Importance of the Secondary Genepool in Barley Genetics and Breeding I. Cytogenetics and Molecular Analysis

Richard PICKERING[1], Paul A. JOHNSTON[1] and Brigitte RUGE[2]

[1]New Zealand Institute for Crop & Food Research Limited, Christchurch, New Zealand;
[2]Federal Centre for Breeding Research on Cultivated Plants, Institute of Agricultural Crops, Gross Lüsewitz, Germany

Abstract*:* There have been no plant breeding developments using species from the tertiary genepool of cultivated barley for breeding or genetics since the VIII[th] International Barley Genetics Symposium in 2000. Hence, the first part of this review describes progress since 2000 in developing and characterising recombinant lines derived from hybridisations between the sole species in the secondary genepool, *Hordeum bulbosum* L., and cultivated barley, *Hordeum vulgare* L. The topics discussed in part I are cytogenetics and molecular analysis of recombinant lines.

Keywords: barley; *Hordeum bulbosum;* chromosome pairing; introgressions; EST; cDNA-AFLP; molecular markers

Cytogenetics

In situ hybridisation, which combines cytogenetic and molecular methods, can be used to identify and locate introgressions transferred from *Hordeum bulbosum* L. into the cultivated barley (*H. vulgare* L.) genome (Pickering *et al.* 2000). The first step is performed on somatic chromosome preparations (e.g. root tip cells) using genomic *in situ* hybridisation (GISH) to identify the site of the introgression on one or more unknown chromosomes. This is followed sequentially on the same chromosome preparation by fluorescent *in situ* hybridisation (FISH) with the microsatellite sequence $(CTT)_{10}$ or $(GAA)_7$ to identify individual barley chromosomes (Pedersen & Linde-Laursen 1994). Although the methods are laborious and require expensive equipment, the chromosome location of introgressions and approximate physical size can be established prior to more critical genetic mapping with molecular markers.

By these means we have identified and characterised more than 70 recombinant lines (RLs) containing introgressions of *H. bulbosum* chromatin on 13 out of the 14 chromosome arms of the *H. vulgare* genome (Table 1). One problem using these RLs in breeding programmes is linkage drag where the introgressed segment cannot be reduced in size by genetic recombination. To overcome this linkage drag it is necessary to reduce the size of the introgression and remove unwanted DNA linked to the locus of interest – a process that necessitates screening large segregating populations. Does this linkage drag result from reduced meiotic pairing between recombinant and non-recombinant chromosomes? To investigate this possibility we used FISH to determine the levels of meiotic chromosome pairing in heterozygous RLs and compared these with homozygous controls.

Molecular analyses

To complement GISH and FISH, molecular methods have been used successfully to characterise RLs derived from *H. vulgare* × *H. bulbosum* hybrids.

Table 1. Numbers of recombinant lines with introgressions on particular chromosomes obtained from *Hordeum vulgare* × *H. bulbosum* hybrids and the identifiable traits associated with the introgressions

Chromosome location of introgression	Number of recombinant progeny	Transferred trait
1HS	1	
1HL	3	resistance to leaf rust
2HS	12	resistance to leaf rust and powdery mildew; glossy spike and leaf sheath
2HL	13	resistance to leaf rust
3HS	1	
3HL	0	
4HS	1	resistance to scald
4HL	13	resistance to Septoria speckled leaf blotch; pubescent leaf and leaf sheath; black aleurone
5HS	1	response to DDT
5HL	7	resistance to leaf rust; very susceptible to powdery mildew; vernalisation requirement
6HS	7	resistance to BaYMV/BaMMV
6HL	5	
7HS	3	resistance to stem rust?
7HL	5	resistance to powdery mildew; resistance to stem rust

An initial screen to identify RLs that contain *H. bulbosum* chromatin introgressed into *H. vulgare* has been developed (Johnston & Pickering 2002). These RLs can then be further characterised with GISH and FISH (Pickering *et al*. 2000) and the genetic size of the introgression determined by molecular markers. Because of the variation between barley and *H. bulbosum* genomic DNA, PCR-based markers such as microsatellites may not amplify DNA sequences of both species. To develop our own markers we have used cDNA-based sequences, which are more likely to be conserved between *H. vulgare* and *H. bulbosum* but still show enough polymorphisms for reliably analysing RLs. We describe here two examples of these procedures; first the production of PCR markers derived from cDNA-based RFLP probes for identifying diagnostic expressed sequence tags (EST) and, second, cDNA-AFLP for developing PCR markers closely linked to loci conferring resistance to the soil-borne virus complex barley yellow mosaic virus (BaYMV) and barley mild mosaic virus (BaMMV).

