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ORIGINAL PAPER

Ryd4^{Hb}: a novel resistance gene introgressed from *Hordeum bulbosum* into barley and conferring complete and dominant resistance to the barley yellow dwarf virus

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Abstract Barley yellow dwarf virus (BYDV) causes high yield losses in most of the major cereal crops worldwide. A source of very effective resistance was detected within the tetraploid wild species of *Hordeum bulbosum*. Interspecific crosses between a resistant *H. bulbosum* accession and *H. vulgare* cv. 'Igri' were performed to transfer this resistance into cultivated barley. Backcrosses to *H. vulgare* resulted in offspring which carried a single subterminal introgression of *H. bulbosum* chromatin on barley chromosome 3HL and proved to be fully resistant to BYDV-PAV, as inferred by ELISA values of zero or close to zero and lack of BYDV symptoms. Genetic analysis indicated a dominant inheritance of the BYDV-PAV resistance factor, which we propose to denote *Ryd4*^{Hb}. The identity and effect of *Ryd4*^{Hb} are discussed in relation to other known genes for BYDV resistance or tolerance, as well as the relevance of this gene for resistance breeding in barley.

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Introduction

The upward trend in the incidence of the barley yellow dwarf virus (BYDV) in cereal production highlights the potential impacts global warming may have on agroecological systems. The barley yellow dwarf disease is caused by different viruses of the family *Luteoviridae* (D'Arcy and Domier 2005). According to the current state of knowledge, viruses formerly referred to as different BYDV strains (Rochow 1969; Rochow and Muller 1971; Zhang et al. 1983) are actually classified into two different virus genera, namely the barley yellow dwarf luteovirus with BYDV-MAV, -PAV and -PAS and the cereal yellow dwarf polerovirus with cereal yellow dwarf virus (CYDV)-RPV (formerly BYDV-RPV) as well as CYDV-RPS. In addition, there are some up to now unassigned species like BYDV-GPV, -RMV and -SGV (Mayo and D'Arcy 1999; Miller et al. 2002; D'Arcy and Domier 2005). In Europe, BYDV-PAV is the most common and severely damaging species being predominantly transmitted by the aphids *Rhopalosiphum padi* and *Sitobion avenae*. Temperatures exceeding 10°C during recent winters in some regions of Germany allowed survival of aphids by anholocyclic overwintering and caused high intensities of aphid migration and virus transmission especially in winter barley. For instance, in Germany regional temperatures in January and February 2007 were up to 5°C above the long-term average and cereal aphids were encountered even by the end of February (Löpmeier 2008). The early infection of winter barley with BYDV led to substantial yield losses in 2007, amounting in some instances to 20–30 dtonnes/ha or even

to total failure in cases where insecticide applications had not been applied timely (Wellie-Stephan 2007). In addition, infected plants are more predisposed to frost injury, water deficiency and fungal diseases (D'Arcy 1995).

In terms of resistance breeding, BYDV has continued to be problematic. One reason for this is that in the present barley breeding germplasm there are, at most, varying levels of tolerance rather than resistance to BYDV; based on this lack of true resistance and on the partial effects of the hitherto known BYDV-tolerance genes, the biological plausibility of immunity-like resistance to BYDV in cereals has even been ruled out (Huth 1995). A second reason is that the expression of tolerance is influenced by environmental conditions as well as allelic variation of the tolerance gene(s), the genetic background and the virus strain (Catherall et al. 1970, 1977; Chalhoub et al. 1995; Ovesná et al. 2002; Qualset 1975; Schaller 1984). As an additional obstacle, there appears to be an influence of the leaf colour (Moericke 1969) and possibly other characters of a given host plant on its relative attractiveness to certain aphids, which—if extrapolated to the barley/aphidic-vector systems—could make direct comparisons in the plant breeder's nursery problematic when the aphids have a choice between more or less attractive entries (J. Großer, KWS-Lochow GmbH, Wetze, Germany, personal communication).

In the past, various levels of tolerance to BYDV have been identified, in the barley cv. 'Rojo' and particularly in Ethiopian landraces. The genes involved were denoted *ryd1* (from cv. 'Rojo'; Suneson 1955), *Ryd2* (Rasmusson and Schaller 1959; Schaller et al. 1963), and *Ryd3* (Niks et al. 2004), respectively. While *ryd1* exerts relatively low tolerance and has not been further used for breeding purposes, the two remaining genes have been of more interest to geneticists and breeders. *Ryd2* provides field tolerance to the virus species of BYDV-PAV, BYDV-MAV, and BYDV-SGV (Baltenberger et al. 1987) and has been introduced in a number of former barley cultivars (Burnett et al. 1995). Schaller et al. (1963) and Ovesná et al. (2000, 2002) tested barley cultivars, breeding lines, and resistance sources from world collections. Most genotypes were found to be susceptible or moderately susceptible to BYDV. Reduced levels of symptom expression were due to the presence of the *Ryd2* gene. They also found tolerance genes that were non-allelic to *Ryd2*. QTLs responsible for effects of BYDV infection on yield components, plant height, and heading date were mapped on chromosomes 2HL and 3HL (Scheurer et al. 2001). Recently, *Ryd3*, a novel major gene for resistance to BYDV-PAV and -MAV, was identified and mapped to chromosome 6H (Niks et al. 2004). In that study, *Ryd3* as a QTL explained about 75% of the phenotypic variance among recombinant inbred lines (RIL).

While the genes mentioned above have been drawn from the primary genepool of barley, the secondary genepool has not yet been used to improve resistance to the BYDV complex. The secondary genepool of barley is represented by the wild species *Hordeum bulbosum* L. Despite existing crossing barriers (Pickering 2000) successful introgressions of agronomical useful genes, e.g., resistance to powdery mildew (Pohler and Szigat 1982; Xu and Kasha 1992; Pickering et al. 1995), leaf rust (Szigat et al. 1997; Pickering et al. 1998) and the soil-borne virus complex (Ruge et al. 2003; Ruge-Wehling et al. 2006) have been achieved and characterized by cytogenetic and molecular marker analysis.

An accession of *H. bulbosum* was identified that confers resistance to BYDV (Michel 1996; Habekuß et al. 2004). Plants of this accession remained ELISA-negative for BYDV after several inoculations with aphids charged with virus isolates BYDV-PAV1 Aschersleben, BYDV-MAV1 Aschersleben and CYDV RPV Dittersbach (Habekuß et al. 2004). Since 1994, yearly tests of this accession for BYDV infestation demonstrated that this resistance has remained effective to date. The present paper reports on recombinant barley lines that were derived from this BYDV-resistant *H. bulbosum* accession and results are presented on the characterization of the introgressed resistance factor that we propose to name $Ryd4^{Hb}$.

