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Identification of polypeptide markers of barley yellow dwarf virus resistance and susceptibility genes in non-infected barley (*Hordeum vulgare*) plants

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Summary. Barley yellow dwarf (BYDV) is a group a closely related viruses which cause economic losses in a wide range of graminaceous species throughout the world. Barley plants can be protected from the effects of BYDV by the 'Yd2' resistance gene. Plants which contain the 'Yd2' gene also contain a constitutively expressed polypeptide which was not found in any plants without 'Yd2'. Conversely, BYDV susceptible plants contain another constitutively expressed polypeptide which was not found in any of the BYDV-resistant lines examined. These two polypeptides appear to have the same molecular weight (as assessed by SDS-PAGE) and only slightly different iso-electric points. They also appear to contain an extensive range of similar antigenic determinants. Both polypeptides were found in F_1 hybrids made from resistant and susceptible plants. We suggest that these two polypeptides are the products of two allelic genes. Analysis of near-isogenic lines showed that the locus which controls the 'Yd2' resistance gene and the locus controlling the synthesis of the two polypeptides may be within ± 9 cM of each other. We have developed a Western blot technique which allows assessment of barley lines, 4-days after seed imbibition, for the presence of the 'Yd2' gene.

Key words: Barley yellow dwarf virus – Resistance gene(s) – High-resolution two-dimensional electrophoresis – Barley – Western immunoblot

Introduction

The group of closely related viruses, collectively known as Barley yellow dwarf (BYDV), are arguably the most economically important viral pathogens of cereal crops, pasture grasses, and other graminaceous species throughout the world (Plumb 1983; Eagling et al. 1991). When infection occurs in young plants, 30% or more of the plants yield may be lost (Burnett 1987). Barley plants can be protected against some serotypes of BYDV by the 'Yd2' gene, which was first identified in Ethiopian lines of barley (Rasmusson and Schaller 1959; Schaller et al. 1963, 1964; Damsteegt and Bruehl 1964; Catherall et al. 1970). This gene appears to reduce the rate of replication of BYDV within barley tissues (Skaria et al. 1985; Arundel et al. 1988). Unfortunately, incorporating this gene into barley plants during breeding programs is difficult and time consuming because the symptoms displayed by plants in response to infection by BYDV vary with genotype and may be confused with the effects of environmental or nutritional stresses (Conti et al. 1987). These problems could be overcome by the identification of a biochemical marker of the 'Yd2' gene which could then be used during plant breeding programs to identify non-infected seedlings containing this gene in either the heterozygous or homozygous state.

There are relatively few reports describing the identification of biochemical markers of plant disease resistance genes in the literature. These studies have generally followed one or the other of two strategies:

(1) The use of isozymes. Bands of specific enzyme activity in electrophoresis gels have been shown to correlate with nematode resistance in tomatoes (Rick and Fobes 1975; Gunther et al. 1988), bean yellow mosaic virus resistance in pea (Weeden et al. 1984), and stripe rust resistance in wild wheat (Nevo et al. 1984).

(2) The use of restriction fragment length polymorphisms (RFLPs). Differences in the restriction pattern of genomic DNA isolated from resistant and susceptible plants (as detected by DNA clones), have been observed in a num-

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ber of experimental systems including insect resistance in tomatoes (Nienhuis et al. 1987), downy mildew resistance in lettuce (Landry et al. 1987), tobacco mosaic virus resistance in tomatoes (Young et al. 1988; Paran et al. 1989; Tanksley et al. 1989), and *Pseudomonas syringae* resistance in *Arabidopsis thaliana* (Debener et al. 1991).

Both these techniques do, however, often require that a large number of experiments be undertaken before a useful enzyme or DNA probe/restriction enzyme combination is found which enables a polymorphism at a particular locus to be observed.