MATERIALS AND METHODS

Cytogenetics

Details of the seven heterozygous RLs and two homozygous RLs are presented in Table 2; full details of each RL, including the original code numbers, can be supplied on request. Each RL contained one or more distal introgressions of *H. bulbosum* chromatin on particular *H. vulgare* chromosomes. Heterozygous RLs were obtained either by crossing together two homozygous RLs containing introgressions on different chromosomes or by backcrossing a homozygous RL to barley cultivars Emir or Golden Promise. The plants were grown in a heated glasshouse maintained at 21/15°C ± 2°C (16 h day/8 h night). Natural daylight was supplemented when necessary with 400 W mercury and sodium vapour lamps to extend the daylength to 16 h.

Spikes were fixed in 3 ethanol:1 acetic acid and conventional meiotic pairing analyses were carried out by squashing a single anther in 1% aceto-car-

Table 2. Recombinant lines used for cytogenetic analyses

Code	*H. bulbosum* parents
E-2HL-4HS	A17/1, HB2032
E-2HL-7HL	A17/1, HB2032
E-4HS-7HL	A17/1
EG-5HL-6HS**	Cb2920/4
E-6HS-7HS	Cb2920/4 x Cb2929/1
G-6HS-7HS**	Cb2920/4
E-2HL-6HS-7HS**	Cb2920/4 x Cb2929/1; HB2032
E-2HL*	HB2032
G-2HL*	A17/1

*denotes homozygous RLs; **denotes RLs used for FISH. E (Emir) and G (Golden Promise) refer to the *H. vulgare* parent followed by the chromosome location of the *H. bulbosum* introgression(s)

mine and examining pollen mother cells (PMCs) at metaphase I. To perform FISH, each anther containing PMCs at metaphase I was incubated on a microscope slide in one drop of 2% pectinase + 2% cellulase (Sigma) for 1 h at 37°C in a humid chamber. The enzyme was removed by carefully blotting around the anther with filter paper and replaced with a drop of 45% acetic-acid and left for 3–5 min at room temperature. Anthers were macerated and squashed under 18 × 18 mm cover slips, which were removed with a scalpel blade after immersion in liquid nitrogen. The slides were stored at 4°C until use. To prepare the probe, BAC T15P10 from *Arabidopsis thaliana* containing 45S rDNA (Lysak *et al*. 2003) was labelled with digoxigenin by nick translation according to the manufacturer's (Roche) instructions. The 45S rDNA probe hybridises to the short arms of barley chromosomes 5H and 6H. Subsequent FISH methods were based on those of Toubia-Rahme *et al*. (2003). Detection of hybridisation signals was performed as described by Houben *et al*. (2001) using anti-digoxigenin rhodamine. Slides were mounted in Vectashield (Vector Laboratories) with 1.0 μg/ml 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) as a counterstain.

Molecular analyses

EST marker development. cDNA-derived barley RFLP distal markers were selected for developing into EST PCR markers. Sequences from these markers were used to identify matching barley ESTs by searching the BLASTn algorithm. From the EST database HarvEST (Wanamaker & Close 2003) contiguous consensus sequences that matched the original RFLP clone were identified. These consensus cDNA sequence contigs were searched against the non-redundant or high-throughput genomic sequencing databases using discontinuous megaBLAST to find genomic matches to the cDNA sequence. Genomic matches were most often recovered from the rice genome. The likely positions on introns in the barley genome could, therefore, be estimated. Primers to amplify across a putative intron were designed using Primer3 (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). A second criterion was that primers should match sequences between the barley cDNA contig and matching wheat ESTs to increase the likelihood of the primers being conserved between barley and *H. bulbosum*. Amplified sequences were examined using single-stranded conformational polymorphism (SSCP) to identify alleles originating from the *H. bulbosum* parental genotype in putative RLs.