Materials and methods

Plant material

A tetraploid ($2n = 4x = 28$) *H. bulbosum* (*Hb*) accession (A17) from the Botanical Garden of Montevideo, Uruguay, was used which had been shown to be resistant to BYDV (Michel 1996; Habekuß et al. 2004). A17 had subsequently been used to as a parent in interspecific crosses and backcrosses with *H. vulgare* (*Hv*) cv. 'Igri' which yielded the diploid ($2n = 2x = 14$) BYDV-resistant BC_1F_1 hybrid BAZ-60.001 carrying terminal introgressions of *Hb* chromatin on four barley chromosomes (Scholz et al. 2008).

Backcrossing of BAZ-60.001 to *Hv* cv. 'Igri' resulted, among others, in a BC_2F_1 individual which carried a terminal introgression on a single barley chromosome as judged by cytological analysis. This individual was selfed to BC_2F_2. Selfing of selected BC_2F_2 and BC_2F_3 individuals gave rise to BC_2F_3 and BC_2F_4 offspring, respectively, which were used for further analysis.

Chromosome preparations

Mitotic chromosome preparations were used for identifying *Hb* chromatin in introgression lines and for chromosomal

localisation of the introgression harbouring the BYDV resistance. Meiotic metaphase I (MI) chromosome preparations were used to detect and analyse 3H chromosome arms either carrying or not carrying the *Hb* introgression.

Excised roots from young seedlings or potted plants were pre-treated in ice water for 24 h. Roots or spikes were fixed in 75% ethanol/25% acetic acid for 24 h and stored in 70% ethanol. The fixed root tips or anthers containing pollen mother cells (PMCs) at diakinesis or MI were washed in deionized water for 30 min and enzymatically digested according to Kakeda et al. (1991) at 37°C. The duration of enzymatic treatment was for 40–50 min for root tips and 60–80 min for anthers. After a short rinse in deionized water the macerated root tips were softened in 45% acetic acid for 1–2 min and squashed. The macerated anthers were softened in 60% acetic acid for 3–4 min and dispersed with a thin needle. The cell suspension was squashed gently by cover glass. The slides were frozen at −84°C for 1 h. After removal of the cover slips and air drying (1–2 days) the slides were stored at −25°C until use.

Fluorescence in situ hybridisation and genomic in situ hybridisation

For the detection of the 5S rRNA-specific genes a 117 bp fragment was amplified and labelled with digoxigenin-11-dUTP or biotin-16-dUTP (Roche Diagnostics) from genomic *Hv* DNA via PCR using primers according to Gottlob-McHugh et al. (1990). The 18/25S rRNA plasmid DNA of the probe VER 17 (Yakura and Tanifuji 1983) was labelled with digoxigenin-11-dUTP or biotin-16-dUTP using a nick translation mix (Roche Diagnostics).

For genomic in situ hybridisation (GISH), genomic DNA probes were isolated from the tetraploid *Hb* accession A17 (labelled with biotin-16-dUTP or digoxigenin-11-dUTP using a nick translation mix). Competitor DNA was isolated from the *Hv* cv. 'Igri' and sheared to give fragment lengths of 100–150 bp by autoclaving for 8 min. The hybridisation mix for a first fluorescence in situ hybridisation (FISH) and GISH in one procedure contained per slide in 20 µl: 10 µl 4× SSC with 20% dextran sulphate, 10 µl deionized formamide, 100 ng digoxigenin/biotin-labelled genomic *Hb* DNA of A17, 6 µg sheared competitor *Hv* DNA of cv. 'Igri' and 3 µg salmon sperm DNA. For mitotic chromosome preparations 200–300 ng biotin-labelled 5S rDNA was used in the hybridisation mix. For repeated FISH/GISH experiments with the same specimen (re-probing), the 5S rDNA was replaced by 180–200 ng of the digoxigenin/biotin-labelled plasmid DNA specific for the 18/25S rRNA gene in the hybridisation mix.

The in situ hybridisation procedure was performed according to Schrader et al. (2000) with the following minor modifications. For both FISH and GISH stringent washes were done three times for 5 min at 42°C in 0.3× SSC. For two-colour FISH, biotin was detected with 6 ng/µl of streptavidin-Cy3 (Dianova) and digoxigenin with 6 ng/µl of anti-digoxigenin-FITC (Roche Diagnostics). For GISH of meiotic chromosomes, digoxigenin was detected with 6 ng/µl of anti-digoxigenin-FITC. After washing of the slides in detection buffer (4× SSC with 0.1% Tween 20) three times for 5 min at 42°C, FITC signals were enhanced by 6 ng/µl of anti-sheep fluorescein (Dianova). Chromosomes were counterstained with 1.0 ng/µl of 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) for 5 min at 23°C. The reprobing procedure was used after the protocol of Schwarzacher and Heslop-Harrison (2000, p. 110) with a low-stringent washing step in the detection buffer (three times for 10 min at 23°C).

Photographs were taken with the microscope AXIO-Imager.Z1 (ZEISS) using a computer-assisted cooled CCD camera (AxioCam, ZEISS). Pseudocoloration, mergence of images and chromosome analysis were done with the ISIS-program (MetaSystems).

Molecular markers

The EST-derived SSR anchor markers *GBM1046*, *GBM1050* and *GBM1059* (Thiel et al. 2003) were kindly provided by A. Graner (Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben). The STS marker *ABC161* from the barley consensus-3H map was obtained using the primers published in Grain Genes (http://www.graingenes.org).

The CAPS marker *TC134544* was based on a tentative consensus (TC) sequence drawn from The Gene Index Project (http://compbio.dfci.harvard.edu/tgi/). *TC134544* was chosen because after in silico mapping of the marker *ABC161* on rice chromosome R1, it was found in close vicinity distal to this marker. For PCR of SSR, STS and CAPS markers 50 ng of genomic DNA was used in a solution containing 1× reaction buffer (Qiagen), 200 µM dNTPs, 5 pmol primers (F-tcgacttcaggagccacttt; R-cttt cgcccaagaaaacaaa) and 0.5 U of Taq DNA polymerase (Qiagen). PCR products were separated on 2.5% agarose gels followed by ethidium bromide staining. A 5 µl aliquot of the PCR was digested with 1 U of *Dpn*II to reveal polymorphism for the CAPS marker *TC134544*.

Testing for susceptibility to BYDV

Plants were inoculated by use of aphids carrying the isolate BYDV-PAV1 Aschersleben (PAV1-ASL). The plants were either 6–7-day-old seedlings (BC_2F_4 families and F_4 progeny tests) or approximately 150-day-old clonal parts which had been vernalized (BC_2F_2 and BC_2F_3 families).

For inoculation, 5–10 viruliferous *R. padi* per plant were used. After 2 days the aphids were killed by the insecticide Confidor® WG 70 (Bayer CropScience AG, Germany). The further cultivation of the plants was carried out in an air-conditioned greenhouse (20°C, 16 h photoperiod, 10 klx). Five to 6-week post-inoculation each plant was tested by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) according to Clark and Adams (1977). The test was carried out by using a polyclonal antisera which we produced from the above-mentioned BYDV-PAV 1 ASL isolate. Virus extinction was estimated at 405 nm on a microtitre plate reader Opsys MR (ThermoLabsystems) 1 h after the addition of the enzyme substrate. Plants with an extinction value of <0.1 were classified as resistant.