In this paper we report the use of an alternative technique which we believe to be useful in rapidly locating polymorphisms of particular gene loci. We have used high-resolution two-dimensional electrophoresis to examine the polypeptides extracted from inbred near-isogenic lines (NILs) of barley which differ in their resistance to BYDV. This technique allows the visualisation of approximately 600 polypeptides in extracts from a range of plant organs. Thus, in one experiment the protein products of up to several hundred genes can be observed and any differences between the inbred NILs noted. This approach has allowed us to locate polypeptide markers of the 'Yd2', BYDV-resistance gene and its susceptibility allele. The locus controlling these marker genes appears to lie within ± 9 cM of the locus controlling the 'Yd2' gene itself.

Materials and methods

Chemicals

All chemicals were purchased from BDH (UK) except for ampholines, which were obtained from Pharmacia (Sweden), and CHAPS {3-[(3-Cholamidopropyl)dimethylammonio] 1-propane sulfonate}, obtained from SIGMA (USA).

Growth of plant material

Barley (*Hordeum vulgare*) seeds were obtained from Mr W. Vertigan, Department of Agriculture, Launceston, Tasmania, Australia; Professor C. Schaller, University of California, Davis, USA; and Dr R. Boyd, University of Western Australia, Perth, Australia. Plants were grown in 6 cm diameter pots containing vermiculite maintained at 25 °C in continuous darkness for 4 days, after which coleoptiles were harvested and either used immediately or stored frozen at −20 °C for up to 3 months.

Extraction of polypeptides and electrophoresis

Polypeptides were extracted from coleoptiles by homogenising 1 g in 5 ml of a buffer containing 50 mM Tris/HCl, 0.5 mM $MgCl_2$, 1 mM disodium EDTA, 2% SDS, 50 mM dithiothreitol, pH 7.8. The homogenate was boiled for 2 min, centrifuged at 15,000 g for 15 min and then the supernatant precipitated by addition of ice cold acetone to a final concentration of 80% (v/v) as described by Holloway and Arundel (1988). High-resolution two dimensional electrophoresis was performed according to Holloway and Arundel (1988) using either n-octyl glucoside or CHAPS as the non-ionic detergent. The isoelectric focussing step was conducted using 2.5 mm inner diameter glass tubes containing a 50/50 (v/v) mixture of pH = 3–10 and pH = 5–8 ampholines. Gels were stained with Coomassie blue (Chua 1980). Polypeptide patterns in gels were compared by overlaying one gel with another on the top of a fluorescent light, X-ray film viewing box.

Preparation of antisera and Western blotting

Antisera were prepared by separating polypeptides from cvs. Proctor and Shannon on multiple high-resolution two-dimensional electrophoresis gels as described above. The Coomassie blue-stained polypeptide spots were excised and dialysed against phosphate buffered saline (PBS) for 24 h. Gel pieces were then homogenised in 5 volumes of PBS, mixed 50/50 (v/v) with Freunds complete adjuvant and injected interperitoneally into rats. Injections were repeated twice more at 2-weekly intervals (using incomplete adjuvant). Rats were bled from tail veins 2 weeks after the last injection. The blood was allowed to coagulate overnight at 4 °C and the serum recovered by centrifugation. Antisera was used without further purification after storage at −20 °C for up to 18 months. Methods used for isoelectric focussing were as described by Holloway and Arundel (1988) above, except that polypeptides were separated on a slab gel 14 cm × 16 cm × 0.75 mm. CHAPS was substituted for n-octylglucoside in these experiments although preliminary investigations had shown that both detergents gave equivalent results. After electrophoresis, gels were equilibrated in a buffer containing 5 mM MgCl, 1 mM EDTA, 5% SDS and 50 mM Tris/HCl pH 7.8 for 20 min. Polypeptides were transferred to nitrocellulose membrane (0.45 μm) in a 'Sartoblot 2' (Sartorius) apparatus according to the manufacturer's instructions (20 mins at 0.5 A, Tris/Glycine/Methanol buffer). Filters were blocked for 1 h in a buffer consisting of 20 mM Tris, 500 mM NaCl, pH 7.5 (TBS) and containing 3% w/v gelatine. Filters were then washed twice with TBS containing 0.05% Tween 20 (TTBS), after which they were incubated in a solution of TTBS containing 1% gelatine and a 1/200 (v/v) dilution of antisera at 37 °C for 3 h. Filters were washed twice in TTBS and subsequently incubated in TTBS containing 1% gelatine and anti-rat-horseradish peroxidase conjugate (Silenus laboratories, Australia) at a dilution of 1/200 (v/v). Filters were again washed twice in TTBS, then once in TBS after which they were placed in a colour development solution consisting of 60 mg 4-chloro-1-naphol dissolved in 20 ml methanol added to 100 ml TBS which contained 60 μl H_2O_2 (30% solution). The antisera used in our experiments reacted strongly with the protein markers of resistance and susceptibility described in this paper. If blots were left for prolonged periods in colour developer, a group of three faint bands became visible at the extreme anode end of the blot. These same bands were also apparent if pre-immune serum was used. These faint bands did not interfere with the analysis of genotypes by Western blotting.

