Theor Appl Genet (2009) 118:1429–1437
DOI 10.1007/s00122-009-0992-7

ORIGINAL PAPER

Marker development and characterisation of *Hordeum bulbosum* introgression lines: a resource for barley improvement

Paul A. Johnston · Gail M. Timmerman-Vaughan · Kevin J. F. Farnden · Richard Pickering

Received: 9 October 2008 / Accepted: 9 February 2009 / Published online: 5 March 2009

Abstract A set of 110 diploid putative introgression lines (ILs) containing chromatin introgressed from the undomesticated species *Hordeum bulbosum* L. (bulbous barley grass) into cultivated barley (*Hordeum vulgare* L.) has been identified using a high-copy number retrotransposon-like PCR marker, pSc119.1, derived from rye (*Secale cereale* L.). To evaluate these lines, 92 EST-derived markers were developed by marker sequencing across four barley cultivars and four *H. bulbosum* genotypes. Single nucleotide polymorphisms and insertions/deletions conserved between the two species were then used to develop a set of fully informative cleaved amplified polymorphic sequence markers or size polymorphic insertion/deletion markers. Introgressed chromatin from *H. bulbosum* was confirmed and genetically located in 88 of these lines using 46 of the EST-derived PCR markers. A total of 96 individual introgressions were detected with most of them (94.8%) extending to the most distal marker for each respective chromosome arm. Introgressions were detected on all chromosome arms except chromosome 3HL. Interstitial or sub-distal introgressions also occurred, with two located on chromosome 2HL and one each on 3HS, 5HL and 6HS. Twenty-two putative ILs that were positive for *H. bulbosum* chromatin using pSc119.1 have not had introgressions detected with these single-locus markers. When all introgressions are combined, more than 36% of the barley genetic map has now been covered with introgressed chromatin from *H. bulbosum*. These ILs represent a significant germplasm resource for barley improvement that can be mined for diverse traits of interest to barley breeders and researchers.

Communicated by A. Graner.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-009-0992-7) contains supplementary material, which is available to authorized users.

P. A. Johnston (✉) · G. M. Timmerman-Vaughan · R. Pickering
New Zealand Institute for Plant and Food Research Limited,
Private Bag 4704, Christchurch 8140, New Zealand
e-mail: johnstonp@crop.cri.nz

P. A. Johnston · K. J. F. Farnden
Department of Biochemistry, University of Otago,
PO Box 56, Dunedin 9054, New Zealand

Introduction

Barley is an important cereal crop grown widely throughout the world and was one of the first crops domesticated by humans (Badr et al. 2000). Extensive breeding combined with an inbred habit has resulted in elite barley cultivars with a relatively low level of genetic diversity (Feuillet et al. 2008). The economic value and quality of barley crops is constantly challenged by a range of environmental stresses including drought, frost, soil toxicity and disease; hence novel sources of genetic improvement for barley are of great importance.

Hordeum bulbosum is the sole member of the secondary genepool of *Hordeum* (von Bothmer et al. 1995), and like barley is endemic to the Fertile Crescent and Mediterranean regions from Morocco and Portugal in the west to Afghanistan and Tadzjikistan in the east (von Bothmer et al. 1995). Unlike cultivated barley, *H. bulbosum* is perennial, an obligate outbreeder (self-incompatible) and includes both diploid and tetraploid cytotypes (von Bothmer et al. 1995), culminating in a species with a high level of heterozygosity and heterogeneity. Hence, *H. bulbosum* represents a valuable source of genetic diversity for barley crop improvement. The first reported introgression lines (ILs) between

barley and *H. bulbosum* were generated more than 26 years ago (Szigat and Pohler 1982) with the presence of *H. bulbosum*-like morphological characteristics in 'modified barley plants' containing 14 chromosomes. Transfer of chromatin from *H. bulbosum* into barley was confirmed with the development and use of molecular techniques such as in situ hybridisation and Southern blotting (Xu and Kasha 1992; Pickering et al. 1995). These ILs are diploid and predominantly feature single recombination events between the two species' chromatin resulting in the transfer of the end of one chromosome arm from *H. bulbosum*, replacing the homoeologous region in barley. Hence, these ILs can be considered as near isogenic lines featuring discrete portions of *H. bulbosum* chromatin in a barley genetic background. Because *H. bulbosum* is a non-host to most barley pathogens, development of this material has focused primarily on the transfer of disease resistance from *H. bulbosum* into cultivated barley (Pickering et al. 1995, 1998; Toubia-Rahme et al. 2003; Walther et al. 2000; Xu and Kasha 1992). However, it is likely that these ILs will contain significant genetic diversity for many traits beyond disease resistance.

Sequential application of the cytological techniques genomic in situ hybridisation (GISH) and fluorescent in situ hybridisation (FISH) have been used to determine the physical chromosomal location of the introgressed chromatin (Pickering et al. 2000). Restriction fragment-length polymorphisms (RFLP) with barley-derived probes were also used to determine the genetic location of introgressed chromatin (Pickering et al. 1995). Due to the labour intense nature of the in situ hybridisation and RFLP methods, material for analysis was pre-selected using a small number of morphological (pubescent leaf sheath, growth habit, agronomic performance) or pathological traits (resistance to powdery mildew, leaf rust, scald) derived from *H. bulbosum*. Further characterisation of the ILs with PCR-based molecular markers developed in barley has been problematic due to amplification failure of *H. bulbosum* alleles and preferential or exclusive amplification of barley alleles in heterozygous or hybrid material (Johnston 2007). The development of a PCR-based screening tool using a retrotransposon-like sequence, pSc119.1 (Johnston and Pickering 2002), provided a significant improvement in the detection of putative ILs. This presence/absence assay allowed putative ILs to be selected for subsequent marker analysis independent of the location of the introgressed chromatin and without relying on readily identifiable traits derived from *H. bulbosum*.