A subset of individuals from the BC_2F_3 families were selfed and the selfed progeny tested as described above to infer the resistance genotypes of selfed parent plants.

Results

Chromosomal localisation of the *Hb* introgression by GISH and FISH

In a first step, we tested the 18S/25S rDNA and the 5S rDNA probes as reported by Brown et al. (1999) for their suitability to identify the specific chromosomes in the genetic background (*Hv* cv. 'Igri') of the material under investigation. Both the subchromosomal positions and intensities of the 5S and 18S/25S rDNA hybridisation signals observed in our plant material agreed with those reported by Brown et al. (1999). Chromosomes 2H, 3H, 4H, and 7H could be differentiated via the positions and relative intensities of 5S-rDNA signals. The most intense signal was observed for 2HL whereas less intense signals were found on chromosome arms 3HL, 4HL, and 7HL (Fig. 1a). The NOR of the two satellite chromosomes 5H and 6H were characterized by FISH with the 18S/25S rDNA (VER 17) probe. Chromosome 6H was distinguished from 5H by its larger satellite. No signals specific for the ribosomal RNA genes were found on chromosome 1H. Altogether, these results which are in accordance to Brown et al. (1999), demonstrate that the seven chromosomes of cv. 'Igri' could be identified via FISH.

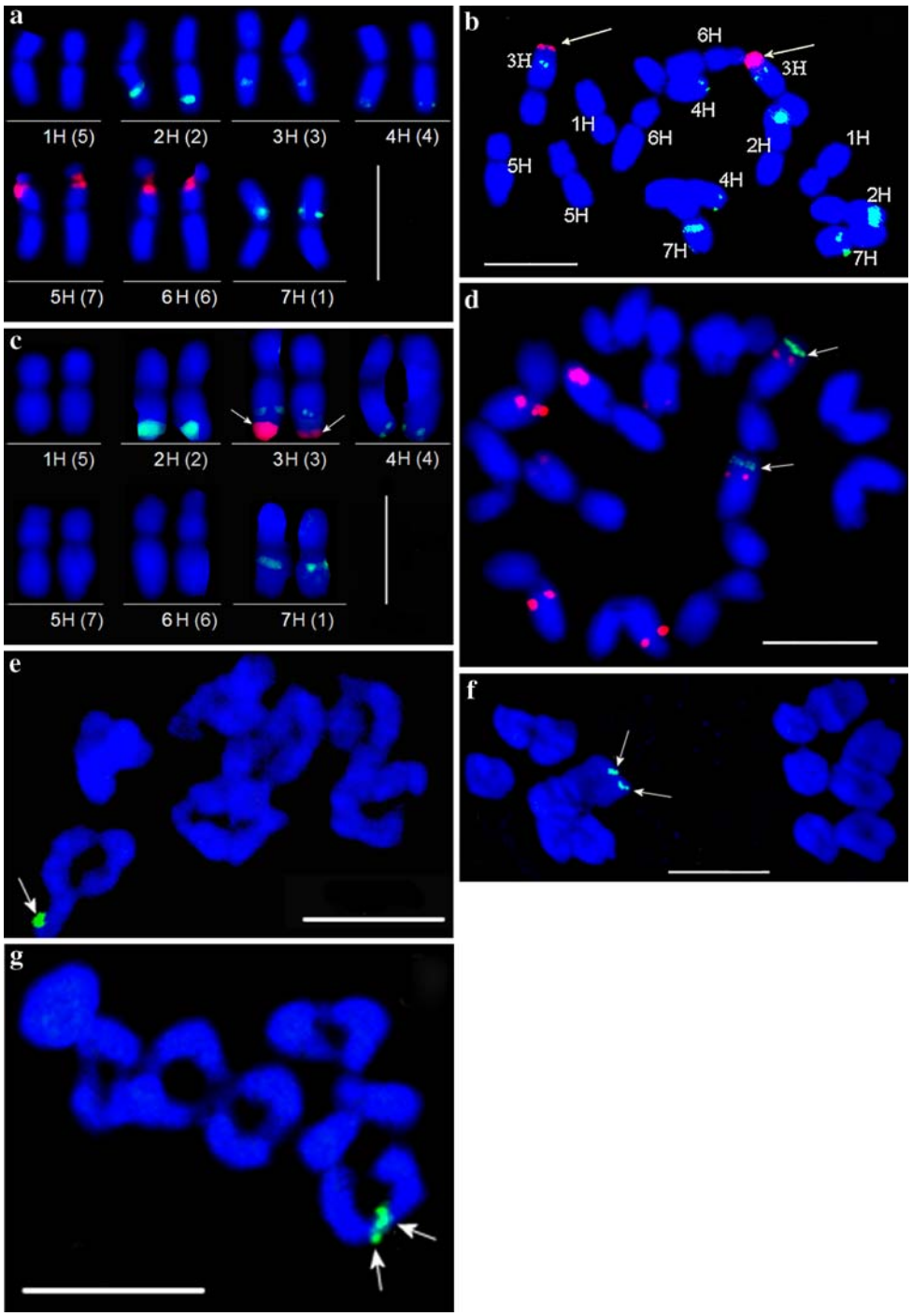
In a second step, we examined mitotic chromosomes of BYDV-resistant plants by a combination of GISH and FISH. The GISH used labelled *Hb* genomic DNA as a probe to visualize the *Hb* introgression while the FISH used the 18S/25S rDNA or the 5S rDNA probes to identify the particular barley chromosomes. Applying the 18S/25S rDNA (VER 17) as a probe, the barley chromosome harbouring the *Hb* introgression did not display any FISH signals (not shown). In contrast, applying the 5S rDNA as a probe generated visible signals proximal to the introgressed *Hb* segment (Fig. 1b). The 5S rDNA signal on this chromosome was weaker in intensity and also more proximal in its position than the 5S rDNA signal on 2HL. Moreover, the 5S rDNA signals of the chromosome under inspection were different from those on chromosomes 7H and 4HL, since the signals on these chromosomes were shown to be located near-centromeric and far distal, respectively (Fig. 1c). In conclusion, the barley chromosome arm which carried the *Hb* introgression conferring BYDV resistance was identified as 3HL.

