CHAPTER 1

INTRODUCTION

1.1 Barley yellow dwarf virus

1.1.1 General features

Barley yellow dwarf virus (BYDV) was first recognised as a pathogen of cereals during a severe epidemic of the disease in California in 1951 (Oswald and Houston, 1953a), and has since become regarded as the most damaging viral pathogen of cereal crops worldwide (Plumb, 1983). BYDV consists of a suite of viruses in the luteovirus group of plant viruses (Randles and Rathjen, 1995). These viruses have been grouped together under the common name of BYDV because of their similar physical and biological properties, their ability to induce stunting and leaf colouration symptoms, and the restriction of their host range to members of the grass (Gramineae) family. BYDV is transmitted by a range of aphid species in a circulative persistent manner, and cannot be transmitted mechanically or by seed (Oswald and Houston, 1953a). Hosts of BYDV include at least 150 species of annual and perennial weed and pasture grass species, and all major cereal crop species (Oswald and Houston, 1953b; D'Arcy, 1995). The virus particles are isometric in shape, 25 to 30 nM in diameter, and contain capsid protein monomers of 24 kDa (Plumb, 1992). The viral genome consists of one single stranded, positive sense RNA species of 5.5 to 5.7 kb, containing six open reading frames (Miller *et al.*, 1995). BYDV virus particles are only found in the phloem cells of infected tissue (Jensen, 1969). It is likely that this contributes to the inability of the virus to be transmitted mechanically, and to the low titre of the virus in infected plant tissues (Rochow and Brakke, 1964).

1.1.2 Isolates of BYDV

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 could be transmitted by each of four aphid vector species (Table 1.1). The five BYDV isolates collected in New York were found to be serologically distinct, based on enzyme immunosorbant assay (ELISA) with antisera

raised against purified virus particles (Rochow and Carmichael, 1979). In addition, nucleic acid probes representing regions of the genome of different BYDV isolates were also found to be capable of distinguishing the isolates (Fattouh *et al.*, 1990). Isolates of BYDV from around the world have been characterised using the four species of aphids shown in Table 1.1 and the antibodies raised against the five New York isolates of BYDV. With a few exceptions, the aphid transmission characteristics and serology of these viruses have been found to be the same as those of the New York isolates (reviewed in Power and Gray, 1995). Accordingly, the names given to the New York isolates, derived from the most efficient aphid vector species of each isolate (Table 1.1), have been widely adopted by researchers for categorising isolates of BYDV.

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
Rhopalosiphum padi L.	+	+++	-	+++	-
Rhopalosiphum maidis Fitch	-	-	-	-	+++
Sitobion avenae Fabr.	+++	++	-	-	-
Schizaphis graminum 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 infested plants that became infected.

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

1.1.3 Relationships between BYDV isolates

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 as shown Table 1.2. This grouping is based on a range of criteria, including serology (Aapola and Rochow, 1971; Rochow and Carmichael, 1979), ultrastructural symptomatology (Gill and Chong, 1979), 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). Recently, the sequencing of the RNA genome of a number of BYDV isolates in part or in full has provided additional information concerning the relationships between BYDV isolates (Miller *et al.*, 1988; Vincent *et al.*, 1990; Vincent *et al.*, 1991; Ueng *et al.*, 1992; Domier *et al.*, 1994). Most significantly, the genome organisation of the subgroup I isolates PAV and MAV was found to differ from the genome organisation of the subgroup II isolate RPV (Miller *et al.*, 1995). In addition, the degree of similarity between the nucleotide sequences of different BYDV isolates, shown by coat protein gene sequence comparisons (Domier *et al.*, 1994) and nucleic acid hybridisation studies (Fattouh *et al.*, 1990) have agreed with the classification of BYDV isolates into two subgroups.

Table 1.2. The classification of BYDV isolates into two subgroups

BYDV subgroup	BYDV isolate
I	MAV PAV SGV
II	RPV RMV

The differences between subgroup I and II BYDV isolates are so great that isolates from the two BYDV subgroups are considered by some to be two separate viruses (Miller *et al.*, 1995). Interestingly, the genome organisation, nucleotide sequence and serology of the subgroup II BYDV isolates are more similar to the luteoviruses beet western yellows virus (BWYV) and potato leafroll virus (PLRV), which infect dicotyledons, than to the BYDV isolates from subgroup I (Rochow and Duffus, 1978; Domier *et al.*, 1994; Miller *et al.*, 1995).

1.1.4 Effects of BYDV on the host

Oswald and Houston (1953a), Burnett (1984) and D'Arcy (1995) have described the visible symptoms of BYDV infection in detail. The most obvious effects include leaf colouration and a restriction of shoot growth. The colour which develops in infected leaves depends on the species and cultivar, and includes yellow, orange, red and purple. Leaf colouration may spread downwards from the leaf tips, or may appear in blotches or stripes. In addition, leaves may become stiff and brittle, and may develop undulations or serrations at the margins. Effects which contribute to reduction in grain yield include a decrease in the number of tillers, floret infertility, and a reduction in the size of heads and grains. Symptoms are sometimes unreliable for diagnosing BYDV infection in the field, as symptoms of BYDV infection resemble those caused by nitrogen and phosphorus deficiency (Paliwal and Comeau, 1987), and under some conditions, BYDV infection may be symptomless (Oswald and Houston, 1953b).

A light microscopy study of BYDV infected grass tissues (Esau, 1957) revealed degeneration of sieve elements, phloem companion cells and phloem parenchyma, in the large and small vascular bundles of leaves and roots. Transmission electron microscopy has revealed BYDV particles in the phloem of infected tissues and the ultrastructural changes that occur within these cells upon BYDV infection (Jensen, 1969; Gill and Chong, 1979). The severe symptoms in oats doubly infected by MAV and RPV isolates of BYDV are accompanied by the appearance of virus particles in xylem tissue as well as phloem tissue (Gill and Chong, 1981).

Physiological changes brought about by BYDV infection are believed to be a direct result of the degeneration of the vascular tissues and the resulting disruption of translocation within BYDV infected plants (Jensen, 1968a). These include an increase in the carbohydrate accumulation and percentage dry weight of leaves, an increased rate of respiration and a decreased rate of photosynthesis (Jensen, 1968b). Other changes within BYDV infected leaves included a decrease in the chlorophyll content and changes in the levels of nitrogenous compounds (Jensen, 1968a, 1968b).

The severity of symptoms caused by BYDV infection depends on the isolate or strain of the virus and the species or variety of the host (Smith, 1967; Rochow, 1969; Baltenberger *et al.*, 1987). Symptoms are sometimes more severe if an efficient rather than inefficient aphid vector species is used, or if greater numbers of aphids are used in the inoculation (Smith and Richards, 1963, Smith, 1967). These effects are believed to be due to the plant receiving a greater amount of virus inoculum (Smith and Richards, 1963). BYDV symptoms, including yield loss, are much greater when plants are inoculated at an early stage of growth and when infected plants are grown in cool conditions (15 to 20°C) which slow plant development (Smith, 1967; Catherall *et al*, 1970). Leaf colouration symptoms are generally more severe in bright lighting (Paliwal and Comeau, 1987; D'Arcy, 1995).

1.1.5 Economic importance and epidemiology of BYDV

BYDV is regarded as the most widespread and economically damaging viral pathogen of cereal crops worldwide (Plumb, 1983; Burnett *et al.*, 1995). The virus is found wherever cereals are grown (Lister and Ranieri, 1995). BYDV causes the greatest economical 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* C. Koch), although significant yield reductions due to BYDV also occur in maize (*Zea mays* L.), rice (*Oryza sativa* L.), triticale (*X Triticosecale* Wittmack) and rye (*Secale cereale* L.) (Pike, 1990; Lister and Ranieri, 1995). BYDV causes considerable losses to rice crops in Italy and Spain, but not elsewhere (Lister and

Ranieri, 1995). In North America, annual reductions in cereal harvests due to BYDV average 1.0 to 3.0%, although losses of 30 to 50% are not uncommon (Burnett, 1984; Paliwal and Comeau, 1987). In Australia and New Zealand, reductions in the value of cereal harvests due to BYDV have been estimated to be worth over US $100 million annually (Johnstone, 1995).