This paper describes the development and implementation of a PCR-based molecular marker set to genetically locate the introgressed chromatin in these putative ILs. This information will allow breeders and researchers to identify *H. bulbosum* introgressions in particular regions of the genome and thus uncover genetic diversity in traits of interest. These tools will also enable fine mapping and targeted backcrossing to reduce the transfer of undomesticated alleles present in chromatin linked with the trait of interest.

Materials and methods

Plant material

Interspecific hybrids between *Hordeum vulgare* and *H. bulbosum* were generated as previously described (Pickering 1988). Diploid progeny were produced from these interspecific hybrids via two methods: (1) triploid interspecific hybrids (VBB, where V and B represent haploid genome components of cultivated barley and *H. bulbosum*, respectively) were backcrossed to the recurrent barley parent and (2) tetraploid interspecific hybrids (VVBB), were allowed to self-fertilise.

Seedlings from previously uncharacterised progeny were directly screened with crude DNA extracts of leaf tissue bulked from up to five individual plants, using the pSc119.1 retrotransposon-based PCR assay (Johnston and Pickering 2002). The pSc119.1 PCR primers (For3: 5′ ATG GGC GCA GCC GAT AGT 3′, Rev2: 5′ ATG CCT CCA TGT ATC TCT GCC 3′) amplify a *H. bulbosum*-specific amplicon (201 bp) from lines containing *H. bulbosum* chromatin, with no amplification product detected from barley cultivars. This PCR was performed in conjunction with primers for a 5.8S rDNA amplicon (329 bp) to control for amplification failure in the crude DNA extracts (Johnston and Pickering 2002). Individual plants from bulk extracts returning a positive pSc119.1 result were then tested to identify those individuals possessing putative introgressions of *H. bulbosum* and DNA was extracted from these positive individuals (Murray and Thompson 1980). All plant lines described in this paper are detailed in Supplementary Table S1.

Marker development

Co-dominant and fully informative EST-based markers were developed using assembled contig sequences from the HarvEST database (Wanamaker et al. 2006) and hence are designated H31_#### referring to that contig number from HarvEST assembly #31. Mapped EST sequences (Kleinhofs et al. 1993; Qi et al. 2004; Sato et al. 2004; Thiel et al. 2003) and cloned *H. bulbosum*-derived cDNA-AFLP (amplified fragment-length polymorphisms) bands were used as BLAST queries to identify matching HarvEST contigs ('stringent' assembly #31). The markers were designed following the principles of exon-primed intron-crossing (EPIC)-PCR (Palumbi 1995). Intron/exon structure was

inferred from matching rice genomic sequence identified using discontinuous megaBLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) against the non-redundant (nr) database and visualised using Spidey (http://www.ncbi.nlm.nih.gov/IEB/Research/Ostell/Spidey/index.html). Primer sequences were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to target exon sequences and to span one or more introns. Due to the absence of introns some markers were developed that consisted entirely of exon sequence. Additional primers (KS markers) were kindly provided by Prof. Kazuhiro Sato [Okayama University, Kurashiki, Japan (Sato et al. 2004)]. All markers were amplified using genomic DNA from four barley cultivars (Emir, Golden Promise, Steptoe and Morex) and four *H. bulbosum* genotypes (2032 (diploid), 2920/4 (diploid), A17 (tetraploid), A17-1 (tetraploid)) commonly used in the breeding programme. Those markers producing a single amplification product were sequenced directly, in both directions, from the PCR amplifications of all eight parental lines using Big Dye® Terminators v3.1 (Applied Biosystems), on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems). Alignments were constructed using SeqScape® (version 2.1, Applied Biosystems) and were visually inspected for accuracy.

Species-specific SNPs and InDels (consistent within each species but differing between species) were used to generate polymorphic markers. The online program BlastDigester (Ilic et al. 2004) was used to select restriction enzymes that discriminated between SNPs from the two species for developing CAPS markers. Markers producing size polymorphisms (due to species-specific InDels) were resolved using agarose gels or on the ABI3100. All single-locus markers were tested against a core set of eight interspecific hybrids (Supplementary Table S1) derived from a range of parental genotypes to ensure amplification of both barley and *H. bulbosum* alleles in a mixed genetic background and to determine whether null alleles were present. Markers showing polymorphisms between cultivars Steptoe and Morex were mapped in the Steptoe × Morex population (Kleinhofs et al. 1993) using the software MapManager QTX (Manly et al. 2001) on default settings.

A basic consensus map was developed by combining the 18 species-specific markers that were mapped here using the Steptoe × Morex population (Supplementary Table S2), with the remaining 28 markers that had their locations previously published in other populations (Qi et al. 2004; Sato et al. 2004; Thiel et al. 2003). The locations of markers on the consensus map were based on their relative chromosomal position according to the mapping population in which they were originally mapped and each chromosome was normalised to a length of 100. For example, the marker KS302 was mapped by Sato et al. (2004) at position 89.5 cM out of a total chromosome length for 4H of 146.8 cM; therefore, is located at position 61 on the consensus map (Fig. 2). This enabled the relative order and position of markers on each chromosome to be visualised (Fig. 2). An estimate of the genetic coverage of these ILs was made by summing the largest introgression (assuming that introgressions detected by only the most distal marker were at least 5% in size) on both arms of each chromosome (e.g. chromosome 2H = 15 + 26 = 41% coverage). The proportion of the entire genetic map length that each chromosome contributed was then determined by averaging across five different mapping populations (Kleinhofs et al. 1993; Ramsay et al. 2000; Sato et al. 2004; Rostoks et al. 2005, Varshney et al. 2007) and the final estimate of genetic coverage was obtained by multiplying the chromosomal coverage by the proportion of the genetic map contributed by that chromosome.

