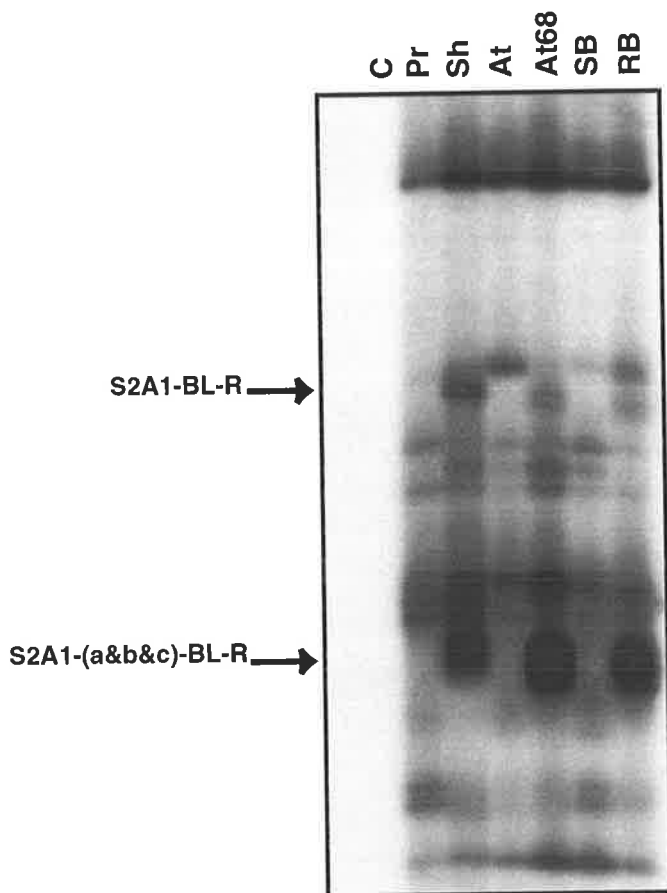
Polymorphisms identified were designated as follows;

- 1)the primer pair used to amplify the band (eg. S2A3).
- 2)whether the polymorphism was between only the NILs (NIL) or the bulks (BL) as well.
- 3)If more than one allele was present, each allele was individually designated as a, b, or c etc.
- 4)Finally each marker was designated as susceptible (S) or resistant (R) associated.

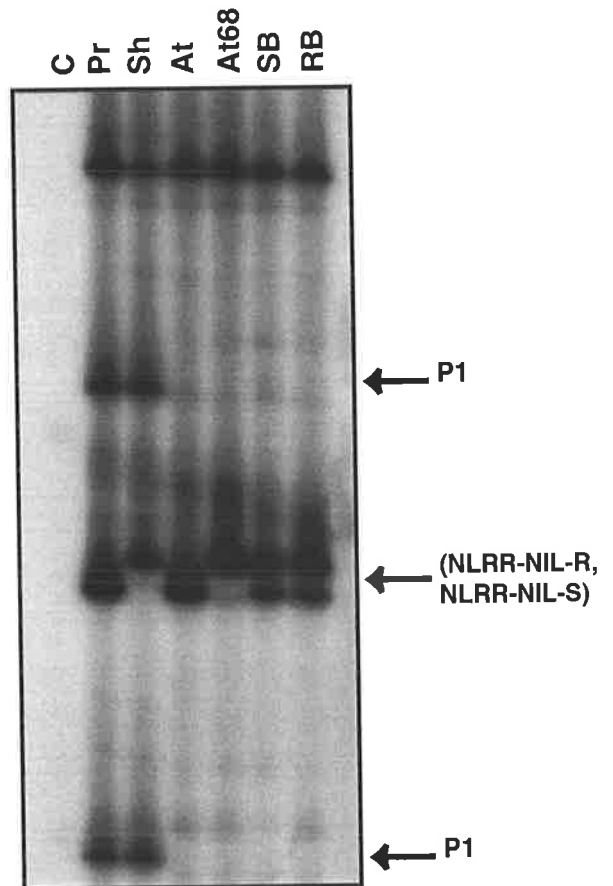
(a) PCR products amplified using the primer pair S2/A3



(b) PCR products amplified using the primer pair S2/A1



(c) PCR products amplified using the primer pair NLRR-F/R



(Yd_2 -minus) versus Atlas68 and Shannon(Yd_2 -plus)} or between the resistant and susceptible bulked pools of DNA (Table 8.3).

Two polymorphisms present between the NILs but not between bulks were identified. A dominant polymorphism found only in both resistant lines Atlas68 and Shannon was amplified using the primer set S2/A3, and was designated **S2A3-NIL-R** (Figure 8.6). The polymorphism amplified using the NLRR-for/rev primer set appeared to be co-dominant, with a higher weight molecular product amplified in the resistant lines (**NLRR-NIL-R**) and a lower weight molecular product amplified in the susceptible lines (**NLRR-NIL-S**) (Figure 8.6). These polymorphisms were considered to be Yd_2 linked and are summarised in Table 8.3.

