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Identification of *Hordeum vulgare* – *H. bulbosum* recombinants using cytological and molecular methods

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy,
School of Biological Sciences
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Barley (*Hordeum vulgare* L. subsp. *vulgare*) is an important crop and ranks fourth in overall production of the major cereal crops in the world. Like other cereal crops, barley suffers from a narrowing of its genetic base and susceptibility to diseases, pests and environmental stresses. *H. bulbosum* is a possible source of desirable genes for introgressing into barley to restore genetic diversity and improve current cultivars. Sexual hybridisation between barley and *H. bulbosum* is the main method for interspecific gene transfer in barley breeding but there are several barriers to overcome. Two of these are reduced recombination and the ability to identify recombinants quickly and efficiently. The aim in this thesis was to gain a better understanding of meiotic chromosomal behaviour in the two species and their hybrids and to improve the characterisation of recombinants from the hybrids.

To study the events during meiosis, synaptonemal complex (SC) analysis was carried out on the two species and two *H. vulgare - H. bulbosum* hybrids. The results indicated that there were interspecific and intraspecific variations in SC length. Mean SC length was positively correlated with recombination frequency but not related to genome size. This suggests that the ratios of mean SC length to genome size (SC/DNA) show divergence among these *Hordeum* examples. An hypothesis based on the conformation of chromatin associated with axial element, which is dependent on SC/DNA ratio, was presented to explain the relationship between SC length and recombination frequency.

Chromosome pairing in the two hybrids was determined by observation at pachytene and metaphase I (MI). Mean percentages of synapses were similar but there were different frequencies of MI pairing between these two hybrids, indicating that different mechanisms may regulate synapsis and MI pairing in the hybrids.
To investigate meiotic recombination, genomic *in situ* hybridisation (GISH) was performed on the two hybrids at MI and anaphase I (AI). It was observed that intergenomic pairing and recombination events occur in distal chromosome segments. A great discrepancy between mean pairing and recombination frequencies was observed in both hybrids and several possible reasons for this discrepancy were discussed. Hybrid 102C2 with high MI pairing had a significantly higher recombination frequency than the low pairing 103K5, suggesting that high MI pairing appears to be associated with high recombination in the hybrids. An interesting finding is that the ratio of recombination to MI pairing in 103K5 (1 : 8.9) is twice as high compared with 102C2 (1 : 17). However, the mechanism for this difference in the ratio between the two hybrids remains unknown.

Sequential fluorescence *in situ* hybridisation (FISH) and GISH were used successfully to localise the introgressions in selfed progeny from a tetraploid hybrid derived from chromosome-doubled 102C2 (102C2/colch). This procedure is fast, cheap and can efficiently detect and locate introgressions. Several disease-resistant recombinants were analysed in more details and leaf rust and powdery mildew resistance was associated with distal introgressions on chromosomes 2HS and 2HL (leaf rust) and 2HS (powdery mildew). It is possible that the leaf rust and powdery mildew resistances were closely linked in the distal region of 2HS. A considerable variation in introgression size was observed at similar chromosomal sites among the different recombinants, which will provide useful information for map-based cloning of genes.
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Abbreviations

μg  Microgram
μl  Microlitre
AFLP  Amplified fragment length polymorphism
AI  Anaphase I
AP  Alkaline phosphatase
bp  Base pair
CTAB  Cetyltriethylammonium bromide
CV  Coefficient of Variation
cv.  Cultivar
DAPI  4’,6-diamidino-2-phenylindole
DIG  Digoxigenin
Fig.  Figure
FISH  Fluorescent in situ hybridisation
GISH  Genomic in situ hybridisation
HNPP  2-hydroxy-3-naphthoic acid-2’-phenylanilide phosphatase
kb  Kilobase
mg  Milligram
MI  Metaphase I
ml  Millilitre
nm  Nanometre
NOR  Nucleolar Organiser Region
PCR  Polymerase chain reaction
pg  Picogram
PI  Propidium iodide
PMC  Pollen Mother Cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PSI</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>PTA</td>
<td>Phosphotungstic acid</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random amplified polymorphic DNA</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SC</td>
<td>Synaptonemal complex</td>
</tr>
<tr>
<td>SC/DNA</td>
<td>Ratio of mean SC length to genome size</td>
</tr>
<tr>
<td>UP</td>
<td>Uranyl acetate and lead citrate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>v/v</td>
<td>Volume per volume</td>
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<tr>
<td>w/v</td>
<td>Weight per volume</td>
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Chapter one

General introduction

1.1 Importance of barley

Barley (Hordeum vulgare L. subsp. vulgare) is a founder crop of Old World Neolithic food production and one of the earliest crops to be domesticated (Harlan & Zohary, 1966; Harlan, 1968). It ranks fourth in overall production of the major cereal crops after wheat, maize, and rice. World production has increased steadily for over 30 years, although recently it has declined, but in Australia production has continued to rise due to the increasing demand for malt and malting barley in China and other countries in Southeast Asia (Edney, 1996). In New Zealand barley outranks wheat in total cultivated area but its contribution to world production is minor (ca 80000 ha). It has three unique characteristics (Poehlman, 1985): 1) broad ecological adaptation, 2) utility as a feed and food grain, and 3) superiority of barley malt for use in brewing. In the future, it may become even more important as the public increasingly becomes aware of its ability to reduce blood cholesterol and consequently the incidence of heart disease (Edney, 1996).

1.2 Origin and adaptability of barley

It is generally regarded that cultivated barley originated from wild barley by mutations that gave rise to the non-brittle rachis (Harlan & Zohary, 1966). Wild barley, H. vulgare subsp. spontaneum (C. Koch) Thell., is an annual two-rowed diploid (2n = 14) with a brittle rachis and rough awns (Takahashi, 1955). Barley was first cultivated in the Fertile Crescent of the Middle East, possibly as early as 10,000 BC (Harlan, 1968). It is among the most widely adapted of the main agricultural crops in the world and well known for its tolerance to low temperatures, poor soils, including saline and alkaline ones, and drought (Poehlman, 1985). This has led to its wide cultivation in many parts of the world. Because of its broad
adaptability and short growing season with a high yield potential, it grows in many different environments, including extremes of latitude and altitude where other crops are not adapted (Harlan, 1976). For instance, it has been found growing on Himalayan slopes at an altitude of 5,000 metres and in the Nile River delta above the salt water table (Fedak, 1994). However, barley is most productive where there is a relatively high rainfall, low relative humidity, and cool temperatures during grain maturation.

### 1.3 Types of barley

Cultivated barley is a highly variable plant. It is primarily a self-pollinated crop although there is around 1% out-crossing per generation in cultivated barley (Allard, 1988). This trait was probably inherited from *H. vulgare* subsp. *spontaneum*, which has considerable variability in out-crossing rate (Brown, 1978). Barley can be divided into two-rowed and six-rowed types based on its kernel row number. It is believed that all cultivated barley originated from two-rowed barley and that six-rowed barley was selected originally from the two-rowed types. Archaeological findings showed that two-rowed barley tended to grow in dry areas whereas the six-rowed type grows in wet climates (Harlan, 1968). In general, two-rowed barley tends to malt better while six-rowed types yield better, although there are some exceptions (Edney, 1996). Barley can also be classified into hulled and hull-less forms depending on whether its kernel has tightly adhering or loose hulls, and into winter and spring types according to its growth habit (Briggs, 1978; Martin, 1976).

### 1.4 The genus *Hordeum*

*Hordeum* is the second largest genus in the tribe Triticeae of the grass family, Poaceae (Bothmer *et al.*, 1991). This tribe contains several economically important annual cereal crops, including wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.) and *X*Triticosecale Wittmack (their interspecific hybrid), along with several important perennial forage grasses such as Russian wild rye (*Psathyrostachys juncea* (Fisch.) Nevski), the crested wheat-grasses (*Agropyron cristatum* (L.) Gaertn), Altai wild rye (*Leymus angustus* (Trinius) Pilger) and

The morphology of *Hordeum* is very specialised in comparison with most other genera in the Triticeae. The typical morphological character of *Hordeum* species is the three one-flowered spikelets, known as triplets, which are borne together at each rachis node. The central spikelet is both pollen and seed fertile. The lateral spikelets may be fertile or sterile. In many wild *Hordeum* species the lateral spikelets are often sterile (Bothmer, 1992). The genus comprises about 30 wild species, both annual and perennial, with a wide range of ploidy levels from diploid to hexaploid (Table 1.1).

Many of the wild species of *Hordeum* are known to contain interesting agronomic traits that have the potential to be used for barley improvement. After extensive screening, resistances to diseases and insects, such as powdery mildew (*Erysiphe graminis*), leaf rust (*Puccinia hordei*), snow mould (*Typhula incamata*), loose smut (*Ustilago nuda* and *U. tritici*), Russian wheat aphids (*Diuraphis noxia*), scald (*Rhynchosporium secalis*), barley yellow dwarf virus, yellow rust and brown rust, have been found in various wild *Hordeum* species (Kuckuck, 1934; Kasha & Sadasivaiah, 1971; Nielsen, 1978; Brown, 1990; Jana & Nevo, 1991; Bothmer, 1992). In addition to resistance, many other important agronomic traits such as earliness, salt tolerance, cytoplasmic male sterility, leaf pubescence, outbreeding habit, high protein and lysine content have been found in this genus (Schooler, 1967, 1974; Orton, 1979; Nevo et al., 1984). The transfer of these useful agronomic traits from wild species into cultivated barley is highly desirable.

The classification of *Hordeum* has to some extent been controversial. Based on the meiotic pairing behaviour of different interspecific hybrids within this genus, *Hordeum* was divided into four "basic genomes" designated "I", "X", "Y" and "H" (Bothmer et al., 1986, 1987, 1991). This classification has been generally accepted.
Genome “T” consists of *H. vulgare* subsp. *vulgare*, *H. vulgare* subsp. *spontaneum* and *H. bulbosum*. *H. vulgare* subsp. *spontaneum* is the only wild *Hordeum* species that is completely cross-compatible and fully interfertile with cultivated barley, and hybrids between them show normal chromosome pairing and segregation in meiosis (Harlan, 1976; Asfaw & Bothmer, 1990). Furthermore, there is a high degree of morphological similarity between the two subspecies but *H. vulgare* subsp. *spontaneum* has a brittle rachis and is always two-rowed (Nevo, 1992). Except for wild barley, *H. bulbosum* is regarded as the most closely related species to cultivated barley on electrophoretic (Jørgensen, 1986), morphological and cytological (Bothmer *et al.* 1981, 1983) grounds. *H. bulbosum* can also be hybridised easily with barley and there is variable meiotic pairing among the hybrids (Kasha & Sadasivaiah, 1971; Bothmer *et al.*, 1983; Thomas & Pickering, 1985). *H. bulbosum* occurs naturally as two cytotypes, diploid (2n = 2x = 14) and tetraploid (2n = 4x = 28). There is no clear-cut morphological difference between the two cytotypes (Katznelson & Zohary, 1967). It is an outbreeding perennial species and its tetraploid cytotype is autotetraploid, which shows a high frequency of multivalents (Lundqvist, 1962).

There has been a suggestion to classify *H. vulgare* and *H. bulbosum* into a genus of their own (*Hordeum* in a narrow sense), all the other species being placed in a separate genus, *Critesion* (Dewey, 1984). However, this classification has not been widely accepted.

Of the two annual Mediterranean species, *H. marinum* and *H. murinum*, the former occurs as diploid and tetraploid cytotypes, the latter as diploid, tetraploid and hexaploid cytotypes. These two species are distantly related to each other and to the genome “T” as well as other *Hordeum* species (Bothmer *et al.*, 1986, 1988, 1989a, b, 1992). Thus the genomes of these two species have been designated as “X” (*H. marinum*) and “Y” (*H. murinum*) by Bothmer *et al.* (1986, 1987). Cytogenetic data has shown a close relationship between the genomes of the polyploid cytotypes within both *H. marinum* and *H. murinum* (Bothmer *et al.* 1988,
1989a, b), suggesting that both species probably contain autoploid races.

The remaining diploid *Hordeum* species (see Table 1.1) are all closely related and share the same basic genome, which is designated as “H”. Nearly all remaining polyploids are segmental allopolyploids in which the genomes are closely related (Bothmer, 1992).

According to Bothmer *et al.* (1983), all hybrids between barley and wild *Hordeum* species from genomes “H”, “X” and “Y” show complete sterility, very low pollen fertility (less than 2%) and a low frequency of chromosome pairing. This indicates that direct sexual hybridisation for gene transfer between cultivated barley and these wild *Hordeum* species is likely to be unsuccessful.

Classification by conventional genome studies has been mainly supported by studies of isoenzyme variation (Jørgensen, 1986), C-banding patterns (Linde-Laursen *et al.* 1980, 1989, 1990), and molecular phylogenesis (Gupta *et al.* 1989; Molnar *et al.* 1989, 1992; Xu *et al.* 1990; Doebley *et al.* 1992; Shcherban' & Vershinin 1992; Svitashev *et al.* 1994; Marillia & Scoles 1996). However, there are minor disagreements with the conventional classification. Hsiao *et al.* (1986) noted that the karyotypes of *H. vulgare* and *H. bulbosum* differed significantly. Furthermore, Gupta *et al.* (1989) pointed out that *H. vulgare* and *H. bulbosum* were not very closely related based on repetitive DNA sequence (pSc119) hybridisation patterns. Molnar *et al.* (1992) proposed that *H. bulbosum* was more closely related to the *H. murinum* complex than to *H. vulgare*. Studies based on hybridisation patterns of six cloned repetitive DNA sequences (Svitashev *et al.*, 1994) and RAPD analysis (Marillia & Scoles, 1996), also supported the above view that *H. vulgare* and *H. bulbosum* are distinctly different.
Table 1.1. The Species/taxa of *Hordeum* (Basic chromosome number \( x = 7 \)).

<table>
<thead>
<tr>
<th>Species/taxon</th>
<th>Ploidy level</th>
<th>Distribution</th>
<th>Life form</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. vulgare</em> L. subsp. <em>vulgare</em></td>
<td>2x (4x)</td>
<td>cultivated</td>
<td>A</td>
</tr>
<tr>
<td><em>H. bulbosum</em> L.</td>
<td>2x, 4x</td>
<td>Mediterranean</td>
<td>P</td>
</tr>
<tr>
<td><em>H. murinum</em> L. subsp. <em>murinum</em></td>
<td>4x</td>
<td>Mediterranean</td>
<td>A</td>
</tr>
<tr>
<td>subsp. <em>glaucum</em> (Steud.) Tzvelev</td>
<td>2x</td>
<td>Mediterranean</td>
<td>A</td>
</tr>
<tr>
<td>subsp. <em>leoporiun</em> (Link) Arc.</td>
<td>4x, 6x</td>
<td>Mediterranean</td>
<td>A</td>
</tr>
<tr>
<td><em>H. maritinum</em> Huds. Subsp. <em>marinum</em></td>
<td>2x</td>
<td>Mediterranean</td>
<td>A</td>
</tr>
<tr>
<td>subsp. <em>gussoniun</em> (Parl.) Thell.</td>
<td>2x, 4x</td>
<td>Mediterranean</td>
<td>A</td>
</tr>
<tr>
<td><em>H. muticum</em> Presl.</td>
<td>2x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. cordobense</em> Bothmer et al.</td>
<td>2x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. chilenense</em> Roem. &amp; Schult.</td>
<td>2x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. stenostachyus</em> Godr.</td>
<td>2x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. erectolium</em> Bothmer et al.</td>
<td>2x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. flexuosum</em> Steud.</td>
<td>2x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. euclaston</em> Steud.</td>
<td>2x</td>
<td>South America</td>
<td>A</td>
</tr>
<tr>
<td><em>H. intercedens</em> Nevski</td>
<td>2x</td>
<td>Western United States</td>
<td>A</td>
</tr>
<tr>
<td><em>H. pusillum</em> Nutt.</td>
<td>2x</td>
<td>North America</td>
<td>A</td>
</tr>
<tr>
<td><em>H. jubatum</em> L.</td>
<td>4x</td>
<td>North America, East Asia</td>
<td>P</td>
</tr>
<tr>
<td><em>H. comosum</em> Presl.</td>
<td>2x</td>
<td>Central Asia</td>
<td>P</td>
</tr>
<tr>
<td><em>H. pumilum</em> Hook. f.</td>
<td>2x</td>
<td>Central Asia</td>
<td>P</td>
</tr>
<tr>
<td><em>H. lechleri</em> (Steud.) Schenck</td>
<td>6x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. procerum</em> Nevski</td>
<td>6x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. arizonicum</em> Covas &amp; Stebbins</td>
<td>6x</td>
<td>Southwest United States</td>
<td>A/P</td>
</tr>
<tr>
<td><em>H. secalinium</em> Schreb.</td>
<td>4x</td>
<td>Europe (North Africa)</td>
<td>P</td>
</tr>
<tr>
<td><em>H. bogdani</em> Wil.</td>
<td>2x</td>
<td>Central Asia</td>
<td>P</td>
</tr>
<tr>
<td><em>H. roshevitzii</em> Bowden</td>
<td>2x</td>
<td>Central Asia</td>
<td>P</td>
</tr>
<tr>
<td><em>H. brevisubulatum</em> (Trin.) Link subsp. <em>brevisubulatum</em></td>
<td>2x, 4x</td>
<td>East Asia</td>
<td>P</td>
</tr>
<tr>
<td>subsp. <em>violaceum</em> (Boiss. &amp; Hohen.) Tzvelev</td>
<td>2x, 4x</td>
<td>Southwest Asia</td>
<td>P</td>
</tr>
<tr>
<td>subsp. <em>turbanianum</em> (Nevski) Tzvelev</td>
<td>6x</td>
<td>Central Asia</td>
<td>P</td>
</tr>
<tr>
<td>subsp. <em>nevskianum</em> (Bowden) Tzvelev</td>
<td>4x</td>
<td>West Asia</td>
<td>P</td>
</tr>
<tr>
<td>subsp. <em>iranicum</em> Bothmer</td>
<td>6x</td>
<td>Western North America, East Asia</td>
<td>P</td>
</tr>
<tr>
<td><em>H. brachyantherum</em> Nevski</td>
<td>4x, 6x</td>
<td>Western United States</td>
<td>A</td>
</tr>
<tr>
<td><em>H. californicum</em> Covas &amp; Stebbins</td>
<td>2x</td>
<td>Western United States</td>
<td>A</td>
</tr>
<tr>
<td><em>H. depressum</em> (Scribn. &amp; Sm.) Rydb.</td>
<td>4x</td>
<td>Central America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. guatemalense</em> Bothmer et al.</td>
<td>4x</td>
<td>South Africa</td>
<td>P</td>
</tr>
<tr>
<td><em>H. capense</em> Thunb.</td>
<td>4x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. parodi</em> Covas</td>
<td>6x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. tetrapioloides</em> Covas</td>
<td>4x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. foetidum</em> Bothmer et al.</td>
<td>4x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td><em>H. patagonicum</em> (Haum.) Covas subsp. <em>patagonicum</em></td>
<td>2x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td>subsp. <em>mustersii</em> (Nicora) Bothmer et al.</td>
<td>2x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td>subsp. <em>santacruzense</em> (Parodi &amp; Nicora) Bothmer et al.</td>
<td>2x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td>subsp. <em>setifolium</em> (Parodi &amp; Nicora) Bothmer et al.</td>
<td>2x</td>
<td>South America</td>
<td>P</td>
</tr>
<tr>
<td>subsp. <em>maggellanicum</em> (Parodi &amp; Nicora) Bothmer et al.</td>
<td>2x</td>
<td>South America</td>
<td>P</td>
</tr>
</tbody>
</table>


A, annual; P, perennial

*H. arizonicum* is sometimes biennial or even with a shorter life cycle.
1.5 Cytogenetic studies on barley

Barley is a diploid plant with a large genome size of approximately 5.5 pg/1C nucleus. Extensive studies by Bennett and Leitch (1995) found no intraspecific variation in its genome size. It has been estimated that 70 - 80% of the barley genome consists of repeated nucleotide sequences; about 6% of the genome consists of inverted repeats ranging from 300 to 3000 bp in size, 10 - 20% as tandem repeats, and 50 - 60% as repeats that are interspersed. It is estimated that there are about 70,000 structural genes in the barley genome (Fedak, 1994).

