Theor Appl Genet (2013) 126:1613–1625
DOI 10.1007/s00122-013-2078-9

ORIGINAL PAPER

Rph22: mapping of a novel leaf rust resistance gene introgressed from the non-host *Hordeum bulbosum* L. into cultivated barley (*Hordeum vulgare* L.)

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Received: 6 August 2012 / Accepted: 23 February 2013 / Published online: 7 March 2013

Abstract A resistance gene (*Rph22*) to barley leaf rust caused by *Puccinia hordei* was introgressed from the non-host species *Hordeum bulbosum* into cultivated barley. The *H. bulbosum* introgression in line '182Q20' was located to chromosome 2HL using genomic in situ hybridisation (GISH). Using molecular markers it was shown to cover approximately 20 % of the genetic length of the chromosome. The introgression confers a very high level of resistance to *P. hordei* at the seedling stage that is not based on a hypersensitive reaction. The presence of the resistance gene increased the latency period of the leaf rust fungus and strongly reduced the infection frequency relative to the genetic background cultivar 'Golden Promise'. An F_2 population of 550 individuals was developed and used to create a genetic map of the introgressed region and to determine the map position of the underlying resistance gene(s). The resistance locus, designated *Rph22*, was located to the distal portion of the introgression, co-segregating with markers H35_26334 and H35_45139. Flanking markers will be used to reduce the linkage drag, including gene(s) responsible for a yield penalty, around the resistance locus and to transfer the gene into elite barley germplasm. This genetic location is also known to harbour a QTL (*Rphq2*) for non-hypersensitive leaf rust resistance in the barley cultivar 'Vada'. Comparison of the 'Vada' and *H. bulbosum* resistances at this locus may lead to a better understanding of the possible association between host and non-host resistance mechanisms.

Abbreviations

IL	Introgression line (*H. bulbosum* chromatin in a barley genetic background)
QTL	Quantitative trait locus
NBS-LRR	A class of resistance gene NBS (nuclear binding site) LRR (leucine-rich repeat)

Introduction

Growers and plant breeders face a constant battle against disease-causing pathogens which annually cause considerable loss of yield and quality in crops. The deployment of plant varieties possessing genetic resistance is considered to be a cost effective and environmentally sustainable strategy to minimise the damage caused by plant pathogens. Genetic resistance to plant diseases can be categorised into either host or non-host resistance. Host resistance is defined as the resistance of some plant genotypes or varieties to a pathogen that has specialised to infect that species, while non-host resistance is classified as the resistance of all genotypes of a plant species to all genotypes of a pathogen species (Heath 1985). Although there are many sources of host resistance used for breeding disease resistant crop varieties there are few examples of

Communicated by A. Graner.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-013-2078-9) contains supplementary material, which is available to authorized users.

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the incorporation of non-host resistance due mainly to the barriers which prevent genetic transfer between species (Baum et al. 1992; Pickering 1992; Fu et al. 2008). In this paper, we outline the mapping of a gene for resistance to barley leaf rust (*Puccinia hordei*) transferred from the non-host species *Hordeum bulbosum* into cultivated barley.

Barley leaf rust is a serious disease which causes significant seasonal crop losses, particularly in the cool temperate regions of the world, where barley is cultivated (Clifford 1985). In North America, average yield losses of 32 % were measured in the susceptible winter barley 'Barsoy' and 6–16 % across all genotypes tested (Griffey et al. 1994). Host resistance to leaf rust in cultivated barley has been characterised into (1) hypersensitive resistance and (2) quantitative or partial resistance. Hypersensitive resistance is conferred by single major genes that have proven in the past to be race-specific and lacking in durability (Parlevliet 2002). In contrast, quantitative or partial resistance is not based on hypersensitivity but leads to a reduced severity of infection despite a susceptible infection type (Parlevliet 1975, 1976, 1978). Partial resistance of barley to leaf rust is conditioned by a number of small-effect genes (prefixed by *Rphq*) of which about 20 have been identified through QTL mapping (Qi et al. 1998, 2000; Marcel et al. 2007a, 2008). This type of resistance is considered to be more durable than hypersensitive resistance genes (Parlevliet 2002). However, the polygenic nature of partial resistance makes them more difficult to handle in breeding programmes than major genes conferring hypersensitive resistance. Accumulation of genes for partial resistance is possible using conventional breeding methods; however, careful quantitative phenotyping is required (Parlevliet and Kuiper 1985). Alternatively, genes for partial resistance may be combined by marker-assisted gene pyramiding; however, this requires markers closely linked to or flanking each of the resistance genes. Breeding cultivars which possess a significant level of partial resistance would be simplified if large-effect genes that confer partial resistance were available. Such genes would potentially provide valuable and durable resistance without the expense and technical difficulty involved in breeding for combinations of small effect genes. Examples exist of durable major-effect and partial resistances such as *mlo* resistance to powdery mildew (*Blumeria graminis* DC. f. sp. *hordei* Em. Marchal) in barley (Büschges et al. 1997) and *Lr34* resistance to leaf rust (*Puccinia triticina*), stripe rust (*Puccinia striiformis*) and powdery mildew (*Blumeria graminis*) in wheat (Krattinger et al. 2009). Neither *mlo* nor *Lr34* resistance is conditioned by NB-LRR (*R*-gene) type hypersensitive response but are both classified as pre-invasion resistances. To date both resistance genes have provided durable and effective control of these pathogens.