Statistics

The frequency of SNPs detected within exon sequence was analysed using a binomial generalised linear model (McCullagh and Nelder 1989) and was compared between those markers that either did or did not possess introns. The comparison was tested within the analysis of deviance done as part of the analysis, using an F test. The results are presented as reciprocals of the estimated proportions, along with 95% confidence intervals. The analysis was carried out with GenStat (GenStat Committee 2006).

Results

Progeny screening with pSc119.1

The diploid progeny, generated from triploid and tetraploid interspecific hybrids, were screened with the pSc119.1 retrotransposon-based PCR assay, revealing a total of 102 (24%) positive lines (putative ILs) from the 425 lines tested (Table 1). The proportion of pSc119.1-positive lines was similar from either triploid (23.1%, $n = 225$) or tetraploid (25%, $n = 200$) interspecific hybrids (Table 1). However, frequencies of pSc119.1-positive progeny varied among the different interspecific hybrids (between 5.4 and 44.4%) (Table 1).

Marker development

A total of 216 amplicons were analysed, comprising 172 markers generated from EST contigs selected from the HarvEST database (Wanamaker et al. 2006) and 44 primer pairs kindly provided by Prof. Kazuhiro Sato (Okayama University, Kurashiki, Japan). After initial screening to remove markers amplifying multiple bands, 126 markers were direct sequenced from four barley cultivars and four

Table 1 Screening of unselected diploid progeny from interspecific hybrids using the pSc119.1 PCR assay

Hybrid code	Pedigree (Hv × Hb)	Positive lines	Positive Individuals	% Positive lines	% Positive individuals
Progeny from triploid (VBB) hybrids					
181P	Emir × (Emir × A17-1)	45/143	152/655	31.5	23.2
203S	Emir × (Emir × A17)	3/8	9/39	37.5	23.1
251 V	Morex × (Morex × 2032)	4/74	9/364	5.4	2.5
		Total = 52/225	Total = 170/1058	Mean = 23.1	Mean = 16.1
Progeny from tetraploid (VVBB) hybrids					
220A	Morex × 2920/4	7/27	14/130	25.9	10.8
228Q1	Morex × 2032	9/23	26/101	39.1	25.7
230H1	Morex × 2032	7/24	19/96	29.2	19.8
230H3	Morex × 2032	1/7	5/33	14.3	15.2
230H13	Morex × 2032	4/16	14/74	25.0	18.9
230H18	Morex × 2032	2/13	8/59	15.4	13.6
230H24	Morex × 2032	6/25	20/108	24.0	18.5
230H25	Morex × 2032	4/30	13/138	13.3	9.4
230S5	Morex × 2032	5/21	18/98	23.8	18.4
231D5	Golden Promise × 2032	1/5	4/17	20.0	23.5
231D8	Golden Promise × 2032	4/9	8/19	44.4	42.1
		Total = 50/200	Total = 149/873	Mean = 25.0	Mean = 17.1
Combined		102/425	319/1,931	Mean = 24	Mean = 16.5

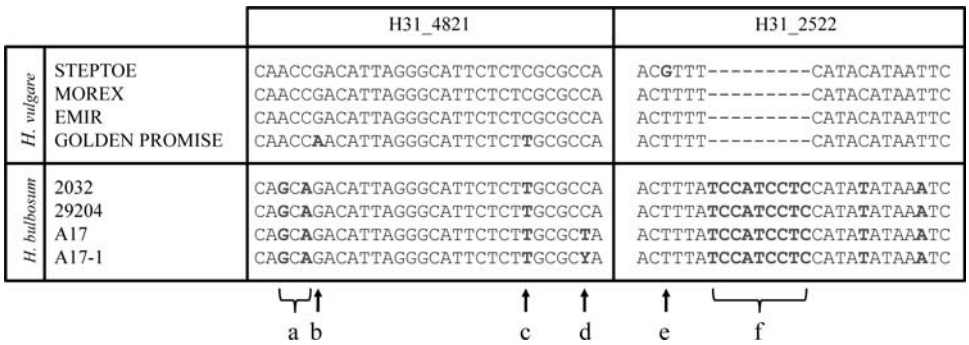

Fig. 1 Composite sequence alignment from the intron regions of markers H31_4821 and H31_2522 indicating; **a** two species-specific SNPs, **b** a genotype-specific SNP in cultivar Golden Promise, **c** a SNP present in all *H. bulbosum* genotypes and barley cultivar Golden Promise, **d** SNP variation within the *H. bulbosum* genotypes, C in diploids 2032 and 2920/4 and T in the tetraploid A17 with tetraploid A17-1 possessing a mixed base (Y = C + T), **e** a SNP between the parents of the Steptoe × Morex mapping population (Kleinhofs et al. 1993) and **f** a species-specific nine base pair InDel

H. bulbosum genotypes. Of these markers, 92 returned good quality alignments consisting of a total of 40.6 kb of trimmed sequence per line, and featured 870 species-specific single nucleotide polymorphisms (SNPs) (Fig. 1a) and 59 species-specific insertions/deletions (InDels) (Fig. 1f). Polymorphism between different barley cultivars (Fig. 1b, c, e) and between or within (observed as mixed bases) the *H. bulbosum* genotypes (Fig. 1d) was also detected in these sequence alignments. However, only the species-specific SNPs and InDels were analysed further.