The extent and severity of BYDV damage to cereal crops determined by a number of factors, including the climate, and the BYDV isolates and aphid vector species that are present (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 Eastman, 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 serve as reservoirs for BYDV (Oswald and Houston, 1953a; Plumb, 1983; Irwin and Thresh, 1990).

In winter barleys (requiring vernalisation to flower), autumn BYDV infection can severely reduce survival during the winter when sub-freezing temperatures and ice encasement are experienced (Paliwal and Andrews, 1990). BYDV infected cereals are also more susceptible to the effects of drought, due to the reduced size of their root systems (Oswald and Houston, 1953a). Synergistic effects have been observed between BYDV and the take-all fungus (*Gaeumannomyces graminis* var. *tritici* Walker) in wheat doubly infected with these pathogens (Sward and Kollmorgen, 1990). A range of fungal pathogens are more likely to affect BYDV-infected than virus-free wheat (Smith, 1962).

1.1.6 Methods of controlling BYDV damage

There are a number of strategies which are currently used to minimise the yield losses in cereal crops due to BYDV infection. Severe damage due to BYDV infection at an early and vulnerable stage of growth by the most concentrated flights of viruliferous aphids can be avoided by adjusting sowing dates (Paliwal and Comeau, 1987; Plumb and Johnstone, 1995). The elimination of field weeds and nearby grasses which serve as

reservoirs for BYDV and vector aphids between growing seasons can reduce BYDV infection of subsequent crops (Osler *et al.*, 1980; Plumb, 1983). The practice of applying insecticides to control BYDV vectors is used in Europe, where it is part of the intensive farming practiced there, but is not an economically viable option for BYDV control elsewhere (Plumb, 1983). In some areas, biological control of BYDV vectors has been extremely successful. For example, in South America, the introduction of several aphid predators and parasites has largely replaced the need for pesticide application (Zúñiga, 1990). In some cereals, sources of resistance to feeding by BYDV vectoring aphid species has been identified, although attempts to use these to limit BYDV infection of crops have not been reported (Irwin and Thresh, 1990).

The use of cereal cultivars which are resistant to BYDV is an effective and cheap means of minimising the losses caused by BYDV (Plumb and Johnstone, 1995). Furthermore, it is likely to be more socially acceptable than the application of insecticides, due to concerns regarding the damage these chemicals may cause to the environment (Plumb, 1992). However, the use of BYDV-resistance to control crop damage is limited by the sources of resistance available, together with problems of incorporating these sources of resistance into new cultivars (Qualset, 1990). Known sources of resistance to BYDV in cereals are described in Section 1.3.

Genes derived from different parts of the genome of a range of viruses have been found to provide resistance against the viruses from which they were derived when genetically transformed into the host. Such artificial resistance genes may prove to be an effective means of producing BYDV resistant cereal cultivars in the future (Miller and Young, 1995). Transgenic potato plants containing the coat protein gene from potato leaf roll virus (PLRV) reduce the replication of PLRV to 10% of the levels of untransformed plants (Kawchuck, 1990). As PLRV is also a luteovirus, these results are an indication that BYDV coat protein transformation may afford protection against BYDV in cereals. According to Burnett *et al.* (1995) and Miller and Young (1995), a number of research groups are currently assessing the effectiveness of BYDV coat protein genes in providing cereals with BYDV resistance. Genetic transformation of

cereals with naturally occurring BYDV resistance genes (such as the Yd_2 gene discussed in Section 1.4) may provide another means of engineering BYDV resistance (Miller and Young, 1995).

1.2 Pathogen resistance in plants

1.2.1 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), characterised by rapid localised cell death at the point of infection (Keen, 1990). The death of the cells in HR lesions is unlikely to be sufficient to arrest the pathogen. Rather, these necrotic cells are thought to produce elicitors which induce processes in the surrounding cells which directly combat infection (Ponz and Bruening, 1986; Keen, 1990). Host proteins that are induced upon infection include pathogenesis related (PR) proteins such as β-1,3-glucanases and chitinases, which have proven anti-microbial properties (Linthorst, 1991), and enzymes for the synthesis of low molecular weight compounds which are toxic to pathogens (Ebel, 1986; Keen, 1990). Infection often induces a systemic acquired resistance (SAR), which provides all parts of the host with increased levels of resistance against subsequent infection by the original pathogen or by other pathogens (Ryals *et al.*, 1994).

1.2.2 Other mechanisms of disease resistance

Not all pathogen resistance in plants can be attributed to an active defence 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 (Prior 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 *et al.*, 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).

1.2.3 The genetics of plant pathogen resistance

Plant pathogen resistance genes often have alleles which allow the specific recognition of pathogen races containing corresponding avirulence genes. 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 resistance 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). Other examples of multiple genes or alleles at single plant disease resistance loci are reviewed by Prior 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).

1.2.4 Molecular characterisation of pathogen resistance in plants

The isolation of plant pathogen resistance genes has been a necessary step toward gaining a complete understanding the molecular basis of resistance gene action in plants. The first resistance gene isolated was the *HMI* 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). Resistance genes which have been subsequently isolated all mediate race-specific resistance involving HR (Table 1.3). Despite the fact that the *RPS2, N, L6* and *RPM1* genes provide resistance to diverse pathogens (a bacterium, virus and a fungus), the predicted protein products each contain leucine-rich repeats and ATP-GTP nucleotide binding motifs, suggesting that these genes may act by similar mechanisms. Leucine-rich repeat motifs are known to participate in protein-protein interactions (Kobe and Deisenhofer, 1994), and may therefore be involved in the perception of the avirulence protein or the activation of regulatory proteins which begin the defence response. Similarly, the *Pto* gene for resistance to *P. syringae* van Hall in tomato encodes a sereine-threonine protein kinase which may activate proteins involved in subsequent stages of the defence mechanism by phosphorylation (Martin *et al.*, 1993).

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 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, 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).

Table 1.3. Isolated plant pathogen resistance genes involved in HR resistance

gene	host	pathogen	leucine rich repeat†	ATP-GTP binding domain†	authority
Pto	tomato§	*P. syringae*	no	no	1
Cf-9	tomato§	*Cladosporium fulvum* Cooke	yes	no	2
RPS2	*A. thaliana* L.	*P. syringae*	yes	yes	3, 4
N	tobacco¥	tobacco mosaic virus	yes	yes	5
L6	flax	*M. lini*	yes	yes	6
RPM1	*A. thaliana* L.	*P.syringae*	yes	yes	7

†These motifs are predicted to occur in the resistance gene products

§Introgressed into *Lycopersicon esculentum* Mill. from *L. pimpinellifolium* (Jusl.) Mill.

¥Introgressed into *Nicotiana tabacum* L. from *N. glutinosa* L.

1. Martin *et al.* (1993) 2. Jones *et al.* (1994) 3. Bent *et al.* (1994)
4. Mindrinos *et al.* (1994) 5. Whitham *et al.* (1994) 6. Lawrence *et al.* (1995) 7. Grant *et al.* (1995)

1.3 BYDV resistance in cereals

1.3.1 The nature of BYDV resistance in cereals

Resistance to BYDV in cereals is characterised by a general reduction in the severity of BYDV symptoms such as stunting and leaf yellowing. Protection against the development of severe BYDV symptoms has sometimes been found to be accompanied by reduced levels of virus accumulation as measured by ELISA, northern dot blot hybridisation and virus particle purification (Jedlinski *et al.*, 1977; Skaria *et al.*, 1985; Xin *et al.*, 1988; Lorens *et al.*, 1989; Gray *et al.*, 1993; Ranieri *et al.*, 1993; Makkouk *et al.*, 1994; Sharma *et al.*, 1995; Larkin *et al.*, 1995; Banks *et al.*, 1995). For many cereal lines showing little or no BYDV symptom expression, reductions in virus titre have not been detected or have not been tested for (Burnett *et al.*, 1995). Cooper and Jones (1983) defined the ability to develop less severe disease symptoms as tolerance, and the ability to limit the pathogen growth as resistance. However, for the sake of simplicity,

resistance will be the term used throughout this thesis to encompass all forms of genetically determined protection against BYDV. Sources of resistance in oat and barley have been found to be effective against some isolates of BYDV but not others (Skaria *et al.*, 1985; Baltenberger *et al.*, 1987; Gray *et al.*, 1993).