In a classical approach to achieve single-gene and potentially more durable resistance to barley leaf rust, introgression lines between cultivated barley and its undomesticated relative *H. bulbosum* were developed and examined for the presence of genes contributing to high levels of partial resistance against barley leaf rust. *H. bulbosum* is the only member of the secondary gene pool of cultivated barley (von Bothmer et al. 1995) and has historically been used for barley improvement as it allows production of doubled haploids through chromosome elimination (Kasha and Kao 1970). However, under certain circumstances true interspecific hybrids can be produced between barley and *H. bulbosum*. Introgression lines (ILs) between barley and *H. bulbosum* were first produced from such hybrids 30 years ago (Szigat and Pohler 1982) and were confirmed using in situ hybridisation and Southern blotting (Xu and Kasha 1992; Pickering et al. 1995). These ILs represent a novel genetic resource for scientific research and barley improvement as they enable access to additional genetic diversity outside the primary gene pool of cultivated barley and *Hordeum vulgare* subsp. *spontaneum* (K. Koch). It is impossible to determine beyond all doubt whether a plant species is a non-host to a potential pathogen species, since this would require testing an infinite collection of both organisms under many environmental conditions (Niks 1987). However, there is considerable evidence for *H. bulbosum* to be considered a non-host to barley leaf rust as 100 isolates of *P. hordei* from *H. vulgare* spp. *spontaneum* were found to be incompatible with all tested accessions of *H. bulbosum* (Anikster 1989). In addition, leaf rust isolates collected from *H. vulgare* (including *H. vulgare* spp. *spontaneum*) and *H. bulbosum* are regarded as different *formae speciales* of *P. hordei* as each is only able to infect their host species and were not able to cross with one another (Y. Anikster and J. Manisterski, pers. comm.). Finally, in glasshouse tests, no visible signs of infection were detected on *H. bulbosum* following inoculation with barley leaf rust (Pickering et al. 1998, 2000; Niks, unpublished data). Hence, for the purposes of this paper we consider *H. bulbosum* to be a non-host to barley leaf rust until evidence to the contrary is uncovered. It is likely that many genetic components are involved in rendering *H. bulbosum* so generally and durably resistant to leaf rust (*P. hordei*) of cultivated barley (*H. vulgare*). Some of these components have been transferred into cultivated barley and have resulted in ILs with quantitative-to-complete resistance to *P. hordei* with or without the occurrence of hypersensitive reactions (Pickering et al. 2004). These ILs represent an ideal resource to identify *H. bulbosum* genes that confer potentially durable resistance against pathogens that are specific to *H. vulgare*, but to which *H. bulbosum* is a non-host.

The subject of this paper, IL '182Q20' (coded as 'G-2HL-b' in Pickering et al.(2004)), was identified in the field as exhibiting a very pronounced 'slow rusting' or partial resistance on adult plants to natural infection of *P. hordei*. 'Slow rusting' or partial resistance is a quantitative resistance that is characterised by a prolonged latency period and reduced infection frequency. Partial resistance is pre-haustorial, since it is based on a high proportion of failed haustorial development (Niks 1986). On seedlings of IL '182Q20', *P. hordei* had a 30 % longer latency period than on its barley genetic background 'Golden Promise' and a 12 % longer latency period than on the partially resistant cv. 'Vada' (Pickering et al. 2004). The resistance in IL '182Q20' was also tested against five different isolates of barley leaf rust (from Spain, Holland and Tunisia), resulting in the same long latency period and low infection frequency due to a very high proportion of early aborting colonies without host cell necrosis (Shtaya et al. 2007).

The yield potential of IL '182Q20' (coded G-2HL-b in reference) in the absence of leaf rust infection (fungicide treated trial) is considerably compromised (25 % reduction) when compared with its susceptible barley parent 'Golden Promise' (Pickering et al. 2004). However, IL '182Q20' (G-2HL-b) yielded identically to cv. 'Golden Promise' in the presence of a natural infection of the leaf rust pathogen (Pickering et al. 2004).

The goal of this study was to develop a population featuring interspecific recombinants to map the non-host-derived resistance locus on a 2HL genetic linkage map specially developed for the *H. bulbosum* introgression within '182Q20'.

Materials and methods

Plant material

The IL '182Q20' was produced by crossing the barley cultivar 'Golden Promise' (diploid VV) with the *H. bulbosum* genotype A17-1 (tetraploid cytotype BBBB) to produce a partially fertile triploid hybrid (VBB) denoted as '161Z4' (where V and B represent a haploid genome equivalent of *H. vulgare* and *H. bulbosum*, respectively). This hybrid was used as the pollen parent and crossed to cv. 'Golden Promise' to produce diploid progeny due to elimination of the *H. bulbosum* chromosomes (Fig. 1). Most of the resulting progeny are normal barley lines except when an interspecific recombination event has occurred to create an introgression line. Although morphologically indistinguishable from cv. 'Golden Promise', the IL '182Q20' was selected in the field due to its 'slow rusting' (partial resistance) response to natural infections of leaf rust. This resistance differed from the response of the genetic background cv. 'Golden Promise'. The resistance was designated *Rph22* to reflect its effectiveness against *P. hordei* and assigned the allele symbol *Rph22.ak*. Genomic in situ hybridisation (Pickering, unpublished) and subsequent application of molecular markers demonstrated that IL '182Q20' possessed a single homozygous *H. bulbosum* introgression on the long arm of chromosome 2H (Johnston et al. 2009).

Mapping population

To map the partial resistance gene in IL '182Q20' genetically, an F_2 mapping population of 550 individuals was first developed by performing a reciprocal cross between cv. 'Golden Promise' and the IL '182Q20' (F_1 coded as 277G and 278D; Fig. 2). An approximately equal number of lines resulting from each cross direction were included in a marker screen for the presence of interspecific recombination within the introgression on chromosome 2HL (Fig. 2). Lines with interspecific recombination were detected by differences in the genotypes of the flanking co-dominant markers k08380 and H35_18000, which marked the known distal and proximal limits of the introgression, respectively (Johnston 2007). DNA from eight F_3 seedlings from each F_2 recombinant line was then screened with the same markers to identify plants that were homozygous for the introgression of reduced size (Fig. 2). If several homozygous F_3 lines were detected from each F_2 recombinant they were retained as independent lines. Seed (F_4) from all homozygous F_3 lines formed the mapping population, henceforth called "182Q20_F4_Popn" (Fig. 2). This population was used for all subsequent experiments with the F_4 sister homozygous recombinant lines (independent lines derived from the same F_2 recombinant) acting as internal controls for both the pathology and genotyping. Genomic DNA was extracted

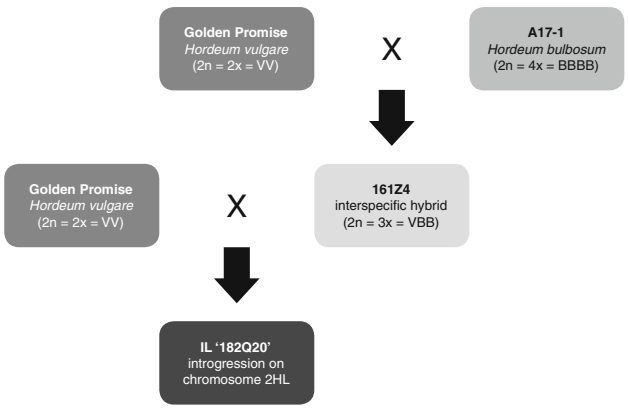

Fig. 1 Crossing plan showing the genetic background of the IL '182Q20' (where V and B refer to haploid genome equivalents (7 chromosomes) of the *Hordeum vulgare* and *Hordeum bulbosum*, respectively)