Introns were identified and located in 64 markers by aligning each genomic marker sequence with the respective barley EST contig from the HarvEST database. Intron sequence accounted for 17 kb or 42.7% of the total marker sequence but contained 61.5% of the species-specific SNPs and 66% of the species-specific InDels. As expected, the frequency of species-specific SNPs was higher in introns (1 per 32.4 bp) than in exons (1 per 69.6 bp). However, the frequency of species-specific SNPs within exon-only markers [1 per 46 bp (95% confidence interval 36,59)] was significantly greater ($P < 0.001$) than within the exon regions of intron-spanning markers [1 per 109 bp (79,149)] (Table 2).

Species-specific InDels (>2 bp) were also detected in 46.7% of the 92 alignments with an InDel detected on average every 445 bp in introns and every 1,166 bp in exons. The InDels that were located in introns were also on

Table 2 Frequencies of species-specific single nucleotide polymorphisms (SNP) between intron and exon sequences

	Intron-spanning markers		Exon-only markers	Overall
	Intron[a]	Exon[b]		
Number of markers	64		28	92
Number of species-specific SNPs	535	126	209	870
Total sequence (bp)	17,357	13,680	9,641	40,678
SNP frequency[c]	1/32.4	1/108.6	1/46.1	1/46.8

[a] Intron sequences from intron-spanning markers were assigned where there was no alignment of genomic marker sequence to the corresponding HarvEST contig sequence (assembly 31)

[b] Exon sequences from intron-spanning markers were assigned by alignment of genomic marker sequence to the corresponding HarvEST contig sequence (assembly 31)

[c] SNP frequency was measured as the mean number of base pairs per species-specific single nucleotide polymorphism

average larger (18.1 bp, range 3–161 bp) than InDels found in exons (9.9 bp, range 3–21 bp).

The Steptoe × Morex mapping population (Kleinhofs et al. 1993) was used to locate markers showing polymorphism between these parents. A total of 25 markers were added to this map (data not shown), as cleaved amplified polymorphic sequence (CAPS) markers (using restriction enzyme digest polymorphisms), electrophoretic size polymorphisms and tetra-primer ARMS PCR (Chiapparino et al. 2004). Genetic mapping of markers provided confirmation of chromosomal location and elucidation of marker order on specific chromosome arms. Of the remaining markers, 52 were monomorphic between Steptoe and Morex, and the remaining 15 could not be easily mapped without additional expense. Marker genotyping of previously characterised ILs also provided additional proof of chromosomal location and marker order where direct mapping was not performed.

A major impediment to the utilisation of these sequence alignments as CAPS markers was the absence of discriminating restriction enzymes or the considerable cost of purchasing all the enzymes required. From 92 informative alignments (each containing at least one species-specific SNP), only 46 fully informative markers were successfully developed to screen the putative ILs. Of these, 40 were developed as CAPS markers and six were markers possessing large size polymorphisms. To be considered 'fully informative' markers had to be co-dominant, generate robust CAPS or size polymorphism between the four barley cultivars and four *H. bulbosum* genotypes used in our program and not to suffer from preferential or exclusive amplification of the barley alleles in heterozygous or hybrid material.

Marker analysis of putative introgression lines

A total of 110 putative ILs (pSc119.1-positive) were analysed using the 46 single-locus markers developed here. The presence of *H. bulbosum*-specific alleles was confirmed in 88 (80%) of the putative ILs with the remaining 22 lines having no introgression detected. Single introgressions were detected in the majority of the ILs, with six ILs possessing two distinct introgressed locations and one IL found to contain three separate introgressions. Of the 96 introgressions detected, 91 (94.8%) extended to the most distal marker of the respective chromosome arm (Fig. 2). The remaining five introgressions were located in interstitial (or sub-distal) regions of the genome and were detected on chromosomes 2HL (×2), 3HS, 5HL and 6HS (Fig. 2). Introgressions were found most frequently on chromosome 2HL (29.2%), with introgressions located on all other chromosome arms with the exception of chromosome 3HL. Introgressions on chromosome 3HL have not previously been detected in any of our material.

A null allele was detected for the marker H31_6035 for all lines derived from the IL 203S11/M2. These lines failed to amplify a *H. bulbosum* allele for this marker despite an introgression being confirmed on chromosome 2HL by flanking markers.

Prior to the adoption of pSc119.1 screening, putative ILs were selected based on the observation of a limited number of *H. bulbosum*-specific phenotypes followed by RFLP marker analysis (Johnston 2007). The chromosomal distributions of introgressed chromatin detected in ILs under these original selection criteria and after the adoption of the pSc119.1 assay were compared. Since using the pSc119.1 assay, a significant reduction in the relative abundance of introgressions on chromosomes 2HS and 4HL has been detected along with a significant increase in the relative abundance of chromosome 2HL introgressions (Fig. 3).

By using the largest introgression on each chromosome arm, a coarse estimate of 36% of the barley genetic map has now been transferred from *H. bulbosum* into cultivated barley (Table 3).

