Restriction Fragment Length Variation/DNA Methylation in Barley Interspecific Hybrids, Addition Lines and Segmental Aneuploids

Thesis submitted for the Degree of
DOCTOR OF PHILOSOPHY

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To

my parents

DECLARATION

The candidate declares that this work has been carried out by him under the supervision of Professor N.C. SUBRAHMANYAM, Dept of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad 500 046, India and that this work has not been submitted for any degree or diploma of any other University.

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CERTIFICATE

This is to certify that the thesis entitled **"Restriction fragment length variation / DNA methylation in barley interspecific hybrids, addition lines and segmental aneuploids"** is based on the results of the work done by **Mr P. Kranthi Kumar** for the degree of **Doctor of Philosophy** under **my** supervision. This work has not been submitted for any degree or diploma of any other University.

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ABBREVIATIONS

A	Adenine
^{6m}A	6-methyl adenine
A_{260}	Absorbance at 260 nm
BARC	Baba atomic research center
bp	Base pairs
BRIT	Board of radiation and isotope technology
BSA	Bovine serum albumin
C	Cytosine
^{5m}C	5-methyl cytosine
$CaCl_2$	Calcium chloride
CHISAM	Chloroform Isoamylalcohol mixture (24:1)
°C	Degree celsius
DNA	Deoxyribo nucleic acid
DH	Doubled haploid
EDTA	Ethylene diamine tetra-acetate
Fig	Figure
g	acceleration due to gravity
h	Hour
HCl	Hydro chloric acid
Kb	Kilo base
LB	Luria broth (Luria Bertani)
	Microgram
µl	Microliter
M	Molar
$MgCl_2$	Magnesium chloride
min	Minutes
mL	milli Liter
mM	milli Molar
$MnCl_2$	Manganese chloride
N	Normality
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	nano meter
NOR	Nucleolar organizer region
ODU	Optical density unit
^{32p}	Phosphorous radio isotope
R/M	Restriction / Modification
rDNA	DNA at *Rrn* loci
RNA	Ribonucleic acid
RNaseA	Ribonulease A
RPC	Reverse phase column
rpm	Revolutions per minute
Rrn	Ribosomal RNA gene loci
rRNA	Ribosomal RNA
SAT	*"Sine Acido Thymonucleicon"*
SDS	Sodium dodecyl (lauryl) sulfate
Sec	Seconds
SSC	Sodiun saline citrate
TE	Tris EDTA
Tris	Hydroxy methyl amino methane
UV	Ultra-violet *(X* 180-340nm)
Vol	Volume

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ABSTRACT

Crosses between diploids of *H. vulgare* and *H. bulbosum* result in haploids of *vulgare* upon embryo rescue *via* selective elimination of *bulbosum* chromosomes. Genetic factors on *vulgare* chromosomes 2 and 3 control elimination process while stability factors present on the *bulbosum* genome play opposite role in overcoming eliminaton leading stability. Parental genomic ratio in the hybrid cell determines commencement of elimination or stability in a given combination although "escapes" at a low frequency are known. Elimination is widespread in *Hordeum* interspecific and intergeneric crosses and a hierarchy of species dominance in selective chromosome elimination is correlated with the suppression of secondary constriction formation (nucleolus organization) at the NOR of the genome which would have otherwise been eliminated at an appropriate genomic ratio in a given combination. Thus it is often considered that these could be different aspects of an overall process of selective silencing of DNA. Structural organization and locus-specific modification can be monitored effectively using rDNA probe. The present investigation was undertaken to identify the nature and extent of changes at rDNA loci in interspecific hybrid(s) / derivatives, addition lines and segmental aneuploids.

Hybrids between *Hordeum vulgare* (2x) and *H. bulbosum* (2x) were obtained following pollinations of *vulgare* with *bulbosum* following embryo rescue technique. F_1 hybrids, amphiploid and doubled haploid derivatives were used for further analysis. Root-tip cells of F_1 hybrids were examined to determine their chromosome number. Cells with the expected complement (14 chromosomes) exhibited two SAT chromosomes instead of the expected three from their parental combination reflecting differential amphiplasty. Root-tip cells from the same hybrid showed different chromosome numbers. While seven chromosome cells were predominant, other cells containing variant chromosome number represent cells at different stages of elimination which included

cells with less than the haploid complement. All these variant cells exhibited single SAT chromosome.

Southern blot hybridization of restriction fragments of genomic DNAs from parents, interspecific hybrids / derivatives were examined to identify locus specific modification at rDNA loci in hybrids / derivatives. *Bulbosum* accessions possessed at least two rDNA repeat lengths (8.6/8.4 or 7.8 Kb) and revealed enzyme/accession specific rDNA repeats. Variation in their rDNA repeat lengths is an indication of heterozygosity of the Nor locus at the gametic level since *bulbosum* is self-incompatible. *Bulbosum* rDNA repeats showed single *Eco RV* site per repeat compared to two sites in *vulgare* rDNA repeats. Hybrids possessed both *vulgare* and *bulbosum* specific rDNA repeats in *Eco RI*, *Eco RV* and *Sac* I digests. Amphiploid and doubled haploid derivatives lacked *bulbosum* specific rDNA repeat units/fragments. Two of the hybrid derivatives lacked full length *bulbosum* specific rDNA repeats but possessed *Sau* 3 *A* and *Hae* III *bulbosum* specific rDNA repeat fragments.

Hybrids (VB251 and VB252), amphiploid (VB19) and double haploid (VDH3) derivatives lacked *vulgare* specific 9 Kb rDNA repeat which is *Rrn 2* specific. This was consistent in all enzyme combinations used. Ratio of 9.0/9.9 Kb repeats deviated from *vulgare* parent indicating loss of part of 9.0 Kb rDNA repeat in hybrids. Ratios of *vulgare/ bulbosum* rDNA repeats also indicated loss of part of the *vulgare* specific 9 Kb rDNA repeat among the hybrids. These results are consistent with the cytological data in the present study where the absence of a second satellite (nucleolar organizer) chromosome was evident. This is the first conclusive evidence for the elimination of a *vulgare* chromosome in *vulgare-bulbosum* hybrids.

Bulbosum accessions possessed at least two rDNA repeat lengths (8.6/8.4 or 7.8 Kb) and revealed enzyme/acc :ssion specific rDNA repeats. Variation in their rDNA

repeat lengths is an indication of heterozygosity of the Nor locus at the gametic level since *bulbosum* is self-incompatible. *Bulbosum* rDNA repeats showed single *Eco RV* site per repeat compared to two sites in *vulgare* rDNA repeats.

Amphiploid and doubled haploid derivatives were elimination products of F_1 hybrids (progeny of a hybrid). The ratios of 9.0/9.9 Kb *vulgare* specific rDNA repeats revealed differential methylation at individual rDNA loci *(Rrn* 1, *Rrn* 2). Differences in *Mbo* I and *Sau* 3 *A*\ gave differential locus specific cytosine / adenine methylations. Hybrids and derivatives showed differential distribution of methylation patterns. Hybrid derivatives displayed methylation at two consecutive *Eco R*\ sites, three *Bgl* II sites and three *Sac* I sites to differing extents in their rDNA at *Rrn* 1 locus. Hybrid derivatives (VB251, VB252) exhibited extensive methylation at *Msp* I and *Hae* III sites and high molecular weight fractions in *Eco R*\ and *Xba* I digests. *Mbo* I and *Sau* 3 *A*\ revealed diffen ntial distribution of adenine / cytosine methylation, whereas *Msp* I and *Hae* III digests revealed CpG bias in methylation pattern.

To examine the over all extent of nucleobase changes among parents and interspecific hybrids, DNAs were hydrolysed and analyzed on reverse phase HPLC for their base composition. Hybrids exhibited narrow variation in 5mC content and 3 to 4- fold variation in 6mA.