Some polymorphisms which were Yd_2 linked were also present between bulked pools of susceptible and resistant DNA. The primer pair S2/A1 amplified a single band resistant associated polymorphism (**S2A1-BL-R**). S2/A1 primer set also amplified a lower molecular weight resistance associated doublet (**S2A1-(a&b)-BL-R**) in Shannon and a triplet (**S2A1-(a&b&c)-BL-R**) in Atlas68 and the resistant Bulk (Figure 8.6). The primer pair S2/A3 amplified a susceptible associated polymorphism (**S2A3-BL-S**) present in both susceptible lines and the susceptible bulk DNA but absent in the resistant lines and resistant bulk DNA. As these polymorphisms were not only present between NILs, but also between the susceptible and resistant bulks, they were considered to be closely linked to Yd_2 .

Polymorphisms identified between NILs and between resistant and susceptible bulks were mapped in each individual critical recombinant to determine the exact genetic linkage of these markers in relation to Yd_2 (Figure 8.7). The two NIL polymorphisms amplified by the primer sets S2/A3 and NLRR-F&R mapped within 1cM of Yd_2 . All three polymorphisms identified between the resistant and susceptible bulks were shown to co-segregate with Yd_2 . Therefore, these new markers could be placed on the Atlas × Atlas68 linkage map (Figure 8.8). All polymorphic products which mapped close to Yd_2 were cloned and sequenced.

Figure 8.7 Mapping of the resistance associated NIL polymorphism amplified using the primer pair S2/A3

The polymorphism identified between the NILs of Atlas (Yd_2 -minus) and Atlas68 (Yd_2 -plus) and Proctor (Yd_2 -minus) and Shannon (Yd_2 -plus) **S2A3-NIL-R**, was mapped in 18 recombinant individuals homozygous for their critical recombination event between the loci *Xmwig952* and *YLM* (Figure 4.7). The resistance associated band was present in 10 of the 18 individuals, placing this loci 0.9cM in genetic distance above Yd_2 and co-segregating with *Xmwig952*. Thereby, identifying a dominant PCR marker proximal to the centromere and above Yd_2 . Atlas (**At**), Atlas68 (**At68**) and a no template control (**C**) were included. Lanes 1 to 18 are individual critical recombinants homozygous for the recombinant chromosome (Section 4.3.5).