Screening programs to identify BYDV resistant cereal lines have been conducted in over 15 countries, including Syria, Mexico, Canada, USA, China, Australia, New Zealand, and Great Britain (Burnett, 1990, and references therein). Searches for BYDV resistance has been most intensive in wheat, barley and oat, as the total cost of BYDV damage is greater for these three cereals than for any others. The following sections describe sources of BYDV resistance available in a number of cereal crops, and their genetic basis where this is known.

1.3.2 Barley

Overall, high levels of BYDV resistance have been found in barley. The main source of BYDV resistance used in barley breeding programs has been the Yd_2 gene, originating from a number of Ethiopian barleys (Rasmussen and Schaller, 1959). This gene has provided substantial levels of resistance against BYDV for a number of years in a range of barley cultivars, and continues to be used by breeders (Qualset *et al.*, 1990; Burnett *et al.*, 1995). The Yd_2 gene is discussed in greater detail in Section 1.3. Suneson (1955) identified a recessive BYDV resistance gene named *yd1* in the commercial barley cultivar Rojo. However, this gene provides less resistance to BYDV than Yd_2, and has not been used widely in breeding (Burnett *et al.*, 1995).

Numerous sources of BYDV resistance have been found among winter barleys (requiring vernalisation to flower). In a screen of winter barleys, Grafton *et al.* (1982) found a high level of BYDV resistance in the winter cultivar Post, and intermediate levels of resistance in the cultivar Perry and four experimental lines. The four winter barley cultivars Surry (Starling *et al.*, 1980a), Henry (Starling *et al.*, 1980b), Monroe (Starling *et al.*, 1980c) and Maury (Starling *et al.*, 1980d), released by the Virginia Polytechnic Institute also possess high levels of BYDV resistance, as do the winter

barley cultivars Acton and Elmira, bred at the University of Guelph, Canada (Burnett *et al.* 1995). Sixteen winter barleys with BYDV resistance greater than or equal to that of Post or the Yd_2 containing cultivar Vixen were identified by Habekuβ and Lehmann (1991).

The spring barley cultivars Windich (Porterman, 1989), bred in Western Australia, and Norbert (Metcalfe and Bendalow, 1981) also show high levels of BYDV resistance (Burnett *et al.*, 1995). However, the resistance in Norbert may be due to the Yd_2 gene, as this variety was bred from the Ethiopian barley CI 5791, found to be BYDV resistant by Gill and Buchannon (1972). In a screen of a worldwide barley collection, the Chinese barley line CI 1113 was found to be as BYDV resistant as Yd_2 containing Ethiopian barleys (Schaller *et al.*, 1963), although the genetic basis of the resistance in this line was not subsequently investigated (Schaller *et al.*, 1964). Other barley cultivars and lines possessing intermediate levels of BYDV resistance have been identified by Damsteegt and Bruehl (1964). Particular sources of BYDV resistance in barley are discussed in more detail in Chapter 8.

Lines derived from interspecific crosses between cultivated barley (*Hordeum vulgare* L.) and *H. brachyantherum* L., *H. bogdanii* Wilensky and *Elymus mollis* Trin. have shown some resistance to BYDV. However, these lines were not as BYDV resistant as lines containing the Yd_2 gene (Melzer *et al.*, 1980).

1.3.3 Oat

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 one, two, three or four 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).

High levels of BYDV resistance have been found in the hexaploid oat species *A. fatua* L. and *A. sterilis* L., and in the diploid oat species *A. stringosa* (Schreber) (Rines *et al.*, 1980; Comeau, 1984a, Jedlinski, 1984). Interspecific hybrids between these species and cultivated hexaploid oat may provide a means of improving BYDV resistance in this cereal (Qualset *et al.*, 1990).

1.3.4 Wheat

Overall, the best sources of BYDV resistance available in wheat are less effective than BYDV resistance known in barley and oat (Burnett *et al.*, 1995). Two sources of BYDV resistance in wheat are represented by the spring wheat cultivar Anza and the Yugoslavian wheat line NS 879/4. In Anza, the BYDV resistance is provided by the incompletely dominant gene *Bdv1*, located on chromosome 7D (Singh, 1993; Singh *et al.*, 1993). Tandon (1990) showed that the BYDV resistance in Anza is conferred by one or two genes. BYDV resistance from other sources in wheat have shown quantitative inheritance, and are therefore likely to be controlled by multiple genes (Cisar, 1982; Qualset, 1992). As in oat, BYDV resistance genes in wheat have additive effects when combined (Qualset *et al.*, 1973). Other spring and winter wheats known to possess BYDV resistance are listed by Burnett *et al.* (1995).

Because of the poor BYDV resistance available in wheat, the potential to introduce BYDV resistance from other grass species into wheat has received much attention. The transfer of BYDV resistance from species closely related to wheat may be achieved by crossing these species to wheat, and obtaining derivatives of the hybrids, consisting of the chromosomes or chromosome segments containing the resistance genes, in a genetic background derived principally from wheat.

Recombination between the chromosomes of wheat and the resistant species may be obtained using homoeologous chromosome pairing mutants or by cell culture (Banks *et al.*, 1995).

A program to introduce the *Yd*$_2$ gene from barley into wheat was described by McGuire and Qualset (1990), but the progress of this program has not been reported since. Other close 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). Progress has been made toward the introduction of BYDV resistance from a number of these species into wheat (Zhong *et al.*, 1994; Sharma *et al.*, 1995; Larkin *et al.*, 1995; Banks *et al.*, 1995). During these programs, individual chromosomes responsible for the BYDV resistance in these species have been identified. In *Thinopyrum intermedium* [(Host) Barkworth and Dewey], BYDV resistance has been shown to be associated with homoeologous group 7 chromosomes in two different accessions (Brettell *et al.*, 1988; Sharma *et al.*, 1995), and homoeologous group 2 chromosomes in a third (Larkin *et al.*, 1995). In *Lophopyron ponticum* (Podp.), BYDV resistance is controlled by chromosomes from one or more of the homoeologous groups 3, 5 and 6 (Zhong *et al.*, 1994). The association of BYDV resistance with individual chromosomes suggests that the resistance from these species is controlled by single genes which will be amenable to introgression.

1.3.5 Rye and triticale

Among the cultivated cereals, rye is the most resistant to BYDV (Oswald and Houston, 1953a). Triticale is a man-made hybrid between wheat and rye, and also demonstrates high levels of BYDV resistance in many lines (Collin *et al.*, 1990). Resistance has been shown to be associated with the rye chromosomes 1R and 2R in the triticale cultivars Muskox and Nord Kivu, respectively (Nkongolo *et al.*, 1992). Genetic studies by Collin *et al.* (1990) suggested that BYDV resistance in a number of triticale cultivars was controlled by a small number of genes.

1.3.6 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 under different growth conditions. The gene has not been named or mapped, but is currently being incorporated into new BYDV resistant rice varieties in Italy.

1.4 The *Yd2* gene for BYDV resistance in barley

1.4.1 Discovery and genetic characterisation of *Yd2*

Schaller *et al.* (1963) screened entries from a worldwide barley collection for resistance to BYDV. Of the lines found to be most resistant, all were from Ethiopia, except for three which were hybrids with Ethiopian parents, and one which was from China. BYDV resistance showed a distinct geographical concentration, as only 9% of the entries from the collection were from Ethiopia. BYDV resistance in Ethiopia was found in both late and early maturing barleys, although resistance was mostly found in early barleys originating from high altitudes (Qualset, 1975). Additional BYDV resistant lines from Ethiopia have been identified by Qualset and Schaller (1969).

The inheritance of BYDV resistance from 16 of the Ethiopian lines was subsequently examined by Rasmusson and Schaller (1959), Damsteegt and Bruehl (1964) and Schaller *et al.* (1964). BYDV resistance from each Ethiopian barley segregated as an incompletely dominant monogenic trait in families derived from crosses between the resistant barleys and barleys that were susceptible. Crosses between pairs of resistant lines yielded no susceptible individuals in over 3,000 F_2 and F_3 progeny. This suggested that the resistance in the different lines was controlled by the same gene, or by a number genes located within 0.92 to 3.24 map units of one another (Rasmusson and Schaller, 1959; Schaller *et al.*, 1964). For the sake of simplicity, the BYDV resistance from the different Ethiopian lines is commonly

referred to as being controlled by a single gene, named *Yd2* (Rasmusson and Schaller, 1959).