Restriction fragment length variation at specific Nor loci was studied in a set of barley addition lines alongwith the background parent *(Triticum aestivum* var *Chinese spring)* and the donor (*Hordeum vulgare* var *Betzes)* of the addition chromosomes. Wheat and barley rDNA generated individual NOR specific repeats. Higher molecular size fractions were not detected for wheat or barley rDNA in wheat background indicating no methylation. However, differential amplification of the barley rDNA repeats was evident as the barley/wheat rDNA ratios of 2.1 in 5H and 0.625 in 6H indicating a

dosage compensation mechanism. Extensive (> 50%) symmetrical methylations could be detected in 5H *i.e.* at *Rrn* 2 locus of barley.

Seven different homozygous duplication lines with the background genotype as diploid control were chosen to assess the effect of differing region which modultes rDNA amounts and / or methylation. Duplication of segments 12 and 14 revealed differential amplification of rDNA repeats, whereas duplication of segments 13, 15, 17 and 19 showed neutralizing effect. Segments 12 and 14 did not show any modification of rDNA repeats at individual *Rrn* loci while duplication of other segments showed various proportion of modifications at individual rDNA loci. Duplication for segment 13 differentiated methylations at *Xba* 1 and *Eco R*\ sites. While duplication for other segments revealed varying proportions of methylation at individual rDNA loci resulting in high molecular weight fractions (trimers or dimers or monomers) in enzyme/ segment specific pattern. Duplication of either 12 or 14 segment(s) alone led to differential amplification. The effects of these segments are masked considerably by other segments. Segments 13, 15, 17 and 19 influenced the extent and distribution of methylated sites in rDNA. Either two consecutive site modifications result in ~30 Kb or alternative site modifications result in -20 Kb in *Eco R*\ and *Xba* I or modification of five consecutive *Bgl* II sites generates ~30 Kb. *Msp* 1 fragments revealed diagnostic segment-/ locus-specific methylation. Duplication for segments 12 and 14 resulted in very few fragments some of which were more intense and lacked some of the 9 Kb *Rrn 2* specific fragments. Other segmental duplications generated a discrete set *of Msp* I fragments indicating modulation in the distribution and extent of methylations at specific loci.

1. Introduction

The increasing demand for food products in the world requires greater productivity of cultivated crops. Wide hybridization is a first and critical step to introduce alien variation, and prior to the transfer of desirable traits from the wild into the cultivated species. Wide hybridization between cultivated species and their wild relatives in interspecific or intergeneric combinations is an important tool in the breeding of cereal crops for transferring agronomically important characters and cytogenetic studies on these plants. Many wild species of Poaceae are known to possess agronomically beneficial genes such as pathogen resistance, salt- and alkaline-tolerance, wide adaptation, cold hardiness, high protein and lysine content and drought tolerance.

The cultivated barley (*Hordeum vulgare* L.) is predominantly self pollinating, (Saghai-Maroof *et. al.,* 1984) whereas *H. bulbosum* is self-incompatible. The genus *Hordeum* belongs to the tribe Triticcae, family Poaceae consists of over 30 species and distributed in a wide climatic conditions (Bothmer, 1992). The basic chromosome number is 7, few species exist as tetraploids and hexaploids in addition to the diploid cytotypes. Barley ranks fourth among cereals after wheat, maize, and rice.

Reciprocal crosses between diploids of *H. vulgare* and *H. bulbosum* result in *vulgare* haploids (Kasha and Kao, 1970; Lange, 1971) upon embryo rescue, through selective elimination *of bulbosum* chromosomes (Subrahmanyam and Kasha, 1973a). Morphological characteristics of the derivatives and chromosome numbers in the resulting embryo further indicate absence of maternal effects on this unique process (Subrahmanyam and Kasha, 1973b). This is akin to mammalian somatic cell hybrids (Handmaker, 1973). The elimination is always of *bulbosum* chromosomes producing haploids of *H. vulgare* (unidirectional). So far neither loss of *vulgare* genome nor recovery of *bulbosum* haploids are reported in the extensively studied *vulgare-bulbosum* cross combination. Diploid crosses results 99% monohaploids and remaining 1 % *vulgare-bulbosum* hybrids, whereas crosses be .ween diploid *vulgare* and tetraploid *bulbosum*

result in 99% of triploid hybrids suggesting that 1 *vulgare* to 1 *bulbosum* genome leads to elimination, whereas 1 *vulgare* to 2 *bulbosum* genomes results in triploid hybrids. Furthermore, increasing the number of *bulbosum* genomes increases the stability and development of the endosperm as depicted in the following order: BBBBV > BBBBVV, BBV > VVBB > VVB (Subrahmanyam and Kasha, 1973a). Further it was shown using trisomics that *H. vulgare* chromosomes 2 and 3 carry at least three factors which control elimination (Ho and Kasha, 1975).

Production of haploids only from *H. arizonicum* by diploid *bulbosum*, hybrids and haploids from *arizonicum* by tetraploid *bulbosum*, and similarly haploids from *lechleri* by diploid *bulbosum*, and only hybrids (2n=35) or near hybrid chromosome numbers in embryos from *lechleri* by tetraploid *bulbosum* crosses, strongly suggest that a balance between the ratio of the parental genomes in each hybrid determines whether predominantly haploid or hybrid progeny are produced (Subrahmanyam, 1977, 1980). For example, a ratio of 3 *arizonicum (arz)* genomes to 1 *bulbosum* (*blb*) genome leads to the elimination of *bulbosum* genome and subsequent formation of *arizonicum* haploids, whereas the 3*arz* : 2*blb* genomic ratio results in some hybrids. Similarly a ratio of 3 *lechleri* (*lch*): 1 *blb* genomes gives haploids of *lechleri*, while the 3 *lch*: *2 blb* results in embryos with hybrid chromosome number. It is well documented in *vulgare-bulbosum* crosses (Subrahmanyam and Kasha, 1973a), and other interspecific cross combinations in *Hordeum* (Subrahmanyam, 1977, 1978, 1979, 1980; Bothmer *et al.*, 1983), that stable hybrids are obtained by increasing *bulbosum* genomes. The formation of haploids from *arizonicum* by diploid *vulgare* crosses and the absence of chromosome instability in embryonic cells from the *arizonicum* by tetraploid *vulgare* cross also indicates that a genome balance effect may be operative in elimination or retention *oivuigare* chromosomes upon hybridization with *arizonicum*. It is thus likely that crosses of tetraploid *vulgare* by *lechleri* (6x) result in stable hybrids unless physiological disturbances, such as dormancy etc., inte • fere with the germination of hybrid embyros (Subrahmanyam,

1980).

Occasionally haploid *vulgare* sectors arise from triploid hybrids *via* sectorial elimination (delayed elimination). Genotypic variation among *vulgare* and *bulbosum* accessions influences elimination/stability. Several studies indicated that some combinations of parental accessions produce haploids/hybrids though the frequency may vary (Fukuyama and Takahashi, 1975) and some of the diploid *bulbosum* accessions on hybridization with a range *of vulgare* genotypes give up to 70% hybrids (Simpson *et al.*, 1980).