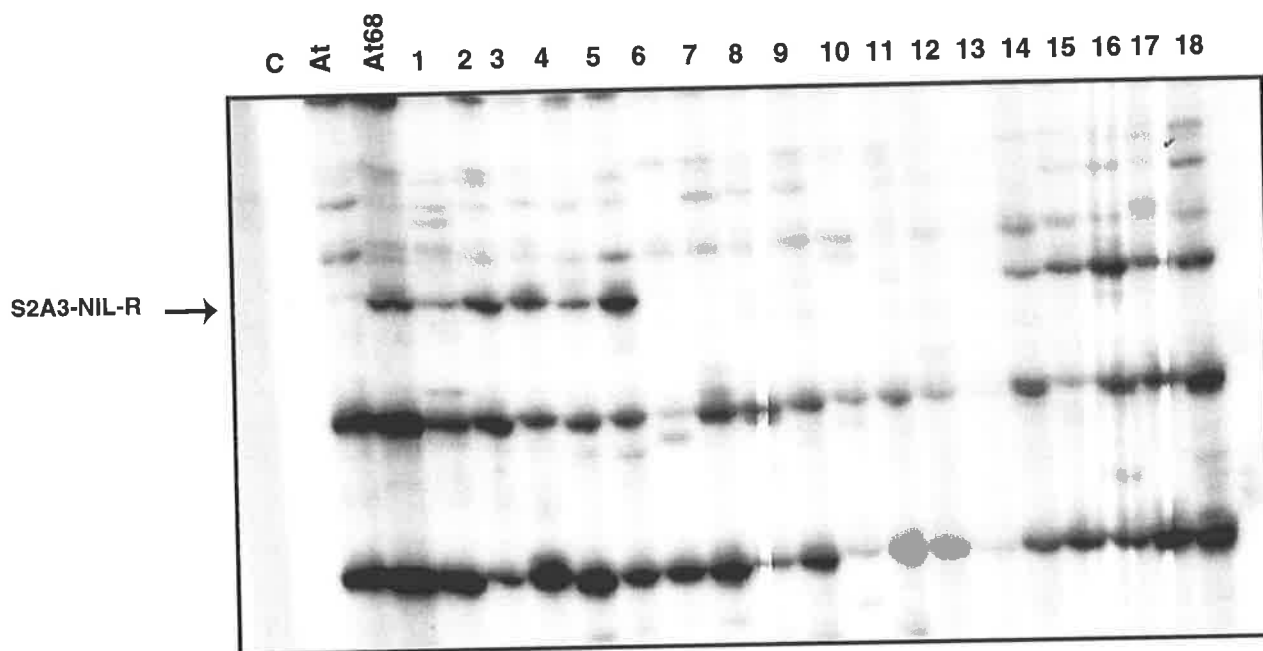
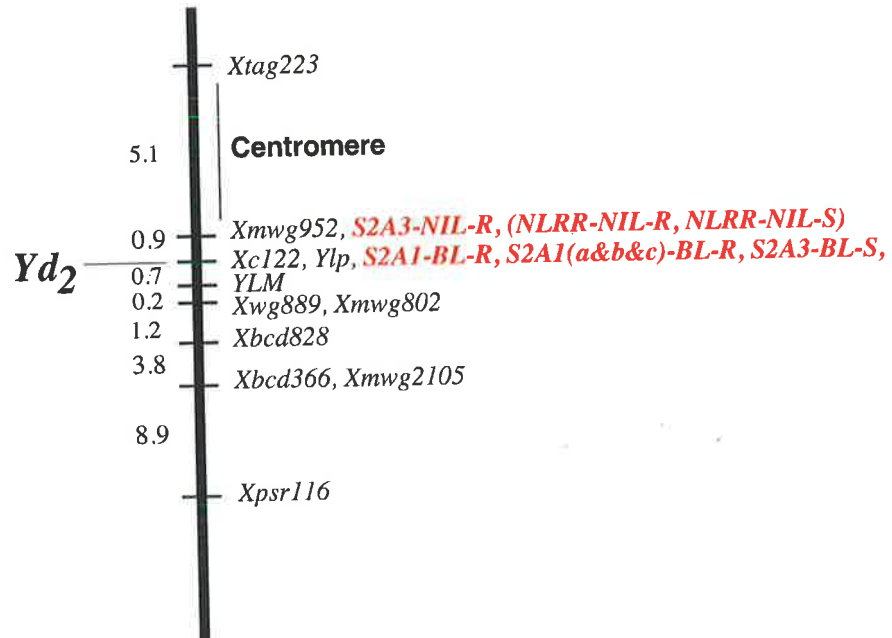


Figure 8.8 Revised linkage map of the Yd_2 region of barley Chromosome 3 in the Atlas × Atlas68 mapping population

A revised linkage map of the Yd_2 region of barley Chromosome 3 in the Atlas × Atlas68 mapping population is presented with 8 new PCR markers highlighted in red. Distances are in centiMorgans.

Atlas x Atlas 68 map



8.3.8 Cloning and sequence analysis of amplified products polymorphic between barley NILs and bulk pools of resistant and susceptible barley DNA

All products polymorphic between NILs and between bulks were targeted for cloning as described in Section 8.2.6. While PCR products polymorphic only between the NILs were successfully cloned, only three of the five products which were polymorphic between the bulks were successfully cloned. These were the susceptible associated bulk polymorphism amplified using the S2/A3 primer pair, **S2A3-BL-S**, and two bands, **a&b** of the triplet resistance associated polymorphism **S2A1-(a&b&c)-BL-R**, amplified using the S2/A1 primer pair. The nucleotide sequence for all the cloned products is presented in Figure 8.9. The other cloned PCR products when checked against the original amplified product were the incorrect size. These clones therefore, contained the incorrect PCR product and were not sequenced.

Pair-wise comparisons of nucleotide sequences showed 97% identity between band **a & b** of **S2A1-(a&b&c)-BL-R** (Figure 8.8), band (**a**) was 121bp, while (**b**) was 120bp. However, no significant stretches of identity existed between **NLRR-NIL-R** and **NLRR-NIL-S**. Therefore, these products may not be resistant and susceptible alleles as first thought. Instead they may represent two different markers associated with both resistance and susceptibility in similar but not the exact same genetic location (Figure 8.8). Pair-wise comparisons of nucleotide sequences showed no significant stretches of identity between any of the other products cloned.

Database similarity searches were carried out on total barley genomic sequences using BLASTN and BLASTX programs on the NR nucleic acid and NR protein database respectively. BLASTN searches on the sequence of bands **a&b** of the triplet resistance associated polymorphism **S2A1-(a&b&c)-BL-R** showed a very significant identity matches (Greater than 90% nucleotide identity over 60% of the total clone length) with a *Hordeum vulgare* degenerate retrotransposon. Both BLASTN and BLASTX searches discovered significant identity matches for the susceptible associated polymorphism **S2A3-BL-S**, with *Alu* repeats. BLASTN and BLASTX searches for all other polymorphic bands cloned however, resulted in either no identity matches or very low probability identity matches with a wide range of target sequences.