Fig. 1 **a** Two-colour FISH karyogram of barley cv. 'Igri' mitotic chromosomes hybridized with 5S rDNA (*green*) and 18S/25S rDNA (*red*) probes. The long arms of chromosome 2H, 3H and 4H and the short arms of chromosome 7H are characterized by 5S rDNA signals in different positions and intensities. The NOR regions of chromosome 5H and 6H are marked by hybridization signals of 18S/25S rDNA (note, the longer chromosome pair 6H had also the largest satellites). **b** Combined FISH (5S rDNA, *green*) and GISH (*H. bulbosum* genomic DNA, *red*) with mitotic chromosomes of a BYDV-resistant BC_2F_2 plant showing terminal introgressions of *Hb* chromatin (*red*; *arrows*) of different sizes on 3 HL homologues. **c** Karyogram of the same cell. **d** Combined FISH (5S rDNA, *red*) and GISH (*H. bulbosum* genomic DNA, *green*) with mitotic chromosome of a homozygous BYDV-resistant plant from family BC_2F_3-1 showing small subterminal introgressions of *Hb* chromatin (*green*; *arrows*) on both 3HL homologues. **e–f** GISH analysis of meiotic chromosome preparations from PMCs of homozygous and heterozygous BYDV-resistant BC_2F_4 offspring. **e** Diakinesis of a heterozygous resistant individual. Note the strong association at the end of the *H. bulbosum* segment (*green*) carrying chromosome in one of the seven ring bivalents. **f** The same PMC after moving of each homologous pair to the opposite ends of the cell at anaphase I. It is obvious that chromatids of only one of the 3H homologues carry a *H. bulbosum* segment, demonstrating the heterozygous genotype of this plant. **g** Metaphase I of a homozygous resistant BC_2F_4 offspring. Note the two green signals on one of the seven ring bivalents (*arrows*). The lengths of scale bars are 10 μm in **a–d** and 5 μm in **e–f** ▶

Identification of the *Hb*-specific segment in backcross families via GISH

Backcrossing of the BC_1F_1 hybrid plant BAZ-60.001 which carried four larger terminal introgressions on different chromosomes (Scholz et al. 2008) gave rise to BC_2F_1 offspring. One plant was cytologically identified which carried a terminal *Hb* introgression on chromosome 3HL (not shown). This plant was selfed to BC_2F_2.

Of the 37 BC_2F_2 offspring, 23 were shown to carry an *Hb* segment while 14 did not display any *Hb*-specific GISH signals. Among the 23 *Hb*-positive plants we observed four triploids ($2n = 3x = 21$). These were not used any further as parents of successive generations. Of the remaining 19 *Hb*-positive diploid BC_2F_2 individuals, 17 plants were heterozygous for the presence of *Hb* introgressions on chromosome arm 3HL, with introgression sizes varying



among the plants (not shown). Two of these were selfed to BC_2F_3-1 and -2 families, which later turned out to segregate with a small subterminal and a larger terminal introgression, respectively (cf. Fig. 3). The remaining two BC_2F_2 individuals carried *Hb* chromatin on both 3H homologues. One of these two plants died at the early seedling stage. The other plant carried terminal introgressions of different sizes (Figs. 1b, c, 3) and survived. This was selfed to family BC_2F_3-3. BYDV-resistant plants from the three BC_2F_3 families were randomly chosen for GISH and FISH analyses. All 16 sampled individuals from family BC_2F_3-1 displayed a small subterminal *Hb* introgression on one or both 3H homologues (Fig. 1d). In contrast, resistant individuals from families BC_2F_3-2 and -3 carried terminal signals which occurred in two different sizes similar to those observed in the BC_2F_2 (not shown; cf. Fig. 1b, c).

Three heterozygous individuals with the small subterminal introgression from the BC_2F_3-1 family were selfed to

Table 1 Mean metaphase I associations per pollen mother cell (PMC) in eight plants from three BC_2F_4 families with (Hb^{+-}, Hb^{++}) or without (Hb^{--}) subterminal *H. bulbosum* introgression on chromosome arm 3HL

Family origin; # plant	Introgression genotype[a]	No. of PMC analysed	No. of II per cell[b]		Types of II observed for 3H chromosomes per cell	
			Ring	Rod	Ring	Rod
BC_2F_4-1-#1	Hb^{--}	25	6.96 (6–7)	0.04 (0–1)	ND	ND
BC_2F_4-1-#2	Hb^{+-}	38	6.99 (6–7)	0.01 (0–1)	+	–
BC_2F_4-1-#3	Hb^{+-}	64	6.98 (6–7)	0.02 (0–1)	+	–
BC_2F_4-1-#4	Hb^{+-}	17	7.0	0.0	+	–
BC_2F_4-1-#5	Hb^{++}	24	6.92 (6–7)	0.08 (0–1)	+	–
BC_2F_4-2-#1	Hb^{+-}	61	6.95 (6–7)	0.05 (0–1)	+	–
BC_2F_4-3-#1	Hb^{--}	22	7.0	0.0	+	–
BC_2F_4-3-#2	Hb^{+-}	26	6.93 (6–7)	0.07 (0–1)	+	–

[a] Hb^{--} introgression lacking from both 3HL homologues, Hb^{+-} heterozygous, Hb^{++} homozygous for introgression

[b] Ranges of observed bivalent (II) numbers in parentheses

create BC_2F_4-1, -2 and -3 families. BYDV resistance in these families occurred in individuals heterozygous or homozygous with regard to the presence of *Hb* introgressions according to GISH (not shown). These families were used for analysis of meiotic chromosome pairing.

Meiotic chromosome pairing

Genomic in situ hybridisation analysis of MI chromosome preparations from 230 PMCs of six BYDV-resistant individuals in the three BC_2F_4 families and 47 PMCs of two susceptible plants from the same families revealed that pairing behaviour of 3H chromosomes was very similar between individuals carrying either two 3H homologues of cv. 'Igri' origin or recombined 'Igri'/*Hb* chromosomes in a homozygous or heterozygous state (Table 1). The mean number of ring bivalents varied between 6.92 and 7.0 (Table 1) whereas the frequency of rod bivalents was low (0.00–0.08). With regard to the recombined 'Igri'/*Hb* 3H chromosomes, ring bivalents were exclusively observed (Table 1). All individuals of the BC_2F_4 families which were analysed displayed very close associations of the 3H chromosomes (Fig. 1e–g), suggesting that the *Hb* introgression was indeed homoeologous to its 'Igri' counterpart and did not impose detectable disturbance of homoeologous pairing in diakinesis.

Inheritance of BYDV resistance

When challenged with BYDV-PAV, the BC_2F_2 family segregated into two distinct groups with markedly differing ELISA extinction values (Table 2). A first group of 14 individuals, which according to GISH were devoid of *Hb* chromatin, gave ELISA extinction values of 1.04 or higher. Plants falling in this group were regarded as ELISA-positive and, thus, susceptible to BYDV. In contrast, a second group of 23 GISH-positive individuals had extinction values close to zero (<0.1; Table 2). This group was considered as representing ELISA-negative offspring resistant to BYDV. According to GISH, all 23 individuals belonging to this group carried the *Hb* introgression on chromosome arm 3HL, most of them (17, including the four triploids mentioned above) in the heterozygous state. The ratio of ELISA-negative versus positive plants (23:14) was consistent with a 3:1 ratio at $P = 0.07$, with a deficiency in ELISA-negative offspring (Table 2).

