Direct measurement of recombination frequency in interspecific hybrids between *Hordeum vulgare* and *H. bulbosum* using genomic *in situ* hybridization

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Two different genotypes of diploid *Hordeum vulgare* × *H. bulbosum* hybrids, which differ in their pattern of meiotic metaphase pairing behaviour, were investigated at MI and AI by genomic *in situ* hybridization (GISH). One hybrid, 102C2, showed a high frequency of bivalents at metaphase I whereas the other, 103K5, showed a high frequency of univalents. The GISH analysis of both hybrids established that pairing occurred only between chromosomes of different parental genomes and revealed that pairing frequency greatly exceeded recombination. Hybrid 102C2 had a significantly higher recombination frequency than 103K5, but in both hybrids recombination involved only distal chromosome regions. However, an interesting finding is that the ratio of recombination to pairing frequency in 103K5 (1:8.9) is twice as high compared with 102C2 (1:17). The hybrids also differed in chromosome stability; little chromosome elimination occurred in 102C2 but 103K5 showed extensive chromosome loss. It appears that the high frequency of bound arms at MI favours retention of *H. bulbosum* chromosomes and maintains stability of chromosome numbers in PMCs. Various ideas are put forward to explain the discrepancy between meiotic pairing frequency and recombination in these hybrids.

Keywords: barley, genomic *in situ* hybridization, *Hordeum bulbosum*, recombination frequency.

Introduction

Diploid and tetraploid cytotypes of *Hordeum bulbosum* L. ($2n = 2x = 14$ and $2n = 4x = 28$) are valuable sources of pest- and disease-resistance genes, which have been successfully transferred into cultivated barley (*H. vulgare* L.) (Xu & Kasha, 1992; Pickering *et al.*, 1995). To achieve this, partially fertile triploid hybrids from the cross between cultivated barley ($2n = 2x = 14$) and *H. bulbosum* ($2n = 4x = 28$) were backcrossed to barley, and recombinants were produced that contained small segments of *H. bulbosum* DNA introgressed into the *H. vulgare* genome (Pickering *et al.*, 1997). However, the number of recombinants and 'chromosomally engineered' plants obtained from crosses between the two species is very low (Lange & Jochemsen, 1975; Pickering, 1992). The reasons for this have been outlined by Pickering (1992) and briefly they are: (i) barley is a diploid species that cannot tolerate much genetic manipulation; (ii) the *H. bulbosum* genome is usually completely eliminated, resulting in haploid barley embryo and plantlet formation; (iii) the interspecific hybrids are often sterile and unstable; and (iv) intergenomic chromosome pairing is variable and genotype-dependent. Apart from the first problem, which is inherent for diploid species, the second and third of these barriers can easily be overcome by manipulating the parental genotypes and crossing environment. However, there still exists the more serious problem of recombination frequencies that are lower than expected in these hybrids (Pickering, 1991), a feature that is seen among interspecific crosses in other genera (Rick, 1969; Causse *et al.*, 1994). Determining the reasons for low frequencies of recombinant progeny from *H. vulgare* × *H. bulbosum* hybrids has been difficult because conventional cytogenetic analyses are not sufficiently refined to draw meaningful conclusions. The use of a paracentric inversion in a diploid *H. vulgare* × *H. bulbosum* hybrid to assess recombination frequencies, by recording the numbers of bridges and fragments at meiotic anaphase (Pickering, 1991), was not efficient because the results only related to one particular chromosome and

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'hotspots' of crossing-over are likely to occur throughout the genome (Pedersen *et al.*, 1995).

Because genomic *in situ* hybridization (GISH) has been used successfully to identify parental chromosomes in *H. vulgare* × *H. bulbosum* hybrids (Schwarzacher *et al.*, 1992b; Anamthawat-Jónsson *et al.*, 1993) and also to visualize introgression of *H. bulbosum* chromatin into *H. vulgare* (Pickering *et al.*, 1997), the objectives in this investigation were to use GISH to assess the extent of crossing-over between parental chromosomes by observations of anaphase I (AI) in pollen mother cells (PMCs) of two diploid *H. vulgare* × *H. bulbosum* hybrids, which differed in their chromosome pairing at metaphase I (MI) of meiosis. In addition, observations on chromosome segregation at AI in PMCs, the frequency of *H. bulbosum* chromosome elimination and any possible relationship between *H. bulbosum* chromosome elimination and MI pairing were made.

Materials and methods

Two different hybrids that have a common female parent (*H. vulgare* cv. Emir) were used, 102C2 (Emir × HB2032) and 103K5 (Emir × Cb3811/5). These hybrids were chosen because earlier observations had shown that the extent of their chromosome pairing at MI was significantly different, with 102C2 having a much higher MI pairing frequency than 103K5.