Figure 8.9 Sequences of barley clones derived from amplified products polymorphic between NILs for *Yd₂* and between resistant and susceptible bulks

The sequences of five clones, cloned from amplified products polymorphic between NILs for *Yd₂* and resistant and susceptible bulks are presented. Lettering in bold black represents the primer sequence at the start and end of each clone.

S2A3-NIL-R

**GAGGGCGAGGGGGAGGCCTGTTGCGTCACTGGGAAGGACAACCTAGAGCTGAAGAGCTCTGGTTATC
TACGGCAGAACATGGCCCACCGTGCTCTGGAGAAGGTCCAAGTTATCCCCATGCCAAAAGATGGGGA
GAGAGTATTTTCATTCCTCATTTTCTTTGGTGTGGGTTTCGTCATCACCCCTTGGTTCAGGGTAT
TATGTTCTCTACGGGCTTGACTTGCATTTTGACATCCCTGGATTAGGCTACAATAACCCCTCTAATC
AAGCTACTGTGATCCCCATGATTAAGCCAATCATGTTACCAAATAGTGATTGATCACTTTGAATTAT
ATATATCATTTCAAATTCACCATTAAAGGCCCTAGAATTTTATTTTGAGAAAATAAACTTCAAAC
AACAAAAGAAAATGTTGCATACTTTTGAAATAATCCAAAACATTTGTTGTTAAGGAAAACAACATC
ACCCTCAAGTAAGTGTGGACCCACCTTCAATAATAGAGCCAAATTGCATTTAAAATGCCCTCTTAA
ATTCAATGGATAATTCAAACAGTTAAAAAATGCTCAAACCTTGTGTGGCATTACAGAATATTGCAAA
GTATTTTATGGGGCAAGTCACTCATTTTTACTATAACAAATAATTGCTAAAAATAAATAACAAAA
GAATAAAAAAACAAAAAGAGAAAAGAAATGAAAAGGGAAATGTGCACTATGCACTTAGGCCGAG
TGCCACAGTGGAAGGCCCAACCCATCTCCCCGTCGTTTCCCCACCCCCC**

NLRR-NIL-S

**TATAAAAAGTGCCGGACTGGGACCTGCCAAGGTAGAGCAGTAGAAAAGCCCTCAACCAGTCTCAGGA
GAAGGAGTGGGCGGGCCCTTGAAAGGCCCATCTACAAAAGGAATCAGGGGAAAACCGACCCTAAGG
AGGAAACATCACGATGCAAGAGGCCCTA**

NLRR-NIL-R

**TATAAAAAGTGCCGGACTCGATTGGATCGGCCTCGGGCCCCACAGCAAGTTATTGATGTAGTACTT
CTCGTGGCAGTCCGAGTCATGAGCCCCGCTGCGTAGCTCCCTGACCTTGGTAGGGCTCCCTTTGAG
AACTCAACTCCACGATGCAAGAGGCCCTA**

S2/A3-BL-S

**GGGGGGGTGGGGAAGACGACTGCTTATTTTTGACAAGGAGAATTGTTCCCTCCCGCCCCTGCCTCCTA
AGTAGCTGGGACTACAGGCACACAGGAGCCTAATTTTTAAAAGTTTTTAATTTTTGTAGAGACAAGG
TCTTACTAGGTTACCTAGGCAGGTCTCAAACCTCGGTCTCAAGTGATCCTTATCCTTGGCCTCCCC
CTCGCCCTC**

S2/A1-T(a)-BL-R

**GGGGGGGTGGGGAAGACGACAAGAGGAGATAGTCTCACATCAACTAGGCGTATCAACGGGCTATGGA
GATACCCATCAATGGATATCAATGTGAGTGAGTAGGGATTGCCACTAGCGTTG**

S2/A1-T(b)-BL-R

**GGGGGGGTGGGGAAGACGACAAGAGGAGATAGTCTCACATCAACTAGGCGTACCAACGGGCTATGG
AGATACCCATCAATAGATATCAATGTGAGTGAGTAGGGATTGCCACTAGCGTTG**

8.3.9 Southern analysis of barley PCR products amplified from barley genomic DNA

DNA inserts from all barley PCR products cloned were oligolabelled and used as hybridisation probes on Southern membranes of restricted barley genomic DNA to determine whether the cloned products detected are single or multiloci. While single major loci were detected by these probes, numerous other loci with weaker hybridisation intensities were also detected inferring these probes contained repetitive sequences.