Segregation of BYDV resistance was also assessed in three BC_2F_3 families (BC_2F_3-1, -2, -3), which were obtained from selfing three BYDV-resistant BC_2F_2 individuals. Segregation patterns among these three families fell into two groups. A first group, represented by family BC_2F_3-1, segregated with the expected 3:1 ratio of resistant versus susceptible offspring (Table 2) and which, according to GISH, carried a small subterminal *Hb* introgression (see previous section, Fig. 1d). The second group was made up of families BC_2F_3-2 and -3 which carried terminal *Hb* introgressions of different sizes (see previous section, Fig. 1b, c) and displayed distorted segregations which were statistically consistent with a 1:1 rather than 3:1 ratio (Table 2). Pooling these two full-sib families gave a segregation pattern of 43 resistant versus 35 susceptible $\chi^2_{1:1} = 0.82$, indicating that one or several segregation-distorting loci (SDL, Vogl and Xu 2000) were linked to BYDV resistance in these two families.

Three BC_2F_4 families obtained from the selfing of *Hb*-heterozygous individuals of family BC_2F_3-1 each segregated with a 3:1 ratio of resistant versus susceptible plants (Table 2). In all segregating families, the ELISA values followed a bimodal distribution where the groups of "negative" and "positive" test results were clearly separated from each other (Fig. 2). The three BC_2F_4 families were pooled to constitute a future mapping population

Table 2 Assessment of BYDV-PAV1 concentration (ELISA extinction) in families segregating with *Hb* introgressions for chromosome arm 3HL

Family	*N*	ELISA grouping[a]		Goodness of fit			
				1 susc.:3 res.		1 susc.:1 res.	
		Positive	Negative	χ^2	*P*	χ^2	*P*
BC_2F_2	37	14 (1.04–2.29)	23 (0.00–0.01)	3.25	0.071	2.19	0.139
BC_2F_3-1	59	18 (1.09–2.31)	41 (0.00–0.04)	0.96	0.328	8.97	0.003
BC_2F_3-2	37	19 (0.37–2.32)	18 (0.00–0.01)	13.70	0.000	0.003	0.869
BC_2F_3-3	41	16 (0.99–1.75)	25 (0.00–0.01)	4.30	0.038	1.98	0.160
BC_2F_4-1	87	21 (1.03–1.86)	66 (0.00–0.02)	0.03	0.853	–	–
BC_2F_4-2	14	4 (1.31–1.89)	10 (0.00–0.01)	0.09	0.758	–	–
BC_2F_4-3	25	6 (0.18–1.97)	19 (0.00–0.02)	0.01	0.908	–	–

[a] Ranges of ELISA values in parentheses

which will be ready for use once all individuals have been characterized for their resistance genotypes via progeny testing.

Upon challenging with viruliferous aphids, segregants heterozygous for molecular markers (see below) of the subterminal *Hb* introgression gave ELISA values as low as homozygous *Hb*-marker genotypes, which demonstrated a dominant expression of resistance conferred by the *Hb* introgression. Among the 126 individuals of the pooled BC_2F_4 population, 123 showed zero or high ELISA values and corresponding presence versus absence, respectively, of the *Hb* introgression according to marker genotypes (see below). One of the 126 BC_2F_4 individuals yielded a high ELISA value whilst being heterozygous with *Hb* alleles at marker loci *ABC161*, *GBM1050*, and *GBM1059* and two plants with zero values were homozygous for *Hv* marker alleles. As results from progeny testing were not yet available for the mapping population, the resistance genotypes of the three plants have not yet been determined.

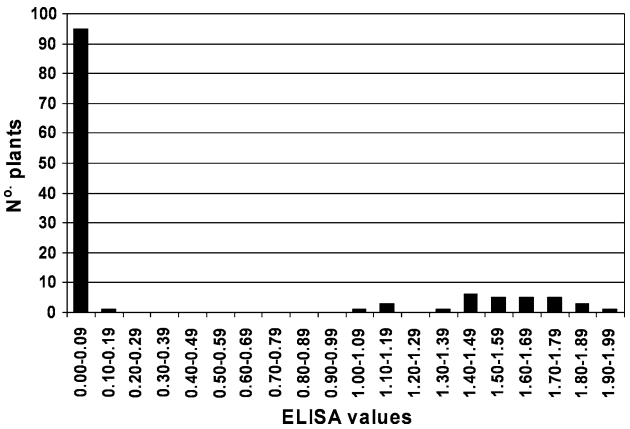


Fig. 2 Distribution of ELISA values obtained with 126 individuals of three pooled BC_2F_4 families

BC_2F_4 individuals classified by molecular marker genotypes as either homozygous or heterozygous for the *Hb* introgression (see below), resulted in distinct phenotypic groups of offspring according to the Kruskal–Wallis test (Table 3). Heterozygous *Hb* recombinants developed into vigorously growing plants without any BYDV symptoms while plants lacking the introgression presented themselves as heavily affected by BYDV, i.e., were stunted with discoloration of leaves or leaf tips. Plant height and the formation of kernels per ear were significantly reduced in susceptible plants lacking the *Hb* introgression (Hb^{-}) as compared to the heterozygous Hb^{+-} plants (Table 3). Infected susceptible plants reached seed maturation about 10 weeks later than heterozygous resistant plants. Notably, offspring homozygous for the presence of the introgression (Hb^{++}) displayed severely depressed growth, with mean plant height and mean kernel formation lower than in susceptible Hb^{-} offspring (Table 3). These plants lagged about 3 weeks behind the heterozygous offspring and started to die off soon after flowering. This suggests that besides BYDV resistance, a recessive sublethality factor was residing on the introgressed subterminal *Hb* segment.