Growing conditions in the glasshouse were maintained at $21 \pm 2°C/15 \pm 2°C$ (day/night), with 16 h daylength. Anthers containing PMCs at MI or AI were fixed in Carnoy's II solution (6 parts ethanol:3 parts chloroform:1 part glacial acetic acid) overnight at 4°C, then transferred to 70% ethanol and stored at 4°C until required.

For GISH, meiotic chromosome preparations from anthers of hybrids were made as follows : anthers containing the appropriate stage were digested in 4% cellulase (Onozuka R-10, Merck) and 1.3% pectolyase (P-3026, Sigma) for 90 min at 37°C. After the enzyme mix was removed with small pieces of filter paper, the anthers were washed twice with distilled water and the contents squeezed out in one or two drops of fresh fixative (3 parts ethanol : 1 part glacial acetic acid) and spread evenly on clean glass slides assisted by a gentle stream of air, then left to air dry. Slides were stored at −20°C until needed.

Genomic DNA, isolated from young leaves of *H. bulbosum*, was labelled with digoxigenin-11-dUTP (Boehringer Mannheim) by nick-translation according to the manufacturer's instructions. Total genomic DNA from *H. vulgare* was broken into 200–500 bp fragments by autoclaving and used as blocking DNA. The method of *in situ* hybridization followed Schwarzacher *et al.*

(1992a) with minor modifications. Forty-five µL of denatured probe mixture, containing 100 ng of DIG-labelled probe and a 50-fold excess of *H. vulgare* blocking DNA, was applied to each slide. The slides were then heated to 80°C for 10 min and the temperature lowered to 37°C for incubation overnight on a thermal cycler (Hybaid, Omnislide). The hybridization mix consisted of 55% formamide, $2 \times SSC$, 10% (w/v) dextran sulphate, 0.15% SDS and 150-fold excess of salmon sperm DNA (200–500 bp). Hybridization was followed by a stringent wash in 50% formamide in $2 \times SSC$ at 42°C. Sites of probe hybridization were detected using the HNPP Fluorescent Detection Set (Boehringer Mannheim). Chromosomes were counterstained with DAPI (1 µg/mL, 4′,6′-diamidino-2-phenylindole) in $1 \times PBS$ buffer. Slides were mounted in antifade mountant (1.8% DABCO in 90% glycerol v/v, 20 mM Tris pH 7.5) and examined under a Zeiss epi-fluorescence photomicroscope. Photographs were taken on Fujichrome colour reversal film (Provia 400), digitized using a Nikon LS-1000 scanner, and the images processed using Adobe Photoshop 3.0.5 software.

Results

GISH analysis of pairing at MI and recombinant chromosomes at AI in hybrids

Using GISH it was possible to distinguish clearly *H. vulgare* and *H. bulbosum* chromosomes and to show that pairing always occurred between chromosomes of *H. vulgare* and *H. bulbosum*. However, the pattern of chromosome pairing in the two hybrids was quite different. There was significantly greater chromosome pairing in hybrid 102C2 than in 103K5 (Table 1; Fig. 1a–e). The former had a mean bivalent frequency of 6.68 per PMC with a range of 5–7 bivalents per PMC, compared with 2.64 bivalents per PMC with a range of 0–5 for the latter. There were also differences in the types of bivalent formed. In 102C2, 35% of the bivalents were ring-shaped (with two bound arms) whereas in 103K5 only 11.7% were ring-shaped. From this it can be seen that the frequency of bound arms was significantly greater in 102C2 than in 103K5 ($\chi_1^2 = 412.1$, d.f. = 1, $P < 0.001$).

Observations on AI cells showed that recombination frequency was significantly higher in 102C2 than in 103K5 ($\chi_1^2 = 7.13$, $P < 0.01$) (Table 2; Fig. 1f,g). In 103K5, only one or two recombinant chromosome arms were seen in any single PMC and 74% of PMCs showed no recombinant arms. In 102C2, the number of PMCs without recombinant arms was similar (71%) but the PMCs that did show recombination had significantly more recombinant arms. One PMC with as many as

Table 1 Comparison of frequencies of MI pairing configurations (I = univalent; II = bivalent) in hybrids between barley and *Hordeum bulbosum*. (PMCs with fewer than 14 chromosomes have been excluded from the analysis)

| Hybrid | No. of PMCs | | I | MI pairing configurations | | Total II | No. of bound arms |
| | | | | II | | | |
				Rods	Rings		
102C2	69		44	299	162	461	623
		Mean	0.64	4.33	2.35	6.68	9.0
		Range	0–4	1–7	0–6	5–7	7–13
103K5	84		732	196	26	222	248
		Mean	8.7	2.33	0.31	2.64	2.95
		Range	4–14	0–4	0–3	0–5	0–6

seven recombinant arms was seen and the mean frequency of recombinant arms in PMCs showing recombination was 1.84 compared to 1.24 in 103K5.