It has a small number of chromosomes (2n = 2x = 14), which are comparatively large (8 - 10 μm at metaphase of mitosis). Giemsa banding patterns based on mitotic chromosome preparations have been used to identify the individual chromosomes and chromosome arms in barley. The common basic C- and N-banding patterns on barley have been reported by many researchers (Linde-Laursen, 1975; Noda & Kasha, 1978; Islam, 1980; Singh & Tsuchiya, 1982a, b; Fukui & Kakeda, 1990). The discrepancies in the reported banding patterns were clarified by Kakeda et al. (1991) who presented detailed descriptions of the C- and N-banding patterns of cultivated barley.

There are five pairs of non-satellited chromosomes and two pairs of satellited chromosomes in barley. The former were traditionally numbered from chromosome 1 to chromosome 5 based on chromosome length, arm ratio and C- or N-banding pattern. Chromosomes 6 and 7 are satellited chromosomes, with the former being shorter in length but with a larger satellite than chromosome 7.

Based on data of barley chromosome length and arm ratios of chromosomes 1 to 5 (Fukui & Kakeda, 1990; Jensen & Linde-Laursen, 1992), chromosome 1 is the third longest chromosome with an arm ratio of 0.97 - 1.04; chromosome 2 is the longest chromosome with
an arm ratio of about 0.83; chromosome 3 is the second largest with an arm ratio of 0.74 - 0.78; chromosome 4 has an arm ratio of 0.81 - 0.9 and is similar in length to chromosomes 1 and 3; chromosome 5 is the shortest with a similar arm ratio of 0.7 - 0.74 to chromosome 3. For the satellited chromosomes, chromosome 6 has an arm ratio of about 0.92 - 1.08 including the satellite. Chromosome 7 has the longest long arm of all the barley chromosomes and an arm ratio of 0.65 - 0.73 including the satellite.

With the successful production of wheat-barley hybrids, Islam and Shepherd (1990) determined homoeology of barley chromosomes to wheat using morphological, protein and isozyme markers. Barley chromosomes 1 - 7 correspond to wheat chromosomes 7, 2, 3, 4, 1, 6, and 5, respectively, and are designated 7H, 2H, 3H, 4H, 1H, 6H, and 5H. This numbering of barley chromosome might eventually be consistently applied throughout the whole Triticeae tribe, which consists of 200-300 species including crop plants and wild relatives.

Recently Pedersen & Linde-Laursen (1994) performed fluorescence in situ hybridisation with an oligonucleotide probe (GAA) to produce banding patterns similar to C- and N-banding which could be used for identifying all the barley chromosome arms. This technique permits a higher resolution of the physical localisation of a DNA probe or alien chromosome segment on individual barley chromosomes using a two-step in situ hybridisation instead of sequential C- or N-banding and in situ hybridisation.

By applying a conventional cytogenetic study of rDNA location using silver staining, Linde-Laursen (1984) proposed that there are minor nucleolar organiser regions on chromosomes in addition to 5H and 6H having major ones. This was verified by molecular methods using rDNA clones pTa71 and pTa794 (isolated from wheat) as probes that hybridised to five other pairs of non-satellited chromosomes as well as to chromosomes 5H and 6H (Leitch & Heslop-Harrison, 1992, 1993; Pedersen & Linde-Laursen, 1994).
Chromosome pairing at metaphase I is a consequence of chiasma formation between homologous chromosomes or chromosome segments. In barley, chromosome pairing has been shown to be initiated at or near the ends of chromosomes as opposed to the centromeric region (Kasha & Burnham, 1965). It has also been shown that there is little variation in chiasma frequency between the different subspecies of barley, and the mean chiasma frequency is close to fourteen (Gale & Rees, 1970; Nilsson & Pelger, 1991). In rye there is a control mechanism preferentially leading to two distally located chiasmata per bivalent, one in each arm (Jones, 1987). Nilsson and Pelger (1991) pointed out that there could be a similar control mechanism existing in barley, which is influenced by the genetic background. If this is confirmed, the cross-over distribution among bivalents is strictly regulated to be distally localised.

1.6 Barley improvement by introgressing genes from H. bulbosum

Barley (H. vulgare subsp. vulgare, hereafter referred to as H. vulgare for convenience), which used to be cultivated as highly heterogeneous landraces with high levels of genetic diversity, has been gradually replaced by higher yielding cultivars over the past 30 years (Nevo, 1992). Successive selection of new cultivars from the crosses between established pure lines has resulted in a marked narrowing of the genetic base of modern cultivars in many advanced agricultural areas. Loss of genetic diversity of barley has accelerated in recent decades and threatens to make the crop increasingly susceptible to diseases, pests and environmental stresses (Harlan, 1976).

In order to restore genetic diversity and improve current cultivars, breeders have tried to introduce important agronomic traits from closely related wild Hordeum species into cultivated barley. Molecular genetic technologies also offer the possibility of transferring alien genes into cultivated barley, but a number of impediments exist to the widespread use of this technology. In general, the procedure is still too inefficient and limited to only a few varieties and as a result, most elite breeding lines have not been used successfully for genetic
transformation. Therefore, conventional sexual hybridisation technology is still essential for barley improvement. *H. vulgare* subsp. *spontaneum*, which is an important source of useful agronomic traits, has been used successfully for gene transfer by interspecific hybridisation (Lehmann & Bothmer, 1988; Ceccarelli, 1989; Ellis *et al.*, 1991). However, despite belonging to the primary gene pool and some successful gene transfer experiments, it is laborious and time-consuming to use this species in practical barley breeding programmes (Bothmer, 1996).

The secondary gene pool, which consists of one species *H. bulbosum*, is another important source of agronomic traits that is attractive to the barley breeder. These include: winter hardiness, disease and pest resistance such as resistance to powdery mildew, yellow rust, brown rust and Russian wheat aphid (Xu & Snape, 1989; Zeller, 1998), allogamy (Szigat & Pohler, 1982), and anther extrusion (Lange & Jochemsen, 1976a; Xu & Snape, 1989). Interspecific hybridisation between *H. vulgare* and *H. bulbosum* remains the best approach for the transfer of useful agronomic traits from *H. bulbosum* into cultivated barley at the current time.

Successful crosses between *H. vulgare* and *H. bulbosum* were first made by Kuckuck (1934), who obtained one sterile triploid hybrid from *H. vulgare* (2n = 2x =14) and *H. bulbosum* (2n = 4x = 28). Since this early work, Konzak *et al.* (1951) produced 11 triploid hybrids using embryo culture techniques to rescue immature embryos and produce plants. Since then a number of successful crosses between these two species have been reported (Morrison & Rajhathy, 1959; Symko, 1969; Kasha & Kao, 1970; Lange, 1971a; Lange & Jochemsen, 1976a; Szigat & Pohler, 1982; Bothmer *et al.*, 1983), but hybrids from these crosses have almost always been completely sterile. Pickering (1988) obtained several triploids (‘VBB’) that showed full dehiscent anthers with 45-79% potential pollen germinability (V = the *H. vulgare* genome and B = the *H. bulbosum* genome). The genomic symbols used here follow Kasha and Sadasivaiah (1971) and Lange (1971b). Recently, a few successes in resistance gene transfer of *H. bulbosum* into *H. vulgare* have been reported (Xu & Kasha, 1992;
Michel, 1995; Pickering et al., 1995). However, there are several problems preventing successful gene transfer by interspecific hybridisation between H. vulgare and H. bulbosum. These include hybrid chromosome instability, variability of inter-genomic pairing, hybrid infertility, and low inter-genomic chromosome recombination (Pickering, 1991).

1.7 Chromosome instability in H. vulgare - H. bulbosum hybrids

Following interspecific hybridisation between barley and H. bulbosum, several authors observed that most progeny did not contain the full complement of both parental chromosome sets (Symko, 1969; Kasha & Kao, 1970). Several hypotheses were put forward to explain this phenomenon including male parthenogenesis (Davies, 1958) and chromosome elimination (Symko, 1969; Kasha & Kao, 1970; Lange, 1971a, b). It is now generally accepted that the absence of one parental chromosome set is due to the elimination of H. bulbosum chromosomes during the development of embryos after fertilisation (Kasha & Kao, 1970). This results in the production of haploid H. vulgare embryos and haploid plants can be obtained by embryo rescue. Fertility can be easily restored in these haploids by colchicine treatment of tillers. Optimisation of the techniques at each stage of the process has enabled doubled haploid production to be extensively used for barley improvement and hastened the release of new cultivars as it is no longer necessary to undertake 6-7 generations of selfing to obtain similar levels of homozygosity (Pickering & Devaux, 1992).

The elimination of H. bulbosum chromosomes is now known to depend on (1) genetic factors (Ho & Kasha, 1975); (2) genome ratios (Subrahmanyam & Kasha, 1973); and (3) temperature after fertilisation (Pickering, 1985). Subrahmanyam (1982) proposed a hierarchical system in Hordeum based on species dominance to explain differences between crosses and therefore chromosomes of the non-dominant species are eliminated.

1. Genetic factors. Ho & Kasha (1975) reported that genes on H. vulgare chromosomes 2 and 3 were involved in the control of elimination of H. bulbosum chromosomes based on an
investigation of crosses between a set of trisomic lines of *H. vulgare* and a tetraploid *H. bulbosum*. Thomas and Pickering (1983) studied two amphidiploid hybrids (‘VVBB’) involving different genotypes of *H. vulgare* (‘Vada’ and ‘Emir’), and found that the chromosome elimination was dependent on the genotype of *H. vulgare*. They concluded that a gene(s) might exist in the genotype of ‘Vada’ that prevents the elimination of *H. bulbosum* chromosomes from the hybrid.

2. **Genome ratios.** In addition to genetic factors, genome ratios also influence chromosome stability. Kasha and Sadasivaiah (1971) and Lange (1971b) observed that different ploidy combinations were likely to favour either haploid or hybrid formation. For hybrid formation, the genome ratios of 1V : >1B are required; and haploid formation by *H. bulbosum* genome elimination often takes place when genome ratios are 1V:1B or 2V:1B. Subrahmanyam and Kasha (1973) suggested that the embryos from the cross combination (‘VV × BB’) showed a similar trend in chromosome elimination to that from the reciprocal cross (‘BB × VV’), whereas triploid embryos having a genomic constitution of ‘VBB’ are relatively stable and most stable endosperm tissues had a genome ratio of 1V:4B. Ho and Kasha (1975) suggested that the genetic factors on *H. vulgare* chromosomes, which control chromosome elimination, are balanced by factors on *H. bulbosum* chromosomes which, when present in sufficient dosage, neutralise the effects of the *H. vulgare* factors. This would explain why the genomic constitution in hybrids with a higher proportion of *H. bulbosum* genomes is more stable than those with lower proportions. However, the rare occurrence of triploid *H. vulgare - H. bulbosum* hybrids with a genome ratio of 2V:1B was reported by Pohler and Szigat (1982). This might depend on the specific cross combination of an *H. vulgare* genotype that is less effective at eliminating *H. bulbosum* chromosomes and an *H. bulbosum* genotype that is more strongly effective in neutralising the effect of eliminating genes in *H. vulgare*.

3. **Temperature effects.** Elimination of *H. bulbosum* chromosomes in the hybrids is also
affected by environmental factors. There have been several reports of the influence of
temperature on the elimination of the *H. bulbosum* genome. Humphreys (1978) found that
chromosome elimination took place more quickly at 30°C than at 25°C in the hybrids.
Pickering and Morgan (1983) noted that more hybrids were produced in winter than in
summer and Pickering (1984) found that a temperature of less than 15°C during the first 5-9
days of embryo development favoured the retention of *H. bulbosum* chromosomes. In further
experiments, Pickering (1985) demonstrated that a temperature below 17°C during the first
few days after pollination was more suitable for obtaining hybrids from diploid cross
combinations than a temperature above 20°C. It was also reported that in *H. vulgare - H. bulbosum*
diploid hybrids, there were slightly greater numbers of degraded chromosomes at
21°C compared with 15°C (Pickering, 1990). Thus, different temperatures appear to have a
marked influence on retention of *H. bulbosum* chromosomes. This might be due to the
effects of temperature on mitotic cycle times.

1.8 Meiotic pairing at metaphase I in *H. vulgare - H. bulbosum*
hybrids

Although the retention of *H. bulbosum* chromosomes and inter-genomic pairing in the hybrids
are equally vital for the efficient transfer of *H. bulbosum* chromatin into barley, less attention
has been paid to pairing. There have been several studies of inter-genomic pairing in the
interspecific hybrids, which showed that the pairing was influenced by parental genotype
(Thomas & Pickering, 1985) and seemed to depend on the interaction of different parental
interspecific hybrids with pairing ranging from an intermediate to a high frequency and
suggested that genetic factors regulated pairing behaviour. Xu and Snape (1988) later
confirmed that both genotypes, especially that of *H. bulbosum*, influenced inter-genomic
pairing in interspecific diploid and triploid hybrids. Furthermore, they noted that *H. vulgare*
chromosomes 2 and 4 were more frequently involved in inter-genomic pairing than the other
chromosomes and hence, might have a closer affinity with homoeologues in *H. bulbosum* than
other chromosomes in the genome.

In addition to genetic factors influencing inter-genomic pairing in the interspecific hybrids, the effect of environmental factors on inter-genomic pairing cannot be ignored. Temperature not only affects the retention of the *H. bulbosum* genome, as mentioned above, but also has an influence on inter-genomic pairing in the hybrid. Pickering (1990) found that meiotic pairing in most interspecific hybrids investigated was significantly greater at 21°C than at 15°C. In diploid hybrids there was a reduction in bivalent formation (especially ring bivalents) and in triploid hybrids ('VBB') a reduction in trivalent formation at lower temperatures. As Xu and Snape (1988) established that the trivalents in triploid hybrids ('VBB') were two *H. bulbosum* chromosomes paired with one *H. vulgare* chromosome, it was apparent that there was a greater inter-genomic pairing frequency in both diploid and triploid hybrids at a higher temperature of 21°C.

High levels of inter-genomic pairing and maximum retention of *H. bulbosum* chromosomes in the interspecific hybrids are both critical for introgressing potentially useful characters from *H. bulbosum* into cultivated barley. Since a higher temperature is desirable for obtaining maximum levels of inter-genomic pairing but is unfavourable for the retention of *H. bulbosum* chromosomes (Pickering, 1984, 1990), a compromise temperature for growing the hybrids has to be determined to obtain high inter-genomic pairing frequency and maximise the retention of *H. bulbosum* chromosomes in gene transfer.

### 1.9 Fertility in the *H. vulgare* - *H. bulbosum* hybrids

A common feature of all diploid and most triploid ('VBB') interspecific hybrids is that the plants are sterile (Bothmer et al., 1986, 1992) and produce few seeds on selfing (Xu & Kasha, 1992). This is one of the main reasons to account for limited success in gene transfer through interspecific hybridisation. Doubling the chromosome number by colchicine treatment of the stable diploid hybrids to produce amphidiploid hybrids ('VVBB') restores
their fertility but preferential homologous pairing results in selfed progeny resembling *H. vulgare* or hybrids (Lange & Jochemsen, 1976a; Thomas & Pickering, 1983). More recently, however, there have been two reports about obtaining barley recombinant plants among selfed progeny derived from fertile tetraploid hybrids (‘VVBB’) (Michel et al., 1995; Pickering et al., 1995), but stable transfers of *H. bulbosum* chromatin into the background of barley are still rare.

Xu and Snape (1988) reported high frequencies of inter-genomic pairing with up to five trivalents per cell in some triploid hybrids (‘VBB’) and showed that gene transfer might be possible throughout inter-genomic recombination during meiosis. However, infertility was frequently encountered in relatively stable triploid hybrids (‘VBB’) (Kasha & Sadasivaiah, 1971; Lange, 1971a), which prevents the use of triploid hybrids for gene transfer. Nevertheless, Pickering (1988) successfully produced partially fertile triploid hybrids (‘VBB’) and used these as a useful way to transfer genes from *H. bulbosum* into *H. vulgare*. For example, by backcrossing these fertile triploid hybrids to *H. vulgare*, backcross progenies comprised haploid and diploid *H. vulgare* plants, chromosome substitution lines (Pickering, 1992) and recombinant lines (Pickering et al., 1997). Xu and Kasha (1992) also reported stable transfer of mildew resistance from *H. bulbosum* into *H. vulgare* using fertile triploid hybrids (‘VBB’) in backcrosses to *H. vulgare*. However, recombinant genotypes among backcross progeny are less than would be expected from the chromosome pairing data.

### 1.10 Inter-genomic recombination in the *H. vulgare* - *H. bulbosum* hybrids

Although it is generally accepted that meiotic pairing at metaphase I (MI) can reflect recombination levels, inter-genomic pairing in interspecific hybrids does not necessarily result in recombination. Orellana (1985) proposed that most of the inter-genomic pairing at MI in wheat-rye hybrids is non-chiasmatic. Later, Benavente et al. (1996) confirmed that pairing levels greatly exceeded recombination frequencies in wheat-rye hybrids. It is possible that
this phenomenon in the wheat-rye hybrids also occurs in the *H. vulgare-H. bulbosum* hybrids.

Production of high pairing stable interspecific hybrids is relatively easy (Thomas & Pickering, 1985), but the most difficult outstanding problem for gene transfer through interspecific hybridisation is low recombination between these two *Hordeum* genomes. Pickering (1991) compared the recombination frequency in barley and *H. vulgare- H. bulbosum* hybrids using a paracentric inversion and found a reduced recombination between the parental genomes in the *H. vulgare - H. bulbosum* hybrids. This is the only report on study of recombination in the *H. vulgare - H. bulbosum* hybrids. However, the method using paracentric inversion to measure recombination was limited because it only involved one particular chromosome whereas recombination sites occur through the whole genome. Therefore, a method for measuring recombination frequency in the whole genome is needed to identify hybrids with high recombination frequencies, which could be used for gene transfer.

### 1.11 Objectives of this study

The three key objectives of this study were:

1. To make detailed observations of chromosome synapsis and synaptonemal complex (SC) length at meiotic prophase I using SC spreading techniques. This should give a better insight into problems associated with different recombination rates in the species and hybrids.

2. To obtain direct measurements of recombination frequency in *H. vulgare- H. bulbosum* hybrids by the application of genomic *in situ* hybridisation (GISH) to the analysis of anaphase I (AI) chromosomes.

3. To characterise putative recombinants from selfed progeny of the high-pairing *H. vulgare-H. bulbosum* hybrid quickly and efficiently using two step *in situ* hybridisation techniques in the mitotic chromosome preparations.
Chapter two

Materials and methods

2.1 Plant materials

The plant materials with their codes, genomic constitutions and pedigrees used in this study are listed in tables 2.1 & 2.2. All the clones and seeds of plants were kindly provided by Dr Richard Pickering, New Zealand Institute for Crop & Food Research Limited, Private Bag 4704, Christchurch.

The plants of barley, *H. bulbosum*, their hybrids and putative recombinants from the selfed progeny of the hybrids were grown in glasshouses at the School of Biological Sciences, the University of Auckland, Auckland. The temperature in the glasshouses was maintained at 20 - 22°C during the day; 16 hours natural light was supplemented when necessary with an irradiance of about 200 - 300 uE/m²/sec light intensity from 400W mercury vapours bulbs and 14 - 15°C during the night (8 hours). Humidity was kept at about 75% in the glasshouses. Regular repotting, watering and application of fertiliser were carried out to keep plants at optimal growth conditions.

For the synaptonemal complex (SC) study, *H. bulbosum* was vernalised at 4°C for 6 – 8 weeks and then 10 – 15°C for 2 weeks in a growth cabinet before being transferred to the glasshouse.
Table 2.1. *Hordeum vulgare* – *H. bulbosum* hybrids and their parental plant materials.