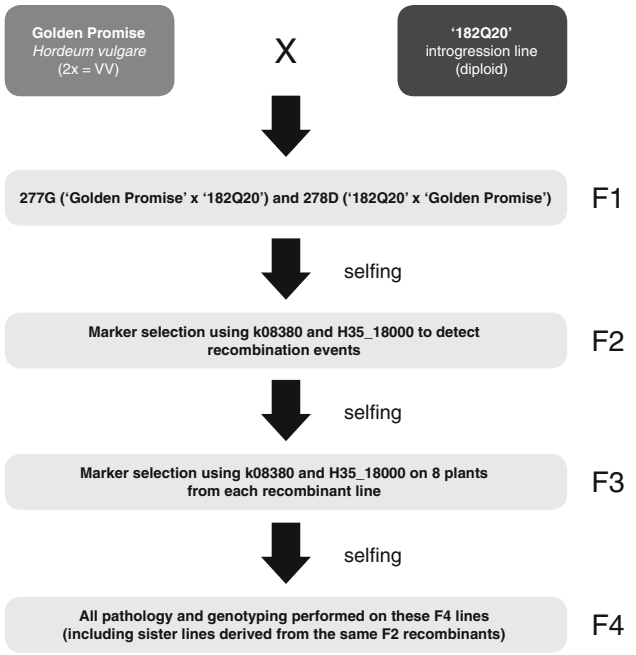

Fig. 2 Diagram showing the development of the "182Q20_F4_Popn"

from leaf material of parental lines using the DNeasy Plant Mini Kit (Qiagen) and from leaf material of the "182Q20_F4_Popn" using the "Wheat and Barley DNA Extraction in 96-well plates" method (Chao and Somers 2012).

Interspecific molecular marker development

In order to locate the *H. bulbosum*-derived leaf rust resistance gene using the population developed above (182Q20_F4_Popn) we needed to generate markers on chromosome 2HL that could discriminate between barley and *H. bulbosum* alleles. Previously developed PCR markers located on chromosome 2HL (Johnston 2007, 2009) were used for initial genotyping of the population. Additional single copy PCR-based markers were developed to discriminate between the four main barley cultivars and the four main *H. bulbosum* genotypes used in our research as per the methods of Johnston et al. (2009). DNA sequences for marker development were selected from a number of sources including: the assembly 35 genetic map from the HarvEST database (version 1.77, http://harvest.ucr.edu) and the published barley genetic maps of Marcel et al. (2007b) and Sato et al. (2009). Markers were named from the source of the original DNA sequence used in marker development. Alternative marker names are given in Supplementary Table 1, where markers that have been developed from the same sequence have appeared in different maps and/or publications (e.g. H35_18000 from the HarvEST database genetic map, k03697 from Sato et al. (2009) and H31_8716 from Johnston et al. (2009) are based on the same DNA sequence).

All markers were amplified in 10 µl PCRs containing 1× ReddyMix PCR Buffer (Thermo Fisher), 0.2 mM dNTPs (Fermentas), 0.2 µM of each primer (Bioneer) and 0.2 U of ThermoPrime Plus *Taq* Polymerase (Thermo Fisher). The basic PCR program conditions were 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, then a final extension step of 72 °C for 5 min. All PCRs were performed on either an Eppendorf Mastercycler pro S or a BioRad C1000 Thermal Cycler. After visualisation on agarose gels, the PCR amplification products with clear single bands were directly sequenced in both directions on an ABI3130xl Genetic Analyser (Applied Biosystems) across four barley cultivars ('Emir', 'Golden Promise', 'Steptoe', 'Morex') and four *H. bulbosum* genotypes ['2032', '2920/4', 'A17-1', IL '182Q20' (homozygous *H. bulbosum* introgression on chromosome 2HL)]. Sequence data were checked visually for discrepancies, assembled and alignments created using the software Geneious Pro™ (Drummond et al. 2011). Diagnostic markers for differentiating between barley and *H. bulbosum* alleles were then generated from the sequence alignments, using restriction enzyme digestion, high-resolution melting (HRM) analysis (Light Scanner, Idaho Technology), size polymorphisms (InDels) or presence/absence (dominant markers). Primer sequences, alternative marker names (from previous publications), variations from the basic PCR conditions and methods for discrimination of alleles can be found in Supplementary Table 1.

Use of synteny between barley and rice genomes for development of markers closely linked to *Rph22* in barley

As the barley genome has not yet been fully sequenced, the rice genome (Goff et al. 2002) was used to identify a region homologous to *Rph22* in that species. A high level of synteny is known to exist between barley chromosome 2 and rice chromosome 4 (Mayer et al. 2011) and hence might be expected to enable the identification of additional markers closely linked to *Rph22* in barley. Thirty-one full-length barley EST contigs (HarvEST assembly 35) that were used to map the *Rph22* locus within the introgressed chromatin were also used as tblastx (Altschul et al. 1997) queries against the rice genome 'Genes in MSU RGAP Release 7–Genomic Sequences' database (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml) using the default settings. All rice coding regions (CDSs) identified in the homologous region to *Rph22* were used to identify additional barley EST contigs which might map to the region of

interest (tblastx search of the 'Barley 35 relaxed' database on the HarvEST website http://www.harvest-web.org/).

Evaluation of resistance

Phenotyping of seedlings for leaf rust resistance/susceptibility was performed in two separate experiments using the same procedure. Experiment (a) was carried out on a subset of the original F_2 seed to determine whether the resistance was dominant or recessive in nature and experiment (b) involved phenotyping the 182Q20_F4_Popn to genetically map the *Rph22* resistance locus. For the mapping experiment one seedling of each F_4 line was evaluated for resistance in a blind trial with F_4 sister lines derived from the same original F_2 recombinant acting as internal controls.