8.3.10 PFGE analysis of barley cloned PCR products amplified from barley genomic DNA

DNA inserts from all PCR amplified barley clones was oligolabelled and used as hybridisation probes on PFGE membranes of restricted HMW barley DNA, to determine if the loci detected by these clones could be shown to be physically linked to loci detected by the DNA probes Ylp and BCD134 which both detect loci genetically very close to *Yd₂* (Chapter 4). However, the hybridisation signals produced by the probes were not discrete bands and the physical distance of these probes in relation the DNA markers Ylp and BCD134 could not be determined.

8.3.11 Northern analysis of infected and noninfected *Yd₂*-minus and *Yd₂*-plus barley cultivars using barley clones amplified from barley genomic DNA.

³²P radiolabelled DNA insert of the barley clones amplified from genomic barley DNA were used to probe Northern membranes of total RNA extracted from Atlas and Atlas 68 infected and noninfected, root and shoot material. However, no hybridisation signals were detected.

8.4 Discussion

8.4.1 The identification of markers linked to Yd_2 using PCR primers designed to amplify RGAs from genomic barley template and a rice YAC syntenous with the Yd_2 region in barley

DNA from three sources was chosen as the template for the amplification of PCR products using primers designed to amplify RGAs to identify markers closely linked to Yd_2 , which may also be considered as possible Yd_2 candidate genes. A rice YAC from the region syntenous with Yd_2 in barley, two pairs of barley NILs for Yd_2 and resistant and susceptible bulks derived from our genetic mapping population of Atlas \times Atlas68 were used as the DNA templates. A number of different products were amplified from the rice YAC and all were shown by PFGE to be physically close to the loci $Xrc122$ and $XrYlp$, syntenous with the loci in barley $Xc122$ and $XYlp$ shown to co-segregate with Yd_2 (Chapter 4). However, due to a lack of polymorphism between Atlas and Atlas68 with the restriction enzymes used they were unable to be mapped as RFLPs in our mapping population. The recent identification of BACs from the Yd_2 region of barley (Chapter 7) however, may provide a better source of DNA template for the amplification of PCR products using primers designed to amplify RGAs. Considering that our closest flanking markers in the Atlas \times Atlas 68 cover a genetic distance of 1.6cM (Figure 8.8) and there are 18 individual recombinants within this genetic interval (Figures 4.5, 4.7), products amplified from the barley BACs linked to Yd_2 may provide new markers which will further delimit the genetic area encompassing Yd_2 .

Radiolabelled PCR products amplified from barley genomic template were resolved using polyacrylamide gel electrophoresis and detected by autoradiography. This technique was found to be much more sensitive in detecting loci than agarose gel electrophoresis and ethidium bromide detection of PCR products. This is consistent with the findings of Chen *et al.* (1998) who detected between 30 and 130 bands in barley with the same PCR primers using denaturing polyacrylamide gel electrophoresis, while only detecting between 1 to 5 bands with ethidium bromide detection of products resolved by agarose gel electrophoresis.

Using the polyacrylamide gel electrophoresis detection system greater than 400 loci were detected using 9 primer pairs in this study. Loci linked to Yd_2 were identified based on a size polymorphism or presence/absence polymorphism between two pairs of NILs for Yd_2 in barley and between susceptible and resistant pools of DNA constructed from our genetic mapping population (Section 4.3.5). Any loci showing polymorphism between the two pairs of NILs or the bulks were then mapped using each individual critical recombinant to confirm linkage to Yd_2 . Using both NIL and bulked segregant analysis 8 new PCR markers were detected within 1cM of Yd_2 , 5 of which were found to co-segregate with Yd_2 . Both NIL analysis (Young *et al.*, 1988) and bulked segregant analysis (Michelmore *et al.*, 1991) have been used previously to successfully identify molecular markers closely linked to a number of resistance genes in a variety of crops (Hinze *et al.*, 1991; Barua *et al.*, 1993; Williams *et al.*, 1994; Borokova *et al.*, 1995). In this study we found NIL and bulked segregant analysis in combination with primers designed to amplify RGAs to be an efficient way of detecting markers closely linked to our target gene.

Importantly, 3 markers were found proximal to Yd_2 , one of which *S2A3-NIL-R*, can easily be detected using agarose gel electrophoresis coupled with ethidium bromide staining (Figure 8.6 a). PCR markers which flank the target gene can help to quickly analyse large numbers of individuals needed for the map-based isolation of plants genes in comparison to other conventional techniques such as RFLP analysis. Chen *et al.* (1998) stated that amplified polymorphic RGAs can be used as reliable molecular markers in genome mapping. Therefore, if there is a need to increase the genetic resolution of our map and expand the mapping population the dominant PCR marker proximal to Yd_2 (*S2A3-NIL-R*) will be available to type recombination events close to Yd_2 in combination with the co-dominant PCR markers, the *YLM*, 1cM distal to Yd_2 (Paltridge *et al.*, 1998) and the *Ylp*-derived CAPs marker which co-segregates with Yd_2 (Ford *et al.*, 1998).