<table>
<thead>
<tr>
<th>Name/Crd</th>
<th>Genomic constitution</th>
<th>Pedigree</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. vulgare</em></td>
<td>VV</td>
<td>Emir</td>
</tr>
<tr>
<td><em>H. bulbosum</em></td>
<td>BB</td>
<td>Cb2920/4</td>
</tr>
<tr>
<td>102C2</td>
<td>VB</td>
<td>Emir (<em>H. vulgare</em>) × HB2032 (<em>H. bulbosum</em>)</td>
</tr>
<tr>
<td>103KS</td>
<td>VB</td>
<td>Emir (<em>H. vulgare</em>) × Cb3811/5 (<em>H. bulbosum</em>)</td>
</tr>
</tbody>
</table>

Table 2.2. Putative recombinants selected from hybrid 102C2 progeny

<table>
<thead>
<tr>
<th>Code</th>
<th>Comments</th>
</tr>
</thead>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>102C2/2/5/1</td>
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<td>102C2/5/3</td>
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<td>5</td>
<td>102C2/7/6</td>
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<td>102C2/13/2</td>
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</tr>
<tr>
<td>21</td>
<td>102C2/18/3</td>
</tr>
</tbody>
</table>
2.2 Methods

All chemical reagents used in this study are given in Appendix I.

2.2.1 Two-dimensional surface spread of SC from plant pollen mother cells (PMCs)

Two-dimensional SC spreads were prepared according to Albini et al. (1984) with some modifications. Fresh unfixed anthers were removed from the inflorescence, which had not emerged from leaf sheaths. One anther from each floret was squashed and stained in FLP orcein to determine the meiotic stage. When an anther was identified as being at pachytene under the light microscope, the remaining two anthers from the floret were collected and stored in distilled water at room temperature until sufficient anthers had been accumulated.

Five to ten fresh anthers with PMCs at pachytene were placed into 30 μl of digestion medium (0.4% cytohelicase) in the depression of a cavity slide. The contents of anthers were squeezed out into the medium, using the end of a brass rod and anther wall debris was removed carefully. The cell suspension was left for about 8 minutes, until the cell walls of the PMCs were digested, to produce a protoplast suspension. This procedure was monitored using phase contrast microscopy. The meniscus of the cell suspension was stirred with a brass rod within the first 4 minutes of digestion until the PMCs had detached from each other to accelerate digestion.

After cell wall digestion, the cell suspension was immediately transferred to 40 μl of 0.5% lipsol detergent solution on a flat microscope slide using a Pasteur pipette. The slide was tilted to mix thoroughly the cell suspension and lipsol, and left for 5 minutes before the addition of 80 μl of 4% paraformaldehyde. Then the mixed solution was spread using a clean Pasteur pipette, taking care to touch only the meniscus, and allowed to dry on the slide overnight in a fume hood.
The slide was washed gently in distilled water for about 30 seconds and then left to dry completely for at least 6 hours. Three staining methods were used in this study. For silver staining, a few drops of 33% or 50% silver nitrate were added to each dry slide and covered with a nylon coverslip (Nybot 3xxx or 68GG-243 fabric, Ure Pacific Traders, Avondale) then incubated in a moist chamber at 37°C or 60°C until the nylon cloth appeared golden-brown colour. After staining, the nylon coverslips were rinsed away with distilled water and the stained slides were washed for 3 minutes in deionised water and then air-dried. For phosphotungstic acid (PTA) staining, slides were immersed in 1% ethanolic PTA from 5 to 20 minutes to determine the optimal staining time, rinsed in 95% ethanol, and air dried. For uranyl acetate and lead citrate (UP) staining, slides were incubated in 1% aqueous uranyl acetate, rinsed in distilled water, incubated in Reynolds’ (1963) lead citrate, rinsed again, then air dried. Both incubation times ranged from 5 to 15 minutes for optimal staining.

Unmounted stained slides were scanned under low-power bright-field light microscopy (LM) to select for the promising slides with well-spread nuclei. The selected slides were immersed in a 0.75% solution of polystyrol in chloroform. The speed of retracting the slide determined the thickness of the coating and optimum thickness was decided by trial and error. The sites of well spread nuclei on the slides were marked using an indelible felt-tipped pen. The polystyrol film was scored around the marked nuclei using a diamond tipped pen. A few drops of 1% hydrofluoric acid were dropped on the scored lines to detach the polystyrol film plus the nuclei from the surface of the slide. The polystyrol film with nuclei was floated on the surface of clean water. Electron microscopy (EM) grids (GCu 50, Probing & Structure) were put carefully on the marked area of polystyrol film making sure they attached completely, then the polystyrol film with the attached grids was picked up by a piece of plastic coated paper (Benchcoat, Whatman). When the paper was dry, the grids plus polystyrol film were picked off and stored in a grid holder in a dry and dark place until required.
The EM grids with well-spread nuclei, which had been transferred successfully, were examined with a transmission electron microscope (Phillips CM12). Photomicrographs were taken at 890, 1150 and 1500x magnifications on a 35mm camera, using Copex PET 10 film. As the size of a nucleus is generally much bigger than the negative size, several photographs had to be taken and combined together to form the intact picture of a nucleus. After developing the film, negatives were scanned using a Nikon Scanner LS-1000 and images digitised. Different parts of the nucleus were combined using ADOBE PHOTOSHOP 3.0.5 software. The SC length measurements were carried out using a SUMMAGRAPHICS graphics tablet and CLARIS CAD software.

2.2.2 Meiotic chromosome preparation using enzyme digestion

Inflorescences, which had not emerged from the leaf sheath, were collected and fixed overnight in Carnoy's II fixative at 4°C. The inflorescences were then transferred to 70% ethanol and stored at -20°C until required.

Under the dissecting microscope, one anther from each floret was dissected out, stained on a slide and squashed in FLP orcein solution to determine the stage of meiosis. When appropriate stages were determined, the remaining two anthers from same floret were kept in the distilled water for later use.

These two anthers were then placed into 20 µl of enzyme mix (4% cellulase and 1.3% pectolyase) on a clean slide, which was then put in a moist chamber at 37°C. After 1.5 hours digestion, anthers were washed twice with distilled water to remove the enzyme mix and a drop of fresh Carnoy's I fixative was added to the partially digested anther. The contents of the anthers were carefully squeezed out in one or two drops of fixative solution and immediately spread out on the slide with the end of brass rod, assisted by a gentle stream of air. The slide was left to air dry before being put into an oven at 37°C overnight and stored in an airtight box at -20°C until required.
2.2.3 Mitotic chromosome preparation using enzyme digestion

Roots from either well-growing seedlings or germinating seeds were collected and pre-treated in iced water for 15-16 hours. The root tips were fixed in Carnoy's I fixative for 2 hours at room temperature. After fixation, roots were transferred to 70% ethanol and stored at -20°C.

Mitotic chromosome preparations were made using the air-drying method of Olin-Fatih & Heneen (1992) with minor modifications in concentration of enzymes and digestion times. One root tip was put into 20μl of enzyme mix (4% cellulase and 1.3% pectolyase) on a clean slide and incubated in a moist chamber in an oven at 37°C for 1.5 hours. The enzyme mix was removed by washing twice with distilled water. Excess water around the root tip was removed with the corner of a small piece of filter paper and one or two small drops of fresh Carnoy’s I fixative were added to the root tip. The softened root tip in the fixative solution was macerated into a suspension and spread over the surface of the slide with a gentle stream of air. The slide was left on the bench to air dry. Alternatively, a small drop of 45% acetic acid was added to the partially digested root tip after washing twice with distilled water following the modified procedure of Anamthawat-Jónsson et al. (1993), then stirred into a suspension. A coverslip was placed over the suspension and squashed firmly under two layers of filter paper. The coverslip was removed by freezing in liquid nitrogen. The slides were then air dried and stored following the meiotic chromosome preparation procedure.

2.2.4 C-and N-banding of mitotic chromosome preparation

For C-banding on mitotic chromosome preparation, a slightly modified protocol of Kakeda et al. (1991), involving an increase of the temperature of the Ba(OH)₂ treatment, was adopted. The air-dried slides were incubated in 0.2M HCl at 55°C for 3 minutes, followed by 5% Ba(OH)₂ solution at 30°C for 5 minutes, then in 2 × SSC solution at 55°C for 20 minutes. For N-banding following Jewell (1981), the air-dried slides were incubated in 1M NaH₂PO₄ solution at 90°C for 3 minutes. In both banding procedures, the slides were rinsed briefly in
distilled water after each step of the treatment.

Following the C- or N-banding treatments, the slides were stained in 2% Giemsa solution (BDH) diluted with 1/30M phosphate buffer (Sorensen's) at pH 6.8 for 0.5 - 2 hours. Slides were monitored under the light microscope and when stained properly, were taken out of Giemsa solution and washed in distilled water, then air-dried.

2.2.5 Estimation of pollen viability

For determining pollen viability, the technique of Heslop-Harrison and Heslop-Harrison (1970) was adopted to test the integrity of the plasma membrane of the pollen grain using fluorescein diacetate (FDA). A 2 mg/ml solution of FDA in acetone was added to a 15% (w/v) sucrose solution until the first permanent milkiness became visible. Fresh pollen was placed into the solution and left to "stain" at least 10 minutes before observation under a Zeiss universal microscope using a UV light source and filter set 02. Pollen grains showing a bright fluorescence under UV light were scored as fertile. At least 1000 pollen grains were counted for each hybrid.

2.2.6 Genomic DNA extraction and purification

Total genomic DNA was extracted from young leaves by the modified CTAB procedures of Murray & Thompson (1980) and Saghai-Maroof et al. (1984) with the following modifications. Two grams of young plant leaves were ground to a fine powder in liquid nitrogen (taking care not to let the plant material thaw). The powder was poured into a 150 ml flask, and 20 ml of 2 x TP buffer was added followed by 400 µl of 2-mercaptoethanol, then mixed well. The flask was incubated in a water bath at 65°C for 20 minutes then allowed to cool down to room temperature. 20 ml of chloroform/isoamyl alcohol (24:1) was added and the flask was put on a rotary shaker for 15 min at room temperature. The contents of the flask were transferred to a 50 ml centrifuge tube and centrifuged at 8,000 rpm for 20 minutes at 20°C. The supernatant was transferred to a clean 50 ml centrifuge tube and an equal
volume of ice-cold 2-propanol added. The tube was inverted carefully several times and the precipitated DNA was hooked out and transferred to a sterile Eppendorf tube. The DNA pellet was washed twice with 70% ethanol and allowed to air dry for 1 hour. 500 μl of TE buffer was added to the Eppendorf tube to dissolve the DNA, then 5 μl of pre-boiled RNase A (10 mg/ml) was added and incubated at 37°C for 1 hour.

The extract was purified following the procedure of Sambrook et al. (1989) with the minor modifications as follows. 500 μl of Tris equilibrated phenol was added to the DNA extract and mixed well before centrifuging at 6000 rpm for 5 minutes. The supernatant was transferred to another clean Eppendorf tube. 250 μl each of phenol and chloroform/isoamyl alcohol (24:1) was added and mixed well. Centrifugation was repeated as in the previous step and the supernatant transferred to another clean Eppendorf tube. 500 μl of chloroform/isoamyl alcohol (24:1) was added, mixed well and centrifuged as above. The supernatant was transferred to another clean Eppendorf tube. Two volumes of 100% ethanol and one tenth volume of 7.5M ammonium acetate were then added and the tube was inverted several times to precipitate DNA. The precipitated DNA was washed twice with 70% ethanol and transferred to a clean Eppendorf tube. The DNA pellet was air-dried and dissolved in TE. The DNA concentration was determined by electrophoresis on a mini-gel by comparison to high DNA mass ladder (GIBCO BRL) (Sambrook et al. 1989).

2.2.7 Plasmid DNA preparation
Transformation of competent E. coli cells (DH5α) and identification of bacterial colonies that contain recombinant plasmids were carried out according to Sambrook et al. (1989).

One colony of the successful transformants was cultured overnight at 37°C in 50 ml of LB medium with 100 μg/ml ampicillin. After amplification of clone pHcV39 in bacterial culture, the bacterial pellet was centrifuged and the recombinant plasmid extracted using the QIA prep Spin Miniprep kit according to the manufacturer’s instructions (QIAGEN).
Restriction analysis of pHvC39 was performed using restriction enzymes BamHI and EcoRI to estimate the size of the insert compared with 100 bp DNA size marker (GIBCO BRL) by mini-gel electrophoresis (Sambrook et al. 1989).

2.2.8 Polymerase Chain Reaction (PCR) to label cloned DNA sequences

PCR was used to produce large quantities of labelled microsatellite DNA using the pUC/M13 universal forward and reverse primer sequences. The procedure followed Leitch et al. (1994) with minor modifications. Fifty µl of PCR reaction mixture contained the following: 5 µl of 10 x PCR buffer, 200 µM of each of dATP, dCTP, dGTP, 140 µM of dTTP, 60 µM of digoxigenin-11-dUTP, 0.5 µM of each of the universal primers, 100 pg of DNA template (pHvC39), 2.5 units of Taq DNA polymerase, then sterile distilled water was added to a total volume of 50µl.

The PCR was performed as follows: firstly, the reaction was denatured at 94°C for 3 minutes before the first cycle. Then, 30 cycles of 30 seconds at 94°C to denature the template DNA, 30 seconds at 55°C to anneal primers to denatured template DNA and 30 seconds at 72°C for primer elongation were carried out. After 30 cycles the PCR reaction mixture was incubated at 72°C for 5 minutes to complete final primer elongation. The reaction was stopped by chilling the tubes to 4°C.

PCR reaction products were purified using High Pure™ PCR product purification kit according to the manufacturer’s instructions (Boehringer Mannheim). The final concentration and size of purified PCR products were estimated by mini-gel electrophoresis and comparing the bands with known DNA standards.

2.2.9 DNA probes and hybridisation mixtures

For genomic in situ hybridisation (GISH), genomic DNA from *H. bulbosum* was labelled with
digoxigenin-11-dUTP by nick-translation (Boehringer Mannheim) according to the manufacturer's instructions. Total genomic DNA from *H. vulgare* was cleaved into 200–500 bp fragments by autoclaving (121°C, 15 PSI) for 10 minutes and used as blocking DNA. The hybridisation mixture followed Schwarzacher *et al.* (1992) with the following modifications. The labelled *H. bulbosum* DNA probe was mixed to a final concentration of 2 µg/ml and a 50-fold excess of *H. vulgare* blocking DNA was added. The concentration of formamide in the hybridisation mixture was adjusted to obtain a hybridisation stringency of 85%.

For fluorescence *in situ* hybridisation (FISH), the clone pHvC39 with a 334 bp insert containing the GAA-microsatellite sequence was kindly provided by Dr Carsten Pedersen, Plant Microbe Symbioses, Plant Biology and Biogeochemistry Department, Risø National Laboratory, Denmark. The insert of clone pHvC39 was labelled with digoxigenin-11-dUTP (Boehringer Mannheim) using the polymerase chain reaction (PCR) on a thermal cycler 9600 (Perkin-Elmer). The probe was mixed to a final concentration of 1.25 µg/ml in the hybridisation mixture (Leitch & Heslop-Harrison, 1992).

For FISH using the oligonucleotide (GAA)₁₀ as a probe, a single-stranded oligonucleotide with the sequence (GAA)₁₀ was synthesised on an automated DNA synthesiser (GIBCO, BRL). A tailing kit (Boehringer Mannheim) was used to end-label (GAA)₁₀ with digoxigenin-11-dUTP. The labelled (GAA)₁₀ was mixed to a final concentration of 0.8 µg/ml in the hybridisation mixture (Pedersen & Linde-Laursen, 1994).

### 2.2.10 *In situ* hybridisation and detection

The procedure for *in situ* hybridisation followed Schwarzacher *et al.* (1992) with the following minor modifications. Hybridisation mixture containing *H. bulbosum* genomic or the clone pHvC39 probes was denatured at 95°C for 10 minutes (this denaturation step was omitted for the (GAA)₁₀ probe) before 45 µl of the mixture was applied to each slide and covered with a plastic coverslip. The slides were then heated to 80°C for 10 minutes and the
temperature lowered to 37°C for incubation overnight (this incubation was reduced to 2–3 hours for (GAA)$_{10}$ probe) on a thermal cycler (Hybaid, Omnislide). Hybridisation was followed by a stringent wash in 50% formamide in 2 × SSC at 42°C for the total genomic probe. For the clone pHcV39 and (GAA)$_{10}$ probes, the most stringent wash with 0.2 × SSC was performed at 45°C for the former and at 37°C for the latter. Sites of probe hybridisation were detected using either HNPP Fluorescence Detection Set or Fluorescent Antibody Enhancer Set for DIG Detection (Boehringer Mannheim). Chromosomes were counterstained with DAPI (1 µg/ml, 4′, 6′-diamidino-2-phenyl-indole) for Texas red signals or with PI (0.1 µg/ml propidium iodide) for fluorescein signals. Slides were mounted in 10 µl of antifade mountant and examined under a Zeiss epi-fluorescence photomicroscope. The whole slide was scanned and all scorable cells were used in the analysis. Photographs of suitable cells with signals were taken on Fujichrome colour reversal film (Provia 400), digitised using a Nikon LS-1000 scanner and images processed using ADOBE PHOTOSHOP 3.0.5 software.

2.2.11 Reprobing

In order to localise the introgressions from *H. bulbosum* chromatin on specific barley chromosomes of recombinants, a two-step *in situ* hybridisation procedure was employed in this study. This required a reprobing technique. Following examination and photography of mitotic chromosome preparations that had been hybridised with the appropriate probe, ISHed slides were washed three times in 4 × SSC with 0.1% TWEEN 20 for 2 hours. The slides were further washed twice in 2 × SSC for 10 minutes each to remove mountant and most detection reagent according to Heslop-Harrison *et al.* (1992). After that, they were incubated in freshly made 4% paraformaldehyde solution for 10 minutes to preserve the chromosome morphology and dehydrated through an ethanol series. Then the hybridisation procedure was repeated from the denaturation step.
Chapter three  
The synaptonemal complexes of *Hordeum vulgare*, *H. bulbosum* and their hybrids

3.1 Introduction

Meiosis is an essential part of the life cycle of sexually reproducing organisms. It is the division process where a diploid cell of the sporophyte generates four haploid gametes through two successive nuclear divisions - meiosis I and II. At the end of meiosis I (reduction division), which comprises the stages of prophase I, metaphase I, anaphase I and telophase I, homologous chromosomes disjoin from each other. At meiosis II (equational division), sister chromatids segregate in the nuclei arising from meiosis I, proceeding through the stages of prophase II, metaphase II, anaphase II and telophase II as in a mitosis. There is often a short interphase between meiosis I and II. Among all these stages, prophase I is the most complex and lengthy. During prophase I two homologous chromosomes (one from each parent) pair and synapse, the synaptonemal complex (SC) is formed and reciprocal recombination (crossing-over) takes place. Prophase I has been divided into five substages depending on the chromosome morphology and behaviour (a detailed description of meiosis in wheat is given in Bennett *et al.* 1973). These are 1) leptotene: the individual chromosomes become visible at this stage as long, thin, single threads; 2) zygotene: this is a time of active pairing of the threads, synapsis starts and two homologous chromosomes pair progressively side by side as if by a zipper; 3) pachytene: synapsis is complete and fully synapsed threads appear to be thicker and more condensed, where nucleoli are often pronounced; 4) diplotene: the homologous chromosomes condense further and start to separate except at the sites of chiasmata; 5) diakinesis: chromosomes proceed through further condensation and bivalents become recognisable under the light microscope.
To ensure precise homologous pairing and proper segregation of a complete set of chromosomes at anaphase I, an important structure, the SC, is usually required at meiosis. The SC is a tripartite, ribbon-like structure of proteinaceous nature comprising a central element, two flanking lateral elements and linking transverse filaments (Moses, 1968; Heyting, 1996). Each SC is surrounded by a halo of chromatin loops that are anchored to the lateral element. Very little DNA passes through the central region of the SC (Vázquez Nin et al., 1993). A diagram of the SC structure is shown in Fig. 3.1. The SC plays a vital role in the meiotic process, because mutations that affect SC formation typically show reduced fertility (Bogdanov et al., 1998; Peirson et al., 1997). However, its role remains somewhat controversial.