cDNA-AFLP. An RL, which was resistant to the soil-borne virus complex BaMMV, BaYMV-1 and -2, was derived from an *H. vulgare* × *H. bulbosum* cross. Inheritance of resistance was determined from segregating F5 and F6 families based on field trials and glasshouse tests using mechanical inoculation. The methods for carrying out the cDNA-AFLP analysis have been published (Ruge *et al*. 2003) but, briefly, RNA was isolated from homozygous resistant and susceptible pooled populations of F_5 plants. cDNA was synthesised from the RNA, fragmented with *Taq1* and adaptors ligated. Sixty-four primer combinations were used to amplify the cDNA. PCR products were separated on polyacrylamide gels and diagnostic fragments cloned into a plasmid vector for later use as RFLP probes on a mapping population.

RESULTS AND DISCUSSION

Cytogenetics

Meiotic pairing using aceto-carmine squashes

Apart from E-6HS-7HS, E-2HL-6HS-7HS and, to a smaller extent G-6HS-7HS, chromosome pairing in the RLs was similar to or even higher than the *H. vulgare* controls (Table 3).

Highest pairing was observed for the homozygous RL, G-2HL and lowest mean ring bivalent formation was in E-2HL-6HS-7HS (5.99) and E-6HS-7HS (6.31). Both of these RLs contained 6HS and 7HS introgressions derived from a common *H. bulbosum* parent (Cb2920/4 × Cb2929/1) but E-2HL-6HS-7HS had an extra introgression on 2HL transferred from *H. bulbosum* genotype HB2032. Another RL (G-6HS-7HS) with a different pedigree also contained 6HS and 7HS introgressions but had higher pairing (6.69 ring bivalents) although somewhat lower than GP.

Hence, chromosome pairing may be influenced by genotype, but introgression size may be another factor. Since chromosome pairing initiation occurs at the telomeres in barley (Kasha & Burnham 1965) large terminal introgressions may be more disruptive to chromosome pairing than small introgressions in heterozygous RLs and interstitial introgressions may allow nearly normal pairing and recombination. We lack precise physical and genetic map information on the introgression sizes to assess these effects.

Meiotic pairing using FISH

In the three heterozygous RLs studied, most of the unpaired chromosomes involved the short arms of satellite chromosomes 5H or 6H (Table 4), but these chromosomes could not be distinguished from each other with the 45S rDNA probe. In E-2HL-6HS-7HS, 25 of the 91 PMCs analysed contained two or more rod bivalents. Despite the absence of an introgression on chromosome 5H in this RL, 36.4% of the rods in these 25 PMCs involved 5HL-5HL and 6HL-6HL pairing, which was much higher than expected by chance. The short arms of both pairs of chromosomes remained unpaired.

Table 3. Mean (range) of univalent (I) and bivalent (II) formation in PMCs of heterozygous and homozygous (*) recombinant lines derived from *Hordeum vulgare* × *H. bulbosum* crosses using aceto-carmine squash preparations

Code	No. of PMCs	I	II	
			rod	ring
E-2HL-4HS	212	0	0.34 (0-2)	6.66 (5-7)
E-2HL-7HL	365	0	0.34 (0-3)	6.66 (4-7)
E-4HS-7HL	206	0	0.38 (0-3)	6.62 (4-7)
EG-5HL-6HS	331	0	0.36 (0-2)	6.64 (5-7)
E-6HS-7HS	265	0.03 (0–2)	0.68 (0-4)	6.31 (3-7)
G-6HS-7HS	442	0	0.31 (0-2)	6.69 (5-7)
E-2HL-6HS-7HS	209	0	1.01 (0-3)	5.99 (4-7)
E-2HL*	236	0.02 (0–2)	0.27 (1-2)	6.72 (5-7)
G-2HL*	184	0	0.07 (0-1)	6.93 (6-7)
Emir	334	0	0.40 (0-3)	6.60 (4-7)
Golden Promise	241	0	0.18 (0-2)	6.82 (5-7)
H. bulbosum (Cb2920/4)	390	0	0.09 (0-2)	6.91 (5-7)

Table 4. Mean (range) of rod and ring bivalents in PMCs of three recombinant lines heterozygous for *H. bulbosum* introgressions and the % rod bivalents involving 5H-5H or 6H-6H pairing as determined by FISH analysis