Results and discussion

In initial experiments, polypeptides were extracted from the coleoptiles of BYDV-resistant barley cv. CM 67 and its near-isogenic, BYDV-susceptible, recurrent parent cv. California Mariout, and then separated by high-resolution two-dimensional electrophoresis. When the gels were examined, the vast majority of the estimated 500–600 polypeptides visualised were represented in both cultivars. However, one polypeptide in cv. California Mariout (s) was not present in cv. CM 67 (r). This polypeptide is shown surrounded by a square in Fig. 1. It has an

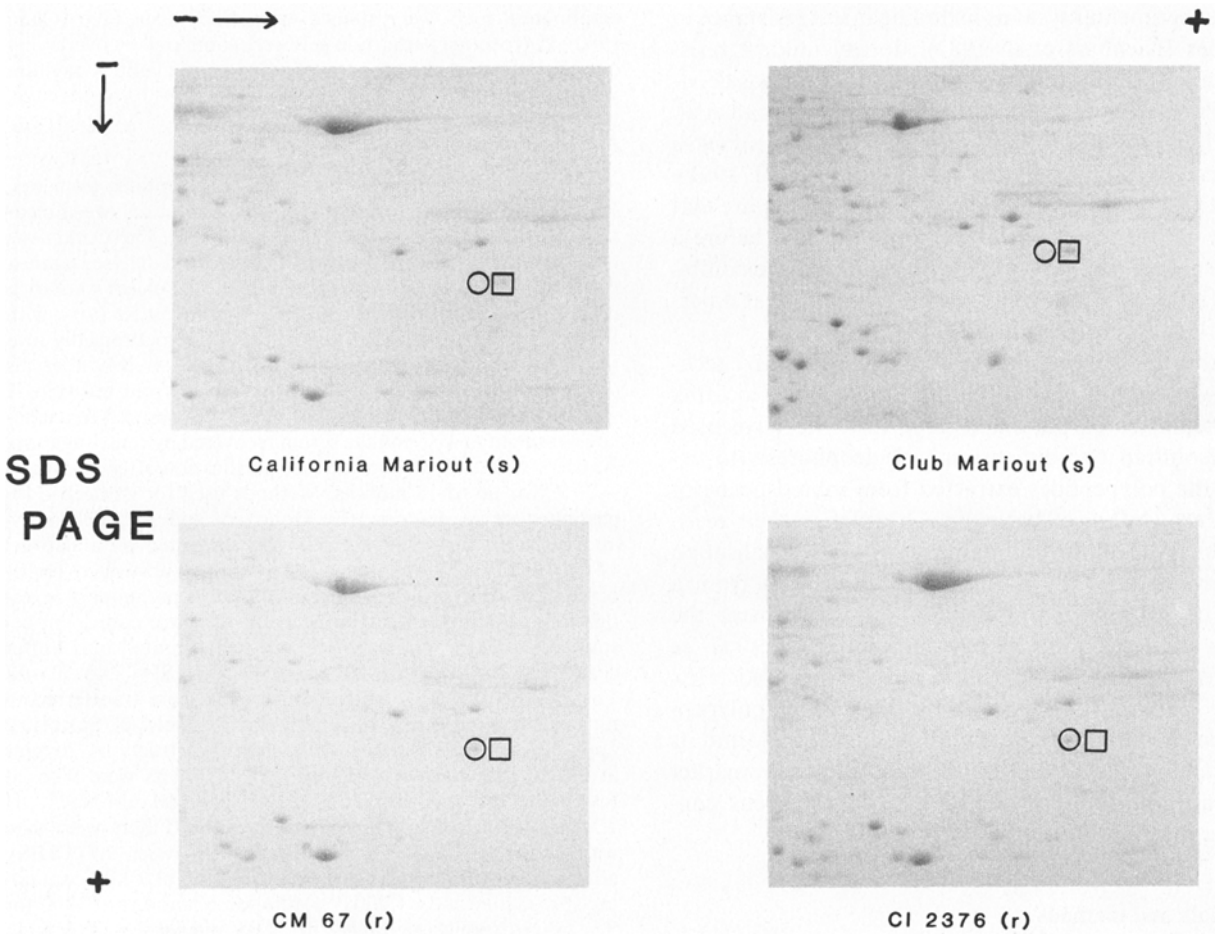

Fig. 1. Photographs of parts of high-resolution two-dimensional electrophoresis gels stained with coomassie blue which show the patterns obtained after electrophoresis of coleoptile polypeptides from four barley cultivars: California Mariout, CM 67, Club Mariout and CI 2376. The location of the YDRP is shown surrounded by a *circle* and the location of the YDSP is shown surrounded by a *square* in each photograph (see text for details). BYDV-resistant cultivars are denoted with the suffix (*r*), and BYDV-susceptible cultivars with the suffix (*s*), after the cultivar name

apparent molecular weight of approximately 40 kDa and an apparent isoelectric point of 7.2.

Conversely, coleoptiles from cv. CM 67 appeared to contain one polypeptide that was not present in cv. California Mariout. This polypeptide is shown surrounded by a circle in Fig. 1. It had the same apparent molecular weight, but a slightly altered isoelectric point (pI = 7.0), compared to the unique polypeptide present in cv. California Mariout. Extraction and electrophoresis of a 1:1 (w:w) mixture of coleoptiles from cvs. California Mariout and CM 