The seeds were sown in 37 × 39-cm plant boxes. In each box we additionally sowed seed from the very susceptible line 'L94', the partially resistant cultivar 'Vada', the recurrent parent 'Golden Promise' and the original IL '182Q20' as references (each represented by one seed). Each seedling was fixed in a horizontal position to the soil by the use of metal ∩-shaped pins, taking care that the adaxial surface of the leaves faced upwards. For each box 3.5 mg of urediospores of *P. hordei* isolate 1.2.1 were diluted 10 times with *Lycopodium* spores to increase the volume of the inoculum, and achieve a homogeneous distribution of the rust spores over the horizontally fixed seedlings. Trays were placed in a settling tower and the inoculum was administered by a powder blower. Inoculation density amounted to about 200 rust spores per cm^2. After incubation overnight (8 h) at 100 % relative humidity in a dark dew chamber at 18 °C, the inoculated seedlings were transferred to a greenhouse compartment at 20 ± 3 °C with 30–70 % relative humidity. Seven days after inoculation the seedlings were evaluated for numbers of mature rust pustules and numbers of pale flecks (representing immature rust infections). Five lines were retested from the mapping experiment since their scores were ambiguous, or unexpected in comparison with the results obtained from the available F_4 sister lines. In that repeat experiment five seedlings were tested per F_4 line. In addition, the "182Q20_F4_Popn" lines were seed-increased in the field in unreplicated rows (summer 2009–2010, Lincoln, New Zealand) and were phenotyped for resistance/susceptibility in response to natural leaf rust infection.

Genotyping and linkage mapping

Genotyping was performed on the "182Q20_F4_Popn" to determine the extent of the introgressed chromatin from *H. bulbosum* in each of the recombinant lines. In addition, we phenotyped the material for one morphological marker on 2HL, the cleistogamy gene (Turuspekov et al. 2004) using microscopic examination of the lodicule size. 'Golden Promise' has very small 'bib' type lodicules compared with the larger 'collar' type lodicules in IL '182Q20' that have been transferred from *H. bulbosum*. The larger lodicules swell and cause the lemma and palea to open resulting in floret gaping at anthesis (open flowering), whereas in 'Golden Promise' the florets do not open nearly as far (closed flowering). A genetic linkage map was developed using the Mapmanager QTX software (Manly et al. 2001). This map was constructed using the data of the F_4 genotyping, combined with the original F_2 data obtained during screening for recombination events (550 F_2 plants). This allowed the genotyping to be confined to the informative recombinants and enabled the map distances to be more easily compared to published barley linkage maps.

Results

Detection of interspecific recombinants

From the F_2 population ('Golden Promise' × IL '182Q20') of 550 individuals, 76 lines with interspecific recombination events were detected using the flanking markers k08380 and H35_18000. Genotyping of eight F_3 individuals from each of these recombinants with the same markers enabled detection of homozygous lines with introgressions of reduced genetic size from each of the F_2 recombinants. A total of 176 homozygous lines were identified with between 1 and 5 independent sister lines derived from each of the 76 F_2 recombinants. F_4 seed from these 176 lines ("182Q20_F4_Popn") were used for all subsequent phenotyping and genotyping.

Evaluation of resistance

Infection frequency, measured in numbers of rust pustules per cm^2 leaf area, was an average of 8.3 on '182Q20', 50.5 on 'Golden Promise' and 38.0 on 'Vada' when screened with four isolates of *P. hordei*. This reduction of infection frequency in IL '182Q20', relative to 'Golden Promise', was associated with more than half of the infections being arrested within the first 24 h after inoculation. About 85 % of the arrested infection units were not associated with plant cell necrosis.

Analysis of an independent set of 61 F_2 seedlings [Experiment (a)] revealed a qualitative segregation, allowing a straightforward classification into resistant versus susceptible plants. The 3:1 (46 resistant:15 susceptible) segregation ratio indicated that the *Rph22* resistance gene was dominant in nature.

The infection phenotypes of the "182Q20_F4_Popn" [Experiment (b)] also allowed a qualitative classification into resistant versus susceptible plants. Resistant plants had clearly fewer macroscopically visible infection sites, of which only few had produced a sporulating pustule at the time of evaluation (Fig. 3). The high level of non-hypersensitive resistance of IL '182Q20' was also seen in the resistant recombinants (Fig. 3). A much higher proportion of pale flecks to mature orange pustules was observed on IL '182Q20' and the resistant recombinants than on cv. 'Golden Promise'. These pale flecks represent *P. hordei* infection units that had not developed to maturity, but did not induce chlorosis or necrosis of the plant tissue. The assignment of resistant or susceptible phenotypes was consistent between all F_4 sister lines that were derived from the same F_2 recombinant with two exceptions. These were (1) line #19, which was scored as resistant, although its three sister lines (with the same genotype) were scored as susceptible; and (2) sister lines #153 and #154 which were scored as susceptible and resistant, respectively. Five additional seeds from lines #19, #153 and #154 were sown and retested. All additional #19 and #153 seedlings were assessed as susceptible, whilst line #154 segregated for resistance/susceptibility. Line #154 was later found to have been incorrectly selected and was actually an F_4 heterozygote. Thus, this line was segregating for the full-size introgression and the smaller recombinant introgression and hence for the resistance gene too. In addition, there were two indeterminate lines #110 and #150, which were later assigned as susceptible and resistant, respectively in agreement with the two independent F_4 sister lines available for each. This resulted in 39 of the original 76 F_2 recombinant families (consolidated results from the independent F_4 sister lines derived from the same F_2 recombinant) classified as resistant and 37 as susceptible (assigning the family containing the sister lines 153 and 154 as susceptible). Disease assessment was also completed in New Zealand (summer 2009–10) for the 176 F_4 lines (in field rows) in the presence of a natural leaf rust infection and resulted in the assignment of 38 resistant and 38 susceptible F_2 families. There was one inconsistency (line 24) between the two assessments (glasshouse and field), which was due to a heterozygous seed lot as indicated by subsequent genotyping.

Genetic linkage map

Genotyping and phenotyping of the "182Q20_F4_Popn" enabled the extent of the introgressed segment to be determined in each recombinant line (Table 1). The *Rph22* locus was mapped to the distal portion of the introgression on chromosome 2HL with the map spanning 7.3 cM between markers k08380 and H35_18000 (Fig. 4).