General features of chromosome elimination in interspecific hybrids of barley are degraded type of chromatin (Subrahmanyam and Kasha, 1973a; Lange, 1971), acentric fragments, loss of whole genome, chromosomes or part thereof (Subrahmanyam and Kasha, 1973a; Subrahmanyam and Bothmer, 1987; Bothmer and Linde-Laursen, 1989; Bennett, 1995). The *in vivo* pattern of elimination could be mimicked *in vitro*, by incubating root-tips with restriction endonucleases (Subrahmanyam *et al*, 1976). The rate of chromosome elimination is known to be influenced by the temperature at which the hybrids are raised (Humphrey, 1978). Elimination can occur in the first zygotic division itself or prolonged for few days. Nevertheless the frequency of cells with haploid (gametic) chromosome complement reaches ~95% by day 11 (Subrahmanyam and Kasha, 1973a; Bennett *et al.*, 1976)

Preferential elimination *of vulgare* chromosomes occurs in an order (7, 6, 5, 4, 3, 2, 1) in crosses of *H. vulgare (Tuleen* 346) with *H. marinum* (Finch, 1983). However, in *vulgare-bulbosum* hybrids chromosome elimination is random and progressive. Different cells in the VB hybrid embryo and different tillers of the same plant exhibited variation in chromosome number (Subramanyam and Kasha, 1973a). Progeny from the crosses between *H. lechleri* and *H.vulgare* exhibit variation in morphology and chromosome number (Subrahmanyam, 1980). Increasing the *vulgare* genomes from 1

to 2 in crosses with *H. lechleri* led to the loss of 3 to 6 chromosomes of *lechleri* parent (Linde-Laursen and Bothmer, 1988). It was shown in *lechleri-vulgare* combination that chromosome 7 is lost first while the order of other chromosomes was more or less random (Linde-Laursen and Bothmer, 1988). In somatic cell hybrids, ordered elimination of human chromosomes was reported (Norum and Migeon, 1974).

Selective chromosome elimination is widespread in *Hordeum* interspecific crosses and occurs in over 30 combinations with either *vulgare* and/or *bulbosum* resulting in haploidy (Subrahmanyam and Bothmer, 1987). A hierarchy of species dominance in chromosome elimination among interspecific cross combinations of barley is evident (Subrahmanyam, 1982). Species eliminating one *vulgare* genome exhibit elimination of up to 2 *bulbosum* genomes.

Hierarhy ofchromosome elimination in *Hordeum* interspecific hybrids*

Group I		Group II		Group HI	
		vulgare (4x)	>	*bulbosum*	(4x)
		(2x)	>		(2x)
arizonicum (6x)					
lechleri (6x)	>	*vulgare* (2x)	>	*bulbosum*	(2x)
procerum (6x)					
		parodii (6x)			
		jubatum (4x)			
		brachyantherum (4x)	>	*bulbosum*	(2x)
		brevisubulatum (4x)			
		depressum (4x)			
		vulgare (2x)	>	*chilense*	(2x)
				flexuosum	(2x)

*The dominant species or group of species is placed on the left of the species whose chromosomes are eliminated. More efficient species within the group is placed on the top.

In *H. marinum* X *vulgare* cross, elimination *of vulgare* genome from endosperm and elimination of *marinum* genome from embryo occur alternatively where double dose of *marinum* genome (from secondory nucleus) in combination with single dose of *vulgare* genome result in the elimination *of vulgare* genome whereas in the embryo 1 *vulgare* to 1 *marinum* genome leads to the elimination of *marinum* genome from this combination (Finch, 1983). Hybridization of tetraploid *marinum* with diploid *vulgare* produced dihaploids of *marinum* (Jorgensen and Bothmer, 1988). Further studies have shown that crosses of *H. marinum* ssp. *marinum* H515 (2x) with *vulgare* (2x) result in haploids of *vulgare,* while *H. marinum* ssp *gussonianum* H588 (2x) with *vulgare* result in haploids of *marinum,* indicating that the direction of elimination is determined by the species/line used. In this combination only haploids of either species were reported. This indicates reversible elimination/stability resides on the genotypic make up of the species used.

Ten combinations of intergeneric hybridizations with *Hordeum* as one of the parental species resulting in elimination have been reported. These include combinations with *Triticum aestivum* var. *Chinese spring* (Miller and Chapman, 1976), *Secale cereale* (Fedak, 1977), *Dasypyrum villosum* (Fedak, 1983), *Aegilops crassa* (Shigenobu and Sakamoto, 1977), *Psathyrostachys fragilis* (Bothmer *et al.*, 1984), *Zea mays* (Laurie and Bennett, 1988a), *Taeniatherum caput-medusae* (Frederickson, 1989), *Elymus pseudonutans* (Lu and Bothmer, 1990).

In other intergeneric crosses *Secale cereale* X *Zea mays* (Zenkteler and Nitzsche, 1984), *Triticum aestivum* var *Chinese spring* X *A vena sterilis* (Zenkteler and Nitzsche, 1984), *Thynopyrum elongatum* X *Agropyron mongolicus* (Wang, 1987), *Triticum aestivum* var *Chinese spring* X *Zea mays* (Laurie and Bennett, 1988b; Chen *et al.*, 1991), *Triticum aestivum* var *Chinese spring* X *Sorghum bicolor* (Laurie and Bennett, 1988c,), *Triticum aestivum* var *Chinese sp ing* X *Pennisetum glaucum* (Laurie, 1989,

Ahmad and Comeau, 1990), *Elymus shandogenesis* X *Triticum aestivum* var *Chinese spring* (Lu and Bothmer, 1990) and *Avena saliva X Zea mays* (Rines and Dahleen, 1990) chromosome elimination leading to haploidy were reported.

Elimination might have evolved as a defense mechanism that carried from prokaryotes and utilized for speciation and serving as potential barrier for silencing against deleterious sequences as a system of defense/protection mechanism (Bestor, 1990; Doerfler, 1991).

Nucleolar organiser (NO) regions which are associated with secodnary constrictons of specific chromosomes, named SAT chromosomes (Heitz, 1931), have been shown to be the sites of 18S, 5.8S and 28S, and 18S, 5.8S, 2S, 26S ribosomal RN A (rRNA) cistrons in *Drosophila* (Ritossa and Speigelman, 1965), *Xenopus* (Wallace and Birnsteil, 1966), maize (Phillips *et al.*, 1971, 1974; Givens and Phillips, 1976; Liang *et a!.*, 1977; Ramirez and Sinclair, 1975; Doerschug, 1976), wheat (Flavell and O'Dell, 1976) and a variety of mammals (Miller *et al.*, 1976 a, b). The multiplicity of r RNA cistrons and the broad variation in the degree of multiplicity have been demonstrated in many organisms (see Subrahmanyam and Azad, 1978 a).

Navashin (1934) described that the SAT chromosomes of two different species brought together by hybridization suffered striking alterations in their individuality and mainly the secondary constriction formation was suppressed in the SAT chromosome from one of the two parental species. The affected chromosome was always from the same parental species in a given cross. This phenomenon was termed as differential amphiplasty (Navashin, 1934). Such differential amphiplasty was reported in *Hordeum* interspecific hybrids (Kasha and Sadasivaiah, 1971; Lange, 1971; Lange and Jochemsen, 1976). Furthermore, a strong correlation exists between the selective chromosomal elimination and the selective suppression of secondary constriction formation in *Hordeum*

interspecific hybrids (Subrahmanyam and Kasha, 1973 a, b; Lange and Jochemsen, 1976) as in mammalian somatic cell hybrids (Croce, 1976; Croce *et al.*, 1977; Miller *et al*, 1976 a, b). A comparison of the number of SAT (secondary constriction) chromosomes in the metaphase cells and the maximum number of nucleoli in interphase cells in barley interspecific hybrids revealed that the chromosomes capable of organizing nucleoli were not always reflected through secondary constriction formation (Jessop and Subrahmanyam, 1984). The presence of a higher number of nucleoli than the number of SAT chromosomes seen and the presence of expected number of rRNA cistrons led Subrahmanyam and Azad, (1978b) to suggest that the suppression of secondary constriction formation in interspecific hybrids represents a complete or partial repression of rRNA genes on chromosomes contributed by one of the two parents. Detection of human rRNA in human-mouse somatic cell hybrids destined to lose mouse chromosomes and mouse rRNA in similar hybrids destined to lose human chromosomes (Croce *et al*, 1977) are consistent with the proposal that the suppression of secondary constriction formation is not due to the selective loss of rDNA form its site and perhaps represents a part of an overall phenomenon of selective silencing of DNA (Sager and Kitchin, 1975).