67 revealed the presence of both of the aforementioned polypeptides in the resulting gel (data not shown). These data strongly suggest that there are indeed two polypeptides which differ in electrophoretic mobility. Furthermore, this difference did not appear to result from a chemical modification of a single polypeptide during sample preparation or electrophoresis.

The two other barley cultivars used in the development of cv. CM67 were also examined for the presence of the two polypeptides. Electrophoresis of a preparation of coleoptile proteins from the BYDV-susceptible cv. Club Mariout showed that this cultivar contained a polypeptide with the same electrophoretic mobility as the one unique to the other BYDV-susceptible cultivar, California Mariout (Fig. 1). No trace of the unique polypeptide in cv. CM 67 was observed. When coleoptile polypeptides from the 'Yd2', BYDV-resistance gene donor CI 2376 were electrophoresed, a polypeptide with the same electrophoretic mobility as the one unique to cv. CM 67 was seen (Fig. 1). No trace of the polypeptide exclusively present in cvs. California Mariout and Club Mariout was observed.

These data imply that the gene controlling the synthesis of the polypeptide exclusive to the BYDV-resistant plants has been transferred from CI 2376 to CM 67, along with the 'Yd2' gene which confers BYDV-resistance. Because cv. CM67 resulted from a pooled sample of 130 BYDV-resistant plants selected from a selfing

Table 1. Pedigrees of BYDV-resistant barley cultivars used during the studies described herein

CM 67: is a composite of 130 BYDV-resistant F_5 generation lines from the backcross:
California Mariout * 5/CI 2376//2 * California Mariout * 6/Club Mariout
(Shaller and Chim 1970 b).