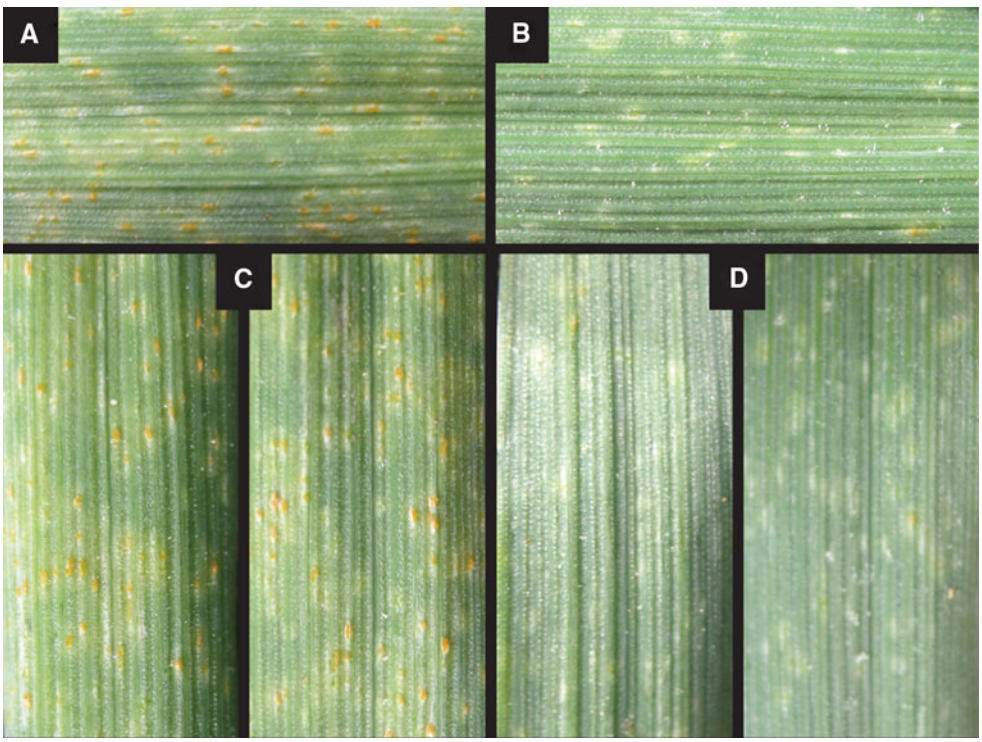

Fig. 3 Parental lines **a** cv. 'Golden Promise' (susceptible) and **b** IL '182Q20' (resistant). **c** two randomly chosen susceptible lines: #101 and #95. **d** Two randomly chosen resistant lines: #79 and #85. Pictures were taken 7 days after inoculation with *Puccinia hordei*

Table 1 Genotype and phenotype data from a set of non-redundant lines and markers (where B and V represent homozygous *H. bulbosum* and barley (*H. vulgare*) genotypes, respectively and R and S refer to resistant and susceptible responses to barley leaf rust (*P. hordei*)

	Marker name (distal to proximal)																					
Plant code	k08380	H35_17700 (k03475)	k08317	H35_19216	k00932 (H35_4033)	H35_15816	H35_26334 (WBE115)	H35_45139	Rph22	H35_13826 (WBE114)	H35_17215	H35_451	H35_17782	H35_1730 (WBE113)	H35_1915	H35_2436	k04190	k06104	H35_1860	k00917	Cleistogamy	H35_15016 (k00484)	H35_18000 (k03697)
36	B	B	B	B	B	B	B	B	R	B	B	B	B	B	B	B	B	B	B	B	B	B	V
50	B	B	B	B	B	B	B	B	R	B	B	B	B	B	B	B	B	B	B	B	V	V	V
170	B	B	B	B	B	B	B	B	R	B	B	B	B	B	B	B	B	B	B	V	V	V	V
145	B	B	B	B	B	B	B	B	R	B	B	B	B	B	B	B	B	B	V	V	V	V	V
29	B	B	B	B	B	B	B	B	R	B	B	B	B	B	B	B	B	V	V	V	V	V	V
10	B	B	B	B	B	B	B	B	R	B	B	B	B	B	B	B	V	V	V	V	V	V	V
106	B	B	B	B	B	B	B	B	R	B	B	B	B	B	B	V	V	V	V	V	V	V	V
149	B	B	B	B	B	B	B	B	R	B	B	B	B	B	V	V	V	V	V	V	V	V	V
3	B	B	B	B	B	B	B	B	R	B	B	B	B	V	V	V	V	V	V	V	V	V	V
75	B	B	B	B	B	B	B	B	R	B	B	B	V	V	V	V	V	V	V	V	V	V	V
140	B	B	B	B	B	B	B	B	R	B	B	V	V	V	V	V	V	V	V	V	V	V	V
67	B	B	B	B	B	B	B	B	R	B	V	V	V	V	V	V	V	V	V	V	V	V	V
138	B	B	B	B	B	V	V	V	S	V	V	V	V	V	V	V	V	V	V	V	V	V	V
94	B	B	V	V	V	V	V	V	S	V	V	V	V	V	V	V	V	V	V	V	V	V	V
82	B	V	V	V	V	V	V	V	S	V	V	V	V	V	V	V	V	V	V	V	V	V	V
28	V	B	B	B	B	B	B	B	R	B	B	B	B	B	B	B	B	B	B	B	B	B	B
38	V	V	B	B	B	B	B	B	R	B	B	B	B	B	B	B	B	B	B	B	B	B	B
55	V	V	V	B	B	B	B	B	R	B	B	B	B	B	B	B	B	B	B	B	B	B	B
24	V	V	V	V	B	B	B	B	R	B	B	B	B	B	B	B	B	B	B	B	B	B	B
159	V	V	V	V	V	V	B	B	R	B	B	B	B	B	B	B	B	B	B	B	B	B	B
15	V	V	V	V	V	V	V	V	S	B	B	B	B	B	B	B	B	B	B	B	B	B	B
47	V	V	V	V	V	V	V	V	S	V	B	B	B	B	B	B	B	B	B	B	B	B	B
44	V	V	V	V	V	V	V	V	S	V	V	V	B	B	B	B	B	B	B	B	B	B	B
130	V	V	V	V	V	V	V	V	S	V	V	V	V	B	B	B	B	B	B	B	B	B	B
4	V	V	V	V	V	V	V	V	S	V	V	V	V	V	B	B	B	B	B	B	B	B	B
70	V	V	V	V	V	V	V	V	S	V	V	V	V	V	V	V	B	B	B	B	B	B	B
7	V	V	V	V	V	V	V	V	S	V	V	V	V	V	V	V	V	B	B	B	B	B	B
23	V	V	V	V	V	V	V	V	S	V	V	V	V	V	V	V	V	V	B	B	B	B	B
121	V	V	V	V	V	V	V	V	S	V	V	V	V	V	V	V	V	V	V	V	V	B	B
26	V	V	V	V	V	V	V	V	S	V	V	V	V	V	V	V	V	V	V	V	V	V	B
Recombination Events	6		1		1		0		1		1		6		1		10		14		2		
		2		1		1		0		3		5		4		6		1		1		9	