Before pairing in early prophase I, each single chromosome develops a common proteinaceous axial core or axial element along its entire length. As synapsis progresses, the axial elements derived from homologous chromosomes are closely connected to each other and arranged in parallel by transverse filaments to become the lateral elements of the SC. Within mature SCs, lateral elements are separated from each other by the central region, which is observed equidistant between the lateral elements. The size of the central region in the SC shows considerable uniformity (in plants it is about 100 nm wide) while the central and lateral element dimension are more variable (Gillies, 1984).
Since Moses (1956) and Fawcett (1956) published the first descriptions of the ultrastructure of “cores” or “fibrils” existing in the prophase I nuclei of invertebrate and vertebrate animals using sectioned material, the SC has been a subject of extensive investigation and characterisation in a number of eukaryotes (Moses, 1968; Westergaard & von Wettstein, 1972; Gillies, 1984; Loidl, 1994; McKim et al., 1998). Originally it was thought that SC formation was a prerequisite for the initiation of recombination between homologous chromosomes. This view was based on cytogenetic observations that chiasmata occurred only in chromosomal regions where an SC was formed (von Wettstein et al., 1984; Loidl, 1994). The SC was thought to provide a framework for initiation and formation of crossovers (Meyer, 1964; Rasmussen, 1975; Jenkins & Rees, 1991). McKim et al. (1998) reported that there was a normal synaptonemal complex formation in two meiotic mutants of the female Drosophila (mei-W68 and mei-P22), which eliminate meiotic crossing-over and gene conversion. These results all support the traditional view of synapsis followed by recombination. However, more recently this has been challenged by genetic and molecular evidence, mainly from yeast (Weiner & Kleckner, 1994). Work on yeast suggests that the initiation of recombination occurs prior to synapsis, and the formation of the SC stabilises the early recombination intermediates and transforms them into functional chiasmata. Various lines of evidence from work on yeast support this view. They are: 1) a gene (RAD51) responsible for repairing double-strand breaks (DSB) is required for SC formation (Alani et al., 1990); 2) recombination intermediates in yeast are observed in advance of SC formation (Padmore et al., 1991); 3) high levels of genetic recombination can be accomplished in the absence of SC formation (Bähler et al., 1993). Thus, the new view was referred to as the yeast view (recombination-synapsis-maturation of recombination products) (Santos, 1999). Earlier observations by Maguire (1972, 1977) also proposed that recombination might precede or be associated with full formation of the SC in maize. The recent identification of a meiosis-specific protein (Zip 2) essential for SC formation, which co-localised with proteins involved in DSB formation and processing, further supports the yeast view (Chua & Roeder,
The yeast view suggests that the SC is a structure that influences the number and distribution of cross-overs, and converts cross-overs into stable and functional chiasmata that can ensure the proper segregation of homologous chromosomes at meiosis.

It is now generally accepted that homologous chromosome pairing is a multistep process (Kleckner, 1996; Schwarzacher, 1999; Sybenga, 1999) that can be divided into three main steps. Briefly they are: 1) chromosome association, where the homologous chromosomes contact at some sites along the axial elements; 2) alignment, where the homologous chromosomes are brought together to within a certain distance, possibly at a distance greater than the SC; 3) synopsis, where the homologous chromosomes are pulled closer together and the tripartite SCs are formed.

Recent work on the SCs has led to a number of new advances in understanding their function. Molecular approaches have been exploited extensively in the analysis of SC proteins and their encoding genes (Moens, 1994; Kobayashi et al., 1994; Klimyuk & Jones, 1997; Chua & Roeder, 1998). Meanwhile, both immunocytology of meiotic proteins and fluorescence in situ hybridisation also have been applied to investigate chromosome association, alignment and synopsis (Weiner & Kleckner, 1994; Barlow & Hultén, 1996; Moens et al., 1997).

However, electron microscopic study on conventionally stained SC spreads remains the chief method for the analysis of SC morphology and the extent of synopsis.

Although the SC function remains controversial (Kleckner, 1996), the relationship between SC length and recombination/chiasma frequency at pachytene-metaphase I has been widely investigated in many eukaryotes. In mice, there was a good correlation between SC length and chiasma frequency where the chiasma frequencies in male and female were very similar (Speed, 1977) and the SC lengths between two sexes were also alike (Speed, 1982; Moses & Poorman, 1984). In contrast, in humans a very pronounced sex difference in SC length has been found where the SC length was twice as long in the oocytes as in the spermatocytes.
Although there was no sex difference in the number of recombination nodules (Bojko, 1983, 1985; Wallace & Hultén, 1985), the genetic map of human autosomes showed almost twice as much recombination in females compared to males (Donis-Keller et al. 1987). A study on oocytes and spermatocytes of the hermaphroditic flatworm Dendrocoelum lacteum has also shown a pronounced difference in SC length that almost exactly parallels their chiasma frequency difference (Jones & Croft, 1989). Using the effect of additional heterochromatin in the short arm of chromosome 9 of maize, Mogensen (1977) found that higher female and lower male chiasma frequencies were associated with longer and shorter SCs, respectively. In addition, there were also several indications of the same relationship in some plant and animal species that were not dependent on sex difference or the presence of additional heterochromatin. For example, De Azkue & Jones (1993) found that mean SC length and mean chiasma frequencies at metaphase I showed positive correlation in wild populations of Crepis capillaris. In tomato, Sherman & Stack (1995) showed that there was a strongly positive correlation between relative SC length and the number of recombination nodules on each SC ($r^2 = 0.96$). They further found that the number of recombination nodules was primarily related to the SC length in euchromatin ($r^2 = 0.95$). A study by Quevedo et al. (1997) on 21 males of Locusta migratoria from three different laboratory families also demonstrated a positive correlation between chiasma frequency at diplotene and mean SC length ($r^2 = 0.71$). Croft and Jones (1986) found that a difference in SC length between two male locust species, Locusta migratoria and Schistocerca gregaria, parallels a difference in chiasma frequency. However, this is quite different from the previously mentioned cases since the SC length and chiasma frequency differences are associated with a 49% difference in genome size between these two species. Overall, positive correlation of SC length and chiasma / recombination frequency appears to be extensive in eukaryotes.

There is also some evidence that suggests that genome size has an influence on total SC length. In humans, Jørgensen and Bak (1981) reported a $1:1$ correspondence between relative SC length and relative genome size for each of the 22 autosomes. By analysing ten
different angiospermous plant species, Anderson et al. (1985) found a strong linear correlation between SC length and genome size ($r^2 = 0.94$). Further, they concluded that there was a positive relationship between these two values in higher plants. This is supported by a study (see above) on the two locust species (Croft & Jones, 1986). However, this is contrary to the suggestion by Mogensen (1977) that there is only a weak correlation between SC length and genome size. In order to establish whether a consistent relationship between SC length and genome size exists in vertebrates, Peterson et al. (1994) investigated this relationship in 18 species of vertebrates from the classes Osteichthyes (bony fish), Reptilia (reptiles), Aves (birds), and Mammalia (mammals) and found that there did not appear to be a correlation between SC length and genome size. Furthermore, when birds were excluded from these data, a linear regression analysis showed that variation in genome size accounted for approximately 50% of the variation in total SC length ($r^2 = 0.47$), suggesting birds were considerably different from the other vertebrates analysed in this relationship. A possible reason for this is that unlike the other vertebrates, birds possess strongly bimodal karyotypes, which show two distinct types of chromosomes that differ in size (Tegelström & Ryttman, 1981). Therefore, although some evidence supports the positive relationship between SC length and genome size, there still exists a divergence in this relationship among different organisms.

Recently Jaffe (1998) reported that recombination frequencies of a set of common markers in *H. bulbosum* were only 0.2-0.3 times those found in *H. vulgar* which resulted in large differences in marker distances on their genetic maps. Since SC length has been found to be positively correlated with chiasma/recombination frequency in many organisms (e.g. Mogensen, 1977; Croft & Jones, 1986; Jones & Croft, 1989; Quevedo et al., 1997), one would expect that mean SC length might be longer in *H. vulgar* than in *H. bulbosum*. However, as both species have almost identical genome sizes, about 5.5 pg/C each (Bennett & Smith, 1976; Bennett & Leitch, 1995), this would suggest that both species might have similar SC lengths according to the conclusion from the data of 10 species of higher plants
(Anderson et al., 1985). Whether the SC lengths between these two species with almost identical genome size differ from each other has not been addressed. Another interesting question that arises from the meiotic analysis of the two *H. vulgare* - *H. bulbosum* hybrids (102C2 and 103K5), is whether there is any difference in the extent of synopsis of the two hybrids and how any differences might be related to their SC length. Although the hybrids are expected to have very similar genome sizes, they differ greatly in their recombination frequencies (see Chapter 4).

There have been extensive karyotypic studies on the mitotic chromosomes of *H. vulgare* and *H. bulbosum* (Lange & Jochemsen, 1976b; Hsiao et al. 1986; Fukui & Kakeda, 1990; Linde-Laursen et al., 1990b; Jensen & Linde-Laursen, 1992; Linde-Laursen et al., 1992). However, there has been little work on the SCs of these two species. The only previous work is one study on haploid barley, which used reconstructions of serial sections from three haploid nuclei (Gillies, 1974). The haploid SCs showed a tripartite structure and either intra- or inter-chromosomal pairing. Due to technical difficulties and the time required for the three-dimensional reconstruction of the nuclei from serial sections, the number of nuclei studied was fairly low. This, together with considerable variation of SC length observed among individual nuclei from the same plant, limited the value of SC studies for karyotypic analysis in haploid barley.

The development and modification of the surface spreading techniques provided the possibility of obtaining larger numbers of two-dimensional SCs for karyotypic analysis (Counce & Meyer, 1973; Gillies, 1981; Albini et al., 1984). The SCs obtained by this method are usually stained with silver nitrate. This stains the proteins associated with these structures and the lateral elements appear as darkly-staining parallel strands. Karyotype studies of SC spreads have shown that the SC karyotypes agree well with those from conventional squashes and serial section reconstructions in maize (Gillies, 1981), and from mitotic metaphases in *Allium* species (Albini & Jones, 1988). Several researchers (Albini & Jones, 1988; Sherman
& Stack, 1995) have reported that the distribution of recombination nodules from conventionally stained SC spreads parallels that of chiasmata and the average number of recombination nodules is strongly correlated with SC length in euchromatin. Sherman et al. (1992) demonstrated that the modified silver staining on SC spreads revealed the same distribution and frequency of nodules as those from UP staining and higher contrast SC morphology in the electron microscope than UP or PTA staining. Therefore, the objectives of this chapter were: 1) to compare different staining methods on SC spreads of H. vulgare and H. bulbosum in an attempt to obtain an effective technique to study their SC karyotypes and recombination nodules; 2) to investigate possible variation in SC length among the species and their hybrids; 3) to study the extent of synapsis in these two hybrids. In addition, observations of pairing sites along axial elements were made in this investigation to determine where pairing is initiated.

3.2 Results

3.2.1 Comparison of different staining methods for the observation of SC morphology in H. vulgare and H. bulbosum

A variety of staining procedures were used to visualise aspects of SC morphology. Ideally, lateral elements plus centromeres, nucleolar organiser regions (NORs) and recombination nodules should be observed, but in Hordeum it was not possible to observe all these features in the same preparation. Staining with 50% silver nitrate solution at 60°C in both species resulted in well-differentiated lateral elements with high contrast morphology (Figs. 3.2 & 3.3). However, with the sole exception of a barley nucleus with incomplete centromere staining, centromeres were not visible. NORs and recombination nodules were not stained either (Figs. 3.2, 3.3, & 3.4). A 33% silver nitrate treatment at 37°C in both species distinctly stained centromeres but this was at the expense of SC morphology as the lateral elements were not differentiated clearly (Figs. 3.5 & 3.6). NORs were differentiated occasionally after the staining, but recombination nodules were not observed.
PTA staining poorly resolved SC morphology. It was not possible to differentiate individual lateral elements and visualise any recombination nodules. However, PTA stained centromeres more heavily than other regions in *H. bulbosum* (Fig. 3.7), although this was not observed in *H. vulgare*. Staining with UP did not show any advantages over PTA staining.

Large, rounded nucleoli, when present in the surface-spread prophase I nuclei, were usually closely associated with the NOR regions of the SC, although they were often lost during the surface spread process. At most one nucleolus per nucleus at pachytene was seen in *H. bulbosum* (Fig. 3.4) whereas two nucleoli per nucleus were seen in *H. vulgare* (Fig. 3.5). One of the difficulties in studying karyotypes of both species is that the SCs were easily broken during the preparation of surface spreads, especially in *H. vulgare*. SC threads treated with 50% silver nitrate at 60°C were more fragile than those subjected to PTA or UP treatments, but despite some disadvantages, 50% silver nitrate at 60°C proved best for the following studies because of the high contrast obtained.

### 3.2.2 SC length in the two species

For SC length measurements, at least 200 spikelets from four different plants of each species were used to make SC preparations. Both species showed variation in SC length (Table 3.1), which probably corresponds to different substages of pachytene occurring in the prophase I nuclei, i.e. early, mid and late pachytene. However, an absence of morphological markers meant that it was not possible to determine the substage of pachytene in this study. Despite this variation, there was a significant difference in the total SC length between these two species (*t* = 6.17, d.f. = 29, *p*<0.01) (Table 3.3). *Hordeum vulgare* had a mean SC length of 500.5 μm per nucleus with a range of 353.3 - 614.4 μm, compared to 353.8 μm per nucleus with a range of 254.8 - 454.6 μm for *H. bulbosum*. Nine out of 18 nuclei analysed in *H. vulgare* had SCs longer than 500.5 μm, while the longest SC in 20 nuclei analysed from *H. bulbosum* was 454.6 μm.
3.2.3 Pairing initiation and unpaired loops in the two species

Pairing initiation occurred predominantly at or near the telomeres, but it was also observed at multiple sites along axial elements in both species (Figs. 3.8, 3.9a & b). SC formation was observed to be asynchronous within the prophase I nuclei, where some axial elements had completed pairing to form the SC but others were not fully paired in the same nucleus (Fig. 3.10). Three out of the 20 H. bulbosum nuclei had unpaired loops (Fig. 3.11a & b) on one or two chromosomes but these loops were rarely seen in H. vulgare nuclei.

3.2.4 SC length and extent of synopsis in the hybrids 102C2 and 103K5

As many of the PMCs of the hybrids were anucleate, at least 400 spikelets from four different plants of each hybrid were used for preparing the SCs. In both H. vulgare – H. bulbosum hybrids that were analysed there was considerable variation in SC length (Table 3.2). 102C2 had a mean SC length of 337.0 μm with a range of 185.6 - 460.8 μm compared to 255.2 μm with a range of 171.6 - 320.2 μm for 103K5. In 102C2, twelve out of the 20 nuclei analysed had an SC length of more than 337.0 μm, while the longest SC in the nuclei of 103K5 analysed was 320.2 μm. The differences in SC length between the two hybrids were statistically significant (t = 4.1, d.f. = 30, P<0.01). The coefficient of variation (CV) in SC length was 22.96% in 102C2 compare to 16.03% for 103K5. Furthermore, the total element length in 103K5 was significantly shorter than that in 102C2 as well as H. bulbosum (t = 3.18, d.f. = 26, P<0.01 & t = 2.5, d.f. = 30, P< 0.05 respectively), while they were very similar between 102C2 and H. bulbosum (t = 0.97, d.f. = 19, P = 0.36).

Full synopsis was rarely observed in the hybrids and in both there was considerable cell to cell variation (Table 3.2). In 102C2 the mean percent synopsis within cells was 82.6% with a range of 53 – 100% but in 103K5 the mean was 71.8% with the range between 34% and 95.1%. Overall, the mean percent synopsis was similar (t = 1.9, d.f. = 27, P = 0.07) but there were big differences in the observed ranges between the two hybrids (Table 3.3). Two out of
the 20 nuclei investigated in the hybrid 102C2 showed full synapsis (Fig. 3.12) but this was not seen in any of the nuclei of the other hybrid 103K5. It is interesting that these two cells differed by almost 25% in their total SC length, 358.3 μm versus 436.6 μm. The coefficient of variation in percent synapsis was 15.6% in 102C2, almost half that observed in 103K5 where it was 27.7%.

Plotting SC lengths against percent synapsis transformed to angles (Fig.3.13) revealed significantly positive correlation in both hybrids ($r_1 = 0.51$, $P<0.05$ & $r_2 = 0.82$, $P<0.01$ for 102C2 & 103K5 respectively).

3.2.5 SC configurations in the hybrids 102C2 and 103K5

The formation of multivalents was frequently observed at pachytene in both hybrids (Figs. 3.14, 3.15a), but it was not possible to study chromosome configurations at prophase I in detail due to the high frequency of SC breakage. Partially synapsed bivalents showed inequality of the unpaired ends and foldback loops at pachytene (Fig. 3.15b & c). Univalents were not observed in the hybrid 102C2 whereas 2 of the 17 cells in 103K5 showed one or two univalents (Fig. 3.14).
Table 3.1. A summary of synaptonemal complex (SC) length and percent synapsis in *H. vulgare* and *H. bulbosum*.

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Table 3.2. A summary of total element length, synaptonemal complex (SC) length and percent synapsis in hybrids.

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<td>673.62</td>
<td>320.2</td>
<td>90.60</td>
<td>72.15</td>
</tr>
<tr>
<td>16</td>
<td>615.1</td>
<td>295.4</td>
<td>92.43</td>
<td>74</td>
</tr>
<tr>
<td>17</td>
<td>560.07</td>
<td>273.0</td>
<td>95.1</td>
<td>77.21</td>
</tr>
</tbody>
</table>

| Mean    | 628.5  | 255.2 | 71.83 | 59.23 |
Table 3.3. A summary of descriptive statistics of SC length and percent synapsis in *H. vulgare*, *H. bulbosum* and their F₁ hybrids.

<table>
<thead>
<tr>
<th>Species/ Hybrids</th>
<th>No. of nuclei</th>
<th>SC length (µm)</th>
<th>Percent synapsis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Range (µm)</td>
</tr>
<tr>
<td><em>H. vulgare</em></td>
<td>18</td>
<td>500.52</td>
<td>353.33 - 614.4</td>
</tr>
<tr>
<td><em>H. bulbosum</em></td>
<td>20</td>
<td>353.77</td>
<td>254.80 - 454.62</td>
</tr>
<tr>
<td>102C2</td>
<td>20</td>
<td>336.97</td>
<td>185.56 - 460.8</td>
</tr>
<tr>
<td>103K5</td>
<td>17</td>
<td>255.21</td>
<td>171.6 - 320.23</td>
</tr>
</tbody>
</table>

CV = coefficient of variation
Figure 3.2. Electron micrograph of an entire surface spread synaptonemal complex of *Hordeum vulgare* stained with 50\% silver nitrate showing full synapsis and incomplete centromere staining. Bar = 10 μm.
Figure 3.3. Electron micrograph of an entire surface spread synaptonemal complex of *Hordeum bulbosum* stained with 50% silver nitrate showing full synapsis. The synaptonemal complex breakage can be seen. Bar = 10 μm.
Figure 3.4. Electron micrograph of an entire surface spread synaptonemal complex of *Hordeum bulbosum* stained with 50% silver nitrate showing well differentiated lateral elements with high contrast and one nucleolus can been seen. Bar = 10 μm.
Figure 3.5. Electron micrograph of an entire surface spread synaptonemal complex of *Hordeum vulgare* stained with 33% silver nitrate showing darkly stained centromeres and two nucleoli can been seen. Bar = 10 μm.
Figure 3.6. Electron micrograph of an entire surface spread synaptonemal complex of *Hordeum bulbosum* stained with 33% silver nitrate showing darkly stained centromeres and NOR region (arrow). Bar = 10 μm.
Figure 3.7. Electron micrograph of an entire surface spread synaptonemal complex of *Hordeum bulbosum* stained with phosphotungstic acid showing darkly stained centromeres. Bar = 10 μm.
Figure 3.8. Electron micrograph of a surface spread prophase I nucleus of *Hordeum vulgare* stained with 50% silver nitrate showing pairing initiation at telomere (arrow). Bar = 10 μm.
**Figure 3.9.** Electron micrographs of surface spread prophase I nuclei of *Hordeum bulbosum* stained with 50% silver nitrate showing (a) pairing initiation at telomere (arrow) (b) multiple initiation sites (arrowhead). Bar = 10 μm.
Figure 3.10. Electron micrograph of an entire surface spread prophase I nucleus of *Hordeum vulgare* stained with 50% silver nitrate showing asynchronous formation of synaptonemal complex. Bar = 10 μm.
Figure 3.11. Electron micrographs (a & b) of surface spread synaptonemal complexes of *Hordeum bulbosum* stained with 50% silver nitrate showing unpaired loops (arrowheads). Bar = 10 μm.
Figure 3.12. Electron micrograph of an entire surface spread synaptonemal complex of hybrid 102C2 stained with 50% silver nitrate showing full synapsis. Bar = 10 μm.
Figure 3. A line graph showing relationships of synaptonemal complex (SC) length and percent synapsis in the hybrids 102C2 and 103K5 ($r_1 = 0.51$, $P < 0.05$ & $r_2 = 0.82$, $P < 0.01$ for 102C2 & 103K5 respectively).
Figure 3.14. Electron micrograph of an entire surface spread synaptonemal complex of hybrid 103K5 stained with 50% silver nitrate showing univalent and a multivalent. Bar = 10 \mu m.
Figure 3.15. Electron micrographs of surface spread synaptonemal complexes stained with 50% silver nitrate showing (a) multivalent (arrowhead) in hybrid 102C2 and (b & c) foldback (arrows) in hybrids 102C2 and 103K5. Bar = 10 µm.
3.3 Discussion

3.3.1 Variation in SC length of *H. vulgare*, *H. bulbosum* and their hybrids

SC lengths in species and hybrids, like most other metrical characters in biological systems, show a defined range of variation. The present study shows that there is approximately a two-fold variation in SC length within the two species and their hybrids. Similar levels of intraspecific variation have been reported for many other plants (Mogensen, 1977; Anderson *et al.*, 1985; De Azkue & Jones, 1993).