Shannon: is the result of two generations of single plant selection for BYDV-resistance from the backcross:
Proctor * 4/CI 3208-1
(Symes 1979)

Prato: is an F_5 selection of BYDV-resistant lines from the backcross:
CM 67/3 * Briggs/4/Briggs * 4/3/California Mariout * 4/CI 1179//2 * California Mariout * 6/Club Mariout
(Shaller et al. 1979).

Atlas 68: is a composite of 140 BYDV-resistant F_5 generation lines from the backcross:
Atlas * 3/CI 3920-1//Atlas 46/3/2 * Atlas * 4/CI 1179// Atlas 57 * 2
(Shaller and Chim 1970 a).

Table 2. The presence (+) or absence (−) of both the YDRP and YDSP (see text) in coleoptile polypeptides extracted from the BYDV-resistant barley cultivars Shannon, Prato and Atlas 68 as well as other cultivars involved in their pedigrees. BYDV-resistant cultivars are denoted with suffix (r), and BYDV-susceptible cultivars with the suffix (s), after the cultivar name. Analysis was by high-resolution two-dimensional electrophoresis (HR2DE) or by Western blotting

Cultivar	HR2DE		Western blot	
	YDRP	YDSP	YDRP	YDSP
Shannon (r)	+	−	+	−
CI 3208-1 (r)	+	−	+	−
Proctor (s)	−	+	−	+
Prato (r)	+	−	+	−
CM 67 (r)	+	−	+	−
Briggs (s)	−	+	−	+
California Mariout (s)	−	+	−	+
Club Mariout (s)	−	+	−	+
CI 1179 (s)	−	+	−	+
Atlas 68 (r)	+	−	+	−
CI 3920-1 (r)	+	−	+	−
Atlas 57 (s)	−	+	−	+
Atlas 46 (s)	−	+	−	+
Atlas (s)	−	+	−	+
CI 1179 (s)	−	+	−	+

population (Table 1), and we have assayed in excess of 100 plants of this cultivar, these data strongly suggest that no recombination of the two genes occurred in any of the lines originally selected. We conclude, therefore, that the 'Yd2' gene and the gene controlling the synthesis of the polypeptide unique to BYDV-resistant plants are linked. For the purposes of further discussion we have called the polypeptide unique to BYDV-resistant plants the 'Yellow Dwarf Resistance Polypeptide' (YDRP) while the unique polypeptide found in BYDV-susceptible plants has been called the 'Yellow Dwarf Susceptibility Polypeptide' (YDSP).

In order to further investigate the putative linkage between the YDRP and BYDV-resistance respectively, coleoptile polypeptides from other barley cultivars were examined by high-resolution two-dimensional electrophoresis. In all cases a BYDV-resistant cultivar was compared with its near-isogenic, BYDV-susceptible recurrent parent as well as with the other cultivars in its pedigree.

The BYDV-resistant cultivar Atlas 68 is a result of a complex backcross between its BYDV-susceptible recurrent parent cv. Atlas 57, other BYDV-susceptible cultivars (Atlas, Atlas 46 and CI 1179) and the source of the 'Yd2', BYDV-resistance gene, CI 3920-1 (Table 1). The polypeptide patterns in the gels which resulted from electrophoresis of these cultivars showed that the BYDV-susceptible lines all exclusively contained a polypeptide which co-electrophoresed with YDSP (Table 2). The BYDV-resistant cultivars, however, contained a polypeptide which co-electrophoresed with YDRP (Table 2). Thus because Atlas 68 originally resulted from a pooled sample of 140 BYDV-resistant lines, the gene controlling the synthesis of YDRP has been independently selected along with the 'Yd2' gene. These data also support the hypothesis that the 'Yd2' gene and the gene for the YDRP are linked.