Pairing frequency was much higher than recombination frequency in both hybrids ($\chi_1^2 = 2107.6$, $P < 0.001$ and $\chi_1^2 = 249.8$, $P < 0.001$ for 102C2 and 103K5, respectively) and a comparison of the mean ratios per PMC of the recombinant arms at AI to the bound arms at MI showed that this was twice as high in 103K5 (1:8.9) compared with 102C2 (1:17).

In both hybrids, synapsed chromosome arms were bound predominantly at distal regions and the recombination events involved only small terminal segments (Fig. 1f,g). No evidence of recombination from double crossovers was observed. Hybrid 102C2 showed an ordered arrangement of bivalents at MI and of segregation at AI whereas 103K5 showed irregular chromosome congression and little evidence of polar migration of chromosomes.

Chromosome elimination in PMCs of hybrids

Differences in chromosome number were seen among PMCs of both hybrids and these only involved the loss of *H. bulbosum* chromosomes. However, there were clear differences in the frequency of chromosome loss in the two hybrid combinations. Elimination of one or two *H. bulbosum* chromosomes was seen in ≈50% of 173 PMCs of hybrid 103K5 (Fig. 1c,d) but elimination was only rarely observed in PMCs of 102C2. PMCs of 103K5 could be classified into three types: type A (48.6%) with the complete chromosome complement (7 *H. vulgare* chromosomes + 7 *H. bulbosum* chromosomes or 7V + 7B); type B (38.7%) with 13 chromosomes (7V + 6B) and type C (12.7%) with 12 chromosomes (7V + 5B). Significant differences were found for the mean number of bound arms per PMC between types A and B ($\chi_1^2 = 18.0$, $P < 0.001$) and

between types A and C ($\chi_1^2 = 13.0$, $P < 0.001$), whereas the difference between types B and type C was not significant.

Discussion

Apart from one report of homoeologous chromosome pairing in PMCs of diploid and triploid *H. vulgare* × *H. bulbosum* hybrids using C-banding techniques (Xu & Snape, 1988), conventional meiotic analysis has not been adopted as an efficient method to establish homoeology between *H. vulgare* and *H. bulbosum* chromosomes and to study recombination frequency in hybrids between the two species. However, genomes from *H. vulgare* and *H. bulbosum*, which are genetically very closely related, can be differentiated by GISH on root-tip chromosome preparations (Schwarzacher *et al.*, 1992b; Anamthawat-Jónsson *et al.*, 1993). GISH has also been shown to be an efficient technique for the study of homoeologous chromosome pairing and recombination in wheat–rye hybrids (Le & Armstrong, 1991; King *et al.*, 1994; Fernández-Calvín *et al.*, 1995; Benavente *et al.*, 1996, 1998), and in other interspecific and intergeneric hybrids (Parokonny *et al.*, 1997; King *et al.*, 1999). However, GISH has not been used previously for studying meiotic behaviour in diploid *H. vulgare* × *H. bulbosum* hybrids. GISH is therefore a valuable additional technique to conventional genome analysis, and has enabled us to readily clarify some aspects of the reduced recombination in hybrids between *H. vulgare* and *H. bulbosum*.

Our observations have confirmed the lower level of meiotic pairing in hybrid 103K5 and shown that there is a higher frequency of recombination as measured by recombinant *H. vulgare* chromosomes at AI in hybrid 102C2. In both hybrids there was more pairing at MI compared with the recombination frequency at AI and there are at least three possible explanations for this discrepancy (see also Benavente *et al.*, 1996, 1998).