Schaller *et al.* (1964) located *Yd2* to barley chromosome 3, by detecting linkage between *Yd2* and morphological markers, a disease resistance locus and translocation break points known to be located on this chromosome. The data of Schaller *et al.* (1964) have been used to position *Yd2* on a genetic map spanning the whole of barley chromosome 3, containing cytogenetic markers, genes for morphological traits, isoenzyme loci and disease resistance loci (Søgaard and von Wettstein-Knowles, 1987).

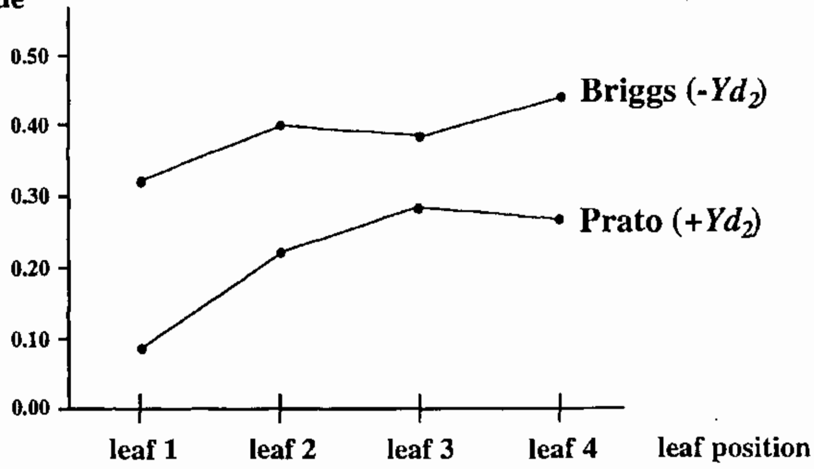
1.4.2 The nature of *Yd2*-mediated BYDV resistance

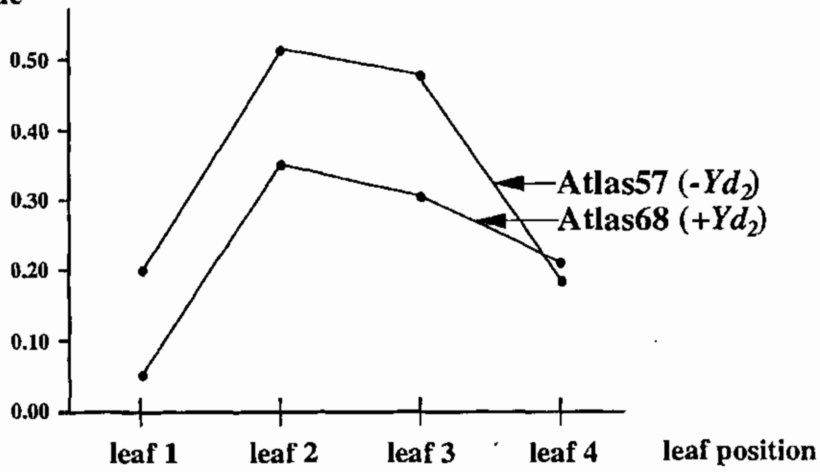
The effects of the *Yd2* gene have been well characterised by comparing closely related barley lines with and without *Yd2*. ELISA studies have shown that *Yd2* limits the level of virus accumulation in infected tissues (Skaria *et al.*, 1985; Pereira and Lister, 1989; Larkin *et al.*, 1991; Ranieri *et al.*, 1993; Makkouk *et al.*, 1994). The *Yd2* gene does not prevent the virus from spreading systemically from the point of infection. In addition, it does not appear to confine the virus to the inoculated leaf to any degree, according to the representation of the data of Ranieri *et al.* (1989) shown in Figure 1.1. From the available evidence, the *Yd2*-mediated resistance appears to act by reducing the rate of BYDV replication within the phloem, rather than by restricting the movement of virus within the phloem sieve tubes or between the sieve tubes, phloem parenchyma and phloem companion cells. The study of Larkin *et al.* (1991) showed that *Yd2* did not reduce the rate of BYDV replication in individual protoplasts. However, this does not necessarily contradict the theory that *Yd2* reduces replication within host cells, as the protoplasts were derived principally from tissues other than the phloem which may have patterns of gene expression differing from that of the phloem tissues in which BYDV normally replicates (Larkin *et al.*, 1991).

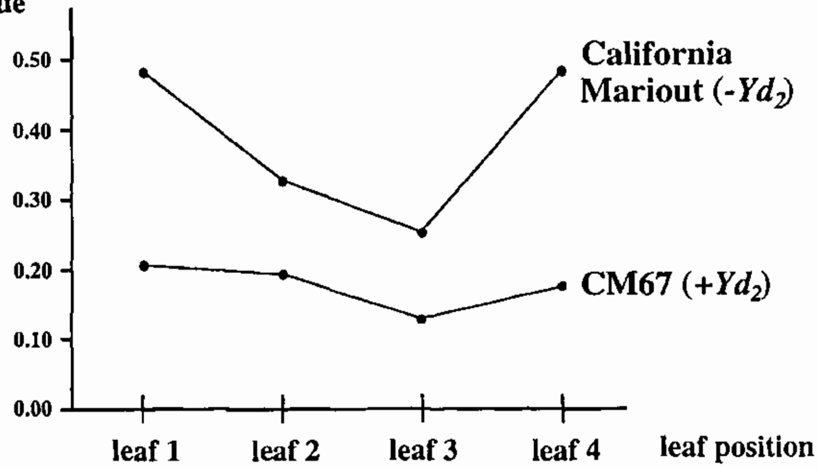
Yd2 has been found to be effective against the BYDV isolates PAV and MAV from subgroup I, but not against the BYDV isolates RPV and RMV from subgroup II, according to measurements of symptoms and viral accumulation (Skaria *et al.*, 1985;

Figure 1.1. Pattern of BYDV accumulation in barley lines with and without Yd_2

In the study of Pereira and Lister (1989), ELISA was used to measure the concentration of BYDV-PAV capsid protein in three pairs of barley cultivars nearly-isogenic for Yd_2, 12 days after infection. The raw data is presented here in graph form to illustrate the possible effects of the Yd_2 gene on the pattern of systemic BYDV-PAV spread. The Yd_2-containing cultivars generally showed a lower level of virus accumulation. Inoculated and systemic leaves showed similar reductions in virus accumulation due to Yd_2, suggesting that Yd_2 does not inhibit long range transport of BYDV through the phloem. Horizontal axis shows leaf position; leaf 1 = inoculated (oldest) leaf, leaf 2 = leaf produced after the inoculated leaf, etc.


ELISA value
0.50
0.40
0.30
0.20
0.10
0.00
Briggs (-Yd_2)
Prato (+Yd_2)
leaf 1
leaf 2
leaf 3
leaf 4
leaf position


ELISA value
0.50
0.40
0.30
0.20
0.10
0.00
Atlas57 (-Yd_2)
Atlas68 (+Yd_2)
leaf 1
leaf 2
leaf 3
leaf 4
leaf position


ELISA value
0.50
0.40
0.30
0.20
0.10
0.00
California Mariout (-Yd_2)
CM67 (+Yd_2)
leaf 1
leaf 2
leaf 3
leaf 4
leaf position

Baltenberger *et al.*, 1987; Pereira and Lister, 1989; Herrera and Plumb, 1989; Ranieri *et al.*, 1993; Makkouk *et al.*, 1994). BYDV-PAV accumulation is significantly reduced by Yd_2 in the roots and the shoots, whereas the accumulation of BYDV-MAV appears to be reduced in the roots only (Skaria *et al.*, 1985; Ranieri *et al.*, 1993). Although the 3′ end of the BYDV genome contains genes which are conserved between all isolates of BYDV, the gene content of the 5′ end of the BYDV genome is fundamentally different in subgroup I and II isolates (Miller *et al.*, 1995). Considering that Yd_2 appears to be effective against BYDV subgroup I isolates only, it is tempting to speculate that the viral gene responsible for sensitivity to the Yd_2-mediated resistance is located in the 5′ end of the genome of subgroup I isolates. This genomic region contains an open reading frame encoding the viral replicase, and another which encodes a protein of unknown function (Miller *et al.*, 1995).