Discussion

Adoption of the pSc119.1 assay has allowed all previously characterised ILs to be detected with high selectivity and sensitivity for *H. bulbosum* chromatin (Johnston and

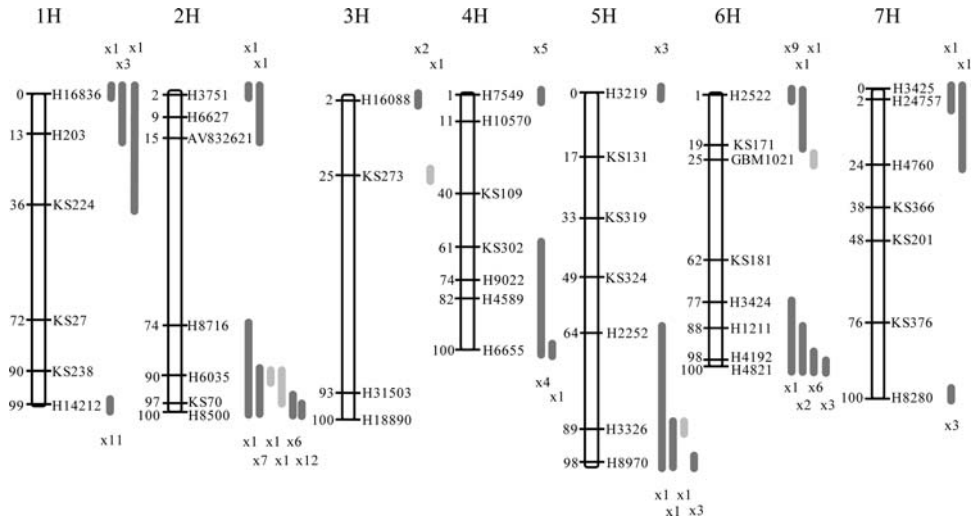

Fig. 2 Chromosomal location and genetic sizes of introgressions from 46 EST-derived PCR markers performed on progeny pre-selected using pSc119.1. The detected introgressions are indicated by *bars to the right* of the genetic chromosome map (*black bars* indicate introgressions that extend to the most distal marker available and *grey bars* indicating interstitial introgressions) with the introgression ends located adjacent to diagnostic markers. Because the markers are derived from a number of different genetic maps their positions are shown as a percentage of the chromosome length from the mapping population in which that marker was mapped (i.e. most distal short arm marker = 0 and most distal long arm marker = 100). The multipliers (i.e. ×3) indicate where multiple versions of an introgression with the same marker results have been detected. Markers designated with an *H* were developed from HarvEST contigs generated from assembly #31 (Wanamaker et al. 2006), markers designated with *KS* were developed by Prof. Kazuhiro Sato (Okayama University, Kurashiki, Japan) and the marker GBM1021 was developed by Dr. Thomas Thiel (IPK, Gatersleben, Germany)

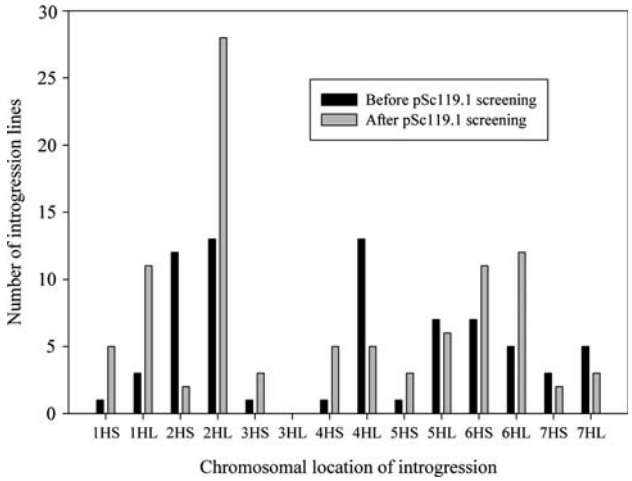

Fig. 3 Chromosomal distributions of chromatin introgressed from *H. bulbosum* into cultivated barley, before and after the adoption of the pSc119.1 PCR assay for progeny screening

Table 3 Estimated coverage of the barley genetic map provided by the largest introgression lines (ILs) on each chromosome arm (assuming a minimum introgression size of 5%)

	Short arm	Long arm	Total	Proportion[a]	Coverage
1H	36	5	41	0.144	5.9
2H	15	26	41	0.148	6.1
3H	10	0	10	0.150	1.5
4H	5	39	44	0.117	5.2
5H	5	36	41	0.176	7.2
6H	24	23	47	0.125	5.9
7H	24	5	29	0.140	4.1
Total					35.9

[a] Proportion of the barley genetic map contributed by each chromosome was averaged from five different linkage maps (Kleinhofs et al. 1993; Ramsay et al. 2000; Sato et al. 2004; Rostoks et al. 2005, Varshney et al. 2007)

Pickering 2002). More importantly, the pSc119.1 assay allowed putative ILs to be selected independently of phenotype. For example, ILs containing introgressions on two chromosome arms (2HS and 4HL) that were phenotypically selected at high frequency were detected at relatively lower frequency after adopting pSc119.1 screening (Fig. 3). These two introgression locations were most likely over-represented in previous analyses due to the presence of easily identified *H. bulbosum*-derived traits for mildew resistance/glossy leaf and sheath (2HS), pubescent leaf sheath and prostrate growth habit (4HL). Progeny selection strategies are limited by the time and cost required to sample material for a finite number of traits. Screening putative ILs at a molecular level will reduce the bias when selecting traits such as disease resistance and will provide material for screening novel traits in future barley breeding programmes.