Molecular marker analysis of *Hb* recombinants

In addition to cytological analysis, molecular markers were used to further characterize the *Hb* recombinants in different BC_2F_3 and -F_4 families. Markers *ABC161*, *GBM1050*, *GBM1059*, and *GBM1046* were chosen which have been reported as anchor markers on barley chromosome 3HL, with *GBM1046* representing the marker at the most distal location (Thiel et al. 2003; Varshney et al. 2006, 2007). In addition to these known markers, a new marker, *TC134544*, was developed that even mapped 10 cM distal to *GBM1046* (B. Ruge-Wehling, unpublished data). These markers proved to be polymorphic between the *Hv* cv. 'Igri' and *Hb* parents and, thus, suitable to

Table 3 Comparisons (pairwise and pooled) of groups of introgression genotypes (Hb^{+-}, Hb^{++}, and Hb^{--}) with regard to plant height and seed set; genotypic groups were analysed in BC_2F_4 families after inoculation by viruliferous aphids

Genotypic group[a]	*N*	Plant height (cm)					Seed set (no. kernels/ear)				
		Range	Mean	KW test[b]: *H* statistic *P* value			Range	Mean	KW test[b]: *H* statistic *P* value		
				Hb^{+-}	Hb^{--}	Hb^{++}			Hb^{+-}	Hb^{--}	Hb^{++}
Hb^{+-}	50	57.8–83.3	72.8	–	45.21	43.76	19.8–27.8	25.3	–	45.21	44.03
					<0.0001	<0.0001				<0.0001	<0.0001
Hb^{--}	22	13.3–41.3	26.9	–	–	10.13	0–10.9	4.5	–	–	15.63
						0.0015					<0.0001
Hb^{++}	21	13.5–31.5	20.3	–	–	–	0–6.5	0.9	–	–	–
All groups				70.82					72.20		
				<0.0001					<0.0001		

[a] As inferred from molecular marker alleles stemming from the *Hb* donor parent

[b] Analysis of variance according to Kruskal and Wallis w/t 1 *df* and 2 *df* per pairwise comparison and pooled comparison, respectively

identify recombined and non-recombined chromatin in the subgenomic region of interest. Marker analysis revealed that *Hb* recombinants differed in the distal boundary of their *Hb* introgressions, thereby distinguishing the groups of families which either segregated with BYDV-resistant and -susceptible offspring in the expected 3:1 or distorted (1:1) ratio (Fig. 3). Families segregating 3:1 carried *Hb* introgressions truncated by recombination to end proximal to marker *GBM1046*. In contrast, in the families segregating with distorted ratios of resistant to susceptible offspring, *Hb* alleles were found at the most distal marker loci *GBM1046* and *TC13454*, suggesting the presence of an SDL distal to *GBM1059*. Furthermore, in family BC_2F_3-3 the distal part of the chromosome was recombined to

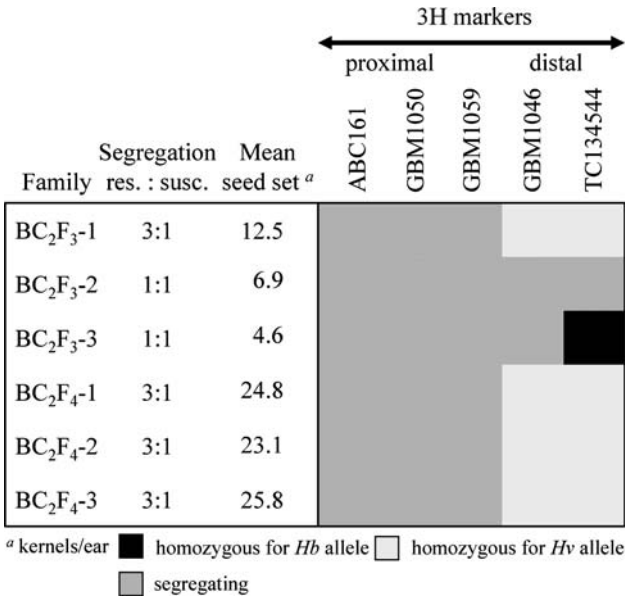


Fig. 3 Segregation at molecular marker loci in BC_2F_3 and BC_2F_4 families showing either disturbed or non-disturbed segregation with regard to BYDV resistance

Hb-homozygosity (marker *TC134544* in Fig. 3), yet showed a 1:1 segregation ratio similar to BC_2F_3-2 which was heterozygous across this chromosome region, indicating that the SDL which explained the distorted segregation was located proximal to *TC134544*. There were also differences between the BC_2F_3 and BC_2F_4 families with respect to seed set (Fig. 3), as demonstrated by the Kruskal–Wallis test (Table 4). Three groups of families could be distinguished which differed in their seed sets, namely BC_2F_3-2 and -3 having the lowest mean numbers of kernels per ear than, family BC_2F_3-1 with medium mean seed set and the BC_2F_4 families, having the highest mean seed sets.

To determine the action (i.e., zygotic or gametic) of the SDL segregating in families BC_2F_3-2 and -3, segregation analysis of codominant markers was performed. Remnant seeds from the two families which had not been completely characterized for their resistance phenotypes were grown and seedlings genotyped for the codominant markers *GBM1059* and *GBM1046* which both segregated in the two families. Among 37 individuals genotyped in family BC_2F_3-2, 21 and 19 plants were homozygous for the *Hv* allele at the respective marker loci while 16 and 17 plants, respectively, were heterozygous. Among 72 plants genotyped in family BC_2F_3-3 the frequencies of *Hv*-homozygous and heterozygous marker genotypes at the two marker loci were 36 in each case. Thus, marker analysis revealed that the distorted 1:1 segregation ratio of resistant versus susceptible offspring in these two families (cf. Table 2) corresponded to complete absence of marker genotypes homozygous for *Hb* alleles, which indicated selection against gametes carrying *Hb* alleles at the respective molecular marker loci.

Marker analysis was also used to roughly estimate the position of the BYDV resistance factor along the *Hb* introgression. Comparing resistant to susceptible offspring of family BC_2F_3-2 revealed that presence of *Hb* chromatin

Table 4 Comparisons (pairwise and pooled) of BC_2F_3 and BC_2F_4 families with regard to seed set after inoculation by viruliferous aphids

Family	*N*	Seed set (no. kernels/ear)						
		Range	Mean	KW test[a]: *H* statistic *P* value				
				BC_2F_3-2	BC_2F_3-3	BC_2F_4-1	BC_2F_4-2	BC_2F_4-3
BC_2F_3-1	18	6.9–16.6	12.5	8.79	24.40	17.80	15.95	11.25
				0.0030	<0.0001	<0.0001	<0.0001	0.0008
BC_2F_3-2	7	0.18–10.6	6.9	–	2.36	11.67	10.64	8.08
					0.1246	0.0006	0.0011	0.0045
BC_2F_3-3	35	0.18–13.3	4.6	–	–	22.82	33.02	12.81
						<0.0001	<0.0001	0.0003
BC_2F_4-1	10	22.3–27.3	24.8	–	–	–	0.53	1.22
							0.4685	0.2703
BC_2F_4-2	23	4.21–30.2	23.05	–	–	–	–	0.15
								0.6965
BC_2F_4-3	5	24.6–26.8	25.79	–	–	–	–	–
All families				68.11				
				0<.0001				

[a] Analysis of variance according to Kruskal and Wallis w/t 1 *df* and 3 *df* per pairwise comparison and pooled comparison, respectively

in the distal part, i.e., at marker loci *GBM1046* and *TC134544* (Fig. 4, *Hb* recombinant types 1 and 2), was not sufficient to confer BYDV resistance. Rather, the resistance factor appeared to be located proximal to *GBM1046*.