Genes coding for the 18S, 5.8S and 25S rRNAs are organized in tandem arrays of repeating units in enormous amounts in higher plants. The number of units per haploid genome varies from 570 in *Arabidopsis thaliana* to over 32,000 copies in *Hyacinthus orientalis* (Ingle *et al*, 1975; Timmis *et al*., 1972; Pruitt and Meyerowitz, 1986). There is also distinct variation within the same species (Birnsteil *et al.,* 1971; Flavell and Smith, 1974; Cullis, 1975, 1976; Cullis and Davies, 1974; Hotta and Micseche, 1974; Phillips, 1978; Shaw *et al,* 1993; Linde-laursen, 1984; Subrahmanyam and Azad, 1978a, b).

The number of arrays of rDNA repeats per genome is usually small, e g., in

barley where chromosomes 6 and 7 carry these two loci (Subrahmanyam and Azad, 1978a; Appels *et al,* 1980). The length of the rDNA repeats varies between species from 7 Kb in soybean (Varsanyi-Breiner *et al,* 1979) to over 12 Kb in wheat (Appels *et al,* 1980). This length variation is due to different amounts of intergenic subrepeats as shown in wheat (Gerlach and Bedbrook, 1979; Appels *et al,* 1980), barley (Gerlach and Bedbrook, 1979; Saghai-Maroof *et al,* 1984), *Allium* (Garrido *et al.,* 1994), cucumber (Zentgraf *et al.,* 1990) and *Vicia faba* (Yakura and Tanifuji, 1983; Rogers *et al,* 1986; Rogers and Bendich, 1987). In spite of the variation for rDNA repeat lengths within species, and even within a plant at individual loci, there is considerable homogeneity for the number of subrepeats of intergenic DNA (Appels *et al,* 1980; Appels and Dvorak, 1982).

Investigations on the location of different restriction sites in cereal rDNA repeats (Gerlach and Bedbrook, 1979; Appels *et al,* 1980) and the rDNA spacer length polymorphism (Saghai-Maroof *et al,* 1984) revealed two rDNA repeat lengths at the two rDNA loci *Rrn*1 and *Rrn*2. The rDNA repeat length in wheat is 9.5 Kb whereas barley carry 9.9 Kb and 9 Kb repeats (Gerlach and Bedbrook 1979) at two different NOR loci (Subrahmanyam *et al,* 1994) while *H. bulbosum* genome has a single SAT chromosome with a rDNA repeat length of ~8.5 Kb (Molnar *et al,* 1989).

Duplication of the Nucleolar Organizer Region (NOR) in maize results in a larger pachytene nucleolus and a double copy number of rRNA genes (Phillips *et al.,* 1971), while the monosomic for the nucleolar organizer (chromosome 6) contains half the number of rRNA genes. In barley, NOR6 contains 1,600 copies and NOR 7 carries 2,600 copies (Subrahmanyam and Azad, 1978a), yet chromosome 6 organizes a larger nucleolus than chromosome 7. This fact suggests that the size of the nucleolus is related to the proportion of total active rRNA genes rather than the absolute number of rRNA genes (Subrahmanyam and Azad, 1978a). This conclusion is supported by the findings

in wheat (Flavell and O'Dell, 1979). Thus, most of the rRNA genes are inactive and they are condensed as heterochromatin (Ramirez and Sinclair, 1975; Givens and Phillips, 1976; Doerschug, 1976; Phillips, 1978).

The active NOR loci in wheat have a higher proportion of rRNA genes with unmethylated cytosine residues in comparison with less active or inactive loci. The proportion of genes with methylated cytosine residues at CCGG sites also increases as the total rDNA increases (Flavell *et al.*,1988). Specific cytosine methylation of subrepeat is correlated with transcriptional repression of the repeat (Flavell *et al.*, 1993; Sardana *et al.*, 1993; Jupe and Zimmer 1993; Thompson and Flavell 1988).

The total number of rRNA genes in barley is known to be influenced by the increased dosage of chromosomes, not only NOR 6 and NOR 7 but also other chromosomes (1,2,4,5) not known to carry any NOR (Subrahmanyam and Azad, 1978a). Subsequently, it was shown that these chromosomes (1,2,4 and 5) carry minor rDNA loci (Peaderson and Linde-Laursen, 1994). In wheat, deleting or duplicating any one of 14 out of the 17 chromosomes with no rDNA loci brings about alteration in the number of nucleoli formed (Longwell and Svihla, 1960; Flavell and O'Dell, 1979). In barley, when both NOR6 and NOR 7 are brought on to the same chromosome through translocation, the activity of NOR 7 is reduced (Anastassova-Kristeva *et al.*, 1980). However, in maize the translocation of NOR to other chromosomes does not prevent its activity (Ramirez and Sinclair, 1975; Givens and Phillips, 1976; Doerschug, 1976; Phillips, 1978).

Size of the nucleolus is correlated with the degree of the NOR expression (Flavell and O' Dell, 1979; Reeder, 1985; Subrahmanyam and Azad, 1978a,b) which is reflected in the size of the secondary constriction. Each NOR has the ability to organize a nucleolus. Despite having less number of rRNA genes, NOR6 organizes a bigger nucleolus than NOR7 in barley. In wheat 1B constitutes 30% whereas 6B constitutes

60% of rDNA, yet 1B organizes a larger nucleolus (Flavell *et al*., 1988). Introduction of *Aegilops umbellulata* chromosome bearing NOR (1U) suppresses wheat NORs and 1U organizes a bigger nucleolus. Substitution / deletion of NOR bearing chromosomes in wheat with non-NOR chromosomes increased the volume of other nucleoli indicating compensation for the deletion (Sardana *et al,* 1993). It was shown that NOR of 1U has more number of 130 bp subrepeats in its rDNA repeat units. In wheat line Cheyenne 6B organizes a larger nucleolus and the rDNA repeats of 6B contain more number of 130 bp subrepeats (enhancer-like) in the repeat (Appels and Dvorak 1980; Sardana *et al.,* 1993).

Attempts to introduce alien genetic variation into cultivated species, led to the designing and deployment of refined techniques for the production of addition lines of barley in wheat background (Islam *et al,* 1981; Islam and Shepherd, 1990; Koba *et al*., 1991). Rye addition lines in wheat, maize addition lines in oat (Rines *et al*. 1995; Riera-Lizaraju *et al.* 1996)) were produced. These are invaluable tools in assigning markers/ genes onto individual chromosomes, studying interaction of genes and construction of high density chromosome specific linkage maps (Klienhofs *et al.,* 1995). Barley addition lines are produced following hybridization between wheat and barley. The amphidiploid on crossing with wheat produces 49 chromosome plants. Such hybrids are pollinated with *H. bulbosum*. During the embryo development, elimination of *bulbosum* chromosomes occurs and on embryo rescue, progeny with 21 wheat chromosomes and 1 chromosome of barley could be obtained. Doubling the chromosome numbers results in disomic barley addition lines in wheat background. These addition lines are useful to delineate locus-specific changes particularly at the two *Rrn* loci in barley.