Fewer than five Ethiopian barleys have been used as the source of Yd_2 in tests to determine the specificity of the Yd_2 gene against different BYDV isolates. Furthermore, most of these tests have been performed using only one or two BYDV isolates. Considering the limited nature of these investigations, the existence of alleles of the Yd_2 gene which differ in resistance specificity cannot yet be ruled out.

1.4.3 Expression of Yd_2-mediated BYDV resistance

The effectiveness of Yd_2-mediated BYDV resistance is strongly influenced by growth rate. This effect can be seen when the Yd_2 gene is introduced into genetic backgrounds of barley lines which differ in their rate of maturity. For example, the Yd_2 gene from an Ethiopian barley CI 3208-2 was ineffective when bred into the genetic background of a slow maturing barley line, and effective when reintroduced into the genetic background of a fast maturing barley line (Jones and Catherall, 1970). Also, in homozygous resistant 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 (Jones and Catherall, 1970). Growth conditions can also bring about 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. Jones and Catherall (1970) suggested that a 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 F_1 or F_2 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 BYDV 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 was also influenced by the growth conditions. For example, the Yd_2 gene from the Ethiopian barley CI 1237 was codominant when plants were grown in a warm glasshouse, and recessive when grown in a cool glasshouse. 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.

1.4.4 The use of Yd_2 in breeding

Five different Ethiopian barleys have been used in the breeding of 16 Yd_2-containing, BYDV resistant barley cultivars in the USA, Wales and Australia (Table 1.4). 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 be desirable to provide cultivars with resistance to a broader range of BYDV isolates (Qualset *et al.*, 1990; Burnett *et al.*, 1995).

At the Welsh Plant Breeding Station, initial attempts to use Yd_2 to produce slow maturing barley lines with sufficient levels of BYDV resistance failed, due to the diminishing effect of slow growing genetic backgrounds on BYDV resistance levels (Catherall *et al.*, 1977). However, this problem was overcome by using the Ethiopian Yd_2 donor barley CI 3906-1, which provides a particularly high level of BYDV resistance (Catherall *et al.*, 1977). This enabled the slow maturing, BYDV resistant barley cultivars Coracle (Catherall *et al.*, 1977) and Vixen (Parry and Habgood, 1986) to be produced. In California, another slow maturing BYDV resistant barley cultivar (Sutter) was bred using the Yd_2 gene from the Ethiopian barley line CI 1237 (Qualset *et al.*, 1990). Success in introducing Yd_2 mediated BYDV resistance into slow maturing winter barley genotypes has also been reported by Delogu *et al.* (1995). Other problems experienced while using Yd_2 in barley improvement are discussed in Chapter 3.

Table 1.4. BYDV resistant barley cultivars containing *Yd2*[†]

variety	source of *Yd2*[§]	region[¥]	reference
Atlas 68	CI 3920-1	USA	Schaller and Chim, 1969a
Coracle	CI 3906-1	Wales	Catherall *et al.*, 1977
CM67	CI 2376	USA	Schaller and Chim, 1969b
CM72	CI 2376	USA	Schaller *et al.*, 1977
Franklin	CI 3208-1	Australia	Vertigan, 1991
Nomini	BYDV resistant Atlas	USA	Starling *et al.*, 1994
Prato	CI 2376	USA	Schaller *et al.*, 1979
Shannon	CI 3208-1	Australia	Symes, 1979; Vertigan, 1979
Sutter	CI 1237	USA	Schaller *et al.*, 1973
UC337	CI 1237	USA	Schaller *et al.*, 1990a
UC476	CI 1237 or CI 2376	USA	Schaller *et al.*, 1990c
UC566	CI 2376	USA	Schaller and Prato, 1973
UC603	CI 2376	USA	Schaller *et al.*, 1990b
Venus	CI 3920-1	USA	Brown *et al.*, 1988
Vixen	CI 3906-1	Wales	Parry and Habgood, 1986
Wysor	BYDV resistant Atlas	USA	Starling *et al.*, 1987

[†]reproduced in part from Burnett *et al.* (1995)

[§]Sources of *Yd2* were BYDV resistant Ethiopian barley lines, unless stated as BYDV resistant Atlas. The source of *Yd2* in the BYDV resistant Atlas was not recorded in the literature.

[¥]region where barley varieties was bred

1.5 The molecular analysis of grass genomes

1.5.1 Genetic maps of cereal chromosomes

Genetic maps of cereal chromosomes have been constructed using morphological, biochemical and cytogenetic loci (Søgaard and von Wettstein-Knowles, 1987; Milne and McIntosh, 1990; Coe *et al.*, 1990). Further development of these maps

are limited by the numbers of morphological, biochemical and cytogenetic loci showing identifiable polymorphisms within these cereal species. In contrast, the numbers of genomic DNA polymorphisms which can be detected using molecular genetic markers is virtually unlimited. The use of molecular markers has led to significant progress in the genetic characterisation of grass genomes. Genetic maps containing molecular markers have been constructed in wheat (Liu and Tsunewaki, 1991; Devos and Gale, 1993a; Nelson *et al.*, 1995a, 1995b; Van Deynze *et al.*, 1995a), barley (Heun *et al.*, 1991; Kleinhofs *et al.*, 1993; Graner *et al.*, 1991, 1994), rye (Baum and Appels, 1991; Devos *et al.*, 1993; Philipp *et al.*, 1994), *Triticum monococcum* L. (Van Deynze *et al.*, 1995a), oat (O'Donoughue *et al.*, 1992, 1995; Rayapati *et al.*, 1994; Van Deynze *et al.*, 1995b), maize (Burr and Burr, 1991; Gardiner *et al.*, 1993), sorghum (Hulbert *et al.*, 1990; Whitkus *et al.*, 1992) rice (Causse *et al.*, 1994; Kurata *et al.*, 1994a), and *Triticum tauschii* (Coss.) Schmal., the D genome progenitor of bread wheat (Gill *et al.*, 1991; Lagudah *et al.*, 1991).

Restriction Fragment Length Polymorphisms, or RFLPs (Botstein *et al.*, 1980; Tanksley *et al.*, 1989) are by far the most common types of molecular markers mapped in cereals. Other types of molecular markers which have had more limited use in the genetic mapping of cereal chromosomes include Random Amplified Polymorphic DNAs, or RAPDs (Williams *et al.*, 1990; Welsh and McClelland, 1990), Amplified Restriction Fragment Length Polymorphisms, or AFLPs (Zabeau and Vos, 1993; Vos *et al.*, 1995; Becker *et al.*, 1995), Sequenced Tagged Sites, or STSs (Olson *et al.*, 1989; Talbert *et al.*, 1994), and microsatellite loci detected by PCR (Becker and Heun, 1995). Each of these marker types have advantages and disadvantages over the others. RFLP markers are appreciated for their reproducability, their ability to show useful levels of polymorphism within species, and their ability to be transferred to different crosses and species.

1.5.2 The evolution of genomic DNA sequences in grasses

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, genome size varies considerably within this family. 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 4.2×10^8 bp per haploid nucleus (Arumuganathan and Earle, 1991). Much of the additional DNA in the larger cereal genomes consists of repetitive sequences. These constitute 83% and 76% of the large genomes of barley and wheat, respectively, but only 50% of the small 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 hybridises 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.*, 1995c). Sequences identified in two grass species by a single cDNA probe are likely to represent genes which have descended from a single ancestral gene (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.