Anderson *et al.* (1985) proposed that the variation of SC length within a species might be attributable to different substages of pachytene, because they found that SC lengths from nuclei judged as early pachytene by squash preparations were generally longer than those judged as late pachytene. To demonstrate the substages of pachytene, Bojko (1985) used the distribution of the telomeres on the nuclear envelope as the criterion. In serial sections of nuclei of human oocytes, the nuclei with a prominent bouquet were classified as early pachytene, and those with a dissolving bouquet and telomeres distributed evenly on the nuclear envelope were classed as mid and late pachytene, respectively. From these nuclei, mean SC lengths did not show much variation throughout different substages of pachytene; 533 μm at early pachytene, 458 μm at mid and 519 μm at late pachytene. However, Moses *et al.* (1977) reported that autosomal SC lengths in spermatocytes of the Chinese hamster decreased from late zygotene to mid pachytene and then increased at late pachytene. Several other researchers (Rasmussen & Holm, 1980; Gillies, 1982) reported a change in SC length with different pachytene substages. This appears to depend on the organism studied.

Anderson *et al.* (1988) described another method for determining pachytene substages in SC spreads by directly comparing chromosomes in squashes with SC spreads of maize nuclei. Based on this method, zygotene to early pachytene stages were characterised by a large, rounded nucleolus which appeared intensively stained; at mid pachytene, the nucleolus was
irregular in size and shape while at late pachytene the nucleolus was dispersed or absent. In the present study, however, telomere distribution and the appearance of the nucleoli could not be employed as the nuclear membrane was digested in the SC spreading process and the nucleoli were often absent from the pachytene nuclei. It is likely that the enzyme digestion technique used in spread preparations affects nucleolus morphology. In this study, SC preparations were made over an extended time period and from a large number of florets and therefore probably represent a reasonable sample of the intraspecific variation.

As far as variation in SC length between species is concerned, there are several possible explanations for this. One major factor is variation in genome size, measured either as variation in chromosome length or nuclear DNA amount. SC length may be correlated with mitotic chromosome length. In *H. vulgare* and *H. bulbosum* information on chromosome length is somewhat contradictory. Two studies that compared the karyotypes of *H. vulgare* and *H. bulbosum* showed that the mitotic chromosome length in *H. bulbosum* was either 71.5% (Lange & Jochemsen, 1976b) or 88% (Hsiao et al., 1986) of that in *H. vulgare*. The mean of these two measurements, approximately 80%, is somewhat greater than the 70.8% differences observed in SC length between *H. vulgare* and *H. bulbosum* in this study but the variation is in the same direction. A similar relationship between relative SC length and mitotic chromosome length has been found in several other plant and animal species (Moses et al., 1977; Gillies, 1981; Kaelbling & Fechheimer, 1983). Moreover, Albini and Jones (1988) reported that SC karyotypes are comparable with those produced from the mitotic metaphases in two *Allium* species. However, it can be seen that in *H. vulgare* and *H. bulbosum* there is a large difference in the ratio of mitotic chromosome lengths between the two reports of Lange and Jochemsen (1976b) and Hsiao et al. (1986). It is not known whether this reflects intraspecific variation in chromosome length or is attributable to some sources of errors in determination of mitotic chromosome length (Bentzer et al. 1971).

As with the measurements of chromosome length, measurements of genome size in *H.*
vulgare and H. bulbosum show some inconsistency between the different reports. Bennett and Smith (1976) and Schwarzacher et al. (1992b) used Feulgen microdensitometry to measure several different cultivars or lines of H. vulgare and H. bulbosum and found that the DNA amount was almost identical between these two species (5.5 pg/C). As there is a good correlation between measurements made by Feulgen microdensitometry and flow cytometry for DNA amounts in both monocotyledons and dicotyledons (Galbraith et al., 1983; Huelgenhof et al., 1988; Arumuganathan & Earle, 1991; Michaelson et al., 1991a, b; Dickson et al., 1992), Bennett and Leitch (1995) used both techniques and confirmed the previous measurements of genome size and the absence of intraspecific variation in H. vulgare. In contrast, Kankanpää et al. (1996) reported a considerable variation in DNA amount among four accessions of barley (3.95 - 4.7 pg/C) and one accession of H. bulbosum (3.7 pg/C). However, their measurements were made using flow cytometry with DAPI relative to chicken or rainbow trout red blood cells as calibration standards, so are probably less accurate. There are two reasons for this. First, DAPI binds preferentially to the AT-rich regions of DNA so is regarded as an unreliable fluorochrome for DNA measurements by many investigators (Michaelson et al., 1991b; Dolezel, et al., 1992). For animal and plant DNA estimation, PI is now the fluorochrome that is most widely used due to its sensitivity and base independent binding to DNA (Arumuganathan & Earle, 1991; Michaelson et al., 1991b; Dolezel et al., 1992, 1994; Figueira et al., 1992). Second, animal calibration standards are considered inferior to plant calibration standards for estimating genome sizes in plants because animal genomes are usually in a very different size range from plant genomes and may show intraspecific variation (Price et al., 1980). Hence, I think that the reports that H. vulgare and H. bulbosum possess almost identical genome sizes are probably more reliable.

Anderson et al. (1985) reported that SC length was strongly correlated with genome size in many of the higher plants. This relationship has also been observed in two locust species where the difference in SC length is almost exactly proportional to the difference in genome size of the two species (Croft & Jones, 1986). However, the results of the present study
show that these two closely related _Hordeum_ species with almost identical genome sizes had an approximately 45% difference in their mean SC length. Mogensen (1977) also reported that there was only a very weak correlation between mean SC length and genome size in a variety of animals and fungi. This was confirmed by a later study on 18 species of vertebrates where there did not appear to be a correlation between these two values (Peterson et al., 1994). Further support for the absence of a relationship between SC length or total element length and genome size comes from the present study of the SCs in _H. vulgare - H. bulbosum_ hybrids. This suggests that SC length or total element length is genotype- rather than genome size- dependent in the hybrids. As there is a significant difference in SC length but very similar genome size between the species as well as hybrids, the ratio of mean SC length to genome size (SC/DNA) shows considerable divergence among the _Hordeum_ examples described here as well as in a wide variety of other species.

It is interesting that the result of the present study and those of Jaffe (1998) appear to be related. Jaffe (1998) found that the linkage maps of _H. vulgare_ and _H. bulbosum_ showed a basically similar order of markers but large differences in genetic distances between them. Recombination frequencies were about three times greater in _H. vulgare_ than in _H. bulbosum_. This parallels the difference in the mean SC lengths of the two species obtained from the present study, with the mean SC length in _H. vulgare_ being about 1.5 times as long as that of _H. bulbosum_. This sort of relationship between SC length and chiasma/recombination frequency has also been observed in the two sexes of a number of animal species (Speed, 1977, 1982; Moses & Poorman, 1984; Bojko, 1983, 1985; Donis-Keller et al., 1987; Jones & Croft, 1989). Examples are also found among individuals of the same sex (Croft & Jones, 1986; De Azkue & Jones, 1993; Quevedo et al., 1997) and also where different amounts of heterochromatin are present in meiocytes (Mogensen, 1977). Likewise, in the present study of hybrids 102C2 and 103K5 with recombination frequencies of 0.53 and 0.33 per PMC respectively (see Chapter 4), the difference (32%) in SC length parallels a difference in recombination frequency between them. Thus, it appears evident that there is a positive
relationship between SC length and recombination frequency.

In the present study mean SC lengths of 102C2 and *H. bulbosum* are similar. In contrast, there are large differences between them in numbers of bound-arms per cell at MI (9.0 and 14 respectively) (see Chapter 4 and Kasha & Sadasivaiah, 1971). As SC length has been observed to be positively correlated with recombination frequency, it is possible that some of the bound-arms in *H. bulbosum* are non-chiasmatic and are remnants of chromosome association at prophase I (see also Orellana, 1985). Hence, it can be suggested that *H. bulbosum* has a lower recombination frequency than the previous assumption of one chiasma per chromosome arm in *Hordeum* species (Kasha & Sadasivaiah, 1971). This would agree with the results of Jaffe (1998) that *H. vulgare* has a significantly higher recombination frequency than *H. bulbosum*. Nevertheless, one cannot rule out the possibility that genetic map inflation has occurred and that there has been an over-estimation of recombination frequency in *H. vulgare*. Several investigators (Nilsson *et al.*, 1993; Sybenga, 1996; Hall *et al.*, 1997) have reported that some sources of error, e.g., a misclassification of RFLP markers, can greatly increase the map length and lead to map expansion.

Several explanations have been put forward to explain the relationship between SC length and recombination frequency. Jones and Croft (1989) suggested that there might be a causal connection between SC length and recombination. As there are two conflicting views about the order of recombination and SC formation (see also Santos, 1999), operational direction of this relationship remains uncertain. According to the traditional view, SC formation confers a framework for recombination events, longer SCs provide more space for homologous contact and result in more recombination initiation sites. Thus SC length has an influence on recombination frequency. In opposition to the traditional view, the yeast view suggests that the initiation of recombination occurs before SC formation and recombination intermediates mature into cross-overs only when an SC is formed (Padmore *et al.* 1991). In this context recombination frequency regulates SC length. This agrees with the hypothesis by Quevedo *et
al. (1997), which proposed that the condition of axial elements and their associated chromatin during early prophase I would play key roles in determining recombination and also be likely to influence SC length at pachytene. However, one cannot rule out the possibility that both SC length and recombination frequency are affected by other factors, in which case the correlation does not indicate any direct causality (see also Dawe, 1998).

An hypothesis that the conformation of chromatin anchored to chromosome axes might play a vital role in recombination has been widely accepted. The results of the present study and those of the previous studies in animals and plants (e.g. Bojko, 1985; Donis-Keller et al., 1987; Jones & Croft, 1989; De Azkue & Jones, 1993) have shown that a higher SC/DNA ratio is associated with higher recombination frequency. The higher SC/DNA ratio would mean less dense DNA compaction, which might facilitate crossing-over (Stack, 1984). Loidl (1994) compared a number of different organisms and concluded that there was a positive correlation between SC/DNA ratio and recombination frequency. Subsequently, Loidl et al. (1995) reported that a human-derived yeast artificial chromosome (YAC) showed the same degree of DNA compaction as endogenous chromosomes in prophase I nuclei of yeast, which is about 20 times less compactly packaged than that in human. Furthermore they suggested that less dense DNA compaction favoured increased recombination frequency by a comparison of human-derived YAC and human DNA in its natural environment. Thus, it is likely that a looser chromatin conformation along axial elements allows more contact between DNA strands of homologues and results in higher recombination. Likewise the degree of chromatin compaction along axial elements might affect SC formation and the looser chromatin conformation might result in longer SC length.

The data from the published mitotic metaphase C-banded karyotypes of these two Hordeum species has shown that H. vulgare has an obviously higher proportion of heterochromatin in its genome than H. bulbosum (Linde-Laursen, 1978; Linde-Laursen et al., 1990b; Kakeda et al., 1991). However, Stack (1984) found that the SC was over-represented in euchromatin
compared to heterochromatin in a study of two angiospermous plants and one mammal. In this context, if the two *Hordeum* species had same degree of DNA compaction in their genomes, it would be expected that *H. vulgare* would have a shorter SC than *H. bulbosum*. This is at variance with the present observations in that mean SC length in *H. vulgare* is significantly longer than that of *H. bulbosum*. A possible explanation for this is that *H. vulgare* genome has overall a looser degree of DNA compaction that might result in longer SCs. In a study of serial reconstructions of metaphase nuclei of *H. vulgare - H. bulbosum* hybrids, Schwarzacher *et al.* (1992b) found that the chromosome volume in *H. bulbosum* is about 85% of that in *H. vulgare*. This supports the explanation that *H. vulgare* has less condensed DNA in its genome than *H. bulbosum*.

3.3.2. Variation in the extent of synopsis in the hybrids

Genome size difference between the parents of an interspecific hybrid is one likely cause of synaptic failure (Jenkins & Rees, 1983; Albini & Jones, 1990). However, in the present study of hybrids with very similar genome size, full SCs were observed in a few cells of hybrid 102C2 but no full SC formation seen in 103K5. This suggests that other genetic factors might play a key role in synopsis of these interspecific hybrids.

It is expected that SC length has been observed to be positively correlated with the percent synopsis in both hybrids. However, the present results showing significant difference in SC length and slight difference in percent synopsis between the two hybrids suggest that different genotypes may be responsible for difference in SC length but exert little influence on their extent of synopsis at pachytene. Alternatively, it is possible that the different genotypes have an influence on the initiation of synopsis, but exert little influence on synaptic extension.

Like SC length, percent synopsis in the two hybrids showed considerable cell to cell variation. The coefficient of variation in SC length is higher in 102C2 (22.96%) than in 103K5 (16.03%), but in contrast, 102C2 has a lower coefficient of variation in percent synopsis
(15.63%) than 103K5 (27.72%). At present there is no ready explanation for this phenomenon. Possibly it is merely a statistical effect and does not imply any relationship between them.

Multivalent formation in these two hybrids shows that synopsis at prophase I occurs not only between homoeologous chromosomes, but also between non-homoeologous chromosomes. This has also been observed in other interspecific hybrids in the Poaceae (Jenkins & White, 1990; Cuñado & Santos, 1999). Since many highly repeated sequences are widely distributed among all chromosomes of plant genomes (Schmidt & Heslop-Harrison, 1998), it is likely that synopsis is initiated at these homologous regions and extends far into non-homologous regions. Nevertheless, multivalents at pachytene do not persist into MI as both hybrids have only univalents and bivalents at MI (see Chapter 4). Thus multivalents are resolved into intergenomic bivalents and univalents when prophase I progresses through MI in the hybrids, as occurs in diploid Lolium, Allium and Aegilops interspecific hybrids (Albini & Jones, 1990; Jenkins & White, 1990; Cuñado & Santos, 1999). From all these results, it would appear that the formation of multivalents might be a common feature at prophase I in interspecific hybrids.

3.3.3 Staining techniques in H. vulgare and H. bulbosum

Silver nitrate treatment (50% solution at 60°C) on the SC spreads of the two species showed high contrast staining which was clearly superior to the other three staining methods that were tried. This is similar to what has been found by the other researchers (Stack & Anderson, 1986; Albini & Jones, 1988; Sherman et al., 1992). Stack and Anderson (1986) suggested that silver staining had more specificity for lateral elements than PTA or UP staining and thus its high contrast staining was suitable for interpreting the pattern of synopsis in SC spreads. However, since the 50% silver staining at 60°C generally fails to stain recombination nodules, PTA or UP staining has been extensively used for the study of nodules on the SC. Due to poor contrast with PTA or UP staining of the SCs, a modified
silver staining method (33% solution at near 40°C) was developed by Sherman et al. (1992). They demonstrated that this method showed higher contrast staining and the same distribution and frequency of nodules as those stained with UP. In the present study the use of 33% silver nitrate, PTA, and UP with SC spreads also indicated that 33% silver nitrate gave higher contrast staining than PTA or UP, but none of them revealed nodules. This does not agree with the suggestion that the nodules can be stained by 33% silver, PTA or UP (Stack & Anderson, 1986; Solari et al. 1988; Albini & Jones, 1988; Sherman et al. 1992). Likewise, a study of locust spermatocyte SCs by Croft and Jones (1986) also found that PTA did not detect the nodules in this species. This suggests that in these species, the recombination nodules might be lost during the spreading procedure, or more likely the nodules might be transiently present at pachytene stage (also see Albini & Jones, 1988).

As there were frequent fragmentation of the SCs and paucity of staining of centromeres in the treatment with 50% silver nitrate at 60°C, using this method made it impossible to construct SC karyotypes in both Hordeum species. Sherman et al. (1992) reported that silver staining at 60°C often resulted in broken SCs, suggesting that 50% silver nitrate at high temperature might have a disruptive effect on the SCs. In the present study 33% silver at 37°C stained both centromeres and NORs well but broken SCs were still frequently seen. However, one cannot rule out the possibility of construction of the SC karyotype in these species. Considering the observed effects of silver treatment at different concentrations and temperatures (Sherman et al. 1992), further experimentation might solve the problem of the frequent broken SCs.

One and two nucleoli can be found attached to the region proximal to the end of some SC bivalents in the nuclei of H. bulbosum and H. vulgare respectively. This agrees with expectations because karyotypes produced from mitotic preparations of both species have shown that H. bulbosum has one pair of satellited chromosomes and H. vulgare has two pairs.
Chapter four

Chromosomal behaviour and recombination frequency in diploid *Hordeum vulgare* – *H. bulbosum* hybrids

4.1 Introduction

Diploid and tetraploid cytotypes of *Hordeum bulbosum* L. are valuable sources of many useful agronomic traits such as pest and disease resistance genes, which have been successfully transferred into cultivated barley (*H. vulgare* L.) (Xu & Kasha, 1992; Pickering *et al.*, 1995). Two main crossing methods have been used to transfer these pest and disease resistance from *H. bulbosum* into barley. First, the recombinants that contain small segments of *H. bulbosum* DNA introgressed into the *H. vulgare* genome were directly selected among selfed progeny of a tetraploid *H. vulgare* - *H. bulbosum* hybrid, which was derived from a colchicine-treated diploid hybrid (Pickering *et al.*, 1995). Second, a partially fertile triploid hybrid from the cross between *H. vulgare* and *H. bulbosum* (2n = 4x = 28) was backcrossed to barley and recombinants were produced from the backcross progeny (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, 1976a; Pickering, 1992). The reasons for this have been outlined by Pickering (1992) and briefly they are: 1) barley is a diploid species that cannot tolerate much genetic manipulation; 2) the *H. bulbosum* genome is usually completely eliminated resulting in haploid barley embryo and plantlet formation; 3) the interspecific hybrids are often sterile and unstable; 4) intergenomic chromosome pairing is variable and genotype-dependent. Apart from the first problem that is inherent for diploid species, manipulating the parental genotypes and crossing environment can easily overcome the second and third of these barriers, and make it relatively easy to obtain stable high pairing *H. vulgare* - *H. bulbosum* hybrids. 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. 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 as the results only related to one particular chromosome and "hotspots" of crossing-over are likely to occur throughout the genome (Pedersen et al., 1995).

Since genomic in situ hybridisation (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 visualise introgressions of *H. bulbosum* chromatin into *H. vulgare* (Pickering et al., 1997), the objectives in this investigation using GISH were 1) to assess the extent of recombination 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); 2) to make a comparison of pairing and recombination frequencies in the hybrids. In addition, observations of chromosome polar migration at MI, chromosome segregation at AI and the frequency of *H. bulbosum* chromosome elimination in the PMCs of the hybrids were made. The possible relationships between these observations and MI pairing were discussed.