Variations in the relative amounts of the two (9.9 and 9 Kb) rDNA repeats were utilized to detect the role of the chromosomal segment in differential amplification and methylation of rDNA repeats in barley (Subrahmanyan *et al,* 1994). It was dem-

onstrated that the relative position of the segment "12 to 16" of chromosome 6 in barley determines differential amplification of the two rDNA repeats while the duplication of the same segment controls methylation and the overall rDNA content (Subrahmanyam *et ah,* 1994).

Scope of the Present Investigation

Crosses between diploids of *H. vulgare* and *H. bulbosum* results in haploids of *vulgare* upon embryo rescue *via* selective elimination of *bulbosum* chromosomes. Genetic factors on *vulgare* chromosomes 2 and 3 control elimination process while stability factors present on the *bulbosum* genome plays an opposite role in overcoming eliminaton leading to stability. Parental genomic ratio in the hybrid cell determines commencement of elimination or stability in a given combination although "escapes" at a low frequency are known. Elimination is widespread in *Hordeum* interspecific and intergeneric crosses and a hierarchy of species dominance in selective chromosome elimination is correlated with the suppression of secondary constriction formation (nucleolus organization) at the NOR of the genome which would have otherwise been eliminated at an appropriate genomic ratio in a given combination. Thus it is often considered that these could be different aspects of an overall process of selective silencing of DNA. Though the factors involved in the elimination were known, details of the mechanism(s) involved in this process are yet to be elucidated.

Several hypotheses were put forward regarding the mechanism of chromosome elimination: (1) Mitotic rhythm/cell cycle timings (Lange, 1971), (2) Spindle abnormalities/ centromere inactivation (Bennett *et al,* 1976; Finch and Bennett, 1983; Bennett, 1995), (3) Genome position - peripheral / central (Laurie and Bennett 1989; Anamthawat-Jonsson *et al.* 1993; Bennett, 1995) and (4) Restriction- modification system (Davies, 1974).

Mitotic rhythms/cell cycle timings - It has been proposed that differing cell cycle timings could lead to elimination of the late replicating genome. In *vulgare* and *bulbosum*, the DNA content and the cell cycle timings are more or less similar, and hence the possibility of mitotic rhythms playing any possible role is ruled out.

The proposed spindle abnormalities and centromere inactivation processes can not account for the presence of acentric fragments and degraded type of chromatin among the cells of the tissues in which elimination commenced. Moreover, the centromeres of eliminated genome and parents did not show any discernible differences in experiment to test this hypothesis (Noda and Shiraishi, 1990).

It has been proposed that peripheral genome in the hybrid is predisposed to elimination (Laurie and Bennett, 1989; Anamthawat-Jonsson *et al.*, 1993; Bennett, 1995,), based on the observations in *vulgare-bulbosum* hybrids the *vulgare* genome is located centrally and the *bulbosum* genome being peripheral. However, in *P. fragilis* x *H. vulgare* and *H. lechleri* x *H. vulgare* combinations the genomes that are centrally located are eliminated (Bothmer *et al.*, 1984; Linde-Laursen and Bothmer, 1988;Linde-Laursen and Jensen, 1991) which is contrary to the proposal of Laurie and Bennett, (1989) and Anamthawat-Jonsson *et al,* (1993).

Davies (1974) proposed that loss of genome in *Hordeum* interspecific hybrids may have a mechanism similar to the prokaryotic restriction modification system (Boyer *et al.,* 1973). Modification plays an important role in countering such restriction was known in phages (Arber, 1974; Bickle and Kruger, 1993). Further it was shown that treatment *ofbulbosum* and *vulgare* root-tip cells with bacterial restriction endonucleases resulted in chromosome degradation (Subrahmanyam *et al,* 1976) which is similar to the occasionally observed degradation of chromosomes in *vulgare-bulbosum* hybrid cells (Subrahmanyam and Kasha, 1973a). The product of *H. vulgare* chromosomes 2 and 3 causes the loss of the chromosomes of *H.bulbosum* (Ho and Kasha, 1975) . There

are recognition sites which allow a distinction to be made between them. To protect the *H. vulgare* genome from degradation of its own chromosomes it must either lack those sites which are susceptible to breakage or it has the capacity to modify such sites. The survival of the triploid hybrids which have two doses of *H. bulbosum* chromosomes could be related to the presence of two copies of this genetic information. Alternatively, the exceptional VB or VVBB hybrids may be due to a modification of the *bulbosum* as well as of the *vulgare* chromosomes *i.e* the sites on the bulbosum chromosomes arc altered such that they are protected.

Since hierearchy of selective chromosome elimination and selective nucleolar organizer suppression are correlated, these can be viewed as part of an overall selective silencing mechanism (Sager and Kitchin, 1975). Structural organization and locus-specific modifications can be monitored effectively using rDNA probe. The present investigation was undertaken to identify the nature and extent of changes at rDNA loci in barley interspecific hybrid(s) / derivatives, addition lines and segmental aneuploids.

2. Materials & Methods

2.1 Plant Materials

Accessions of diploid *H. vulgare* and *H. bulbosum* used in the present study are listed in Table 1. Plants were grown in glasshouse with 16 h daylight and 8 h dark regime at 24 ± 1°C. Shoots from 3-week-old-seedlings of *vulgare* and shoots from *bulbosum* accessions 3-weeks after pruning were collected for DNA extractions.

2.2 Morphology of the Plants:

While *vulgare* parent (Fig. 1b) has upright habit, annual, low tillering, predominantly self-pollinating, *bulbosum* parent (Fig. 1 a) has prostrate, perennial, profuse tillering, pubescent leaves and self-incompatible with winter habit. F_1 hybrids had prostrate growth habit, curly leaves, dark green with hair on leaves and excessive tillering (Fig. 1d)

F_1 hybrids were the exceptional hybrids obtained by embryo rescue (Kasha *et al.*, 1978) following interspecific hybridization of heterozygous lines of diploid *H. vulgare* (anonymous) with bulk pollen of the available diploid *bulbosum* lines. The *vulgare-bulbosum* (VB) hybrids constitute < 2% of the seedlings following embryo culture, and were grown in glasshouse at 16 h day light, 8 h night regime at 24 ± 1 °C. All the seedlings were treated with 0.1% colchicine. Seeds from each of them were harvested separately and the seed set on such plants represent sectorial doubling of chromosomes (Subrahmanyam and Kasha, 1975). Among such progeny, *vulgare*-like plants were doubled haploids while those with hybrid morphology some of which also set seed represent "amphidiploid" whose seed weight and size were much smaller than the doubled haploids. Plants raised from the seeds obtained from single "VB" hybrid are designated as derivatives (Table 1, Fig. 2).

All the derivatives used were from the same parental genotypic combinations. VDH3 and VDH2 were doubled haploids derived from two different zygotic embryo:

Table 1: Origin of the accessions of *Hordeum* species, of interspecific hybrids and hybrid derivatives

Species	Source
H. bulbosum (2n=14) 112, 113, 395, 397	Prof. R.von Bothmer, (The Swedish Univ. of Agric. Sci. Svalov, Sweden)
H. bulbosum (2n=28) 134, 353, 2122, 3024, 3032.	
H. vulgare (2n = 14) var *Clipper* (DH)	Prof .N.C. Subrahmanyam

Interspecific F_1 hybrids	Parentage *
VB I	VV (6477) x BB113
VBII	VV (6478) x BB113
VBIII	VV (6478) x BB112
VB IV	VV (6476) x BB 109
VB7885	VV (7885) x BB mixed pollen
VB2457	VV (2457) x

Hybrid derivatives **

VDH 2	(22, 1.10g)	VV (7887) x BB (mixed pollen)
VDH 3	(22, 1.75g)	
VB1(VB19)	(11,0.18g)	
VB251	(15, 0.13g)	
VB252		

* VV - *H.vulgare,* BB - *H. bulbosum*

** Seed set on the Colchicine doubled sectors and weight are in parenthesis

Figure 1. Parental species and interspecific hybrids between *Hordeum vulgare* and *H. bulbosum:* A) *H. bulbosum;* B) *H. vulgare;* C) Triploid hybrid (VBB) with haploid *{vulgare)* sector; and D) Diploid hybrid (VB).