Code	No of PMCs	II		% of rods occurring as 5HL-5HL or 6HL-6HL*
		rods	rings	
G-6HS-7HS	59	0.19 (0-1)	6.81 (6-7)	72.7
E-2HL-6HS-7HS	91	1.09 (0-4)	5.91 (3-7)	69.7
EG-5HL-6HS	66	0.24 (0-2)	6.76 (5-7)	81.3

*denotes pairing between the long arms with the short arms remaining unpaired

Reduced pairing between homologous satellite chromosomes in *H. vulgare* has been described previously. Stoinova (1994) reported that in eight cultivars most of the open ring bivalents at diakinesis involved the satellite chromosomes, probably the short arms. Burnham *et al*. (1954) also observed the frequent occurrence of one rod and six ring bivalents and that the rod was most likely the chromosome pair with the small satellite (i.e. 5H). In two trisomic series of *H. spontaneum* and *H. vulgare* greatest numbers of Y-type trivalents were observed for chromosome 5H (Tsuchiya 1960, 1967). Y-type trivalents arise from chiasmata restricted to one of the two arms and we can speculate that pairing was reduced between the 5HS arms.

In conclusion, reduced chromosome pairing probably contributes to linkage drag, but will depend on the size and location of the introgressed segment. Other factors such as reduced recombination between paired chromosomes (Zhang *et al.* 1999), parental genotype and certation effects are also likely to be important. None of these impediments to exploiting RLs in breeding programmes will be easily overcome.

Molecular analyses

EST marker development

Recombination between *H. vulgare* and *H. bulbosum* chromosomes usually takes place distally and arises from a single crossover. EST markers were, therefore, developed for the distal regions of most chromosome arms where suitable cDNA-derived RFLP markers already exist (Figure 1). By carefully designing primers, almost all the EST markers in both species were amplified. Anchoring the PCR primers in conserved cDNA sequences but amplifying across less conserved introns maximised the production of codominant polymorphic markers. This strategy also allows many markers to be developed *in silico* from large public databases and reduces the cost of developing new markers.

cDNA-AFLP

Resistance to the BaYMV–BaMMV complex in European cultivars is based on the recessive resistance genes *rym4* and *rym5* on barley chromosome 3 (Weyen *et al.* 1996; Graner *et al*. 1999). However, the inheritance of the resistance in the RL, designated *Rym14*^{Hb}, was dominant and positioned on chromosome 6HS using a set of barley anchor markers. Hence the locus is non-allelic with *rym4* and *rym5* and provides breeders with a novel source of resistance. To develop markers linked with *Rym14*^{Hb}, cDNA-AFLP analysis was carried out. One of 64 primer combinations amplified a differentially expressed 250 bp transcript, which was only observed in the resistant populations. Southern hybridisation with the cloned fragment revealed a single-copy hybridisation pattern that displayed a codominant polymorphism between resistant and susceptible genotypes. The marker cosegregated with *Rym14*^{Hb} and a BLAST search against the Genbank database (NCBI Blast Homepage) found no sequence similarities for the amplified 250 bp cDNA-AFLP fragment. The RFLP revealed by use of this fragment was converted to a PCR-based STS marker, which was also codominantly inherited, and is being used in a backcross breeding programme. The size of the introgression has been reduced following further recombination in a segregating population.

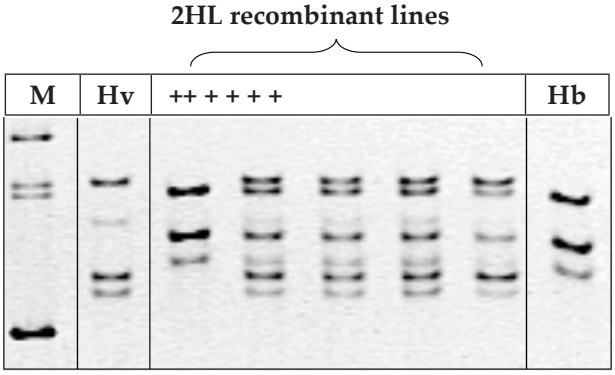

Figure 1. HarvEST contig 5825 (assembly #31) derived from RFLP probe cMWG720 (2HL). Single stranded conformational analysis of parental (*H. vulgare* – Hv and *H. bulbosum* – Hb), heterozygous (+) and homozygous (++) recombinant lines. M = dsDNA marker as control

In conclusion, we have shown that useful PCR-based markers can be developed for marker assisted selection and breeding programmes without incurring a huge investment in time or equipment. Our aim is also to produce these markers for each chromosome arm so we can locate introgressions of *H. bulbosum* chromatin prior to carrying out more precise molecular mapping.