There was a wide range in the frequencies of pSc119.1-positive progeny detected from the interspecific hybrids

(Table 1, between 5.4 and 44.4%). This confirmed previous data indicating that efficient IL generation was influenced greatly by the parental genotypes used in hybrid formation (Pickering 2000). However, even hybrids generated from the same parental genotypes were quite variable, with small populations of Morex × 2032 tetraploid hybrids yielding between 13.3 and 39.1% putative ILs. The use of tetraploid hybrids (VVBB) for producing introgression lines was previously considered inefficient due to the absence of identifiable *H. bulbosum* traits in the progeny (Pickering, unpublished). However, the pSc119.1 analysis has revealed that both triploid (VBB) and tetraploid (VVBB) interspecific hybrids generate a similar frequency of putative ILs (Table 1).

As *H. bulbosum* is a highly heterozygous and heterogeneous species and possesses significant sequence divergence from cultivated barley (Table 2), it was critical to develop a set of conserved markers for detecting introgressed chromatin. In this study, we focused on the detection of species-specific SNPs between four barley cultivars and four *H. bulbosum* genotypes (Fig. 1a) that could be converted into robust PCR-based markers. Additional SNPs between the individual genotypes were often detected (see Fig. 1b–e) along with genotype-specific InDels. All this polymorphism accounts for the difficulties associated with using PCR markers (e.g. SSRs and EST-SSRs) designed from barley sequences for detection of *H. bulbosum* introgressions (Johnston 2007). A comparable study in cultivated barley, detected a frequency of one SNP every 130 bp in randomly selected EST sequences across seven barley genotypes, with 89% of SNPs appearing in more than one genotype (Kota et al. 2007). In our markers, conserved interspecific SNPs were detected 2.15 times more frequently in intron sequence than in exon sequence. A similar ratio of 2.11 (intron SNPs/exon SNPs) was also detected by Zhu et al. (2003) from 115 sequences across 25 soybean genotypes reinforcing the value of using intron-spanning markers to increase polymorphism detection.

In our programme, markers need to be 'fully informative', i.e. capable of detecting multiple *H. bulbosum* alleles in a barley genetic background without preferential amplification. The presence of a null allele in one IL family for the marker H31_6035 (2HL) (later resolved by primer redesign) indicated that there are possibly additional lines escaping detection. This further emphasises the need to locate PCR primers in regions of conserved sequence between the species and to take into account variation between and within the individual *H. bulbosum* genotypes. When the frequency of species-specific SNPs was examined solely in exon sequence, a large difference was detected between exon sequence from intron-spanning markers (1 SNP per 109 bp) and exon sequence from exon-only markers (1 SNP per 46 bp) (Table 2). One reason for this discrepancy may be that the exon sequences sampled in intron-spanning markers are enriched for sequences located near exon–intron junctions. Exon sequences flanking introns evolve at half the rate of exon centres from the same genes in humans and mice (Parmley et al. 2007). Therefore, PCR primers sited close (~70 bp) to these exon–intron junctions or within small exons may provide a way of maximising conserved primer design for cross-species applications.

Most (94.8%) of the detected introgressions extended to the most distal marker available, indicating the importance of having distally located markers to maximise the detection of small introgressions. The high frequency of introgressions located on chromosome 2HL relative to other chromosomal locations was not reflected in the relative level of chromosome association detected between homoeologous chromosomes from two diploid interspecific hybrids (Pickering et al. 2006). This may instead be attributed to a number of factors including: the chromosomal distribution of pSc119.1, intensive marker development on chromosome 2HL, greater sequence similarity between the barley and *H. bulbosum* in this region, recombination hotspots and/or a greater ability for 2HL introgressions from *H. bulbosum* to compensate for the substituted regions of cultivated barley.

Despite the development of both the pSc119.1 assay and the set of single-locus markers described here, ILs possessing *H. bulbosum* introgressions located on chromosome 3HL had, until recently, still not been detected. However, a relatively high frequency of homoeologous chromosome association was detected between 3H and $3H^b$ in two interspecific hybrids (Pickering et al. 2006). This may indicate that chromosome association does not necessarily correlate with interspecific recombination frequency and that perhaps post-pollination events are responsible for a lack of 3HL introgressions (Pickering et al. 2006). Since the submission of this manuscript a single 3HL IL was identified using GISH/FISH (Pickering, unpublished) but when tested using the 3HL markers H31_31503 and H31_18890 developed here, this IL returned only the barley genotype. This may be due to a failure of the markers to detect the specific *H. bulbosum* alleles introgressed in this case (i.e. a null allele).

Twenty-two putative ILs were positive for the pSc119.1 assay but no *H. bulbosum*-specific alleles were detected in the single-locus marker analysis. It is possible that the marker coverage was not sufficient to detect very small introgressions, that there were a large number of null alleles or that IL detection using pSc119.1 may in some cases have arisen independently of *H. bulbosum* introgression.

It was hoped that pre-screening using the pSc119.1 assay would result in an increased number of proximal and interstitial introgressions being detected, but this has not

eventuated. It is likely that constraints on recombination between these two species are driving the distal location of most introgressions. In general, the barley genome is characterised by suppressed recombination in the proximal/centromeric regions and more frequent recombination in the distal chromosomal regions (Künzel et al. 2000). *H. bulbosum* has an even more marked contrast in recombination frequency between distal (high) and proximal (low) chromosomal regions (Jaffé et al. 2000; Salvo-Garrido et al. 2001). The combination of low recombination frequencies in the proximal chromosomal areas of both species and further reduced recombination between the two species may explain the paucity of *H. bulbosum* introgressions in the more proximal chromosomal locations (Fig. 2). This phenomenon does have important implications for the ability to access *H. bulbosum* for traits located in these proximal or centromeric regions of the genome.