First, there may be a limitation in the ability of GISH detection in the degree of resolution of fluorescence microscopy on meiotic chromosome preparations at AI, as chromosomes of these hybrids at AI are more highly condensed than at MI. Secondly, the size of introgressed segments is related to their ease of detection. Jiang &
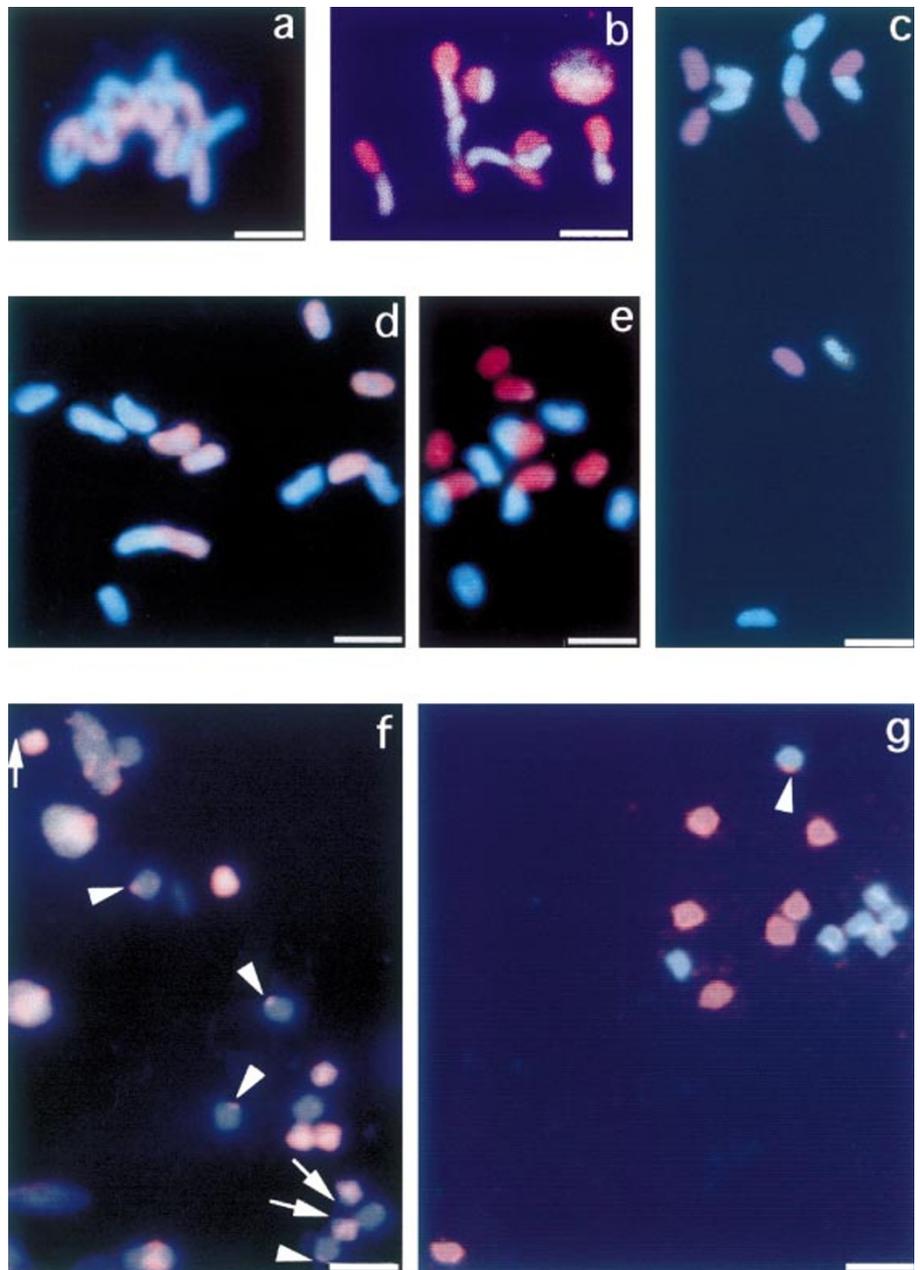

Fig. 1 GISH using *Hordeum bulbosum* DNA as probe on meiotic metaphase I (a – e) and anaphase I (f, g) chromosome preparations in PMCs of the barley × *H. bulbosum* hybrids 102C2 and 103K5. The labelled *H. bulbosum* chromosomes appear red and barley chromosomes are blue with the DAPI counterstain. Arrowheads indicate recombinant barley chromosomes. Arrows indicate recombinant *H. bulbosum* chromosomes. (a) Hybrid 102C2 with four ring and two rod bivalents; (b) hybrid 102C2 with two ring and five rod bivalents; (c) a 12 chromosome cell of hybrid 103K5 with two rod bivalents; (d) a 13 chromosome cell of hybrid 103K5 with two rod bivalents; (e) complete chromosome complement of hybrid 103K5 at MI with one ring and two rod bivalents; (f) hybrid 102C2 with four recombinant barley chromosomes (arrowheads) and three recombinant *H. bulbosum* chromosomes (arrows); (g) hybrid 103K5 with one recombinant barley chromosome (arrowhead). Bars represent 10 μm.