8.4.2 Are the products amplified from the rice YAC and barley genomic template RGAs?

The most significant advantage of RGAs over arbitrary DNA markers is that they are likely to represent potential target genes. Although not all RGAs are functional disease resistance genes, they all contain the conserved sequences of a LRR with or without a NBS or a kinase with or

without a LRR. A number of the PCR products cloned from the barley genomic template using primers designed to amplify RGAs were identified as being closely linked to Yd_2 or co-segregating with Yd_2 . However, none showed significant sequence identity with other previously cloned RGAs. Instead, they were identified as retrotransposons or other barley repetitive elements. Retrotransposons constitute a large percentage of the barley genome (Kleine *et al.*, 1993), and their high copy number in barley is likely to have interfered with the generation of specific amplification products from the barley genomic DNA. This is consistent with the findings of Leister *et al.*, (1999) whom also found that using barley genomic DNA as a template yielded no candidate resistant gene fragments unless subtractive procedures or RT-PCR were first used to reduce the complexity of the template genomic DNA.

Chen *et al.*, (1998) stated that there is a high probability that products amplified using these 9 primers pairs and resolved using polyacrylamide are involved in signal transduction pathways. However, the results found in this study which are consistent with results found by Leister *et al.*, (1999) do not support this hypothesis. It is essential to consider that while Chen *et al.*, (1998) amplified products linked to a number of resistance genes none of their products were sequenced to determine whether these products were RGAs or repetitive elements linked to the gene of interest. While the technique of RGA amplification from genomic DNA is an efficient method for the identification of markers closely linked to the target gene based on size or presence/absence polymorphisms, it may not be a useful tool for identifying RGAs in the vicinity of a target gene in plants with a large genome, such as barley. Instead, it may be better to use these primers to amplify potential RGA products from subtracted DNA or cDNA (Leister *et al.*, 1998;1999). Therefore, RT-PCR on mRNA from BYDV infected and noninfected NIL lines for Yd_2 may help identify possible RGAs linked to Yd_2 . Secondly, the identification of BACs encompassing the Yd_2 region would provide a target template of less complexity from which to amplify specific RGA products.

Amplification of PCR products from the rice YAC with primers designed to amplify RGAs did not yield any products with sequence identity to RGAs. Instead, the products which were cloned and sequenced showed sequence identity to genes or transcripts not identified as RGAs in the database searches undertaken (Table 8.2). In contrast to total genomic barley DNA, the

complexity of the DNA target for the amplification of products from the rice YAC is greatly reduced. Therefore, as retrotransposons and repetitive elements do not constitute as large a percentage of the rice genome (Flavell *et al.*, 1974; Deshpande and Ranjekar, 1980) they should not interfere with the amplification of specific products. Importantly non-subtracted rice genomic DNA has previously yielded RGAs (Leister *et al.*, 1998). Therefore, the possible reasons for products which have been cloned which do not show identity with RGAs include the following;

1) As discussed in Section 6.4.1, there may be a breakdown in the level of colinearity at the microsynteny level. This has been demonstrated by comparative mapping of rice and barley NBS-LRR homologues in rice, barley and foxtail millet which indicate a faster gene diversification for NBS-LRR genes than the rest of monocot genomes (Leister *et al.*, 1998). Therefore, RGAs which may be present in barley close to the Yd_2 region may be absent in the syntenous region of rice.

2) Secondly as indicated by previous RGA mapping studies there may be no RGAs in this region of the genome in both barley and rice (Leister *et al.*, 1999). The Yd_2 gene may have no sequence similarity to known resistance genes. Therefore, attempting to identify Yd_2 by the amplification of RGAs by the use of degenerate primers designed to amplify the conserved regions of R genes will not succeed.

Although no RGAs were amplified using this approach in rice, a number of other interesting products were amplified using the less stringent PCR conditions (Section 8.4.3).

8.4.3 Are the products amplified from the rice YAC and barley genomic templates candidates for the Yd_2 resistance gene?

With the exception of the products **S2A1-a&b-BL-R** which were identified as retrotransposons, all other clone products showed no or very low probability identity matches with a wide range of subjects and therefore, most likely represent repetitive noncoding DNA. However, it is possible that Yd_2 is a novel gene and as such is unable to be identified by database searches. To address

this possibility, Northern analysis using the cloned products was conducted on BYDV infected and noninfected root and shoot material from resistant and susceptible cultivars in an attempt to identify possible gene transcripts. However, no hybridisation signals were detected and there is a strong possibility that these clones represent noncoding genomic DNA.