The initial introgressions of *H. bulbosum* chromatin transferred into the barley genome can be reduced in size by interspecific recombination during backcrossing to the recurrent parent. However, a general reduction in recombination frequency (relative to barley) has been identified, which probably depends on the chromosomal location of the introgression. Korzun et al. (1999) examined a 15.8-cM marker interval (between psb43 and mwg616) on 4HL in barley that was reduced to 0.5 cM in the corresponding region introgressed from *H. bulbosum*. Ruge et al. (2003) also detected a similar reduction on chromosome 6HS (between psr167 and mwg573) from 12.8 cM in barley to 1.8 cM in the introgressed segment. Large backcross populations and the development of markers closely linked to traits of interest within the introgressed chromatin will be required for accessing *H. bulbosum* traits for barley improvement.

Molecular marker analysis of ILs between barley and *H. bulbosum* demonstrated the transfer of *H. bulbosum* chromatin into the cultivated barley genome. Using the locations of the most proximal markers that detect each *H. bulbosum* introgression for genetic sizing and assuming a minimum introgression size of 5% for introgressions detected by a single distal marker, a coarse estimate of 36% of the barley genetic map has now been introgressed from *H. bulbosum* into cultivated barley. Each introgression probably extends more proximally than the detecting marker and this should, therefore, be considered a conservative estimate. These ILs provide an important resource for barley improvement and the molecular markers described will facilitate genetic map-based selection of ILs for a wide variety of agronomic and quality traits.

Acknowledgments The authors would like to thank Professor Kazuhiro Sato for providing primer sequences of mapped EST markers, Mrs. Ruth Butler for statistical analysis of SNP frequencies and Dr. Samantha Baldwin for helping construct the figures for this manuscript. Comments from journal reviewers made a significant improvement to this manuscript.

References

Badr A, Muller K, Schafer-Pregl R, El Rabey H, Effgen S, Ibrahim H, Pozzi C, Rohde W, Salamini F (2000) On the origin and domestication history of barley (*Hordeum vulgare*). Mol Biol Evol 17:499–510

Chiapparino E, Lee D, Donini P (2004) Genotyping single nucleotide polymorphisms in barley by tetra-primer ARMS-PCR. Genome 47:414–420

Feuillet C, Langridge P, Waugh R (2008) Cereal breeding takes a walk on the wild side. Trends Genet 24:24–32

GenStat Committee (2006) The guide to GenStat Release 9—parts 1–3. In: Payne R (ed) VSN International, Oxford

Ilic K, Berleth T, Provart N (2004) BlastDigester—a web-based program for efficient CAPS marker design. Trends Genet 20:280–283

Jaffé B, Caligari P, Snape J (2000) A skeletal map of *Hordeum bulbosum* L. and comparative mapping with barley. Euphytica 115:115–120

Johnston P (2007) Molecular characterisation of chromatin introgressed from *Hordeum bulbosum* L. into *Hordeum vulgare* L. PhD Thesis, Department of Biochemistry. University of Otago, Dunedin, New Zealand, p 340

Johnston P, Pickering R (2002) PCR detection of *Hordeum bulbosum* introgressions in an *H. vulgare* background using a retrotransposon-like sequence. Theor Appl Genet 104:720–726

Kleinhofs A, Killian A, Saghai-Maroof M, Biyashev R, Hayes P, Chen F, Lapitan N, Fenwick A, Blake T, Kanazin V, Ananiev E, Dahleen L, Kudrna D, Bollinger J, Knapp S, Liu B, Sorrells M, Heun M, Franckowiak J, Hoffman D, Skadsen R, Steffenson B (1993) A molecular, isozyme and morphological map of the barley (*Hordeum vulgare*) genome. Theor Appl Genet 86:705–712

Korzun V, Malyshev S, Pickering R, Borner A (1999) RFLP mapping of a gene for hairy leaf sheath using a recombinant line from *Hordeum vulgare* L. × *Hordeum bulbosum* L. cross. Genome 42:960–963

Kota R, Varshney R, Prasad M, Zhang H, Stein N, Graner A (2007) EST-derived single nucleotide polymorphism markers for assembling genetic and physical maps of the barley genome. Funct Integr Genomics 8:223–233

Künzel G, Korzun L, Meister A (2000) Cytologically integrated physical restriction fragment length polymorphism maps for the barley genome based on translocation breakpoints. Genetics 154:397–412

Manly K, Cudmore R, Meer J (2001) Map Manager QTX, cross-platform software for genetic mapping. Mamm Genome 12:930–932

McCullagh P, Nelder J (1989) Generalized linear models, 2nd edn. Chapman and Hall, London

Murray M, Thompson W (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4326

Palumbi S (1995) Nucleic acids II: the polymerase chain reaction. In: Hillis D, Moritz C (eds) Molecular systematics, 2nd edn. MA Sinauer Associates Inc., Sunderland, pp 205–247

Parmley JL, Urrutia AO, Potrzebowski L, Kaessmann H, Hurst LD (2007) Splicing and the evolution of proteins in mammals. PLoS Biol 5:e14

Pickering R (1988) The production of fertile triploid hybrids between *Hordeum vulgare* L. (2n = 2x = 14) and *H. bulbosum* L. (2n = 4x = 28). Barley Genet Newsl 18:25–29

Pickering R (2000) Do the wild relatives of cultivated barley have a place in barley improvement? In: Logue S (ed) Barley genetics. In: VIII Proceedings of the 8th international barley genetics symposium, Adelaide, Australia, pp 223–230