Table 2 Comparison of recombination frequencies at AI in hybrids between barley and *Hordeum bulbosum*

Hybrid	No. of PMCs	No. of recombinant *H. vulgare* chromosome arms per PMC									Mean of all cells	Mean of cells showing recombination
		0	1	2	3	4	5	6	7	Total		
102C2	273	194	37	26	12	2	1	0	1	145	0.53	1.84
103K5	110	81	22	7	0	0	0	0	0	36	0.33	1.24

Gill (1994) reported that in the successful cases of mapping low- or single-copy sequences, the targeted DNA sequences on chromosomes with reliable frequency of signal detection were all more than 10 kb long. Thus small introgressed segments may go undetected. The third possible explanation to account for this discrepancy is that a majority of bound arms between chromosomes of different parental genomes at MI in hybrids may be attributable to remnants of prophase pairing which is not related to crossing-over. We believe that the third explanation is the most likely and is consistent with the observations in a study of wheat–rye hybrids by Orellana (1985), and if proven, would establish that the paucity of recombinant progeny from *H. vulgare* × *H. bulbosum* hybrids results largely from low levels of crossing-over between chromosomes of different parental genomes.

In 102C2 and 102K5, rod bivalents predominate (Tables 1 and 3), but even in 102C2, which had almost complete bivalent formation, there is less chromosome pairing at MI than in cultivated barley. In barley seven ring bivalents are usually observed in most cells (Nilsson & Pelger, 1991). Recombinant segments at AI confirmed that crossing-over occurs distally, as is the case in barley (Pedersen *et al.*, 1995), and suggested that there was a reduced recombination in the proximal regions of the chromosome arms. GISH results on root-tip chromosome preparations of recombinants from the progeny of *H. vulgare* × *H. bulbosum* hybrids also revealed that introgressions of *H. bulbosum* chromatin are located distally (Pickering *et al.*, 1997; Pickering, unpubl. obs.).

Although the high-pairing hybrid 102C2 showed higher recombination frequency than the low-pairing hybrid 103K5, when a comparison is made of the recombination frequency in comparison to the number of bound arms at MI, recombination frequency is then higher in 103K5 than 102C2. In other words, when the chromosomes of the hybrids are paired at MI, they are then more likely to undergo recombination in 103K5 than 102C2. This seems at variance to what might have been expected and at present we have no ready explanation for the phenomenon. It is possible that the localization of chiasmata is less precisely controlled in 103K5, which results in the formation of more interstitial chiasmata and the exchange of larger segments, which are then more readily detected by GISH (see above).

We have also found that of the three types of PMCs in 103K5, type A had a significantly higher proportion of bound arms than types B and C, as would be expected, but there was no significant difference in the number of bound arms between types B and C. It is possible therefore that a high formation of bound arms promotes the retention of *H. bulbosum* chromosomes and maintains the stability of chromosome numbers and pairing. Support for this suggestion came from the analysis of 90 different genotypes of diploid *H. vulgare* × *H. bulbosum* hybrids (Pickering, unpubl. obs.), where a negative correlation ($r = -0.528$, $P < 0.01$) between the number of univalents and mean chromosome number was found.

In summary, the occurrence of chromosome pairing provides a basis for recombination in *H. vulgare* × *H. bulbosum* hybrids, albeit at a lower frequency than in *H. vulgare* itself. Because high meiotic pairing seems to be associated with high recombination in the hybrids, selection of high-pairing hybrids has resulted in the development of recombinant barley plants with introgressed *H. bulbosum* chromatin. Several of these recombinants possess resistance to fungal and/or viral

Table 3 Comparison of frequencies of MI pairing configurations in PMCs of *Hordeum* hybrid 103K5 with different chromosome numbers

Type of PMCs	No. of PMCs	Percentage (%)	Mean bound arms per PMC	Mean bound arms per potential II	MI pairing configurations			
						II		
					I	Rods	Rings	Total II
A	84	48.6	2.95	0.42	732 (8.7)*	196 (2.33)	26 (0.31)	222 (2.64)
B	67	38.7	1.95	0.33	631 (9.4)	109 (1.63)	11 (0.16)	120 (1.71)
C	22	12.7	1.69	0.34	198 (9.0)	29 (1.32)	4 (0.18)	33 (1.5)

Type A, PMCs with 14 chromosomes containing 7 *Hordeum vulgare* chromosomes (7 *V*) and 7 *H. bulbosum* chromosomes (7*B*).
Type B, PMCs with 13 chromosomes containing 7 *V* and 6*B*.
Type C, PMCs with 12 chromosomes containing 7 *V* and 5*B*.
*Mean MI pairing configurations per PMC in parentheses.

pathogens and will provide breeders with a novel source of resistance genes.

Acknowledgements

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