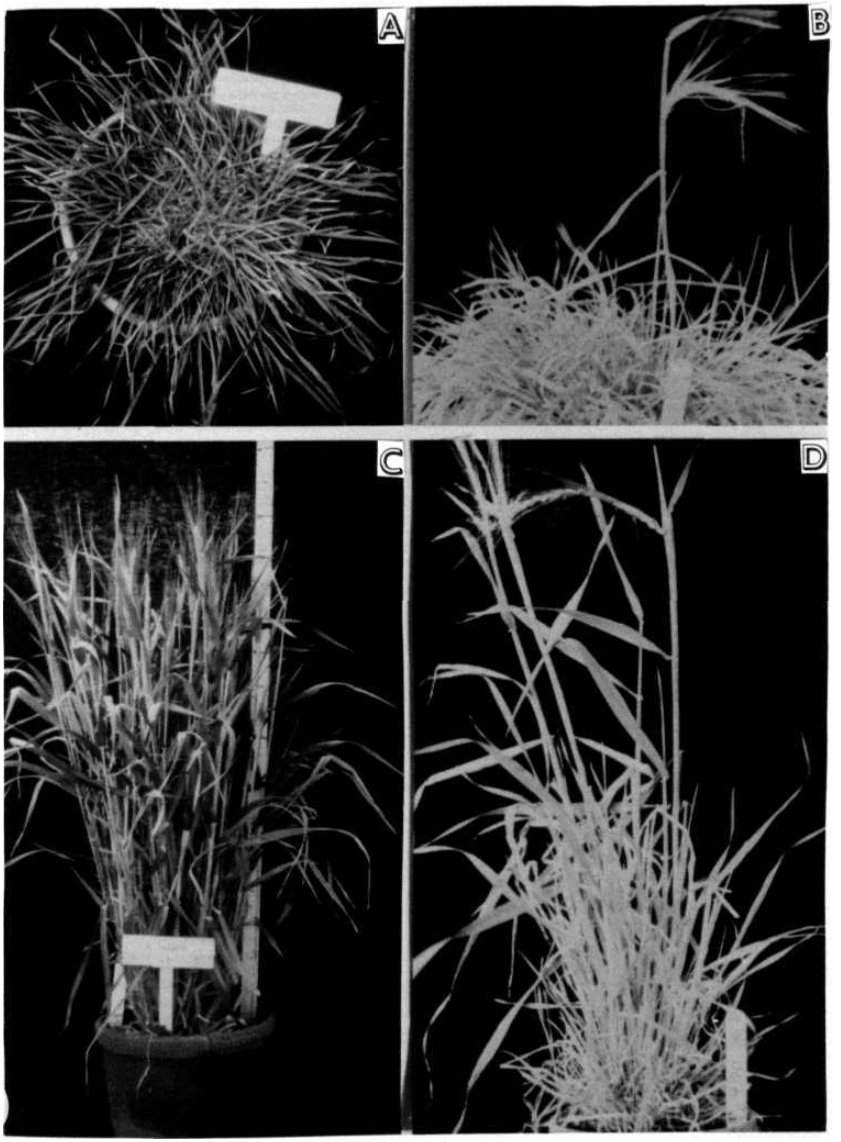

A
B
C
D

Table2: Origin and composition of barley addition lines and segmental aneuploids

Species /Cytogenetic stocks	Chromosomal/Segmental composition	Source
Hordeum vulgare var *Bonus*	**2n** =2x =14	Prof. A. Hagberg
Triticum aestivum var. *Chinese Spring*	2n = 6x = 42	Dr. A.K.M.R. Islam
Hordeum vulgare var *Betzes*	2n = 2x = 14	Waite Agric. Res.
Addition lines		
1HS	21 pairs of Wheat + a pair of barley 1HS	Inst. Adelaide,
2H	**21** + a pair of barley 2H	South Australia
3H	**21** + of barley 3H	
4H	**21** + of barley 4H	
5H	**21** + of barley 5H	
6H	**21** + of barley 6H	
7H	**21** + of barley 7H	
Segmental aneuploids	Segments duplicated	
D31	7-13 and 68-69	Prof. Hagberg
D32	7-17 and 68	Institute of Plant
D33	7-14 and 68-75	Breeding Resources,
D34	7-12 and 68-73	The Swedish Agric.
D35	7-19 and 68-71	Sciences, Svalov
D36	7-15 and 68-74	Sweden
1)8	12-16	

from the same parental genotypes (Table. 1) The "derivatives" were raised from different zygotic embryos following hybridization of a heterozygous line (7887) of *H. vulgare* with pollen mixture of *bulbosum* accessions. VDH2 and VDH3 are doubled haploids progeny resulting from chromosome doubled sectors on *vulgare*-like progeny. VB251 and VB252 were derived from the amphiploid sectors of one F_1 hybrid while VB19 was derived from amphiploid sector of another F_1 hybrid.

The morphology of the addition lines resembles more to their wheat parent. The segmental duplications D31 to D36 (Hagberg and Hagberg, 1991) differ in their segmental composotion (Table. 2).

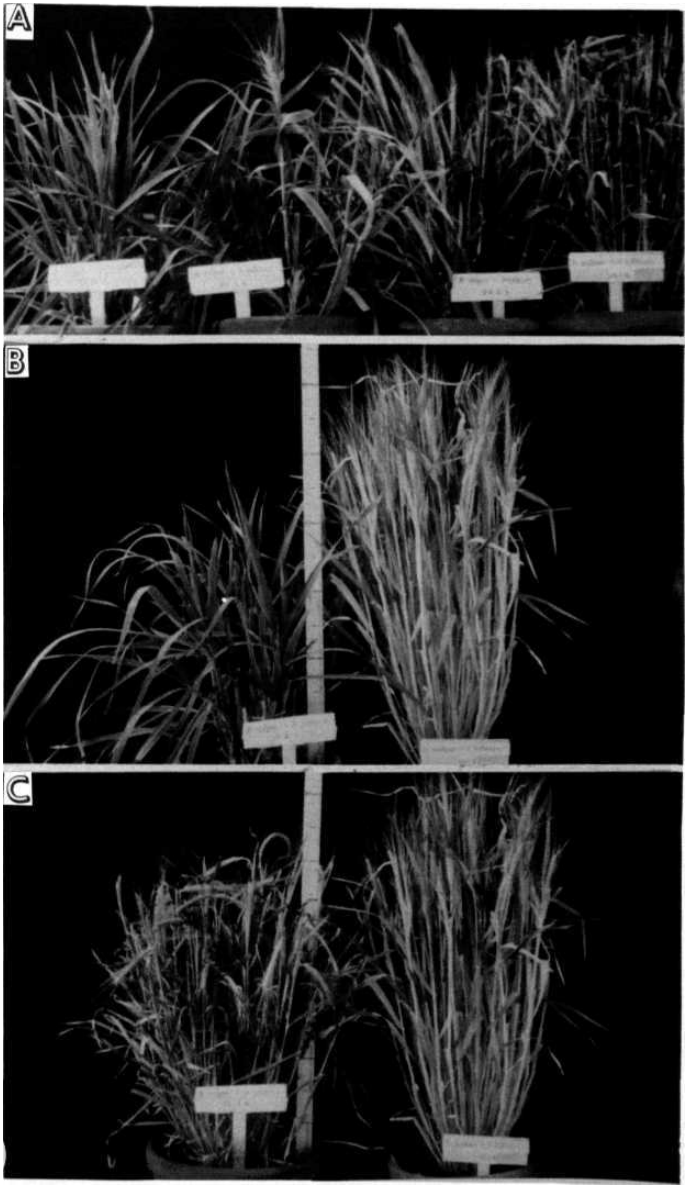
2.3 Cytology

Root-tips were collected during the forenoon in prechilled water and kept at 4°C overnight followed by fixing in 1:3 acetic alcohol for up to 24 h at room temperature (25°C). Then transferred to 70% ethanol for future use. Root-tips were treated with 0.05% pectinase at room temperature (25°C) for 1 h, stained in 2% acetocarmine to determine the chromosome numbers.