Rphq2

The numbers below each adjacent marker pair indicate the number of F2 recombination events between the markers. The darker shaded region indicates the likely position of *Rph22* between H35_15816 and H35_13826. The shaded bar at the bottom shows the corresponding location of *Rphq2* from cv. 'Vada' [between markers WBE114 and WBE115 (Marcel et al. 2007b)]

The best matching rice homologues from 24 of the 31 barley EST contigs identified a distal region of rice chromosome 4 (between 32.4 and 35.4 Mb) confirming conservation of synteny at the distal end of the chromosome with some inversions of homologue order at the proximal end of the introgressed region (data not shown). Two of the barley flanking markers [one recombination event distal (H35_15816) and proximal (H35_13826)] of *Rph22* matched rice coding sequences LOC Os04g59150 and LOC Os04g59330, respectively. This region of rice chromosome 4 spanned 88.3 kb and contained 17 intervening coding regions (LOC_Os04g59160 to LOC_Os04g59320), including

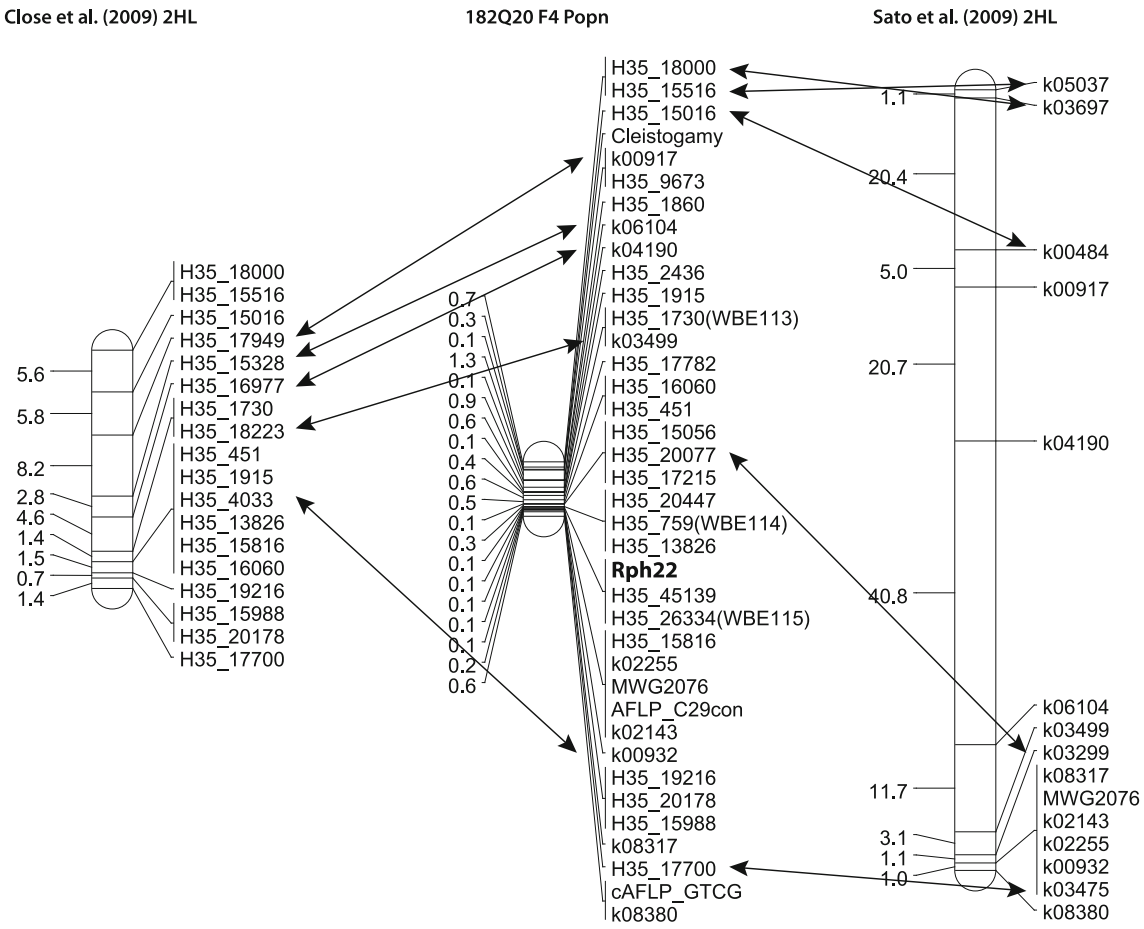

Fig. 4 Genetic linkage map of the introgressed region of IL '182Q20' from *H. bulbosum* on barley chromosome 2HL (*centre*) with only the common markers shown from the high density maps HarvEST assembly 35, on the *left* (Close et al. 2009) and 'Haruna Nijo' × 'H602', on the *right* (Sato et al. 2009). Markers which exist on all three maps have been indicated with *arrows* linking the different names (more information can be found in Supplementary Table 1). Linkage maps were constructed using MapChart v2.2 (Voorrips 2002) (numbers to the *left* of the *bars* indicate cM between mapped loci)

nine genes (featuring a cluster of five peroxidase genes), seven retrotransposon elements and a transposon (Table 2). Markers developed from barley EST contigs homologous to three of these rice coding regions were successfully mapped in the 182Q20_F4_Popn using diagnostic restriction digests. Marker H35_45139, a barley homologue of LOC_Os04g 59320 co-segregated with the *Rph22* gene. Two additional barley HarvEST contigs, H35_20447 and H35_759 that are homologous to members of the peroxidase cluster (Os04g59160, 190, 200, 210, 260) were located proximal to the *Rph22* gene. The markers developed from the remaining homologues failed to amplify, amplified several products or revealed no sequence polymorphisms between 'Golden Promise' and '182Q20', and hence were not pursued further.

The genetic linkage map of the introgressed region of IL '182Q20' was developed by converting the genotypic data from the F_4 individuals to that of the original F_2 lines (thus including the non-recombinant lines). The map featured 38 loci and covered a total length of 7.3 cM (Fig. 4). Two markers, H35_26334 [identical to WBE115, Marcel et al. (2007b)] and H35_45139 (best barley match to rice LOC_Os04g59320; Table 2), co-segregated with the resistance gene *Rph22*. The inclusion of three markers, WBE115 (H35_26334), WBE114 (H35_759) and WBE113 (H35_1730), identified an overlapping marker interval (Table 1) encompassing both the resistance gene *Rph22* identified in this present study and *Rphq2* from cv. 'Vada' (Marcel et al. 2007b).