**4.2 Results**

**4.2.1 GISH analysis of pairing at MI and recombination at AI in hybrids**

Using GISH it was possible clearly to distinguish *H. vulgare* and *H. bulbosum* chromosomes and to show that pairing always occurred between the 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 4.1; Figs. 4.1a, b & 4.2a, b, c). 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 these data it was calculated that the frequency of bound arms was significantly greater in 102C2 than in 103K5 ($\chi^2 = 412.1, P < 0.001$).

From observations on AI cells it was found that recombination frequencies were significantly higher in 102C2 than in 103K5 ($\chi^2 = 7.13, P < 0.01$) (Table 4.2; Figs. 4.3a, b, c & 4.4a, b). 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 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 ($\chi^2 = 2107.6, P < 0.001$ and $\chi^2 = 249.8, P < 0.001$ 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, homoeologous chromosome arms were bound predominantly at distal regions and the recombination events involved only small terminal segments (Figs. 4.3a, b, c & 4.4a, b). Mean numbers of bound arms per PMC were 9.2 and 2.95 for hybrids 102C2 and 103K5 respectively, both of which were lower than that of either of their parents where the number was approximately 14. No evidence of recombination from double cross-overs was observed.
4.2.2 Chromosome elimination in PMCs of hybrids

Differences in chromosome number were seen among PMCs of both hybrids (Table 4.3). By using GISH it was possible to show that this was due to the selective loss of \textit{H. bulbosum} chromosomes. Furthermore, there were clear differences in the frequency of chromosome loss in the two hybrid combinations. Elimination of one or two \textit{H. bulbosum} chromosomes was seen in approximately 50% of 173 PMCs of hybrid 103K5 (Figs. 4.2a & b) 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 \textit{H. vulgare} chromosomes + 7 \textit{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 ($\chi^2 = 18.0$, $P < 0.001$) and between types A and C ($\chi^2 = 13.0$, $P < 0.001$), whereas the difference between type B and type C was not significant. Chromosome degradation occurred occasionally in 103K5, but not in 102C2. Deletions in \textit{H. bulbosum} chromosomes were observed in a few 103K5 PMCs with incomplete chromosome complement (Fig. 4.5a).

4.2.3 Chromosome segregation

Hybrid 102C2 showed a bipolar orientation of chromosomes in bivalents at MI and an ordered arrangement of segregation at AI in most of the PMCs examined (Figs. 4.1a & 4.3b) whereas 103K5 showed irregular chromosome congression and little evidence of polar migration of chromosomes (Figs. 4.2a, b & c, Fig. 4.4b). A comparison of chromosome polar orientation at MI and segregation pattern at AI in 102C2 showed that these were closely correlated ($\chi^2 = 0.314$, no significance). Furthermore, the chromosome segregation pattern in 102C2 showed a binomial distribution ($\chi^2 = 0.006$, no significance). In the PMCs examined at MI and AI of 102C2 (Table 4.4), the highest proportion of PMCs had a segregation pattern of $3B + 4V / 4B + 3V$ (52.3-57%) (Fig. 4.5b), followed by PMCs with $2B + 5V / 5B + 2V$ and $1B + 6V / 6B + 1V$ (31-34 % and 11-12.3% respectively).
Approximately 1% of PMCs had $0B + 7V / 7B + 0V$ (Fig. 4.6).

**4.2.4 Pollen viability**

Pollen viability was estimated using fluorescein-diacetate (FDA) staining and showed that there was a very low level of pollen stainability in both hybrids. 102C2 had three stained pollen grains from examination of 1636 pollen grains, while only one pollen grain was stained from a total of 1140 pollen grains for 103K5.
Table 4.1. Comparison of frequencies of MI pairing configurations (I = univalent; II = bivalent) in the *H. vulgare – H. bulbosum* hybrids (PMCs with fewer than 14 chromosomes have been excluded from the analysis).

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>No. of PMCs</th>
<th>MI pairing configurations</th>
<th>No. of bound arms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rods</td>
<td>Rings</td>
</tr>
<tr>
<td>102C2</td>
<td>69</td>
<td>44</td>
<td>299</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>0.64</td>
<td>4.33</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0-4</td>
<td>1-7</td>
</tr>
<tr>
<td>103K5</td>
<td>84</td>
<td>732</td>
<td>196</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>8.7</td>
<td>2.33</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>4-14</td>
<td>0-4</td>
</tr>
</tbody>
</table>
Table 4.2. Comparison of recombination frequencies at AI in the *H. vulgare* – *H. bulbosum* hybrids.

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>No. of PMCs</th>
<th>No. of recombinant <em>H. vulgare</em> chromosome arms per PMC</th>
<th>Total No. of the recombinant chromosome arms</th>
<th>Mean of all cells</th>
<th>Mean of cells showing recombination</th>
</tr>
</thead>
<tbody>
<tr>
<td>102C2</td>
<td>273</td>
<td>194 37 26 12 2 1 0 1</td>
<td>145</td>
<td>0.53</td>
<td>1.84</td>
</tr>
<tr>
<td>103K5</td>
<td>110</td>
<td>81 22 7 0 0 0 0</td>
<td>36</td>
<td>0.33</td>
<td>1.24</td>
</tr>
</tbody>
</table>
Table 4.3. Comparison of frequencies of MI pairing configurations in PMCs of the hybrid 103K5 with different chromosome numbers.

<table>
<thead>
<tr>
<th>Type of PMCs</th>
<th>No. of PMCs</th>
<th>Percentage (%)</th>
<th>Mean bound arms per PMC</th>
<th>Mean bound arms per potential II</th>
<th>MI pairing configurations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rods</td>
</tr>
<tr>
<td>A</td>
<td>84</td>
<td>48.6</td>
<td>2.95</td>
<td>0.42</td>
<td>732</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(8.7)*</td>
</tr>
<tr>
<td>B</td>
<td>67</td>
<td>38.7</td>
<td>1.95</td>
<td>0.33</td>
<td>631</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(9.4)</td>
</tr>
<tr>
<td>C</td>
<td>22</td>
<td>12.7</td>
<td>1.69</td>
<td>0.34</td>
<td>198</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(9.0)</td>
</tr>
</tbody>
</table>

Type A: PMCs with 14 chromosomes containing 7 H. vulgare chromosomes (7V) and 7 H. bulbosum chromosomes (7B).

Type B: PMCs with 13 chromosomes containing 7V and 6B.

Type C: PMC with 12 chromosomes containing 7V and 5B.

*: Mean MI pairing configurations per PMC in brackets.
Table 4.4. Chromosome segregation patterns at MI and AI in the hybrid 102C2. Expected values were obtained from the binomial expectation \((p + q)^9\) where \(p =\) possibility for presence of barley (or \(H.\ bulbosum\)) chromosome in one pole and \(q =\) possibility for absence of barley (or \(H.\ bulbosum\)) chromosome in this pole.

<table>
<thead>
<tr>
<th>Segregation patterns and number of PMC (percentage)</th>
<th>Total</th>
<th>0B + 7V / 7B + 0V</th>
<th>1B + 6V / 6B + 1V</th>
<th>2B + 5V / 5B + 2V</th>
<th>3B + 4V / 4B + 3V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed values MI</td>
<td>65</td>
<td>1 (1.5%)</td>
<td>8 (12.3%)</td>
<td>22 (34.0 %)</td>
<td>34 (52.3%)</td>
</tr>
<tr>
<td>Observed values AI</td>
<td>263</td>
<td>2 (0.76%)</td>
<td>29 (11%)</td>
<td>82 (31.0%)</td>
<td>150 (57.0%)</td>
</tr>
<tr>
<td>Expected values</td>
<td></td>
<td>1.56%</td>
<td>10.94%</td>
<td>32.8%</td>
<td>54.7%</td>
</tr>
</tbody>
</table>

0B + 7V / 7B + 0V:
7 \(H.\ vulgare\) chromosomes migrate to one pole;
7 \(H.\ bulbosum\) chromosomes to the opposite pole.

1B + 6V / 6B + 1V:
1 \(H.\ bulbosum\) chromosome and 6 \(H.\ vulgare\) chromosomes migrate to one pole;
6 \(H.\ bulbosum\) chromosomes and 1 \(H.\ vulgare\) chromosome to the opposite pole.

2B + 5V / 5B + 2V:
2 \(H.\ bulbosum\) chromosomes and 5 \(H.\ vulgare\) chromosomes migrate to one pole;
5 \(H.\ bulbosum\) chromosomes and 2 \(H.\ vulgare\) chromosomes to the opposite pole.

3B + 4V / 4B + 3V:
3 \(H.\ bulbosum\) chromosomes and 4 \(H.\ vulgare\) chromosomes migrate to one pole;
4 \(H.\ bulbosum\) chromosomes and 3 \(H.\ vulgare\) chromosomes to the opposite pole.
Figure 4.1. Meiotic metaphase I in
(a) a PMC of hybrid 102C2 with four ring and two rod bivalents.
(b) a PMC of hybrid 102C2 with two ring and five rod bivalents.

*H. vulgare* chromosomes are blue with DAPI counterstain and the labelled *H. bulbosum* chromosomes appear red with the HNPP fluorescent detection system. Bar = 10 μm.
Figure 4.2. Meiotic metaphase I in

(a) a twelve chromosome PMC of hybrid 103K5 with two rod bivalents.
(b) a thirteen chromosome PMC of hybrid 103K5 with two rod bivalents.
(c) a complete chromosome complement PMC of hybrid 103K5 with one ring and two rod bivalents.

*H. vulgare* chromosomes are blue with DAPI counterstain and the labelled *H. bulbosum* chromosomes appear red with the HNPP fluorescent detection system. Bar = 10 µm.
Figure 4.3. Meiotic anaphase I in

(a) a PMC of hybrid 102C2 with four recombinant barley chromosomes and three recombinant *H. bulbosum* chromosomes.

(b) a PMC of hybrid 102C2 with two recombinant barley chromosomes and two recombinant *H. bulbosum* chromosomes.

(c) a PMC of hybrid 102C2 with two recombinant barley chromosomes.

*H. vulgare* chromosomes are blue with DAPI counterstain and the labelled *H. bulbosum* chromosomes appear red with the HNPP fluorescent detection system.

Arrowheads indicate recombinant barley chromosomes. Arrows indicate recombinant *H. bulbosum* chromosomes. Bar = 10 μm.
Figure 4.4. Meiotic anaphase I in
(a) a PMC of hybrid 103K5 with two recombinant barley chromosome and one recombinant
*H. bulbosum* chromosomes.
(b) a PMC of hybrid 103K5 with one recombinant barley chromosome.

*H. vulgare* chromosomes are blue with DAPI counterstain and the labelled *H. bulbosum*
chromosomes appear red with the HNPP fluorescent detection system.
Arrowheads indicate recombinant barley chromosomes. Arrows indicate recombinant *H.
bulbosum* chromosomes. Bar = 10 μm.
Figure 4. 5.

(a) a PMC of hybrid 103K5 at MI with a deleted *H. bulbosum* chromosome segment (arrow).

(b) a PMC of hybrid 102C2 at AI with the segregation pattern of 3B + 4V/4B + 3V.

*H. vulgare* chromosomes are blue with DAPI counterstain and the labelled *H. bulbosum* chromosomes appear red with the HNPP fluorescent detection system. Bar = 10 μm.
Figure 4. 6. A PMC of hybrid 102C2 at AI with the segregation pattern of 0B + 7V/7B + 0V.

*H. vulgare* chromosomes are grey and the labelled *H. bulbosum* chromosomes appear black in the black and white photo taken under bright field of microscopy. Bar = 10 μm.
4.3 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 readily to clarify some aspects of the reduced recombination in hybrids between *H. vulgare* and *H. bulbosum*.

My observations have confirmed the lower level of meiotic pairing in hybrid 103K5 when compared to 102C2, and have 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. Second, the size of introgressed segments is related to their ease of detection. Jiang & 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 homoeologous chromosomes at MI in hybrids may be attributable to remnants of prophase pairing which is not related to crossing-over. The last, and most likely, explanation to account for this discrepancy is consistent with the observations in a study of wheat-rye hybrids by Orellana (1985), and if proved, would establish that the paucity of recombinant progeny from *H. vulgare* - *H. bulbosum* hybrids results largely from low levels of crossing-over between homoeologous chromosomes.

In 102C2 and 103K5, rod bivalents predominate (Tables 4.1 and 4.3), 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 suggests that there is 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, 2000 & Chapter 5).

Although the high-pairing hybrid 102C2 showed a 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 I have no ready explanation for the phenomenon. It is possible that the localisation of chiasmata is less precisely controlled in 103K5, which results in the formation of more interstitial chiasma and the exchange of larger
segments, which are then more readily detected by GISH (see above).

It was 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 high numbers 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, unpublished), where a negative correlation ($r = -0.528, P < 0.01$) between the number of univalents and mean chromosome number was found. Although type C PMCs did not differ statistically in the number of bound arms from type B, the result did show that there was a lower number of bound arms in type C than in type B. Since only 22 type C PMCs were involved in this study compared with 84 and 67 PMCs involved for types A and B, respectively, more type C PMCs needs to be analysed to establish the significance of this difference between types B and C.

Observation of chromosome segments in a few PMCs of 103K5 confirmed that the degraded chromosomes, which have been reported by Finch (1983) and Thomas (1988), were from the *H. bulbosum* genome. At present it is not possible to show whether the same chromosomes are eliminated preferentially or chromosomes are eliminated at random in this material.

The chromosomal behaviour at MI and AI stages of 102C2 conforms to that in barley. Only 102C2 had an obvious tendency toward chromosome polar migration and the pattern of chromosome segregation appeared to be at random. It seems likely that bivalent formation between homoeologous chromosomes is responsible for normal chromosome polar migration at MI and regular segregation of chromosomes at AI, and a lack of full bivalent formation in the hybrids could cause a disturbance in the ordered behaviour of the chromosomes. This result agrees with the idea that bivalent formation is normally an integral part of successful
segregation in meiosis (John, 1990). The observations of chromosomal behaviour of 102C2 at MI and AI showed that chromosomes from different parental genomes did not segregate to the opposite poles in about 99% of PMC examined. Approximately 1% of the PMCs showing the segregation pattern of 0B + 7V/7B + 0V possibly provides an explanation for the low level of pollen viability in the hybrid (0.18% FDA stainability in 1636 pollen grains).

In summary, the occurrence of homoeologous chromosome pairing provides a basis for recombination in H. vulgare - H. bulbosum hybrids, albeit at a lower frequency than in H. vulgare itself. Since high meiotic pairing seems to be associated with high recombination and regular chromosomal segregation at AI of the hybrids, selection of high-pairing hybrids has resulted in the development of recombinant barley plants with introgressed H. bulbosum chromatin.
Chapter five

Characterisation of recombinants from selfed progeny of
the hybrid 102C2/colch

5.1 Introduction

The introgression of agronomically important genes into crops from related species has been a
major goal for crop improvement because it enlarges the gene pool available for selection
(Gale & Miller, 1987; Lehmann & Bothmer, 1988; Pickering et al., 1995; King et al. 1998;
Chetelat & Meglic, 2000). There are two procedures for transferring alien chromatin into
crops through the process of sexual hybridisation. One is by using radiation-induced
translocations in hybrids between the targeted crop and closely related species to transfer
alien chromatin. Radiation-induced breakpoints occur at random intra- or inter-
chromosomally in the progeny of wheat-alien hybrids (Friebe et al., 1991, 1993; Mukai et al.,
1993). Another method for gene transfer is by crossing the targeted crop with closely related
species to obtain hybrids in which homoeologous recombination occurs without radiation.
This has been recently used in barley improvement (Xu & Kasha, 1992; Pickering et al.
1995). One advantage of the latter is that the transfer of alien chromatin occurs between
homoeologous chromosomes and can compensate for the loss of the missing chromatin
segment of the targeted crop.

Although preferential homologous pairing in amphidiploid H. vulgare - H. bulbosum hybrids
(VVBB) reduces inter-genomic recombination and results in most of the selfed progeny either
resembling diploid barley plants or hybrids (Lange & Jochemsen, 1976a; Thomas &
Pickering, 1983), the advantages of selecting recombinants from the selfed progeny of fertile
amphidiploid hybrids VVBB is attractive to barley breeders because selfed seeds are readily
formed and no backcrossing is required. Nevertheless, stable introgression of H. bulbosum
chromatin into the barley background has been rare and there are only two reports of the confirmed transfer of *H. bulbosum* resistance genes into barley by means of selfing amphidiploid hybrids (Michel *et al.*, 1995; Pickering *et al.*, 1995). However, a high-pairing sterile diploid *H. vulgare* - *H. bulbosum* hybrid 102C2 (VB) has recently been screened by R. Pickering at Crop & Food Research New Zealand, and has almost complete bivalent formation at MI of meiosis and a relatively high recombination level (see Chapter 4). Its fertility was restored after colchicine treatment to obtain an amphidiploid hybrid denoted as 102C2/colch (VVBB). Unlike other amphidiploid *H. vulgare* - *H. bulbosum* hybrids with fairly low seed setting (Thomas & Pickering, 1983), its seed setting was 36% after selfing. Furthermore, 74% of the 159 progeny after selfing of the 102C2/colch showed some *H. bulbosum* characters as well as powdery mildew and leaf rust resistance in the glasshouse and field (Pickering *et al.*, 1999).

5.1.1 *Methods for characterising recombinants*

In order to further characterise the recombinants from *H. vulgare* - *H. bulbosum* hybrids, GISH and molecular analyses with single or low-copy restriction fragment length polymorphism (RFLP) probes, amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) have been exploited successfully (Xu & Kasha, 1992; Pickering *et al.*, 1995, 1997). However, these procedures for characterising recombinants have been expensive, time-consuming and laborious. Jiang and Gill (1993) successfully identified and localised breakpoints of wheat-rye translocations T1AL·1RS and T4BS·4BL-6RL using sequential N-/C-banding and GISH techniques, but with some loss of resolution of *in situ* hybridisation. Pederson and Linde-Laursen (1994) successfully performed fluorescence *in situ* hybridisation (FISH) with a labelled oligonucleotide (GAA) to show hybridisation patterns similar to barley N-banding patterns and identified individual barley chromosome arms with a higher resolution than could be obtained with Giemsa N-banding. These reports show the possibilities for further fast and efficient characterisation of recombinants using *in situ* hybridisation and banding techniques. Furthermore, Pickering *et
al. (2000) established that GISH followed by FISH with (CTT)\textsubscript{10} (complementary to GAA sequence) detected and localised the introgressed \textit{H. bulbosum} chromatin in barley.

### 5.1.2 Applications of in situ hybridisation and molecular mapping

By applying \textit{in situ} hybridisation techniques to recombinants from the selfed or backcrossed progeny of \textit{H. vulgare} – \textit{H. bulbosum} hybrids, it is possible to establish which chromosomes or chromosome arms are mostly involved in inter-genomic recombination. Combined with the data from pathological tests on the recombinants, it enables the localisation of the introgressed genes for disease/pest resistance, and will provide useful information for cytogenetic analyses and breeding programmes.

By comparing the physical maps of introgressions with genetic maps using molecular markers, cytogenetically-based physical maps can be constructed that define the subregional distribution of the introgression and their closely-linked molecular markers. These map constructions will be a critical first step for the map-based cloning of agronomically useful genes within the introgressed segments, especially from crops with large genomes (e.g. barley).

### 5.1.3 Size determination of introgressions

The exchanged chromosome segments in the \textit{H. vulgare} – \textit{H. bulbosum} hybrid 102C2 are located in the distal regions of chromosomes (see Chapter 4), and correspond with the distal location of the introgressions in recombinant progeny from this hybrid. Distally located introgressions were also seen in recombinants from other \textit{H. vulgare} – \textit{H. bulbosum} hybrids (Pickering et al., 1997, 2000). However, it is not known whether the different introgressions found in similar chromosomal regions show variation in size. If the sizes do vary, it will facilitate positioning the introgressed genes in smaller chromosomal regions using a series of recombinants. This can provide more accurate physical positions for the genes of interest within introgressed segments.
Thus, the main objectives of the present study were:

1) to establish a cheap, fast and efficient method for detecting and localising the introgressions of *H. bulbosum* chromatin into barley by comparing two procedures, namely a sequential C-/N-banding followed by GISH with labelled total genomic *H. bulbosum* DNA compared with a combination of the GISH and FISH with labelled (GAA)$_{10}$; 2) to locate the introgressions mediating the same or different agronomic traits among the recombinants; 3) to estimate the relative sizes of the introgressions and use the information for assessing the possibilities for map-based cloning.