2.4 Extraction and Purification of DNA

Total genomic DNA was extracted from leaf tissue according to Sharp *el al.*, (1988) with minor modifications. Shoots were frozen in liquid nitrogen and ground to a fine powder using pestle and mortar. The powder was transferred into a conical flask and suspended in 5 mL of extraction buffer (100 mM Tris-Cl, pH 8.5, 100 mM NaCl, 50 mM EDTA, 2% SDS was replaced with 2% (w/v) sodium sarcosine) per gram leaf tissue and incubated at 37°C for 2h. An equal vol of 1:1 of Tris-buffered phenol:CHISAM (24:1 chloroform to isoamyl alcohol) was added into the lysate, stirred for 10 min and centrifuged at 12,000 *g* for 15 min. The supernatant was collected and DNA was precipitated with 0.6 vol of isopropanol. DNA was spooled, washed in 70% ethanol,

Figure 2. Morphology of rare hybrid derivatives from *vulgare-bulbosum* cross: A) hybrid derivatives B) hybrid derivative and amphiploid and C) hybrid derivative and doubled haploid.


A
B
C

dried under vacuum and dissolved in 1X TE (10 mM Tris-Cl, 1 mM EDTA, pH 8). DNA was treated with RNase A (50 ug mL' final concentration) at 37°C for of 1 h, followed by proteinase K (100 ug ml', final concentration) at 37°C for 2 h. DNA was re-extracted twice with equal volume of Tris-buffered phenol:CHISAM and once with an equal volume of CHISAM alone. DNA was precipitated by adding one-tenth vol of 3 M sodium acetate (pH 5.2) and 2 vol of chilled absolute ethanol (-20°C). DNA was spooled, washed with 70% ethanol, briefly air-dried and dissolved in 1X TE. To this NaCl (2.5 M, final concentration) was added, the contents were mixed and 2 vol of chilled (-20°C) ethanol was added to precipitate DNA. DNA was transferred to eppendorf vial, air-dried and dissolved in TE, dialysed against 0.1X TE, precipitated with 2 vol of chilled ethanol (-20°C) and vacuum-dried for 5 min and dissolved in 0.1X TE.

The purity of DNA samples were determined spectrophotometrically by measuring the absorbance at 280, 260 and 230 nm. DNA was quantified considering 1 ODU at 260 nm as 50 ug double stranded DNA. The A_{260}/A_{280} ratio was found to be > 1.8. The quality and digestibility of the DNA was checked on 0.8% agarose gel.

2.5 Maintenance and preparation of the probe:

LB Liquid Medium : 1% bacto-tryptone, 0.5% yeast extract and 0.5% NaCl were dissolved in double distilled water, adjusted to pH 7.2. Suitable aliquots were dispensed into conical flasks, plugged with cotton and autoclaved.

LB Agar Plates: To LB liquid medium 1.5% bacto-agar was added and autoclaved. While it cooled to 50°C added filter-sterilised tetracycline (25 ug mL^{-1}, final concentration) and 25 mL each was dispensed into petriplates. The plates were kept for 20 min, sealed with parafilm and stored at 4°C.

2.6 Preparation of Competent *E. coli* Cells and Transformation

E. coli JM 109 cells were made competent as described by Slightom and Quemeda

(1989), Cohen *et al.*, 1973 (described in Sambrook *el al.*, 1989). Single colony of *E. coli* JM 109 was inoculated in 10 mL of 2x YT (2% bactotryptone, 1% yeast extract, 0.1% NaCl, 0.2% glucose, pH 7.2) medium and incubated at 37°C on a platform shaker at 250 rpm. Hundred ml of 2x YT medium was inoculated with 100 uL of fresh over-night culture and incubated at 37°C for 4 h on a platform shaker at 250 rpm. The cells were chilled for 30 min on ice-cold water and centrifuged at 3000 rpm (Sorval SS34 rotor) at 4°C for 5 min. The bacterial pellet was suspended in 25 ml of 2x competent cell buffer (100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM sodium acetate[pH 5.5] and incubated on ice for 30 min. The cells were again centrifuged at 3000 rpm at 4°C for 5 min. The pellet was dissolved in 2.5 ml of 2x competent cell buffer and 1.15 mL of 80% glycerol was added.

Dispensed 300 uL to the glass vials to which recombinant plasmid DNA (pACYC184 containing barley 9.9 Kb ribosomal DNA insert *i.e.* pHV294, (see Subrahmanyam *et al.*, 1994) was added, mixed and kept on ice for 30 min. Heat shock was given to the mixture at 42°C for 90 sec, cooled on ice for 2 min and 0.9 mL of SOC (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KC1, 10 mM $MgCl_2$, 20 mM glucose) was added, the contents were mixed and kept at 37°C on rotary shaker platform at 200 rpm for 1 h to recover. Tetracycline was added (10 ug mL^{1} final concentration) and further incubated for 45 min on shaker platform at 200 rpm. Then 200 uL of transformed culture was plated on agar-tetracycline plates, sealed and covered with black paper and incubated at 37°C for 12 h.

2.7 Amplification and Extraction of the recombinant Plasmid

Plasmid DNA was extracted by the alkaline lysis method (Birnboim and Doly, 1979 described in Sambrook *et al.*, 1989). Five hundred mL of LB medium (1 % tryptone, 0.5% yeast extract, 1% NaCl, pH 7.2) containing 25 ug mL^{-1} tetracycline was inoculated with an isolated colony of transformed bacteria covered with black paper and incubated

at 37°C for 12-16 h. The culture was harvested at 5000 rpm (Sorval GSA rotor) at 4°C for 10 min, the pellet was suspended in STE (0.1 M NaCl, 10 mM Tris-Cl, pH 8, 1 mM EDTA) and was centrifuged at 5000 rpm for 10 min. The bacterial pellet was dissolved in 10 mL of Solution I (50 mM glucose, 25 mM Tris-Cl, 10 mM EDTA, pH 8), mixed well and 2 mL of (10 mg mL^{-1}) lysozyme was added, mixed gently and incubated on ice for 10 min, 20 mL of Solution II (0.2 N NaOH, 1% SDS) was added and mixed rapidly by inverting the bottle and kept at 25°C for 10 min. The suspension was vortcxed after adding 30 mL of solution III (5M potassium acetate, glacial acetic acid) and further incubated on ice for 10 min, centrifuged at 5000 rpm (Sorval GSA rotor) for 10 min at 4°C. The aqueous phase was filtered through cheese cloth and 0.6 vol of isopropanol was added, the contents were mixed, kept at 25°C for 30 min and centrifuged at 5000 rpm for 15 min at 20°C. The DNA pellet was rinsed with 70% ethanol, air-dried for 5 min, dissolved in TE, treated with RNase A (50 ug mL^{-1} final concentration) at 37°C for 1 h followed by treatment with proteinase K (50 ug mL^{-1} final concentration) at 37°C for 2 h. Equal vol of phenol:CHISAM mixture was added and extracted twice. The final extraction was done with CHISAM. To the final aqueous phase one-tenth vol of 3 M sodium acetate (pH 5.2) and 2 vol of chilled ethanol (-20°C) were added and centrifuged. The DNA pellet was dissolved in TE.