Exactly the same pattern was obtained for the BYDV-resistant cultivar Shannon (Table 1), as well as for its BYDV-susceptible recurrent parent (cv. Proctor) and the 'Yd2' gene donor CI 3208-1 (Table 2). Similarly, the BYDV-resistant cultivar Prato, its BYDV-susceptible recurrent parent cv. Briggs, and other cultivars involved in its pedigree (Table 1), also showed this same distribution pattern for the YDRP and the YDSP (Table 2). These data also strongly support the hypothesis that the gene controlling the synthesis of YDRP and the 'Yd2' resistance gene are linked.

In order to confirm the distribution of the YDSP and YDRP between different barley cultivars, polyclonal antibodies were raised against them. These antisera were used to develop a Western-blot assay, after isoelectric focussing of coleoptile extracts, for the two polypeptides. Preliminary experiments showed that antisera raised against the YDSP, bound to a polypeptide with the same isoelectric point as the YDSP in extracts from BYDV-susceptible plants. However this same antisera also bound to a polypeptide with the same isoelectric point as the YDRP in extracts from BYDV-resistant plants (Fig. 2). Identical results were observed for antisera raised against the YDRP (data not shown). We conclude from these data that either of the antisera could be used to specifically detect both the YDRP and the YDSP in coleoptile extracts from BYDV-resistant and BYDV-sus-

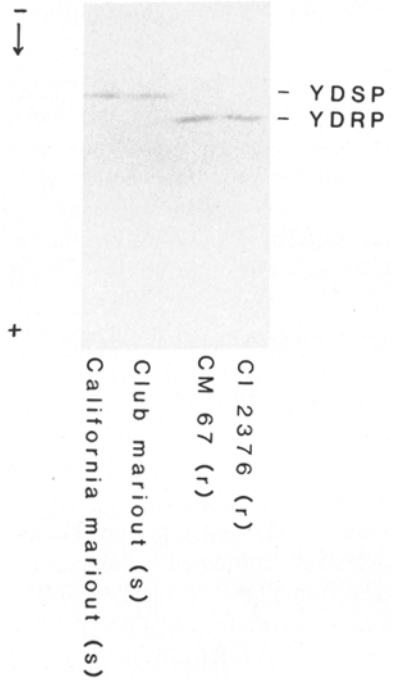

Fig. 2. Photograph of a Western "immunoblot" showing the results obtained when coleoptile polypeptides isolated from cv. CM 67, and the barley lines involved in its pedigree, cultivars California Mariout, Club Mariout and CI 2376 (see Table 1), were separated by isoelectric focussing, transferred to nitrocellulose and subsequently probed with antisera raised against the YDRP. The location of the YDSP and the YDRP are indicated. BYDV-resistant cultivars are denoted with the suffix (*r*), and BYDV-susceptible cultivars with the suffix (*s*), after the cultivar name

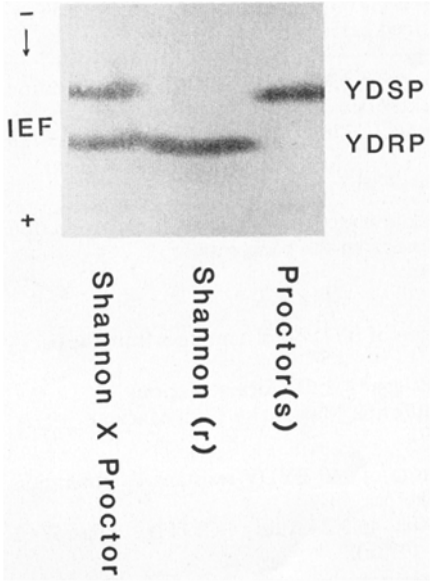

Fig. 3. Photograph of a Western "immunoblot" showing the results obtained when coleoptile polypeptides isolated from cvs. Shannon (BYDV-resistant), Proctor (BYDV-susceptible) and an F_1 hybrid between cvs. Shannon and Proctor were separated by isoelectric focussing, transferred to nitrocellulose and subsequently probed with antisera raised against the YDRP. The locations of the YDSP and the YDRP are indicated

ceptible cultivars respectively using this Western blotting procedure.