It is possible that the evolution of the Yd_2 resistance is the result of a transposable element disrupting or terminating a functional gene of the host plant, which may be needed by BYDV to replicate, resulting in a reduced viral titre in resistant plants. This is an interesting possibility considering that DNA clones **S2A1-a&b-BL-R** co-segregated with Yd_2 . Although Northern analysis, did not detect any mRNA the transcript may be of low abundance and unable to be identified by the Northern analysis conditions used in this study.

A number of products amplified from rice showed low identity matches with genes or transcripts which could be considered as possible candidate genes orthologous to Yd_2 (Table 8.2). Chitinases (Fric and Huttova, 1993; Schickler and Chet, 1997; Wu-GuSui *et al.*, 1997), Extensins and Hydroxproline rich glycoproteins (Wojtaszek *et al.*, 1995; Brownleader *et al.*, 1997; Li *et al.*, 1999), Lipoxygenases (Esquerre-Tugaye *et al.*, 1992; Slusarenko *et al.*, 1992; Veronesi *et al.*, 1996; Feussner *et al.*, 1997), Serine Proteases (Tornero *et al.*, 1996) and Phenylalanine ammonia lyases (Cui *et al.*, 1996; Orczyk *et al.*, 1996; Zeyen *et al.*, 1995) have all been implicated in resistance to plant pathogens. Therefore, it is possible that if any of the DNA clones amplified from the rice YAC represent such a gene, this gene may be orthologous to Yd_2 . Importantly, these clones were shown by PFGE to map physically close to the loci *Xrc122* and *XrYlp*, syntenous with the barley loci *Xc122* and *XYlp* which co-segregate with Yd_2 providing some evidence that these clones may represent a possible candidate gene/s. However, when Northern analysis was conducted on BYDV infected and noninfected root and shoot material from resistant and susceptible cultivars no transcripts were detected. It is possible that the transcripts from the Yd_2 gene and its orthologous counterpart in rice, are in low abundance and are not detected by Northern analysis. Therefore, a more sensitive method such as screening cDNA libraries with these probes may be more fruitful. It is also possible that like the products cloned from barley these clones represent noncoding genomic DNA.

CHAPTER 9

GENERAL CONCLUSIONS

9.1 Prospects for the map-based isolation of the *Yd₂* gene from barley

As a result of the work described in this thesis, considerable progress has been made towards the map-based isolation of the *Yd₂* gene for BYDV resistance. With regard to isolating this gene, the most significant achievements were;

- 1) the development of a reliable resistance assay screen to accurately characterise the *Yd₂* genotype.
- 2) finalisation of the genetic maps of the *Yd₂* region of barley Chromosome 3 and the identification and characterisation of a number of individuals with a recombination event between our closest flanking markers and the *Yd₂* gene.
- 3) the demonstration of close physical linkage between a marker genetically distinct from *Yd₂* and a marker co-segregating with the *Yd₂* gene, thereby demonstrating that chromosome walking in this region of the genome is an appropriate strategy to cloning *Yd₂*.
- 4) the identification of large insert genomic barley clones encompassing a co-segregating marker and a genetically distinct marker with the demonstration of physical overlap between these clones, thereby establishing a partial contig across the *Yd₂* gene.

The continuing effort in our laboratory involves PCR amplification of BAC-specific end probes for the identification of more BACs from the *Yd₂* region of barley Chromosome 3 in order to create a series of overlapping clones covering the entire *Yd₂* region. Overall, the author is satisfied that satisfactory progress is being made, and a complete contig will be established across the *Yd₂* region in the immediate future.

Once a series of overlapping clones is established across the *Yd₂* gene then the entire BAC contig covering this region can be sequenced. Computer analysis of this sequence can be undertaken using gene prediction programs to identify putative candidate genes. Probes derived from the BAC contig based on this information can be used for cDNA capture and Northern analysis. These probes can also be used for Southern analysis to delimit the physical area around the *Yd₂* gene using individuals identified as recombinant between our

two closest flanking markers in the Atlas × Atlas68 mapping population. The delimitation of the physical area will reduce the number of candidate genes to be analysed. It is envisaged that proof of function of any candidate gene will be confirmed through complementation of the phenotype by either transformation or mutagenesis.

Through genetic transformation, the *Yd₂* gene could then be introduced into new barley cultivars or even allow the *Yd₂* gene to be introduced into other cereal species. The cloning of *Yd₂* will also allow the characterisation of *Yd₂* and the mechanism through which it acts, and contribute important information to our general understanding of plant disease resistance.