1.5.3 Comparative mapping in the Gramineae

Genes and other genomic sequences which are derived from a single sequence in an ancestral species are referred to as being orthologous (Hart, 1987). The relative positions of orthologous sequences in different grass species have been determined by mapping these sequences as RFLP markers, using common sets of probes. Comparative

mapping of orthologous RFLP loci has been performed among the following grasses: wheat, barley, rye, oat, rice, maize, sorghum, *T. monococcum* , and *T. tauschii* (Hulbert *et al.*, 1990; Whitkus *et al.*, 1992; Wang *et al.*, 1992; Devos *et al.*, 1993; Devos and Gale, 1993b; Ahn *et al.*, 1993; Ahn and Tanksley, 1993; Kurata *et al.*, 1994b; Nelson *et al.*, 1995a, 1995b; Van Deynze *et al.*, 1995a, 1995b, 1995c). The chromosome positions of orthologous loci in these grasses have shown varying degrees of conservation. In general, if loci were closely linked to one another in one species, their orthologous counterparts were also found to be closely linked in other species. Furthermore, orthologous markers showing conserved linkage were nearly always located in the same order. Breaks in the collinearity of RFLP markers represent points at which chromosome translocations and other rearrangements occurred during the evolutionary divergence of the grasses from a common ancestral species (Whitkus *et al.*, 1992; Devos *et al.*, 1993). These chromosome rearrangements were more numerous between distantly related species than between closely related species. Together with changes in chromosome ploidy, these rearrangements account for the differences in chromosome number among grass species (Ahn *et al.*, 1993).

Wheat, barley and rye are examples of closely related cereals which show a relatively high degree of conservation in chromosome structure. They are all members of the Triticeae tribe, which has a basic haploid chromosome number of 7. Barley and rye are diploid (2n = 14), and wheat is an allohexaploid (2n = 6x = 42) containing three sets of 7 chromosome pairs, designated A, B and D. A high degree of RFLP marker collinearity has been observed between the chromosomes of these species, and between the three genomes of hexaploid wheat, prompting the construction of consensus RFLP maps for each of the 7 Triticeae chromosomes (Devos and Gale, 1993b; Van Deynze *et al.*, 1995a, 1995c; Nelson *et al.*, 1995a, 1995b). The close relationships between the genomes of these three species are consistent with the results of chromosome compensation, chromosome pairing and isoenzyme studies (Sears, 1966; Hart *et al.*, 1987; Naranjo and Fernandez-Rueda, 1991).

1.6 The isolation of genes of interest from cereals

1.6.1 Gene isolation strategies in cereals

The strategy that is best suited to the isolation of any given cereal gene depends on the nature of the target gene and the species from which the gene is being isolated. Purification of the gene products (protein or mRNA) can provide one approach to isolating a gene of interest (Bennetzen and Jones, 1992; Schulze-Lefert, 1995). Obviously, this approach requires a criterion by which these gene products can be identified, such as a tissue specific pattern of expression, or a biochemical activity for the protein. In the absence of such information regarding the gene products, other options must be taken.

In the absence of information regarding the gene product or expression of the gene, tagging the gene using T-DNA or a transposon can be considered (Feldmann, 1991; Gierl and Saedler, 1992). The T-DNA tagging approach relies on the production of large numbers of independent transformants using *Agrobacterium*. Therefore, rice may be the only cereal in which such an approach may be feasible, as it is the only one for which a relatively effective *Agrobacterium*-mediated transformation system is available (Hiei *et al.*, 1994). Maize is the only cereal containing well characterised endogenous transposons, and as a result, is the only one from which genes have been isolated using transposon tagging (Schulze-Lefert, 1995). Transposon tagging systems based on maize transposons have been developed in rice, and may be used to facilitate the isolation of desirable rice genes in the near future (Izawa *et al.*, 1991; Li and Murai, 1995).

Subtractive hybridisation can be used to isolate genes of interest, provided that deletion mutants of the gene are available (Straus and Ausubel, 1990; Chasan, 1992; Sun *et al.*, 1992). Deletion mutations can be induced in plants by exposing seed to ionising radiation such as X-rays and fast neutrons (Wilkinson and Crawford, 1991; Oppenheimer *et al.*, 1991; Shirley *et al.*, 1992; Okubara *et al.*, 1994). Subtractive hybridisation has been used to isolate deleted sequences from wheat (Clarke *et al.*,

1992), but has not yet been used successfully to isolate specific genes of interest from cereals.

A map-based approach based on the chromosomal position of the gene is another approach that can be taken to isolate genes without prior knowledge of their products (Section 1.6.2).

1.6.2 Map-based gene isolation in cereals

In theory, any gene which can be genetically mapped can be isolated by a map-based approach (Wicking and Williamson, 1991; Tanksley *et al.*, 1995). In this approach, molecular genetic markers are mapped relative to the gene of interest and the closest marker used to identify a large insert clone from a genomic DNA library. If this clone does not contain the gene, a series of overlapping clones can be identified until one containing the gene is obtained (a process known as chromosome walking). Likely candidates for the gene of interest can be identified within the genomic DNA clone, and the target gene identified by virtue of its ability to impart the expected phenotype upon its introduction into a plant line by genetic transformation (Wicking and Williamson, 1991; Tanksley *et al.*, 1995).

A large size and high ratio of physical to genetic distance are characteristic of the genomes of many economically important cereals such as barley and wheat. Therefore, the map-based gene isolation of genes from cereals such as these is likely to require the analysis of considerable numbers of molecular markers and large mapping populations. In contrast, a small genome size and other factors are likely to make rice particularly amenable to positional cloning efforts (Ronald *et al.*, 1992; Causse *et al.*, 1994). The development of RFLP maps of cereals, the characterisation of the orthologous relationships between cereal genomes, and the development of rice as a tool to assist in the map-based isolation of cereal genes are some of the most significant factors that are improving the prospects for the map-based isolation of genes from cereals with larger genomes. These and other aspects of positional cloning in cereals are discussed in detail throughout the thesis.

1.7 This study

The aim of this study is to characterise the genetic basis of naturally occurring BYDV resistance in cereals. The study aims to achieve this in the following three ways:

- by isolating the *Yd2* gene for BYDV resistance from barley using a map-based approach

- by determining if a BYDV resistance gene in rice is orthologous to *Yd2*

- by determining if BYDV resistance in non-Ethiopian barleys is controlled by alleles at the *Yd2* locus

Ultimately, it is hoped that the findings of this study will assist in the production of BYDV resistant cereal cultivars and thereby help minimise the substantial economic damage this virus causes to cereal crops worldwide.

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 H_2O, 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, poly vinyl pyrillidone (PVP, 40,000 molecular weight), *E. coli* t-RNA, salmon sperm DNA, dithiothreitol (DTT): Sigma Chemicals (USA). • dextran sulphate, ficoll 400: Pharmacia (USA) • deoxyribonucleotide triphosphates (dATP, dTTP, dGTP and dCTP), ATP: Promega (USA). • phenol: Wako Industries (Japan). • NaCl, NaOH, Na_2EDTA, $MgCl_2$, 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: • mung bean nuclease: New England Biolabs. • calf intestinal alkaline phosphatase (CIAP): Boehringer Mannheim (Germany). • 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).

Oligodeoxyribonucleotides: Synthetic olligodeoxyribonucleotides were made on an Applied Biosystems (USA) Model 380B DNA synthesiser 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).

Radionucleotides: α-^{32}P-dCTP (10 μCi/μl): Bresatec (Australia).

Molecular weight markers, and cloning vectors: • SPPI DNA cut with *Eco*RI, λ DNA cut with *Hin*dIII, and pUC19 DNA cut with *Hpa*II: Bresatec (Australia).
• pBluescipt SK- : Stratagene (USA).

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

Agaroses: • low melting point agarose: BRL (USA). • other agarose (NuSeive GTG grade): FMC Bioproducts (USA).

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

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

Bacterial strains: *Escherichia coli* DH5α: Stratagene (USA).

Kits: • Bresa-Clean: Bresatec (Australia). • 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. 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 25 cm 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 bacterialogical 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 10 ml tubes containing 3.0 ml 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 100 µg/ml or 50 µg/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 l LB culture inoculated using 10 ml of an overnight culture of *E. coli* strain DH5α (BRL, USA) was grown to an optical density (λ_{600}) of 0.9) and the flask chilled on ice for 15 to 30 min. The culture was transferred to 200 ml pots and the cells pelleted in a GSA rotor at 3,000 rpm for 15 min at 4°C. The supernatant was discarded and the cells gently resuspended in 0.5 l of ice-cold 10% glycerol solution. The cells were then pelleted as above, the supernatant discarded, and resuspended in 20 ml of ice-cold 10% glycerol solution. Cells were transferred to 30 ml tubes, pelleted in a HB4 rotor at 4,000 rpm for 15 min at 4°C, and resuspended in 2.0 ml of ice-cold, 10% glycerol solution. The electrocompetent cells were transferred to 1.5 ml Eppendorf tubes in aliquots of 140 µl, snap frozen in liquid nitrogen, and stored at -80°C until use.