In the experiments described below, data is shown from analyses carried out using the antisera raised against the YDRP, although antisera raised against the YDSP gave identical results (data not shown). Polypeptide extracts from all of the barley cultivars previously analysed by high resolution two-dimensional electrophoresis were separated on slab isoelectric-focussing gels and analysed by Western blots using the antisera we prepared. Figure 2 shows the results obtained for cv. CM67 and the three cultivars which make up its pedigree. As can be clearly seen, the two BYDV-resistant cultivars, cvs. CM67 and CI 2376, both showed binding of the antibody to the YDRP. Conversely, both BYDV-susceptible cultivars, cvs. California Mariout and Club Mariout, showed binding of the antibody to the YDSP. This pattern was repeated when all of the other barley cultivars listed in Table 2 were analysed using this same technique. Thus, an identical pattern of the distribution of the two polypeptides was observed using both high-resolution two-dimensional electrophoresis and Western blotting.

In the experiments described above, all of the YDRPs and all of the YDSPs from different cultivars appeared to display the same electrophoretic mobilities. This was confirmed by electrophoresis of mixed extracts from different resistant or susceptible plants. These data, considered together with the extensive immunological cross-reactivity of the antisera towards all of the YDRPs and all of the YDSPs, strongly suggests that there is extensive, if not complete, homology between each of the YDRPs and each of the YDSPs.

Furthermore, because both the YDRP and YDSP have such similar electrophoretic mobilities and immunological properties, it is possible that the slight difference observed in their isoelectric point may result not from the transcription of two separate genes per se, but from some difference in the post-translational processing of the products of a single gene. However, when polypeptides from an F_1 hybrid between the BYDV-susceptible cultivar Proctor and the BYDV-resistant cultivar Shannon were analysed by Western blotting, both the YDSP and the YDRP were found to be present in this hybrid (Fig. 3). This result strongly suggests that the polypeptides are the products of two different genes.

Because of the electrophoretic and immunological similarity between the two polypeptides, and the fact that they are both founbd in an F_1 hybrid constructed from resistant and susceptible plants, but were never found together in any inbred lines of barley, we propose that the

YDRP and the YDSP are the products of two allelic genes controlled by one locus (the YDRP/YDSP locus). The relationship between the polypeptides is currently being pursued by peptide mapping and gene sequencing studies.

The data we have presented suggest that because in all cases the gene coding for the YDRP has been transferred along with the 'Yd2', BYDV-resistance gene, there appears to be linkage between the two loci where these genes are located. Although how closely linked these two loci might be was not specifically explored in our experiments, some preliminary estimate of the linkage can be gained by an examination of the pedigree of one of the BYDV-resistant lines used in this study.

Prato is the result of a cross between cvs. California Mariout and CI 2376 and the subsequent backcrossing to a range of BYDV-susceptible cultivars to give first cv. CM67 (after seven generations) and then, with four more generations of backcrossing to other BYDV-susceptible cultivars, cv. Prato. Thus, the 'Yd2' gene and the gene for the YDRP have remained linked for a total of 11 generations of backcrossing (Table 1). This not only strongly suggests that the locus at which the gene for the YDRP is found is located on the same chromosome as the locus controlling the 'Yd2' gene but that these two loci may be located within ± 9 cM of one another, assuming a chromosome of 100 cM in length (Stam and Zeven 1981). It is of course tempting to speculate that the YDRP polypeptide may indeed be the product of the 'Yd2' gene itself. The linkage between the two loci is presently being studied using a population of F_2 plants which are being screened for the presence of the YDRP and also for their BYDV resistance.

The Western blotting technique we have employed is currently being extensively evaluated as a means of rapidly selecting BYDV-resistant and BYDV-susceptible plants from populations of non-infected barley during an ongoing breeding program. Its utility as a tool for assisting with the transfer of the 'Yd2' gene from barley into wheat, through the interspecific hybridisation of these two species, is also currently under investigation.

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