Discussion

Effect of introgression on recombination frequency

It is noteworthy that the genetic map of the '182Q20' introgression was considerably condensed relative to the

Table 2 Identification of 17 coding sequences from rice chromosome 4 (35.19–35.28 Mb) between homologues of *Rph22* flanking markers H35_13826 and H35_15816

Rice location	Gene description	Best matching harvEST contigs	Mapping relative to *Rph21*
LOC_Os04g59150	**Peroxidase precursor**	**H35_13826**	**Proximal flanking marker**
LOC_Os04g59160	Peroxidase precursor	H35_760,20447,759,13772,13826	H35_760 no sequence differences
LOC_Os04g59170	Retrotransposon protein	No significant homologue	–
LOC_Os04g59180	Retrotransposon protein	No significant homologue	–
LOC_Os04g59190	Peroxidase precursor	H35_759,761,14346,760,13772,13856	H35_759 proximal to *Rph22*
LOC_Os04g59200	Peroxidase precursor	H35_761,759,14346,760,13856,13772	H35_759 proximal to *Rph22*
LOC_Os04g59210	Peroxidase precursor	H35_759,761,14346,13772,13856,760	H35_759 proximal to *Rph22*
LOC_Os04g59220	Retrotransposon protein	No significant homologue	–
LOC_Os04g59230	Retrotransposon protein	No significant homologue	–
LOC_Os04g59240	Transposon protein	No significant homologue	–
LOC_Os04g59250	Retrotransposon protein	No significant homologue	–
LOC_Os04g59260	Peroxidase precursor	H35_759,761,14346,13772,13856,13826	H35_759 proximal to *Rph22*
LOC_Os04g59270	Hypothetical protein	No significant homologue	–
LOC_Os04g59280	Retrotransposon protein	No significant homologue	–
LOC_Os04g59290	Retrotransposon protein	No significant homologue	–
LOC_Os04g59300	Strictosidine synthase	H35_23304(weak match)	Failed to amplify
LOC_Os04g59310	Phospholipase C	H35_18976	No polymorphism across 1,650 bp sequenced
LOC_Os04g59320	Protein kinase	H35_45139	Co-segregates with *Rph22*
LOC_Os04g59330	**Expressed protein**	**H35_15816**	**Distal flanking marker**

same region of chromosome 2HL from barley linkage maps. For example, the HarvEST map (for assembly 35) between contigs H35_18000 (most proximal marker within the introgression) and H35_17700 (most distal marker on the HarvEST map) covered 32 cM on a consensus map of four barley mapping populations (Close et al. 2009), however spanned only 6.7 cM in '182Q20_F4_Popn' (Fig. 4). The map of an F_1 doubled haploid population between cv. 'Haruna Nijo' and *H. vulgare* var. *spontaneum* 'H602' covered 103.8 cM between markers k03697 and k08380 (Sato et al. 2009) but only 7.3 cM in '182Q20_F4_Popn' (Fig. 4). This implies that there is a 5- to 14-fold reduction in recombination frequency between barley and *H. bulbosum* (interspecific recombination) relative to the intraspecific recombination of barley in this region of chromosome 2HL.

The size of the original introgression appears to play a major role in the detection of subsequent interspecific recombination and hence the ability to map and/or break linkage of *H. bulbosum* alleles in these lines. For example, IL '38P18' (derived from cv. 'Emir' × *H. bulbosum* genotype '2032') contains a *H. bulbosum* introgression that only constitutes 1 % of the barley genetic length of chromosome 2H. When that IL, denoted as 'E-2HL-a' in Pickering et al. (2004), was backcrossed to cv. 'Emir' to map the *Rph18* gene, only two recombinants were detected for the region spanned by markers k08380 and H35_17782 in the F_2 population of 715 individuals (Johnston 2007). Within this very same marker interval, 22 recombinants were identified from a population of 550 F_2 plants in the current study of IL '182Q20' (20 % of the genetic length of barley chromosome 2H). Although this comparison involves different barley and *H. bulbosum* genotypes, suppression of interspecific recombination in genetically small introgression lines has been detected previously in crosses between cultivated tomato and *Solanum lycopersicoides* (Canady et al. 2006).

This phenomenon has important implications for introducing traits of interest from wild germplasm. For fine-mapping of desirable trait genes within an introgression, the use of genetically large introgressions will result in relatively more recombination events and hence a higher resolution with which to map the position of the gene of interest. Recombination events are also very important to enable separation of genes of interest (*Rph22*) from undesirable genes (linkage drag) that may also be present in the introgression (e.g. the yield penalty in '182Q20'). The relatively large size of the introgression within '182Q20' made the mapping of *Rph22* relatively straightforward (76 recombinants from 550 individuals). However, mapping in genetically small introgressions will require much larger population sizes, to identify the rare interspecific recombination events.

The use of resistance genes from a non-host in barley breeding

Leaf rust resistance genes have also been identified from other *H. bulbosum* introgression lines at several chromosomal locations (1HL, 2HS, 4HL, 5HL, 6HS and 7HL) and these feature both hypersensitive and quantitative resistance mechanisms (Pickering et al. 2004). Hence, the resistance gene *Rph22* is likely to be one of several genetic components that together shape the non-host resistance of the *H. bulbosum* species towards *P. hordei*. When combined, these components result in a durable and complete protection of *H. bulbosum* against the barley leaf rust. Transfer of single resistance genes from the non-host species *H. bulbosum* into a host species like cultivated barley may increase the risk that these genes will eventually be overcome by adaptation of the pathogen. Alternatively it may be that the *Rph22* resistance is intrinsically durable over time, e.g. by requiring the pathogen to undergo a gain-of-function mutation to break the resistance (Niks and Marcel 2009). However, if a loss-of-function mutation is sufficient for the pathogen to breakdown this resistance, as is the case with the typical *R*-gene resistance based on hypersensitivity, then a better strategy is required for the use of novel *H. bulbosum* resistance genes in breeding programs. Deployment of these genes in combination with other established barley resistance genes (*Rph* or *Rphq*) or by pyramiding resistance genes from the different *H. bulbosum* introgressions (i.e. accumulation of more components of *H. bulbosum* non-host resistance) should help prolong their effectiveness. Both of these strategies rely on having molecular markers closely flanking or linked to the different resistance genes, to enable them to be pyramided into elite cultivars. This study provides the initial tools, consisting of molecular markers and genotypes with a reduction in the size of the introgressed chromatin, to enable this novel resistance gene (*Rph22*) from *H. bulbosum* to be transferred into elite barley cultivars. For example, lines 67 and 159 already have a recombination event close to *Rph22* (Table 1), allowing retention of the resistance gene, but eliminating a large part of the introgression.