Tranformation of electrocompetent cells with plasmids was performed according to the recommendations supplied with the Gene-Pulser. Electrocompetent cells (40 µl) were combined with 1.0 µl milliQ H_2O containing 5.0 ng plasmid DNA or 60 ng of DNA from a ligation reaction. The mixture was transferred to an ice-cold,

disposable electroporation cell (0.1 cm electrode gap, supplied with the Gene-Pulser), and subject to electroporation using a Gene-Pulser (Bio-Rad), set at 1.8 kV, 125 μFD and 200 Ω. Immediately following electroporation, the cells were mixed with 1.0 ml LB broth without antibiotic, and grown at 37°C in a 1.5 ml Eppendorf tube for one hr on a shaker. Two-hundred μl of culture were then plated onto each plate of solid media containing antibiotic, and grown at 37°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 1.5 ml Eppendorf tube and the cells pelleted by centrifuging at 14,000 rpm for 30 sec. The supernatant was discarded and the cells resuspended in 100 μl ice-cold GET buffer (50 mM glucose, 25 mM Tris-HCl, 10 mM Na_2EDTA, pH 8.0) by vortexing for one min. Two-hundred μl of freshly made, ice-cold 0.2 M NaOH, 1.0% SDS was added and mixed in by gentle inversion of the tube. One-hudred and fifty μl of 3.0 M KOAc, 11.5% glacial acetic acid was added to the lysate and the tube vortexed gently before placing the tube on ice for three to five min. The tube was then centrifuged for five min at 14,000 rpm and 350 μl of the supernatant transferred to a new tube. Nucleic acid was precipitated by combining the supernatant with 35 μl of 3.0 M NaOAc, pH 5.2, and 400 μl ice-cold iso-propyl alcohol, and pelleted by centrifugation at 14,000 rpm at 4°C. Pellets were washed in 1.0 ml ice-cold 70% ethanol, dried completely, and resuspended in 20 μl of 10 mM Tris-HCl, 0.1 mM Na_2EDTA, pH 8.0, containing 40 mg/ml pancreatic RNase A.

2.2.5 Agarose gel electrophoresis

Large-scale agarose gels were cast from 100 ml of 0.9 to 1.3% ($^w/_v$) molten agarose solution containing 1 × TAE buffer (0.04 M Tris-acetate, 1.0 mM Na_2EDTA,

pH 8.0), using a 15 × 20 cm mould and a comb for making wells of 15 μl volume (for Southern analysis) or 30 to 50 μl 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 50 V and 18 to 50 mA in 1 × TAE buffer. DNA size markers made from *Hin*dIII cut λ DNA, *Eco*R1 cut SPP-1 DNA, or *Hpa*II cut pUC19 DNA (0.2 to 0.5 μg) were run alongside the sample DNAs when needed. Gels were soaked in 0.5 mg/l solution of ethidium bromide for 10 min, destained by rinsing in water for 10 min and photographed for future reference using UV light of wavelength 302 nm (preparative gels) or 260 nm (gels for Southern analsyis).

Mini agarose gels were cast by pouring 15 ml of 0.9 to 1.5% ($^w/_v$) molten agarose solution containing 1 × TBE buffer (0.045 M Tris-borate, 1.0 mM Na_2EDTA, pH 8.0) onto a 6.0 × 7.0 cm glass plate with the appropriate comb set obove it. DNA samples were mixed with 0.2 volume 6 × ULB loading buffer (40% ($^w/_v$) sucrose, 4.0 M urea, 0.25% ($^w/_v$) bromophenol blue, 0.25% ($^w/_v$) xylene cyanol, 10 mM Na_2EDTA) prior to electrophoresis at 80 to 180 mA in 1 × TBE buffer. Mini agarose gels were stained and photographed as described for large scale agarose gels.

2.2.6 RFLP analysis of cereal genomic DNA

2.2.6.1 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 14,000 rpm in an Eppendorf bench centifuge at room temperature. For each DNA preparation, approximately 0.4 g leaf tissue was harvested from one or more seedlings that were two to four weeks old. Tissue was placed inside a 2.0 ml snap-top Eppendorf tube, frozen by dropping the tube into liquid nitrogen, and pulverised with a spatula to make 0.8 ml of powder. The powder was stirred with 0.75 ml extraction buffer (0.1 M NaCl, 0.1 M Tris-HCl, 10 mM Na_2EDTA, 1.0% ($^w/_v$) sarkosyl, pH 8.5), 0.75 ml phenol:chloroform:iso-amyl alcohol (25:24:1; phenol was equilibrated

with 0.1 M Tris-HCl, pH 8.0 prior to use) added, and the tube shaken vigorously for one min prior to leaving it on a rotator for 15 min. Tubes were centrifuged for 10 min and the aqueous phase transferred to a new tube. The aqueous phase was then extracted with 0.8 ml chloroform as above, and nucleic acid precipitated by combining the aqueous phase with 0.1 volume 3.0 M NaOAc, pH 5.2 and 1.0 volume iso-propyl alcohol. Nucleic acid was pelleted by centrifugation for five min, and the pellet washed in 1.0 ml 70% ethanol for 15 min on an rotator, recovered by centrifugation, dried completely, and resuspended in 45 µl of 5.0 mM Tris-HCl, 0.05 mM Na_2EDTA, pH 8.0, containing 40 mg/mL pancreatic RNase A.

2.2.6.2 DNA restriction, electrophoresis and Southern transfer

For RFLP analysis, 7.0 µl of genomic DNA preparation (approximately 3.0 µg DNA) was digested at 37°C for three to five hours in 11 µl reactions containing 1.0 mg/ml BSA, 1.3 mM spermidine and 1 × concentration of the buffer supplied with the enzyme. Genomic DNA digests were electrophoresed on large-scale agarose gels (Section 2.2.5), 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 the Hybond N^+ membrane. Each gel was shaken gently in 300 ml denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 min, rinsed briefly in water, and shaken gently in 300 ml neutralising solution (1.5 M NaCl. 0.5 M Tris-HCl, 1.0 mM Na_2EDTA, pH 7.2) for a further 30 min. DNA was transferred from the gels to Hybond N^+ membrane (Amersham) by Southern transfer (Southern, 1975) overnight, using 20 × SSC (3.0 M NaCl, 0.3 M 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 0.5 M NaOH. After 20 min, membranes were shaken in a solution of 2 × SSC for at least five min.

2.2.6.3 Purification of RFLP clone inserts

To excise insert DNA from RFLP clones, 10 µl of plasmid minipreparation was digested for three hr at 37°C, in 20 µl reactions containing 20 U of the appropriate restriction enzyme, 1.0 mg/ml BSA, 1.3 mM spermidine and 1 × 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 µl in volume, and contained 0.5 U *Taq* DNA polymerase, 0.25 µM of each primer, 0.2 mM (each) dTTP, dATP, dCTP and dGTP, 0.8 to 3.0 mM $MgCl_2$, 1.0 µl of $^1/_{500}$ dilution of plasmid DNA minipreparation, and 1 × reaction buffer supplied with the *Taq* enzyme (67 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 200 µg/ml gelatin, pH 8.8). Reactions were overlaid with mineral oil and subject to 25 cycles of (95°C for 1 min, 50°C for 1 min, 72°C for 2 min) and a final incubation at 72°C for 10 min, in a PTC-150 Mini Cycler (MJ Research, USA). A $MgCl_2$ concentration of 1.3 mM was effective for the amplification of most inserts, although inserts larger than 1.5 kbp generally required the $MgCl_2$ concentration to be optomised.

Plasmid digests and PCR rections were electrophoresed in large scale agarose gels (Section 2.2.5), 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 2.2.5), alongside known amounts of DNA markers in order to verify the sizes of the inserts and to estimate their concentration.