Pickering R, Hill A, Michel M, Timmerman-Vaughan G (1995) The transfer of a powdery mildew resistance gene from *Hordeum bulbosum* L. to barley (*H. vulgare* L.) chromosome 2 (2I). Theor Appl Genet 91:1288–1292

Pickering R, Steffenson B, Hill A, Borovkova I (1998) Association of leaf rust and powdery mildew resistance in a recombinant derived from a *Hordeum vulgare* × *Hordeum bulbosum* hybrid. Plant Breed 117:83–84

Pickering R, Malyshev S, Kunzel G, Johnston P, Korzun V, Menke M, Schubert I (2000) Locating introgressions of *Hordeum bulbosum* chromatin within the *H. vulgare* genome. Theor Appl Genet 100:27–31

Pickering R, Klatte S, Butler R (2006) Identification of all chromosome arms and their involvement in meiotic homoeologous associations at metaphase I in 2 *Hordeum vulgare* L. × *Hordeum bulbosum* L. hybrids. Genome 49:73–78

Qi LL, Echalier B, Chao S, Lazo GR, Butler GE, Anderson OD, Akhunov ED, Dvorak J, Linkiewicz AM, Ratnasiri A, Dubcovsky J, Bermudez-Kandianis CE, Greene RA, Kantety R, La Rota CM, Munkvold JD, Sorrells SF, Sorrells ME, Dilbirligi M, Sidhu D, Erayman M, Randhawa HS, Sandhu D, Bondareva SN, Gill KS, Mahmoud AA, Ma XF, Miftahudin, Gustafson JP, Conley EJ, Nduati V, Gonzalez-Hernandez JL, Anderson JA, Peng JH, Lapitan NLV, Hossain KG, Kalavacharla V, Kianian SF, Pathan MS, Zhang DS, Nguyen HT, Choi DW, Fenton RD, Close TJ, McGuire PE, Qualset CO, Gill BS (2004) A chromosome bin map of 16,000 expressed sequence tag loci and distribution of genes among the three genomes of polyploid wheat. Genetics 168:701–712

Ramsay L, Macaulay M, degli Ivanissevich S, MacLean K, Cardle L, Fuller J, Edwards KJ, Tuvesson S, Morgante M, Massari A, Maestri E, Marmiroli N, Sjakste T, Ganal M, Powell W, Waugh R (2000) A simple sequence repeat-based linkage map of barley. Genetics 156:1997–2005

Rostoks N, Mudie S, Cardle L, Russell J, Ramsay L, Booth A, Svensson JT, Wanamaker SI, Walia H, Rodriguez EM, Hedley P, Liu H, Morris J, Close TJ, Marshall DF, Waugh R (2005) Genome-wide SNP discovery and linkage analysis in barley based on genes responsive to abiotic stress. Mol Genet Genomics 274:515–527

Ruge B, Linz A, Pickering R, Proeseler G, Greif P, Wehling P (2003) Mapping of *Rym14*^{Hb}, a gene introgressed from *Hordeum bulbosum* and conferring resistance to BaMMV and BaYMV in barley. Theor Appl Genet 107:965–971

Salvo-Garrido H, Laurie D, Jaffe B, Snape J (2001) An RFLP map of diploid *Hordeum bulbosum* L. and comparison with maps of barley (*H. vulgare* L.) and wheat (*Triticum aestivum* L.). Theor Appl Genet 103:869–880

Sato K, Nankaku N, Motoi Y, Takeda K (2004) A large scale mapping of ESTs on barley genome. In: Spunar J, Janikova J (eds) 9th International barley genetics symposium, Brno, Czech Republic

Szigat G, Pohler W (1982) *Hordeum bulbosum* × *H. vulgare* hybrids and their backcrosses with cultivated barley. Cereal Res Commun 10:73–78

Thiel T, Michalek W, Varshney R, Graner A (2003) Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). Theor Appl Genet 106:411–422

Toubia-Rahme H, Johnston P, Pickering R, Steffenson B (2003) Inheritance and chromosomal location of *Septoria passerinii* resistance introgressed from *Hordeum bulbosum* into *Hordeum vulgare*. Plant Breed 122:405–409

Varshney RK, Marcel TC, Ramsay L, Russell J, Röder MS, Stein N, Waugh R, Langridge P, Niks RE, Graner A (2007) A high density barley microsatellite consensus map with 775 SSR loci. Theor Appl Genet 114:1091–1103

von Bothmer R, Jacobsen N, Baden C, Jorgensen R, Linde-Laursen I (1995) An ecogeographical study of the genus *Hordeum*, 2nd edn. International Plant Genetic Resources Institute (IPGRI), Rome

Walther U, Rapke H, Proeseler G, Szigat G (2000) *Hordeum bulbosum*—a new source of disease resistance—transfer of resistance to leaf rust and mosaic viruses from *H. bulbosum* into winter barley. Plant Breed 119:215–218

Wanamaker S, Close T, Roose M, Lyon M (2006) HarvEST: Barley EST database version 1.44; http://harvest.ucr.edu/. Cited 10 November 2006

Xu J, Kasha KJ (1992) Transfer of a dominant gene for powdery mildew resistance and DNA from *Hordeum bulbosum* into cultivated barley (*H. vulgare*). Theor Appl Genet 84:771–777

Zhu YL, Song QJ, Hyten DL, Van Tassell CP, Matukumalli LK, Grimm DR, Hyatt SM, Fickus EW, Young ND, Cregan PB (2003) Single-nucleotide polymorphisms in soybean. Genetics 163:1123–1134