5.2 Results

5.2.1 Comparison of C-/N-banding followed by GISH and combination of GISH and FISH

Sequential C-/N-banding and GISH were initially carried on the recombinants. The results showed that the GISH signals following C-/N-banding were poor and fluorescent counterstaining was much weaker than usual. Modified 0.2 M HCl treatment at 37°C and room temperature for C-banding, which caused some loss of resolution in banding sites, did not improve the intensity of GISH signals. Weak introgressions were occasionally seen, but were too faint to be recorded and photographed.

The GAA-repetitive sequence used as a probe for FISH produced a hybridisation pattern resembling that of C-/N-banding (Pedersen *et al.*, 1996). There were two types of FISH probes used. One was the GAA-satellite sequence clone pHvC39, which is primarily composed of GAA repeats. The other comprised oligonucleotide GAA repeats. These two probes were hybridised to barley chromosomes and results showed that the hybridisation sites with (GAA)$_{10}$ probe were more diagnostic and reproducible than with pHvC39. With pHvC39, insufficient hybridisation sites on barley chromosomes 2H and 7H made
identification difficult despite the use of different washing and hybridisation stringencies. Therefore, a combination of GISH and FISH with (GAA)$_{10}$ was the better choice for detecting and localising introgressions in the recombinants.

Since GISH analysis on the diploid hybrid 102C2 established that recombination events only involved distal segments (see Chapter 4) and hybridisation sites with (GAA)$_{10}$ were proximal and interstitial (Pedersen & Linde-Laursen, 1994; also see Fig. 5.1), the introgression and GAA hybridisation sites were not co-localised in the 102C2 recombinants. Thus, the reporter molecule digoxigenin (DIG) and the same detection system (Fluorescein Antibody Enhancer Set for DIG Detection) were applied to both GISH and FISH. First, sequential GISH and FISH procedures were used in attempts to characterise recombinants. However, cross-hybridisation signals after GISH frequently blocked the GAA banding sites even after trying to remove most of GISH-probes with the standard washing procedure (Heslop-Harrison et al., 1992), and also with a higher washing stringency at 42°C + 0.2% Tween-20. This made the sequential GISH and FISH procedure inefficient. When a sequential FISH and GISH procedure was adopted, detection and localisation of introgressions was greatly improved. After identifying chromosomes by FISH and carrying out a standard wash for reprobing, introgressions in the recombinants could be detected with GISH on 'FISHed' slides despite the simultaneous presence of FISH and GISH signals on chromosomes (see Fig. 5.2a & b).

5.2.2 Characterisation of the putative recombinants with resistance to leaf rust and powdery mildew

From the pathological tests performed on selfed progeny of the hybrid, twelve putative recombinants were selected. All of these were resistant to leaf rust and two of them were also resistant to powdery mildew (Table 5.1). These putative recombinants were further characterised using sequential FISH and GISH analyses. Apart from recombinants 102C2/10/7 and 102C2/18/3, in which no introgressions were detected, the ten remaining recombinants all showed distal signals. Of these ten recombinants, eight that were resistant to
leaf rust but susceptible to powdery mildew had 2HL distal introgressions (Fig. 5.2b, Figs. 5.3a, b, c & d, Figs. 5.4a, b &c). Among these eight recombinants, 3HS and 1HL introgressions were detected in 102C2/13/2 (Fig. 5.3c) and 102C2/16/2 (Fig. 5.2b), respectively. From these data it was concluded that 2HL introgressions were associated with leaf rust resistance and 3HL and 1HL did not confer any leaf rust resistance. No segregation for leaf rust resistance was observed in recombinant populations containing two 2HL introgressions, while there was segregation for leaf rust resistance in selfed populations derived from 102C2/13/2 and 102C2/15/1, each of which possessed only a single 2HL introgression. In 102C2/16/2 carrying a pair of 2HL introgressions and a single 1HL introgression, there was no segregation of leaf rust resistance observed among its selfed progeny. 102C2/14 segregated for leaf rust resistance (Pickering, personal communication) but a selfed selection from this plant (102C2/14/1) was homozygous for leaf rust resistance and had two 2HL introgressions. All these results established that a 2HL introgression was associated with leaf rust resistance and that two signals on 2HL were associated with homozygosity at the locus for leaf rust resistance.

Two remaining recombinants (102C2/18/2/2 and 102C2/18/2/12) (Fig. 5.5a & b), both of which were resistant to leaf rust and powdery mildew and were derived from the same F$_2$ plant (102C2/18/2), carried terminal 2HS introgressions. It appeared, therefore, that leaf rust and powdery mildew resistances were associated with loci in the distal region of 2HS. 102C2/18/2/2 also showed semi-sterility, but no other introgression was detected in this recombinant.

5.2.3 Characterisation of recombinants with features of semi-sterility and short-straw

Apart from screening for resistance, nine putative recombinants were also selected among the selfed progeny on the basis of their morphological traits. These putative recombinants were either short-strawed or semi-sterile and were subsequently characterised by sequential FISH
and GISH analyses. From the results, it can be concluded that three of them carried a single or a pair of distal signals (Fig. 5.5c, d & e), but no signals were detected in the other six plants. Of the three established recombinants, 102C2/12/1 had a pair of 4HL introgressions and was short-strawed whereas 102C2/16/1/1-10 had a single 7HS introgression but was semi-sterile, while 102C2/17/2 with a pair of 6HL introgressions was short-strawed and semi-sterile. From these data, it was not possible to determine the location of the genes responsible for short straw or semi-sterility.

5.2.4 Introgression size in the recombinants

There was considerable variation in introgression size both among the different chromosomal sites (1HL, 2HS, 2HL, 3HS, 4HL, 6HL and 7HS) and among different recombinants with introgressions at similar chromosomal sites. Mean relative lengths (% of the total haploid chromosome complement) of introgressed segments at different locations ranged from 0.68 (1HL) to 2.20 (2HL) (Table 5.2). Regarding the most frequently observed introgressions (2HL), the smallest and largest introgressions had mean relative lengths of 0.92 (range = 0.72 - 1.12) and 2.20 (range = 1.95 - 2.52), respectively (Table 5.2). The size of the introgressions on 2HL differed significantly among some recombinants but not others (F = 44.23, P< 0.01; Table 5.3).
Table 5.1. Results of FISH and GISH analyses on 102C2 recombinants.

<table>
<thead>
<tr>
<th>Code number</th>
<th>Comments</th>
<th>Number and location of introgressions</th>
<th>Introgression size relative to the recombinant chromosome (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>102C2/11/1/2</td>
<td>leaf rust resistant</td>
<td>a pair of homologous 2HL introgressions</td>
<td>6.2</td>
</tr>
<tr>
<td>102C2/11/3</td>
<td>leaf rust resistant</td>
<td>a pair of homologous 2HL introgressions</td>
<td>6.8</td>
</tr>
<tr>
<td>102C2/13/2</td>
<td>leaf rust resistant (maybe heterozygous)</td>
<td>a single 2HL introgression</td>
<td>14.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a pair of homologous 3HS introgressions</td>
<td>9.8</td>
</tr>
<tr>
<td>102C2/13/3</td>
<td>leaf rust resistant</td>
<td>a pair of homologous 2HL introgressions</td>
<td>12.8</td>
</tr>
<tr>
<td>102C2/14/1</td>
<td>leaf rust resistant</td>
<td>a pair of homologous 2HL introgressions</td>
<td>8.0</td>
</tr>
<tr>
<td>102C2/15/1</td>
<td>segregating for leaf rust resistant</td>
<td>a single 2HL introgression</td>
<td>12.7</td>
</tr>
<tr>
<td>102C2/16/2</td>
<td>leaf rust resistant</td>
<td>a pair of homologous 2HL introgressions</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a single 1HL introgressions</td>
<td>5.6</td>
</tr>
<tr>
<td>102C2/16/2/1-10</td>
<td>leaf rust resistant</td>
<td>a pair of homologous 2HL introgressions</td>
<td>7.9</td>
</tr>
<tr>
<td>102C2/18/2/2</td>
<td>leaf rust, partial mildew resistant and semi-sterile</td>
<td>a pair of homologous 2HS introgressions</td>
<td>8.0</td>
</tr>
<tr>
<td>102C2/18/2/12</td>
<td>leaf rust and mildew resistant</td>
<td>a pair of homologous 2HS introgressions</td>
<td>7.1</td>
</tr>
<tr>
<td>102C2/12/1</td>
<td>short-strawed</td>
<td>a pair of homologous 4HL introgressions</td>
<td>7.3</td>
</tr>
<tr>
<td>Entry</td>
<td>Description</td>
<td>Introgression</td>
<td>Value</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------------------------------</td>
<td>---------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>102C2/16/1/1-10</td>
<td>leaf rust susceptible, semi-sterile</td>
<td>a single 7HS introgression</td>
<td>5.7</td>
</tr>
<tr>
<td>102C2/17/2</td>
<td>short-strawed, semi-sterile</td>
<td>a pair of homologous 6HL introgressions</td>
<td>5.5</td>
</tr>
<tr>
<td>102C2/2/3/1</td>
<td>semi-sterile</td>
<td>No introgression detected</td>
<td></td>
</tr>
<tr>
<td>102C2/2/5/1</td>
<td>semi-sterile</td>
<td>Ditto</td>
<td></td>
</tr>
<tr>
<td>102C2/4/5</td>
<td>semi-sterile</td>
<td>Ditto</td>
<td></td>
</tr>
<tr>
<td>102C2/5/3</td>
<td>semi-sterile</td>
<td>Ditto</td>
<td></td>
</tr>
<tr>
<td>102C2/7/6</td>
<td>small plant</td>
<td>Ditto</td>
<td></td>
</tr>
<tr>
<td>102C2/9/3/3</td>
<td>semi-sterile</td>
<td>Ditto</td>
<td></td>
</tr>
<tr>
<td>102C2/10/7</td>
<td>leaf rust resistant in field but susceptible in glasshouse test</td>
<td>Ditto</td>
<td></td>
</tr>
<tr>
<td>102C2/18/3</td>
<td>leaf rust resistant (may be segregating)</td>
<td>Ditto</td>
<td></td>
</tr>
</tbody>
</table>
Table 5.2. Measurements of the relative length of introgression size in the recombinants.

<table>
<thead>
<tr>
<th>Code number</th>
<th>Size of introgression</th>
<th>Chromosomal location of introgressions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative length in different cells (%)</td>
<td>Mean (%)</td>
</tr>
<tr>
<td></td>
<td>1.06 1.10 1.12</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>1.40 1.41</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>2.05 2.20 2.23</td>
<td>2.20</td>
</tr>
<tr>
<td></td>
<td>1.38</td>
<td>2HL</td>
</tr>
<tr>
<td></td>
<td>1.16</td>
<td>3HS</td>
</tr>
<tr>
<td></td>
<td>1.94</td>
<td>2HL</td>
</tr>
<tr>
<td></td>
<td>1.41</td>
<td>2HL</td>
</tr>
<tr>
<td></td>
<td>1.56</td>
<td>2HL</td>
</tr>
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<td></td>
<td>1.36</td>
<td>1HL</td>
</tr>
<tr>
<td></td>
<td>1.26</td>
<td>2HL</td>
</tr>
<tr>
<td></td>
<td>1.17</td>
<td>2HS</td>
</tr>
<tr>
<td></td>
<td>1.04</td>
<td>2HS</td>
</tr>
<tr>
<td></td>
<td>1.04</td>
<td>4HL</td>
</tr>
<tr>
<td></td>
<td>0.77</td>
<td>7HL</td>
</tr>
<tr>
<td></td>
<td>0.79</td>
<td>6HL</td>
</tr>
</tbody>
</table>

Relative length of the introgression is calculated as length of introgression signal relative to total length of haploid chromosome complement.
Table 5.3. A comparison of the size of 2HL introgressions among recombinants.

<table>
<thead>
<tr>
<th></th>
<th>102C2/13/3</th>
<th>102C2/15/1</th>
<th>102C2/16/2</th>
<th>102C2/16/2/1-10</th>
<th>102C2/14/1</th>
<th>102C2/11/3</th>
<th>102C2/11/1/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>102C2/13/2</td>
<td>N.S.</td>
<td>N.S.</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>102C2/13/3</td>
<td>N.S.</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>102C2/15/1</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>102C2/16/2</td>
<td>N.S.</td>
<td>*</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td>**</td>
<td></td>
</tr>
<tr>
<td>102C2/16/2/1-10</td>
<td>N.S.</td>
<td>N.S.</td>
<td>**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>102C2/14/1</td>
<td>N.S.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>*</td>
</tr>
<tr>
<td>102C2/11/3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>N.S.</td>
</tr>
</tbody>
</table>

N.S.: No significant difference.
* : Significant difference at 0.05 level.
** : Significant difference at 0.01 level.
Figure 5.1. FISH with the (GAA)$_{10}$ oligonucleotide to mitotic metaphase chromosomes of *Hordeum vulgare* cv. Emir. Overall, the hybridisation pattern is similar to Giemsa N-banding pattern and permits the unequivocal identification of the chromosomes.

The chromosomes of *Hordeum vulgare* are red with PI counterstain and hybridisation signals appear green with the Fluorescent Antibody Enhancer for DIG detection system. Bar = 10 μm.
Figure 5.2.

(a) FISH with the (GAA)$_{10}$ oligonucleotide to mitotic metaphase chromosomes of *Hordeum vulgare* – *H. bulbosum* recombinant 102C2/16/2.

(b) GISH with total genomic DNA from *H. bulbosum* to the same metaphase plate after reprobing washes, showing one pairing of 2HL introgressions (arrows) and a single 1HL introgression (arrowhead).

The chromosomes of *Hordeum vulgare* are red with PI counterstain and hybridisation signals appear green with the Fluorescent Antibody Enhancer for DIG detection system. Bar = 10 µm.
Figure 5.3. Sequential FISH and GISH to mitotic metaphase chromosomes of *Hordeum vulgare* – *H. bulbosum* recombinant showing:

(a) A pair of 2HL introgressions (arrows) in the recombinant 102C2/11/1/2.

(b) A pair of 2HL introgressions (arrows) in the recombinant 102C2/11/3.

(c) A single 2HL introgression (arrow) and a pair of 3HS (arrowheads) introgressions in the recombinant 102C2/13/2.

(d) A pair of 2HL introgressions (arrows) in the recombinant 102C2/13/3.

The chromosomes of *Hordeum vulgare* are red with PI counterstain and hybridisation signals appear green with the Fluorescent Antibody Enhancer for DIG detection system. Bar = 10 μm.
**Figure 5.4.** Sequential FISH and GISH to mitotic metaphase chromosomes of *Hordeum vulgare*—*H. bulbosum* recombinant showing:

(a) A pair of 2HL introgressions (arrows) in the recombinant 102C2/14/1.

(b) A single 2HL introgression (arrow) in the recombinant 102C2/15/1.

(c) A pair of 2HL introgressions (arrows) in the recombinant 102C2/16/2/1-10.

The chromosomes of *Hordeum vulgare* are red with PI counterstain and hybridisation signals appear green with the Fluorescent Antibody Enhancer for DIG detection system. Bar = 10 μm.
Figure 5.5. Sequential FISH and GISH to mitotic metaphase chromosomes of *Hordeum vulgare* – *H. bulbosum* recombinant showing:

(a) A pair of 2HS introgressions (arrows) in the recombinant 102C2/18/2/2.
(b) A pair of 2HS introgressions (arrows) in the recombinant 102C2/18/2/12.
(c) A pair of 4HL introgressions (arrows) in the recombinant 102C2/12/1.
(d) A single 7HS introgression (arrow) in the recombinant 102C2/16/1/1-10.
(e) A pair of 6HL introgressions (arrows) in the recombinant 102C2/17/2. NOR on chromosome 6HL is indicated by arrowhead.

The chromosomes of *Hordeum vulgare* are red with PI counterstain and hybridisation signals appear green with the Fluorescent Antibody Enhancer for DIG detection system. Bar = 10 μm.
5.3 Discussion

5.3.1 Approaches to identification of H. vulgare - H. bulbosum recombinants

Although Jiang and Gill (1993) detected translocations of wheat-rye chromosomes using sequential C-/N-banding and GISH, this procedure was unsuitable for detecting introgressions in H. vulgare - H. bulbosum recombinants. GISH signals on C-/N-banded chromosome preparations were too weak to be recorded. Modified Giemsa C- or N-banding techniques reduced the reproducibility of banding patterns, but did not improve the detection of GISH signals. There are two possible reasons for this. First, barley chromosomes might be more sensitive to acidic, alkaline or high temperature treatment. Second, detection of smaller introgressions requires higher quality mitotic chromosome preparations compared with the larger translocated segments reported by Jiang & Gill (1993). Acidity, alkalinity or high temperatures in the phosphate buffer can greatly affect chromosome quality and result in reduced GISH resolution, which was also found by Jiang and Gill (1993). Heslop-Harrison and Schwarzacher (1996) pointed out that acid treatment solubilised and depurinated DNA and thus had an influence on DNA in situ hybridisation. This study shows that there is much weaker fluorescent counterstaining on C-/N-banding treated chromosomes, suggesting that C-/N-banding treatments cause over-denaturation of the barley mitotic chromosomes.

Pickering et al. (2000) demonstrated that identification of introgressed H. bulbosum chromatin in barley can be accomplished using GISH with H. bulbosum genomic DNA followed by FISH with a oligonucleotide (CTT)$_{10}$. The present study confirmed the efficiency of these methods compared with sequential C-/N-banding and GISH. Furthermore, in the present study, FISH with the oligonucleotide (GAA)$_{10}$ produced better diagnostic signals for all barley chromosome arms compared with the clone pHvC39, which could not reliably identify all barley chromosomes. It is probable, therefore, that although the insert of pHvC39 is mainly composed of repetitive GAA sequence it does not show perfect repeats of GAA.
The sequential FISH and GISH procedure to detect and localise introgression was also found to be more efficient than GISH followed by FISH. Cross-hybridisation signals occur to some extent on ‘GISHed’ chromosomes of the recombinants because *H. vulgare* and *H. bulbosum* share many conserved DNA sequences in their genomes (Schwarzacher *et al.*, 1992b). It was not possible to remove all cross-hybridisation signals on the barley chromosomes from the first round of GISH when followed by FISH and consequently the GISH signals frequently blocked some of the FISH hybridisation sites. Thus, sequential FISH with (GAA)$_{10}$ followed by GISH appears to be the most efficient method when comparing different combinations of C-/N-banding, FISH and GISH in the present study.

GISH combined with molecular analyses such as RFLP, AFLP and RAPD was successfully used to detect and localise introgressions from *H. bulbosum* chromatin into barley chromosomes (Xu & Kasha, 1992; Pickering *et al.*, 1995, 1997). Although this method is a powerful tool to analyse the recombinants, it requires many experimental steps and is lengthy, laborious and expensive. These requirements include: 1) the purification and digestion of genomic DNA with restriction enzymes, 2) electrophoretic separation of the DNA fragments and the transfer of the digested DNA fragments to membranes, 3) isotopic probe labelling and long exposure time for detection, 4) serial PCR reactions and the associated high cost of *Taq* polymerase. Furthermore, RAPD techniques can also suffer from inconsistencies and unreliability (Chetelat & Meglic, 2000). However, sequential FISH and GISH can detect and localise introgressions in the recombinants with technical simplicity in only 2-2.5 days, depending on the amplification steps adopted for detection. Thus, this method is a fast, efficient and inexpensive alternative to GISH combined with molecular analyses as long as the introgression size falls into the limits of the resolution of GISH.

5.3.2 Introgression of *H. bulbosum* chromatin into barley background

Sequential FISH and GISH provides a direct, fast and efficient method to identify
introgressions in recombinants within the limits of GISH resolution. Using this method, thirteen of the 21 putative recombinants from selfed progeny of 102C2/colch were found to contain distal introgressions, while the other eight putative recombinants proved negative. It is possible that if there were introgressed segments in these eight lines, they remained undetected because the introgressed segments were too small for detection by GISH. A second and more likely explanation is that there were no introgressions in some of these putative recombinants.