2.2.6.4 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 (20 ng) was combined with 6.0 µl random sequence 9-mer oligonucleotide (0.1 mg/ml), and the mixture incubated at 95°C for three min to denature the DNA. The mixture was cooled on ice for five min and combined with 10 µl probe labelling buffer (0.5 M HEPES, 0.125 M Tris-HCl, 12.5 mM DTT, 12.5 mM $MgCl_2$, 1.0 mg/ml BSA)[1], 2.5 µl dNTP mixture (0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP), 1.0 U Klenow fragment, 3.0 µl α-^{32}P-dCTP (10 µCi/µl), and enough milliQ H_2O to make the total volume 25 µl. Probe labelling reactions were incubated at room temperature overnight.

To make spun columns, 1.0 ml 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 (0.1 M NaCl, 1.0 mM Na_2EDTA, 10 mM Tris-HCl, pH 7.2) and centrifuged at 3,000 g for four min to remove excess TEN. Probe reactions were combined with 120 µl TEN solution and passed through the column by centrifugation at 3,000 g for four min to remove unincorporated dNTPs.

2.2.6.5 Hybridisation and autoradiography

The prehybridisation and hybridisation procedures used were based on protocols supplied with the Hybond N^+ membrane (Amersham). Each 10 ml of prehybridisation/hybridisation solution was made by combining 3.0 mL of 5 × HSB solution (3.0 M NaCl, 0.1 M PIPES, 25 mM Na_2EDTA, pH 6.8), 2.0 mL of 50 × Denhardt's solution (2.0% ($^w/_v$) BSA, 2.0% ($^w/_v$) ficoll 400, 2.0% ($^w/_v$) PVP), 3.0 ml 25% ($^w/_v$) dextran sulphate, 2.0 ml milliQ H_2O, and 200 µl of 10 mg/ml salmon sperm DNA which had been denatured at 95°C for 10 min prior to its addition. Membranes were placed in hybridisation bottles containing 5.0 to 20 ml of prehybridisation/hybridisation solution (up to 10 membranes per bottle) and

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[1] The 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.

prehybridised for three to five hr at 65°C. Following prehybridisation, probes was denatured by heating for 10 min at 95°C, cooled on ice for five min, added to the hybridisation mixture, and hybridisation performed at 65°C for 12 to 20 hours. Membranes were then washed (i) three times for five min in 2.0 × SSC, 0.1% SDS at room temperature, and then (ii) two times for 10 min in 0.2 × SSC, 0.1% SDS at 65°C. Two final washes of 10 min 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 1.0 l of boiling 0.1% SDS solution. The lunchbox was left on a shaker at room temperature for at least 10 min before replacing the solution with fresh 0.1% SDS at room temperature. Membranes were stored in this solution at 4°C until their reuse.

2.2.7 Map construction and RFLP locus nomenclature

Linkage analysis was performed using the computer program MAPMAKER (Lander *et al.*, 1987). Markers were firstly grouped using the 'two-point' command with a LOD threshold value of 3.0 and a θ value of 0.25, and ordered using the 'try' command. The order of the mapped markers was confirmed using the 'ripple' command, and the Kosambi Mapping Function (Kosambi, 1944) chosen to convert recombination fraction values to centiMorgans.

RFLP loci detected using the TAG clones were given the prefix *Xglk* in accordance with the nomenclature specified by Liu and Tsunewaki (1991), while the locus detected using the YLP clone was named *XYlp*. The names used for all other RFLP loci were italicised, and consisted of the name of the clone used to detect them (in lower case) preceded by an '*X*'. Locus names were sometimes followed by an '*a*' or a '*b*' to distinguish two loci mapped using the same RFLP probe.

2.2.8 Aphid and BYDV cultures

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 in the oat (*Avena sativa* L.) cultivar New Zealand Cape or Stout grown in a glasshouse at 18 to 25°C in aphid proof cages made from fine nylon mesh. Seeds were sown in 13 cm pots (five seeds per pot) at four week intervals, and the plants used for aphid or virus multiplication when approximately three weeks old. To renew the virus cultures, virus-free aphids were fed for two days on detached leaf segments from infected plants, and five to ten aphids transferred to each virus-free plant. Aphids were allowed to feed for two days before they were killed with Rogor insecticide (Hortico, Australia). BYDV dot blot hybridisation analysis (Section 2.2.10) was used periodically to confirm that the virus was present in the virus cultures and absent in the plants used to raise virus-free aphids.

2.2.9 Barley BYDV resistance assays

To obtain large numbers of viruliferous aphids for BYDV resistance assays, aphids were applied to BYDV infected oats and allowed to multiply for approximately three weeks. Aphids were harvested by shaking oats over a large plastic tray dusted lightly with talcum powder to prevent the aphids sticking together. Harvested aphids were used immediately in resistance assays or were stored for up to seven days in lunch boxes at 4°C if necessary.

Barley plants to be assayed for BYDV resistance were grown in UC soil in 13 cm pots, at a density of four plants per pot. To allow for uneven seed germination, five seeds were sown in each pot, and the extra seedlings removed later if necessary. When

seedlings were seven days old, enough viruliferous aphids were applied to the base of each seedling to allow 30 to 50 aphids to walk onto each seedling. Aphids were allowed to feed for two days before they were killed by spraying with Rogor insecticide (Hortico, Australia). Infected plants grown in the glasshouse at 18 to 25°C or outside between May and September, and the BYDV symptoms scored visually or by measuring the fresh shoot weight four weeks after infection (Chapters 3 and 7). BYDV northern dot blot hybridisation analysis (Section 2.2.10) and RFLP analysis of the infected individuals were used to assist in the resistance assays (Chapter 3).

2.2.10 BYDV northern dot blot hybridisation analysis

Individual plants were subject to northern dot blot hybridisation analysis to determine if they were BYDV infected. The 707 bp DNA probe template was 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 was PCR-amplified from a full-length cDNA clone of the genome of this isolate. This clone was previously described by Young *et al.* (1991), and was generously supplied by Mark Young (CSIRO Division of Plant Industry, Canberra). PCR reactions of 50 µl contained 5.0 ng of the BYDV clone DNA, 0.25 µM of each of the oligonucleotide primers PAV1 (5′- TTA ATG TCA CCG GAC ATT CTG TCG CC -3′) and PAV2 (5′- CAC AAC GCG CTT GTG GCA GTG GA -3′), 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase, 0.2 mM (each) dTTP, dATP, dCTP and dGTP, and 1 × reaction buffer supplied with the *Taq* enzyme (67 mM Tris-HCl, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 200 µg/ml gelatin, pH 8.8). Reactions were overlaid with mineral oil and subject to 25 cycles of (95°C for 1 min, 50°C for 1 min, 72°C for 2 min) and a final incubation at 72°C for 10 min, in a PTC-150 Mini Cycler (MJ Research, USA). The DNA fragment was gel-purified, quantified and used to synthesise radioactively labelled DNA probes using the same procedures described for the preparation of probes for Southern analysis (Section 2.2.6).

To prepare cerial nucleic acid samples for northern dot blot hybridisation analysis, the same procedure described for cereal genomic DNA isolation (Section

2.2.6.1) was used, except that the chloroform extraction step was not performed, and RNase A was omitted from the nucleic acid resuspension buffer. Five µl samples of nucleic acid were dotted 1.0 cm apart onto Hybond N^+ membrane (Amersham, USA). The spots were allowed to dry and the nucleic acid fixed to the membrane by laying the membrane nucleic acid side up onto a pad made from three sheets of Watmann 3MM paper soaked in 0.05 M NaOH. After five min, the membrane was rinsed in 2 × SSC for at least five min.

Prehybridisation and hybridisation conditions used for BYDV northern dot blot hybridisation analysis were the same as those described for Southern analysis (Section 2.2.6), except that a temperature of 68°C was used. Following hybridisation, membranes were washed three times for five min in 2.0 × SSC, 0.1% SDS at room temperature, and then two times for 10 minutes in 0.1 × SSC, 0.1% SDS at 68°C. Autoradiography was performed for one to 12 hours at -80°C with Konica X-ray film and an intensifying screen.