Link between *Rph22*, *Rphq2* and *Rph18* on chromosome 2HL

The map position of *Rph22* appears to be closely linked to, or identical with that of the *Rphq2* QTL on chromosome 2HL, from the barley cultivar 'Vada' (Marcel et al. 2007b). *Rphq2* was reported to be located to a 0.11-cM interval between WBE114 and WBE115 (Marcel et al. 2007b). *Rph22* co-segregates with H35_26334 (WBE115) and is distal to the marker H35_759 (barley homologue for a member of the rice peroxidase family and a sequence match for the primers for WBE114), further strengthening the case for a very close genetic link between the two resistance loci. Interestingly, both the *Rphq2* gene in 'Vada' and the present *Rph22* gene decrease the rate of successful haustorium formation by *P. hordei*, prolong the latency period and reduce the infection frequency of the rust. However, the effect of the *H. bulbosum*-derived *Rph22* gene is much stronger than the *Rphq2* gene from 'Vada'. An additional resistance gene derived from *H. bulbosum* (*Rph18* from IL '38P18') was located in a neighbouring region (between H35_759 (WBE114) and H35_1730 (WBE113)) of chromosome 2HL (P.A. Johnston, unpublished). However, '38P18' was immune at the seedling stage with 96 % of the *P. hordei* infection units aborted within 24 h in association with plant cell necrosis (R.E. Niks, unpublished). Thus, when this region of chromosome 2HL is introgressed from different *H. bulbosum* genotypes ('38P18' from the diploid '2032' and '182Q20' from the tetraploid 'A17-1'), it possesses different mechanisms (hypersensitive and non-hypersensitive) for leaf rust resistance.

The nature of non-host resistance

It is believed that non-host resistance arises through a combination of pathways that are involved in host resistance, namely pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI, also known as basal or quantitative resistance) and effector-triggered immunity (ETI) (Schweizer (2007); Schulze-Lefert and Panstruga (2011)). The balance of the pathways employed by a non-host plant species is considered to be dependent on the evolutionary distance between the pathogen's natural host and a more diverged target species (Schulze-Lefert and Panstruga 2011). PTI relies on plant receptors that have evolved to recognise conserved and functionally important microbial epitopes, such as chitin, xylanase or flagellin and then trigger innate immune responses (Nürnberger et al. 2004). Pathogens which have co-evolved with host plant species have thus developed methods to avoid or suppress the host's PTI system by secreting effector molecules into the host cells, which then interact with the host defence systems (Chisholm et al. 2006). For a specific pathogen species to successfully infect a specific host plant species, it needs to have developed, via rare gain-of-function mutations, plant species-specific effectors which can reprogramme the plant cell's gene expression and suppress the host's PTI system (Niks and Marcel 2009). In the corresponding interaction between a pathogen and a non-host plant species, there is likely to be several redundant layers of functional defence. Due to genetic divergence between the natural host plant species and a non-host

species these targets may or may not be suppressed by the same effectors. Each of the unsuppressed defensive layers would require the prospective pathogen to undergo a similar gain of function mutation in order for it to (better) infect the plant species (Niks and Marcel 2009).

In addition to the PTI type of basal resistance, some members of the plant host species develop ETI systems consisting of specific *R*-genes which either detect the pathogen effector itself or guard the host protein targeted by the effector and respond to changes. These *R*-genes then trigger a second wave of defence response in a gene-for-gene manner. This ETI-type resistance depends on the presence or absence of the corresponding *R* gene for a specific effector from a specific pathogen that targets a particular allele of the host defence machinery (Chisholm et al. 2006).

The interactions between *P. hordei* and its non-host *H. bulbosum* are consistent with the postulate that non-host resistance is a mixture of PTI and ETI systems. Resistance genes that have been transferred from *H. bulbosum* into cultivated barley appear to confer either a non-hypersensitive defence (like *Rph22*) or a hypersensitive defence (like *Rph18*). We assume that the former represents a case of PTI and the second a case of ETI (hypersensitive resistance)-based defence. *H. vulgare* and *H. bulbosum* are thought to have diverged from a common ancestor approximately seven million years ago (Blattner 2004). Their respective closely related leaf rust species *P. hordei* (of *H. vulgare*) and *P. hordei-bulbosi* (of *H. bulbosum*) have developed host specialisation and are not pathogenic to each other's host (Anikster 1989). The close links in both location and function, between *Rphq2* and *Rph22* may indicate that *H. vulgare* and *H. bulbosum* still share conserved elements of their PTI with different levels of effectiveness. Further investigation of the genomic sequences spanning the *Rphq2* and *Rph22* loci may increase our understanding of the relationships between resistance mechanisms involved in non-hypersensitive host and non-host interactions. It is possible that *Rphq2* and *Rph22* encode allelic forms of the same gene with the presence of each allele resulting in a different level of response to leaf rust infection.

Conclusion

This study describes a major step forward in the use of the *H. bulbosum* introgression lines for understanding the genetic basis of non-host resistance and for use in barley disease resistance breeding. Two genetic markers (H35_26334 and H35_45139) co-segregate with the disease resistance and together with additional flanking markers will aid in the transfer of *Rph22* into more advanced barley breeding lines, while simultaneously reducing the linkage drag of other unfavourable alleles present in the original introgression. Preliminary evidence suggests that the yield penalty associated with the IL '182Q20' maps to the proximal end of the introgression and hence is not closely linked to the leaf rust resistance gene *Rph22*. Additional field trials are being undertaken to confirm this result.

Public resources: A collection of 154 ILs (featuring chromosomal segments of *H. bulbosum* within a barley genetic background) has been recently deposited with NordGen for preservation in the Svalbard Global Seed Vault (Pickering et al. 2010).

Acknowledgments The authors wish to thank Mrs. Merle Forbes for crossing and maintaining all plant material. Also thanks to Dr. David Chagné, Dr. Soonie Chng and Dr. Sue Gardiner for critical reading of this manuscript. Funding for this work was provided by the New Zealand Foundation for Research Science and Technology contract number C06X0810.

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