5.3.2.1 Location of the introgressions
Recently leaf rust resistance has been successfully transferred from H. bulbosum into barley using an interspecific tetraploid hybrid (Pickering et al., 1998) and partially fertile triploid hybrids (Pickering et al., 2000). The two leaf rust resistance genes that were transferred are non-allelic and located on barley chromosomes 2HS (recombinant “81882”) and 2HL (recombinant “38P18”). Sequential FISH and GISH analysis on the ten recombinants with leaf rust resistance, combined with observations on their progeny (Pickering, personal communication), confirms that the two leaf rust resistance genes from H. bulbosum chromatin were transferred into the distal regions of barley chromosomes 2HS and 2HL. In the present investigation, there were also 2HS distal introgressions in two recombinants with resistance to both leaf rust and powdery mildew. This result supports the conclusion of Pickering et al. (1998) that there is a strong linkage between powdery mildew and leaf rust resistance loci on chromosome 2HS in the recombinant “81882”, which was derived from irradiated selfed progeny of the tetraploid hybrid “Vada VB1”. Kasha et al. (1996) reported that a powdery mildew resistance gene from H. bulbosum was located on chromosome 2HL of recombinant “BC1-2”, which is non-allelic to the powdery mildew resistance gene on the chromosome 2HS in the report by Pickering et al. (1998). These results, together with the report by Pickering et al., (1999) where the most frequent introgressions occur on barley chromosomes 2HS and 2HL, indicate that using interspecific H. vulgare – H. bulbosum hybrids is an effective approach to transferring leaf rust and powdery mildew resistance genes from H.
bulbosum into barley background.

Along with data from pathological test and plant morphology, the present study indicates that genes controlling short-straw and fertility are not located on the distal region of chromosome 2HS and 2HL, instead they appear to be located on chromosomes 4HL, 6HL and 7HS or other chromosomal region that are not detected at present. Introgessions were detected in three putative recombinants with short straw and/or semi-sterility, but which were susceptible to leaf rust and powdery mildew pathogens. These three recombinants had introgressions at different sites and will be characterised further with more sensitive methods such as RFLP or AFLP techniques to more accurately define their location in the genome.

5.3.2.2 Variation of introgression size

Size variations of 2HL introgressions among recombinants can be attributed partly to differences in the quality of cells in chromosome preparations. Some cells tend to be surrounded with more cytoplasm than others, which results in a variable signal intensity that affects the estimated sizes of the introgressed segments. This could be minimised by screening the slides by light microscopy before performing in situ hybridisation. However, variation in chiasma localisation is also more likely to be responsible for some of the observed variation in introgression size, especially as there were significant differences between different recombinants.

It is already known that the size of introgressed segments varies among progeny derived from wide hybrids. For example, Friebe et al. (1993) reported a wide range in the size of 7Ai#2L introgressed chromatin segments associated with leaf rust resistance in five wheat-A. intermedium translocation lines. The sizes differed by a factor of two. In the present study, although variability of introgression sizes could be partly related to preparation quality, it is clear that the relative lengths of the introgressions vary considerably among different 2HL recombinant lines. It is possible, therefore, to establish a more precise location for the leaf
rust resistance gene by combining data from GISH and molecular mapping. Since
102C2/11/1/2 contains the smallest introgression, it would be the most suitable recombinant
line for map-based cloning of the resistance gene.
Chapter six

Summary

H. bulbosum has the potential to be an important donor species for barley breeding as it shows many useful agronomic traits. There is, therefore, a desire to combine valuable agronomic traits such as disease and pest resistance with high yield and grain quality by interspecific hybridisation. Although the stable H. vulgare – H. bulbosum hybrids are now relatively easy to produce, the production of the recombinants from these hybrids is not straightforward and one major barrier has been the low recombination rate of these hybrids. In order to hasten progress in the transfer of genes from H. bulbosum, further investigation of H. vulgare - H. bulbosum hybrids, the parental species and characterisation of recombinants from the progeny of the selfed hybrid 102C2/coltch were conducted in this study. This chapter will summarise the three main results of the present study and briefly discuss the directions of future research.

6.1 Variation in SC length

SCs have not been studied in any detail in the genus Hordeum apart from one report on haploid barley (Gillies, 1974). This is in contrast to the many observations on both mitotic karyotypes and chromosomal behaviour at metaphase I in many species including H. vulgare and H. bulbosum as well as their hybrids (Lange & Jochemsen, 1976b; Fukui & Kakeda, 1990; Linde-Laursen et al., 1992; Bothmer et al., 1983, 1986). Several key points have emerged from the present study. In both the species and their hybrids it is clear that SC has a defined range of length variation. This supports previous observations on various plants that SC length shows extensive intraspecific variation, which is presumed to reflect stage-related differences in length during pachytene (Anderson et al., 1985; De Azkue & Jones, 1993).
Jaffe (1998) reported that *H. vulgare* had a recombination rate that was approximately three times greater than *H. bulbosum*, and in this study there was a similar relationship, though not so extreme, for SC length. The hybrids 102C2 and 103K5 that were studied also show differences in SC length and recombination rate. Thus the longer SCs are associated with the higher recombination rates in the species and their hybrids. A similar relationship has also been found in humans, as well as other animals and plants (Bojko, 1983, 1985; Donis-Keller *et al.*, 1987; Jones & Croft, 1989; De Azkue & Jones, 1993; Quevedo *et al.*, 1997). This has led to the conclusion that SC length is positively correlated with recombination frequency.

Genome size variation has also been implicated in the variation of SC length. There is a positive relationship between SC length and genome size in some plants and animals (Jørgensen & Bak, 1981; Anderson *et al.*, 1985), but this relationship has not been observed in many other plants, animals and yeast (Mogensen, 1977; Peterson *et al.*, 1994). The results of the present study do not support this positive relationship and show that there is extensive divergence of the ratio of mean SC length to genome size (SC/DNA).

The mechanism for the association of longer SC with higher recombination rate is unknown at present. Loidl (1994) compared SC length and genome size in many species of plants, animals and yeast and found the ratio of SC/DNA was positively correlated with recombination frequency. Since different SC/DNA ratios reflect different degree of coiling of chromatin, one would expect that the conformation of chromatin along axial element might regulate recombination events. The hypothesis put forward by Loidl (1994) for this is that the conformation of chromatin anchored to axial elements has an influence on recombination rate. The mechanism for this might be that less dense DNA compaction facilitates more DNA contact between homologues and subsequently higher recombination rate occurs between them. Support for this hypothesis came from the comparison of human-derived YACs and human DNA in its natural environment, which found that looser DNA packaging conferred higher recombination frequency (Loidl *et al.*, 1995). Likewise, it is possible that SC length.
might be affected by the condition of chromosome axes and their associated chromatin at very early meiotic prophase I as suggested by Quevedo et al. (1997) because the initial events of recombination occur prior to SC formation in some organism like yeast (Padmore et al., 1991).

6.2 Meiotic pairing and recombination in the hybrids

In early prophase I in both hybrids, there is extensive formation of bivalents, multivalents and a paucity of univalents but only univalents and bivalents were seen at metaphase I. This leads to the suggestion that there is extensive non-homologous pairing at prophase I, which also have been reported in other interspecific hybrids in the Poaceae (Albini & Jones, 1990; Jenkins & White, 1990; Cuñado & Santos, 1999). Furthermore, although there is about 2.5-fold difference in the number of bivalents at metaphase I between these two hybrids, their percentages of synopsis at pachytene are similar. This suggests that there are different mechanisms regulating synopsis at prophase I and chiasma formation at metaphase I in the hybrids.

Recombination frequency in the hybrids is the most critical factor affecting gene transfer from *H. bulbosum* into the barley background following sexual hybridisation. The results of the present study and other previous studies (Le & Armstrong, 1991; Benavente et al., 1998) indicate that GISH is a powerful tool for the direct determination of cross-overs in interspecific hybrids. Moreover, this study has shown that the high pairing hybrid has a significantly higher recombination rate than the low pairing one. Thus, selection of the higher pairing *H. vulgare – H. bulbosum* hybrids could result in more recombinants in their progeny, which should facilitate more effective gene transfer.

Genes that control chromosome pairing have been found in wheat and wheat-alien hybrids (Sears, 1981; Benavente et al., 1998; Chen et al., 1994) and in *H. vulgare – H. bulbosum* hybrids there may be a similar genes that control pairing. The results of this study as well as
earlier ones (Thomas & Pickering, 1985; Xu & Snape, 1988) have shown that the genotype of *H. bulbosum* has a key influence on homoeologous pairing, which results in different recombination frequency in the *H. vulgare – H. bulbosum* hybrids. Pickering *et al.* (1999) reported that 102C2 had more recombinants from its selfed progeny and high seed setting compared to other available *H. vulgare – H. bulbosum* hybrids. This suggests that 102C2 is a useful cross combination and that its *H. bulbosum* parent (HB2032) carries a pairing-promoting gene.

### 6.3 Characterisation of recombinants

Using sequential F/GISH it is possible to localise introgressed segments (introgressions) on particular chromosome of potential recombinants from progeny of *H. vulgare – H. bulbosum* hybrids. The results of this study and the previous ones (Pickering *et al.*, 1995, 2000) have revealed that this method provides higher resolution compared to sequential C-/N-banding and GISH procedures and proves to be cheaper, faster, and more efficient within GISH detection ability than the procedure using GISH and molecular analysis (RFLP and AFLP).

The results of this study and a previous one using wheat-*Agropyron intermedium* derivatives (Friebe *et al.*, 1993) indicate extensive variation in size of the introgressed segment. This study has also shown that there is a considerable variation in the size of introgressions associated with leaf rust resistance among the *H. vulgare – H. bulbosum* recombinants. This feature can be used to estimate more precisely the location of the gene of interest and consequently provides useful information for map-based cloning of the gene.

Recombination has been reported to be distally localised in barley (Pedersen *et al.*, 1995). This is further confirmed by the present study in the *H. vulgare – H. bulbosum* recombinants. It might be expected that there would be very low levels of recombination in the proximal regions of barley and *H. bulbosum* chromosomes because heterochromatin, which has a suppressing effect on recombination in the surrounding euchromatin (John, 1988), is
distributed around proximal region in both species. However, it is possible that there are genes of interest in interstitial or proximal region and irradiation may be an effective option for transferring gene of interest from such locations. In wheat-alien hybrids radiation-induced breakpoints in chromosomes appear to be random (Friebe et al., 1991; Mukai et al., 1993) and this may be a way of obtaining recombinants with genes from these areas.

There is a big discrepancy in the number of recombinant chromosomes seen at AI in 102C2 and in its progeny. At AI up to seven recombinant barley chromosomes per cell were observed but the maximum number of introgression found in the progeny is two. There are at least three possible reasons to account for this discrepancy. First, some introgressed segment may be too small to be detected by GISH as they might fall below the limits of GISH detection ability. Second, since barley is diploid it cannot tolerate extensive chromosomal manipulation, too many recombinant chromosomes may disrupt the viability of gamete and cause sterility. Third, many of recombinant seedlings do not survive beyond this stage as they are genetically unbalanced.

6.4 Future directions

Abnormality in the SC has been reported to be associated with reduced fertility in several plants such as Arabidopsis thaliana and rye that show meiotic mutation (Peirson et al., 1997; Bogdanov et al., 1998). For example, Bogdanov et al. (1998) reported that there were bar- and thorn-like abnormalities in the SCs of a mutant rye (Ms6), which was characterised by partially reduced fertility. As many of recombinants from 102C2/colch hybrid studied here show semi-sterility, it would be interesting to investigate whether these semi-sterile recombinants show abnormalities in the SCs compared to the recombinants with normal fertility. This work will lead to an advance in better understanding of the meiotic process.

Due to the limit of GISH for the detection of alien introgressions on mitotic chromosomes, higher resolution of GISH is highly desirable. Pachytene chromosomes, which are about 10
times as long as mitotic chromosome, provide an alternative target for GISH. FISH on pachytene chromosome has successfully detected highly repeated DNA sequences in rye, *Aegilops uniaristata* and *Muscari comosum* (Albini & Schwarzacher, 1992; Cuñado & Santos, 1998) and single / lower copy sequences in tomato (Peterson *et al.*, 1999). This approach should also be applicable to *Hordeum* species and their hybrids. Recently, Brown *et al.* (1999) reported new landmarks for the identification of barley chromosomes. They found that using a combination of the 18S rDNA from *Aedes aegypti* and the 5S rDNA from *Matthiola incana* in a simultaneous FISH, produced a single hybridisation band per chromosome (apart from no signal for chromosome 1H) that permitted easily visual identification and eliminated the requirement for reprobing. Therefore, it should be possible to simultaneously perform multi-colour *in situ* hybridisation with much higher resolution on pachytene chromosome to characterise introgressions of the recombinants. This will greatly increase the sensitivity of GISH detection and show the potential for inexpensive, fast and more efficient characterisation of recombinants.

Several recombinants with resistance to important diseases such as powdery mildew and leaf rust have been selected. This will provide breeders with a novel source of resistance. The physical mapping of these recombinants, together with screening of flanking molecular markers should allow map-based cloning and characterisation of the genes of interest to be carried out. This is a critical springboard for transformation work in an attempt to improve the efficiency of barley breeding.


between genome size and synaptonemal complex length in higher plants. 


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Appendix I: Chemical reagents used in this study

Anti-fade Mountant pH 7.5
1.8% DABCO (Sigma) + 90% glycerol (Riedel-de Haën) v/v in 20mM Tris·HCl.

Aqueous uranyl acetate (1%)
Add 1 g of uranyl acetate (BDH) to distilled water and make up to 100 ml of solution.

Aqueous silver nitrate solutions (30% & 50%)
Add 30 g or 50 g of silver nitrate (Scientific Supplies Ltd) to distilled water then make up to 100 ml with more distilled water. This can be kept in fridge for several months.

Ba(OH)$_2$ solution (5%)
Add 5g of Ba(OH)$_2$ (Scharlau) in 100 ml of distilled water and stir at 60°C until dissolved.

Borate buffer
Dissolve 3.1 g of boric acid (BDH) in 250 ml of distilled water and add 30 ml of 1M NaOH (Scharlau) to make borate buffer at pH 9.

Carnoy’s I fixative
Freshly prepare 3 parts of 95% ethanol (BDH) : 1 part of glacial acetic acid (BDH).

Carnoy’s II fixative
Freshly prepare 6 parts of 95% ethanol : 3 parts of chloroform (BDH) : 1 part of glacial acetic acid.
Cytohelicase solution (0.4%)
Make up 10 ml by adding 0.04g of cytohelicase (IBF, Biotechnics) to 10ml of sterile distilled water. Stir for 2.75 hours at 4°C.
Add 0.1 g of polyvinylpyrroldone MW40,000 (Sigma) and 0.15 g of sucrose (Sigma), stir for a further 15 minutes. Aliquot to several Eppendorf tubes and store in fridge.

Enzyme mix (use for chromosome preparation)
Dissolve 0.013 g of pectolyase (P-3026, Sigma) and 0.04 g of cellulase (Onozuka R–10, Merck) in 1 ml of distilled water. Store in freezer until required.

EDTA (0.1 M) pH 8.0
Dissolve 37.2 g of disodium ethylenediaminetetraacetate 2H2O (EDTA) (BDH) in 800 ml of distilled water. Adjust the pH to 8.0 with NaOH and then make up 1 liter of solution with distilled water.

FLP Orcein
Mix 25 ml of 20% formic acid (Ajax Chemicals PTY Ltd.), 25ml of 80% lactic acid (May and Baker Ltd.), 25 ml of 99% propionic acid (BDH), 25 ml of distilled water and 0.92 g of orcein (Serva) together and stir in a beaker on a heating block at 60°C, then cool down and filter through Whatman filter paper.

Hydrofluoric acid (1%)
Add 2.5 ml of 40 % stock solution (BDH) to 97.5 ml of distilled water, then store in a plastic bottle in fridge.

Labelled nucleotides
1mM digoxigenin-11-dUTP (Boehringer Mannheim).
Lipsol (0.5%)
0.5% Lipsol Liquid Concentrate (LIP Ltd) in pH 9 distilled water.

NaH₂PO₄ (1M)
Dissolve 120 g of NaH₂PO₄ (Scharlau) in distilled water to make up 1 litre of solution.

Paraformaldehyde (4%) (for use in *in situ* hybridisation)
Add 4 g of paraformaldehyde (Riedel-de Haën) in 80 ml of distilled water and heat to at 60-80°C for 20 minutes then clear with NaOH, cool down and add 10 ml of 10 × PBS. Add distilled water to 100 ml.

Paraformaldehyde (4%) (for use in synaptonemal complex preparation)
Stir 4 g of paraformaldehyde (Riedel-de Haën) in 100 ml of distilled water at 60-80°C for 20 minutes then add borate buffer to clear the solution. 1.5 g of sucrose (Sigma) is added to solution and stirred for another 15 minutes. This solution can be kept in fridge for about two months. Pale silver staining indicates weakening of the fixative.

PBS (Phosphate Buffered Saline) (10 ×) pH 7.4
1.3 M NaCl (Scharlau), 70mM Na₃HPO₄ (Scharlau), 30mM NaH₂PO₄ (Scharlau). Adjust pH to 7.4 with NaOH/HCl (BDH).

Phosphorate buffer (Sorensen's)
Solution A: Sodium phosphate (Alkaline):
Na₂HPO₄·2H₂O (Scharlau)  1M = 17.8 g make up to 100 ml with distilled water.

Solution B: Potassium phosphate (Acidic):
KH₂PO₄ (Scharlau)  1M = 13.6 g make up to 100 ml with distilled water.
To make 50 ml of 1M buffer at a given pH, combine the following volumes of solutions A and B:

<table>
<thead>
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<th>pH</th>
<th>6.4</th>
<th>6.6</th>
<th>6.8</th>
<th>7.0</th>
<th>7.2</th>
<th>7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>13.2</td>
<td>18.7</td>
<td>24.5</td>
<td>30.5</td>
<td>36.0</td>
<td>40.5</td>
</tr>
<tr>
<td>Solution B</td>
<td>36.7</td>
<td>31.2</td>
<td>25.5</td>
<td>19.5</td>
<td>14.0</td>
<td>9.5</td>
</tr>
</tbody>
</table>

**Phosphotungstic acid (PTA) (1%)**

Mix three parts of 95% ethanol and one part of 4% aqueous Phosphotungstic Acid (P6395, Sigma).

**Polystyrol in chloroform (0.75%)**

Add 0.75 g of Falcon petri dish to 100 ml of chloroform (BDH), and leave at room temperature until the pieces of petri dish have dissolved completely.

**Reynolds' lead citrate**

Add 1.33 g of Pb(NO₃)₂ (BDH) and 1.76 g of Na₃(C₆H₅O₇)·2H₂O (BDH) in 30 ml of distilled water in a 50 ml volume flask, stir for 1 minute and stand with intermittent shaking in order to insure complete conversion of lead nitrate to lead citrate. After 30 minutes, add 8 ml of 1 M NaOH to the suspension and dilute with distilled water to 50 ml of staining solution.

**RNase A (DNase free) (1%)**

Dissolve 10 mg of RNase A (Sigma) in 1 ml of 15 mM NaCl, 10 mM Tris·HCl pH 7.5 and incubate in boiling waterbath for 15 minutes, slowly cool down and store in freezer.

**SSC (20 ×) pH 7.0**

3 M NaCl, 0.3 M C₆H₅Na₃O₇ (Scharlau). Adjust pH to 7.0 with NaOH/HCl.
**Taq DNA polymerase**

5 units per μl (Boehringer Mannheim).

**Tris-HCl (1 M) pH 8.0**

Dissolve 121.1 g of Tris (Gibco BRL) in 800 ml of distilled water. Allow the solution to cool down before making final adjustments to the pH 8.0 with concentrated HCl. Then make up 1 liter of solution with distilled water.

**TE buffer (100 x) pH 8.0**

1M Tris-HCl (pH 8.0), 0.1 M EDTA (pH8.0).

**TP buffer (2 x)**

2% CTAB (SERVA), 100 mM Tris-HCl (pH8), 20 mM EDTA, 1.4 M NaCl.

**Universal primers**

20 μM universal forward / reverse primers (Gibco BRL).

**Unlabelled nucleotides**

100 mM of each dATP, dCTP, dTTP and dGTP solution pH 7.5 (Gibco BRL).

**Water pH 9**

Add borate buffer to distilled water until pH value reaches 9.