Acknowledgements: We thank Ruth Butler (Crop & Food Research, New Zealand) for advice on statistical analysis, and Professor Ingo Schubert (Institute of Plant

Genetics, Gatersleben, Germany) for allowing R. Pickering to perform part of this research in his laboratory. The programme in New Zealand is supported by the Foundation for Research, Science and Technology.

References

Burnham C.R., White F.H., Livers R. (1954): Chromosomal interchanges in barley. Cytologia, **19:** 191–202.

Graner A., Streng S., Kellermann A., Schiemann A., Bauer E., Waugh R., Pellio B., Ordon F. (1999): Molecular mapping and genetic fine-structure of the *rym5* locus encoding resistance to different strains of Barley Yellow Mosaic Virus complex. Theor. Appl. Genet., **98**: 285–290.

Houben A., Field B.L., Saunders V.A. (2001): Microdissection and chromosome painting of plant B chromosomes. Meth. Cell. Sci., **23:** 115–124.

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

Kasha K.J., Burnham C.R. (1965): The location of interchange breakpoints in barley. II. Chromosome pairing and the intercross method. Can. J. Genet. Cytol., **7:** 620–632.

Lysak M.A., Pecinka A., Schubert I. (2003): Recent progress in chromosome painting of *Arabidopsis* and related species. Chromosome Res., **11:** 195–204.

Pedersen C., Linde-Laursen I. (1994): Chromosomal locations of four minor rDNA loci and a marker microsatellite sequence in barley. Chromosome Res., **2:** 65–71.

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

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.

Stoinova J. (1994): Characteristics of meiosis in some winter barley varieties. Cytologia, **59:** 423–426.

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

Tsuchiya T. (1960): Cytogenetic studies of trisomics in barley. Jap. J. Bot., **17:** 177–213.

Tsuchiya T. (1967): The establishment of a trisomic series in a two-rowed cultivated variety of barley. Can. J. Gen. Cytol., **9:** 667–682.

Wanamaker S., Close T. (2003): HarvEST. http://harvest.ucr.edu.

Weyen J., Bauer E., Graner A., Friedt W., Ordon F. (1996): RAPD-mapping of the distal portion of chromosome 3 of barley, including the BaMMV/BaYMV resistance gene *ym4*. Plant Breeding, **115**: 285–287.

Zhang L., Pickering R., Murray B. (1999): Direct measurement of recombination frequency in interspecific hybrids between *Hordeum vulgare* and *H. bulbosum* using genomic *in situ* hybridization. Heredity, **83:** 304–309.

Received for publication May 25, 2004
Accepted September 21, 2004

Abstrakt

Pickering R., Johnston P. A., Ruge B. (2004): **Význam sekundárního genofondu pro genetiku a šlechtění ječmene. I. Cytogenetika a molekulární analýza**. Czech J. Genet. Plant Breed., **40**: 73–78.

Od 8. Mezinárodního sympozia o genetice ječmene nedošlo k rozvoji šlechtění s využitím druhů z terciárního genofondu kulturního ječmene pro šlechtění nebo genetiku. Proto v první části tohoto přehledu je popsán pokrok od roku 2000 ve vývoji a charakteristice rekombinantních linií odvozených z hybridizace mezi jediným druhem v sekundárním genofondu, *Hordeum bulbosum* L. a kulturním ječmenem Hordeum vulgare L.. Temata v I. části jsou cytogenetika a molekulární analýzy rekombinantních linií.

Klíčová slova: ječmen; *Hordeum bulbosum*; párování chromozomů; introgrese; EST; cDNA-AFLP; molekulární markery

Corresponding author:

Dr. Richard Pickering, New Zealand Institute for Crop & Food Research Limited, Private Bag 4704, Christchurch, New Zealand
tel.: +64 3 3256400, fax: +64 3 3252074, e-mail: pickeringr@crop.cri.nz