9.2 Cloning a *Yd2* orthologue from rice

Rice was investigated as possible subgenomic cloning vehicle to clone the *Yd₂* gene. The rationale behind this was simple, the rice genome is approximately ten-fold smaller than the barley genome while maintaining a high level of both gross colinearity and micro-synteny between rice and barley. Therefore, it was envisaged that rice genomic clones may be used to bridge the gap between *Yd₂* and its closest flanking markers. Thus, markers which flank *Yd₂* by several megabases in barley may span only several hundred kilobases in rice, the approximate size of a YAC or BAC clone. More importantly a rice orthologous gene may be identified which could then be used to identify the *Yd₂* gene in barley. While rice genomic clones of the *Yd₂* region were identified, and a close physical linkage was demonstrated between our two co-segregating markers, possible breakdowns in micro-synteny and gaps in the rice contig were a major limitation to this approach. Therefore this avenue was discontinued.

To-date, the gaps in rice contig in this area have been covered. More importantly BAC clones are available instead of the much maligned YAC clones for use in establishing a series of overlapping large insert genomic clones across the syntenous region of rice. Therefore, it is now possible to use rice as a subgenomic cloning vehicle to clone *Yd₂*. However, with the imminent sequencing of the rice genome across the region of Chromosome 1, the area syntenous with the *Yd₂* region of barley Chromosome 3, this may not be required. The rice sequence freely accessible from the Japanese Rice Genome Project public database will soon

be able to search for possible Yd_2 orthologous genes. However, as previously discussed a Yd_2 orthologue may have moved to a non-syntenic position in the rice genome, secondly a gene orthologous to Yd_2 may not exist in rice. Therefore, the most prudent option for us was to proceed with a map-based approach in barley.

The sequence data generated from the Rice Genome Project will be immensely valuable in enabling many genes in large genome cereals such as barley and wheat to be identified through a synteny approach in rice. This will be quicker and cheaper than the previous arduous methods of map-based cloning as described in this study. However breakdowns in micro-synteny must be considered when undertaking such a strategy.

9.3 Cloning Yd_2 by homology to characterised plant disease resistance genes

As mentioned in Chapter 1 (Section 1.3.3), more than twenty different plant disease resistance genes have, at the time of writing, been cloned, characterised and described in the literature. Interestingly, the deduced proteins encoded by many of these genes share structural similarities, and regions of close homology at the amino acid level (Baker *et al.*, 1997; Hammond-Kosack and Jones, 1997).

The finding that regions within distinct resistance genes are conserved has prompted a number of research groups to use a PCR-based strategy in their efforts to clone new resistance genes. In this approach, degenerate primers designed using sequence information from characterised resistance genes have been used for the isolation of resistance gene homologues (Kanazin *et al.*, 1996; Leister *et al.*, 1996; Yu *et al.*, 1996; Leister *et al.*, 1998). These are then mapped, either in the plant species from which they were isolated, or in related plant species. If a resistance gene homologue maps to the same locus as a known resistance gene, then the resistance gene homologue is a candidate for that gene.

A PCR approach to cloning Yd_2 was undertaken, however while a number of products amplified were shown to be genetically close to Yd_2 , sequencing of these products showed no identity to previously cloned resistance genes. To-date there are no reports in the literature identifying any RGAs close to the Yd_2 region. Thus, a PCR-based approach to the cloning of resistance genes in barley does not appear to have succeeded in identifying Yd_2 at this stage.

Therefore, the possibility exists that the structure of Yd_2 could be different to that of any cloned resistance gene (as was the case with *Mlo*), or indeed any other plant disease resistance gene. Hence, the cloning of Yd_2 using a PCR-based approach may not be possible at all.

9.4 Structure and function of the Yd_2 gene

Plant pathogen resistance genes isolated so far were discussed in Section 1.3.3. The majority 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.

9.5 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 in rice (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 isolation of BYDV resistance genes such as Yd_2 may provide the opportunity to produce BYDV resistant cereal cultivars by genetic transformation. 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. 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.

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Errata

On pages 139, 140 and Figure 8.2 it is reported that five distinct clones were identified after sequencing PCR products from the rice YAC 2733 derived from the *Yd₂* syntenic region. Examination of the five sequences as presented in Figure 8.2, fails to support the existence of five distinct sequences. Clones N11 and N12 are a reverse complement of the same sequence, thus are 100% identical contrary to the statement there was no significant identity. The region defined by the internal duplication of the primers in clone N10 is a reverse complement of the corresponding region of N13 and N14.

Pages 142, 146, 147, Table 8.3. In the absence of segregation data, it is not appropriate to refer to the number of amplified fragments resolved in a gel as being equal to the number of loci. Referring to them as fragments is suffice.

Page 20 *Bdv1* is considered to be a tolerance rather than resistance gene to BYDV.