Discussion

Using *H. bulbosum* accession A17 as a resistance donor, *Hv* cv. 'Igri' as genetic background and BYDV-PAV1 ASL as a pathogen we found that the introgressed resistance (1) is dominantly expressed, (2) is inherited by a factor residing on a subterminal introgression of limited size on chromosome 3HL, and (3) confers true resistance as judged by ELISA values of zero or close to zero. Considering the genes *ryd1*, *Ryd2* and *Ryd3* which have so far been identified in the primary genepool of barley, we propose to name the novel BYDV resistance factor $Ryd4^{Hb}$.

At present, we do not know the resistance mechanism underlying $Ryd4^{Hb}$. The invariably low ELISA values obtained with $Ryd4^{Hb}$ carriers suggest, though, that in the sense of Cooper and Jones (1983), this gene confers either immunity, i.e., lack of infectability, or some type of resistance such as restriction of virus infection, multiplication or invasion. Preliminary results using electrical penetration graph suggest that phloem feeding of *R. padi* might be impaired on $Ryd4^{Hb}$ carriers (Habekuß et al. 2004; E. Schliephake, Quedlinburg, personal communication). In the present study, we have demonstrated that $Ryd4^{Hb}$ is effective against the isolate BYDV-PAV1 Aschersleben. Previous evaluations demonstrated that the

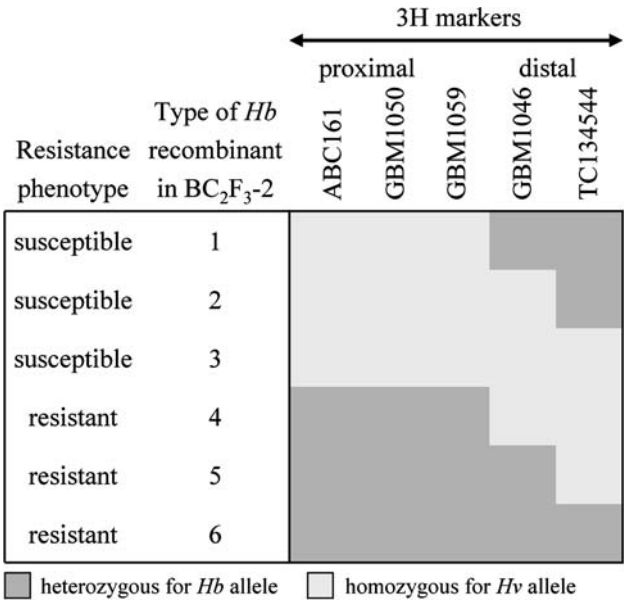


Fig. 4 Types of BYDV-resistant and susceptible *Hb* recombinants and their molecular marker genotypes observed in family BC_2F_3-2

original *Hb* accession, which was used as $Ryd4^{Hb}$ donor, was also resistant to BYDV-MAV1 Aschersleben as well as to CYDV-RPV Dittersbach (Habekuß et al. 2004). Therefore, there is good chance that $Ryd4^{Hb}$ may confer resistance to these other viruses, too, although this remains to be demonstrated in a more direct way.

The present report on BYDV resistance introgressed from *H. bulbosum* into barley is based on virus concentration measurement via DAS-ELISA and on the assessment

of visual symptoms such as discoloration, stunting and reduced kernel formation. With regard to the assessment of BYDV tolerance, ELISA has been questioned as a reliable measure in the literature (Huth 1995; Scheurer et al. 2000; Scheurer et al. 2001) because the virus titre is not always correlated with tolerance in terms of relative kernel yield. For instance, Huth (1995) reports on ELISA extinction values not significantly different among tolerant (including 'Mokusekko', 'Post', and 'Vixen') and sensitive barley cultivars 3 week post-inoculation. Using double-haploid lines from crosses of cvs. 'Post', 'Ogra18', 'Muju covered 2', 'Vixen', 'Nixe', and others, Scheurer et al. (2000) found that when challenged with BYDV-PAV BS, the groups of high and low-yielding DH lines did not show substantially different ELISA values, the latter of which were in the range of 0.98–1.93 60 minutes after substrate addition. This means that genotypes which react tolerant to BYDV in their yield performance may contain relatively high virus titres and, thus, screening based on ELISA may fail to identify tolerant phenotypes. As to the present study, the relevance of the introgressed *Ryd4*^{Hb} gene for relative yield performance under BYDV infection pressure certainly needs to be assessed in future field-plot experiments. Apart from this aspect, though, the situation in the present study was different from that addressed by Huth (1995) and Scheurer et al. (2000) in that ELISA values obtained in the present study differed in a qualitative manner between groups of genotypes either carrying or not carrying the resistance gene and were practically zero in the former. As far as assessed by us via cytology or molecular markers, there was tight correlation of the presence versus absence of the *Hb* introgression and ELISA values of <0.1 versus much higher values, respectively. To conclude, the two groups of ELISA values obtained in the present study may, indeed, be considered indicative for resistance (or immunity) conferred by *Ryd4*^{Hb} and susceptibility, respectively, to BYDV-PAV.

To date, two major BVDV-resistance genes have been reported which may confer relatively high degrees of host tolerance or resistance to certain BYDV/CYDV isolates, namely *Ryd2* and *Ryd3*, which both were derived from Ethiopian barley accessions (Schaller et al. 1964; Niks et al. 2004). *Ryd2* has been located on the long arm of chromosome 3H in close proximity to the centromere and was reported to be inherited as a monogenic, incompletely dominant or recessive trait with varying degrees of resistance. The resistance gene *Ryd2* appears just to be effective against BYDV-PAV but not to all isolates of BYDV-MAV and CYDV-RPV (Baltenberger et al. 1987; Banks et al. 1992; Jefferies et al. 2003). Furthermore, *Ryd2* effects on resistance have been shown to be reduced by *Ryd2*-heterozygosity as well as by growth conditions or genetic backgrounds that result in a slow growth (see Catherall et al. 1970, 1977; Collins et al. 1996; Damsteegt and Bruehl 1964; Jones and Catherall 1970; Parry and Habgood 1986; Rasmusson and Schaller 1959; Schaller et al. 1964). Besides simple inheritance of *Ryd2*-mediated reaction to BYDV, as has been inferred in these studies from symptom expression, a *Ryd2* allele derived from the slow-maturing cv. 'Vixen' was reported to exert, besides other QTLs, a partial, quantitative effect if tolerance to BYDV was assessed in terms of relative kernel yield (Scheurer et al. 2001). Considering its near-centromeric location on chromosome 3HL, *Ryd2* appears not to be orthologous to *Ryd4*^{Hb}, the latter of which has clearly been determined by the present study to be located in a subterminal position via GISH analysis as well as by using 3HL anchor markers. The very distal location of the *Hb* introgression harbouring *Ryd4*^{Hb} is corroborated by an additional marker, *MWG883*, which segregated with *Hb* and *Hv* alleles in the BC_2F_4 families and mapped less than 1 cM away from *ABC161* (B. Ruge-Wehling, unpublished data). *MWG883* was reported to cosegregate at position 127 cM with the marker *MWG902* (Thiel et al. 2003), the latter of which was placed in the most distal subregion of the physical map of chromosome arm 3HL (Künzel and Waugh 2002). A more detailed investigation will be possible once *Ryd4*^{Hb} has been arranged in a molecular marker map.

The second major BVDV-resistance gene is *Ryd3* residing on chromosome 6H. This gene was reported to confer resistance rather than tolerance as inferred from ELISA values close to zero (Niks et al. 2004), which appears similar to the effect of *Ryd4*^{Hb}. In contrast to the present study where almost all plants carrying *Ryd4*^{Hb} proved to be resistant, *Ryd3* carriers did, however, not entirely remain uninfected by the virus. For instance, 20% of the individuals of the *Ryd3*-donor accession L94 developed symptoms, and these had virus concentrations similar or only slightly lower than in susceptible accessions (Niks et al. 2004). With regard to *Ryd4*^{Hb}, we did not observe high incidence of ELISA-positive *Ryd4*^{Hb} carriers. Among 95 segregants of the pooled BC_2F_4 population which carried *Hb* alleles at molecular marker loci in a homozygous or heterozygous state, a high ELISA value (1.67) was recorded for one single heterozygous individual. At present, we cannot exclude genetic recombination between *Ryd4*^{Hb} and molecular markers or measurement error as potential causes of this single deviant. To conclude, the penetrance of *Ryd4*^{Hb} appears to be comparatively high. However, direct comparisons of the effects of *Ryd4*^{Hb} with those of other BYDV resistance genes must remain tentative as long as the effects of varying growth conditions and genetic backgrounds on the expression of *Ryd4*^{Hb} have not been assessed.

Besides *Ryd4*^{Hb}, an SDL was found to reside on the original, large *Hb* introgression which prevented the formation of *Ryd4*^{Hb} homozygotes due to gametic selection

either on the male or female side in the BC_2F_2 and some BC_2F_3 families. We have demonstrated that this SDL could readily be separated from *Ryd4*^{Hb} via recombination, such that resistant versus susceptible offspring occurred in the expected 3:1 (rather than 1:1) ratio and codominant molecular markers linked to resistance segregated in the expected 1:2:1 (rather than 1:1:0) ratio among BC_2F_4 progeny. Recently, recombinative elimination of an SDL preventing the formation of homozygous resistant offspring has also proven feasible in the case of a *Hb* introgression carrying the dominant resistance gene to barley yellow mosaic virus (BaYMV), *Rym16*^{Hb}, on barley chromosome 2HL (Ruge-Wehling et al. 2006).

As can be concluded from Fig. 3, distal parts of the *Hb* introgression exerted a negative effect on fertility. Seed set increased when the original introgression was truncated via recombination proximal to marker *GBM1046*. A further increase in fertility obtained with family BC_2F_4-1 (as compared to BC_2F_3-1) suggests that other *Hb* chromatin with negative effects on fertility had remained in BC_2F_3-1, which was not traceable in the present study via molecular markers yet could be eliminated during an additional round of recombination from BC_2F_3 to BC_2F_4.

A major drawback, though, which currently compromises the use of *Ryd4*^{Hb} in breeding programmes is posed by a recessive sublethality factor which after elimination of the gametic SDL mentioned above, still remains on the recombined subterminal version of the *Hb* introgression in the BC_2F_4 families and causes pronounced growth retardation of homozygous resistant offspring. Whether linkage of *Ryd4*^{Hb} to this factor can also be broken via recombination will have to be seen in future mapping experiments. The fact that according to meiotic MI analyses, associations of 3HL chromosome arms were not different between susceptible cv. 'Igri' plants and heterozygous resistant *Hb* recombinants led us to assume that the *Hb* introgression is homoeologous to its *Hv* counterpart on the original *Hv* 3HL chromosome, and that chromosome pairing and recombination in this region are not severely impaired in *Hb* recombinants. This assumption is corroborated by the occurrence of different sizes of introgressions among selfed BC_2F_3 (cf. Fig. 4) and -F_4 offspring. Thus, there is reason to believe that the subterminal introgression obtained in the present study can be further tailored via additional rounds of recombination in a marker-assisted approach such that the growth-depressing factor would be separated from *Ryd4*^{Hb}. Besides linkage with a growth-depressing factor, a pleiotropic effect of *Ryd4*^{Hb} itself on plant growth may be considered as alternative explanation. For wheat it is known that the effects of a number of disease-resistance genes, e.g., *Lr34*, *Yr18* and *Bdv1* on chromosome arm 7DL, are correlated with a phenotype called leaf-tip necrosis (LTN) and inherited together with LTN as a single Mendelian locus, which suggests that the *Ltn* gene conferring LTN is pleiotropic to some resistances (Singh 1992, 1993; Schnurbusch et al. 2004). There are, however, striking phenotypic and genetic differences between LTN in wheat and the growth depression observed in the present study. Most obviously, the growth-depressing factor is inherited as a recessive trait, i.e., plants heterozygous for *Ryd4*^{Hb} did not exhibit any growth depression yet were completely resistant to BYDV. In contrast, *Ryd4*^{Hb} is dominantly inherited, and there is no close correlation of the resistance and growth-depression phenotypes. As a conclusion, we assume genetic linkage of separate genes as the more plausible explanation for our observation.

Further studies will be needed to judge what potential *Ryd4*^{Hb} may have for breeders wanting a highly effective and easy-to-use type of resistance. Firstly and most importantly, linkage between *Ryd4*^{Hb} and the growth-depressing factor has to be eliminated (see above). Secondly, the influence that the genetic background may have on the expression of *Ryd4*^{Hb} will have to be assessed. Thirdly, it will have to be checked in field-plot experiments whether *Ryd4*^{Hb} carriers also remain unaffected in their yield performance when challenged by BYDV.

To date, a number of recombinant lines (RL) have been generated in barley which carry *Hb* chromosomal segments. Zhang et al. (2001) and Pickering et al. (2004) report on 72 RL which altogether, represent *Hb* introgressions on all *Hv* chromosome arms except for 3HL. The 3HL-RL described in the present study now completes this series.

It has been demonstrated that a variety of traits such as resistances to leaf rust, stem rust, scald, Septoria speckled leaf blotch, powdery mildew, the soil-borne barley yellow mosaic virus complex, glossy spike and leaf sheath, black aleurone, response to DDT and others can be introgressed into cultivated barley using *H. vulgare* × *H. bulbosum* hybrids (Pickering et al. 2004). In the present study, we demonstrate that resistance to BYDV can as well be transferred from *H. bulbosum* to cultivated barley via a subterminal introgression on chromosome arm 3HL. This result adds to the evidence for the potential of the secondary genepool as a genetic resource in barley breeding.

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