The recombinant plasmid was restricted with *Eco R*\ to excise the insert, size-fractionated on low melting point agarose gel (BRL) and stained in 0.5 µg mL^{-1} ethidium bromide for 15 min to confirm the insert size. The 9.9 Kb insert portion of the gel was excised and stored at -20°C for labelling.

2.8 Southern Blot Hybridization of Genomic DNA

DNA samples were digested with 5U of the selected restriction enzymes (Table 3) per µg DNA at 37°C for 4 h and size fractionated on 0.8 or 1.2% agarose gel (Southern, 1975) vacublotted (LKB Vacugene Vac uum Blotting Unit, Pharmacia) onto

Table. 3 Restriction **endonulceases:** recognition **sequences** and sensitivity to **site** specific methylation **#**

Restriction enzyme	Recognition sequence	**Sites sensitive**	Sites resistant
***Bgl* II** **	AGATCT	AG^{m6}ATCT	AGAT^{m5}CT AGAT^{hm5}CT
Dpn I ***	G^{m6}ATC	G^{m6}ATC G^{m6}AT^{m5}C G^{m6}AT^{m4}C	GATC GAT^{m4}C GAT^{m5}C
Eco **RI** *	GAATTC	GAATT^{hm5}C	G^{m6}AATTC GA^{m6}ATTC GAAATT^{m5}C
Eco RV *	GATATC	GATAT^{m5}C	G^{m6}ATATC GAT^{m6}ATC
Hae III *	GGCC	GGC^{m5}C	GG^{m5}CC
Hind III*	AAGCTT		^{m6}AAGCTT AAG^{m5}CTT AAG^{hm5}CTT
*Kpn**	GGTACC	GGTA^{m5}CC GGTAC^{m5}C GGTA^{m5}C^{m5}C	GGT^{m6}A^{m5}CC GGTAC^{m4}C
Mbo I **	GATC	GAT^{m4}C GAT^{m5}C	G^{m6}ATC GAT^{hm5}C
Msp I **	CCGG	^{m4}CCGG C^{m4}CGG C^{m5}CGG	^{m5}CCGG ^{hm5}C^{hm5}CGG
Sac I*	GAGCTC	G^{m6}AGCTC	GAG^{m5}CTC
***Sau*3AI** **	GATC	G^{m6}ATC	GAT^{m5}C GAT^{m4}C GAT^{hm5}C
*Xba**	TCTAGA		TCTAG^{m6}A T^{m5}CTAGA T^{hm5}CTAGA

* Bangalore Genie ** Pharmacia *** Boehringer-Manheim
Nelson and McClelland, (1991)

nylon membranes (NEN-DuPont) according to manufacturers' instructions, the blots were UV cross-linked (A_{302}nm) for 90 sec and baked at 80°C in a vacuum oven for 2 h.

Prehybridization was carried out in a polythene bag containing prehybridization solution (250 raM phosphate bufffer, pH 7.4, 7% SDS, 1 mM EDTA,1%BSA, 100 ug mL^{-1} denatured salmon sperm DNA) at 65°C for 4 h. The 9.9 Kb cloned rDNA insert was radiolabelled (α-^{32}P dCTP) according to BRIT random prime labelling protocol. The probe was denatured at 95°C for 5 min, snap cooled on ice-bath and added to the prehybridization solution and hybridization was carried out at 65°C in Haake's shaker water bath (100rpm) for 16 h.

The blots were washed twice in Solution I at 65°C (1x SSC, 0.1% SDS) for 15 min each and twice in Solution II (0.1x SSC, 0.1% SDS) at 65°C in the shaker water bath. The blots were air-dried, wrapped in Saran wrap and X-ray film was exposed to hybridized blots in X-ray casettes (Sigma) at-70°C and the autoradiograms were developed.

2.9 Removal of the Probe from the hybridized blot

Membranes were dipped in boiled O.lx SSC, 0.1% SDS solution and kept at 37°C at 50 rpm for 30 min. The above step was repeated again to ensure removal of the probe from the blots which were air-dried, Saran wrapped and stored at 4°C.

Hybridization signal distribution in each lane from the selected autoradiograms was determined by the gel documentation system (UVP Inc San Gabriel) the relative signal intensity distribution within each lane were used to assess the nature of changes in each hybrid / derivative in comparison with their parental genome. Close proximity bands in a lane of an autoradiogram were "zoomed" in UVP Gel documentation by adjusting the camera for intensity measurements.

2.10 **Preparation** of standard stock and clutants

Stock solutions of cytosine, adenine, thymine, 6m-adenine (^{6m}A) and 5m-cytosine (^{5m}C) (Sigma,USA) were prepared in 0.01N HC1 at 10 mM concentrations, filtered through 0.22 um millipore membrane and stored at -20°C until use. Guanine stock solution was prepared with 1N NaOH and was neutralized with 1N HC1. The elution solvent A was 100 mM ammonium acetate (pH 4.5, adjusted with acetic acid) and solvent B was 100 mM ammonium acetate (pH 4.5, with 40% acetonitrile and 0.75% (v/v) tetrahydrofuran). Elutants were filtered through 0.45 µM Saurtorius membrane, sonicated and degassed under vacuum.

2.11 High Performance Liquid **Chromatography** (HPLC)

HPLC (Hewlett Packard, model 1090M,USA) with PV5 ternary solvent delivery system, 7125 Rheodyne injector, 1040 multiple wavelength detector operating at 280 nm with 20 nm bandwidth was used for routine analysis. Data was collected in H.P 79994 ALC work station using 79995A operating software. The reverse phase column (250 mm x 4.6 mm) was prepacked (DuPont, USA) with Zorbax RPC 18 and analyses were performed with a prepacked gaurd column (12.5 mm x 4.6 mm) with Zorbax RPC 18.

2.12 Sample **Preparation**

DNA (10-20 µg) samples were taken in borosilicate vials. 100 µL of 98% formic acid was added to each sample and vials were sealed under flame. The DNA samples were hydrolysed at 180°C (oil bath) for 30 min and were frozen at -180°C in liquid nitrogen before cutting open. Samples were lyophilized and redissolved in 0.01N HC1 and filtered through 0.22 um nitrocellulose filter and stored at -80°C until use.

2.13 Sample Analysis

Aliquots of 20 µL of the sample were injected into the column, analyses were

carried out at a flow rate of 1 mL min^{-1}. The elution profile consists of an initial isocratic phase of solvent A (4 min) linear gradient of 0-50% of solvent B (6 min), 50% A and B solvent (5 min), a descending gradient of B 50-0% (5 min) and a final wash of column for 5 min with solvent A before the next analysis. Each chromatogram was recorded and evaluated by retention time (Fig. 3A), spectral analysis (200-400 nm), peak purity (Fig. 3B) and co-chromatogrpahy. Separation data were integrated using area normalization. Extent of mcthylation was determined as the percentage of the area of each methylated base out of the combined area under the methylated and its corresponding normal nucleobases (Pradhan and Subrahmanyam, 1995). Two batches of the DNA extractions and two replicates were used for each sample. Data represent average (± standard error) of four independent replicate estimates for each hybrid/ species.

Figure 3. Chromatographic scans of standard nucleobases: (A) separation of nucleobases by Reverse phase High Performance Liquid Chromatography. Bases are **Cyt-** cytosine; **^{5m}C-** 5-methylcytosine; **Gua-** guanine; **Thy-** thymine; **Ade-** adenine; **^{6m}A-** 6-methyladenine and (B) Spectral scan of the **^{6m}A**.

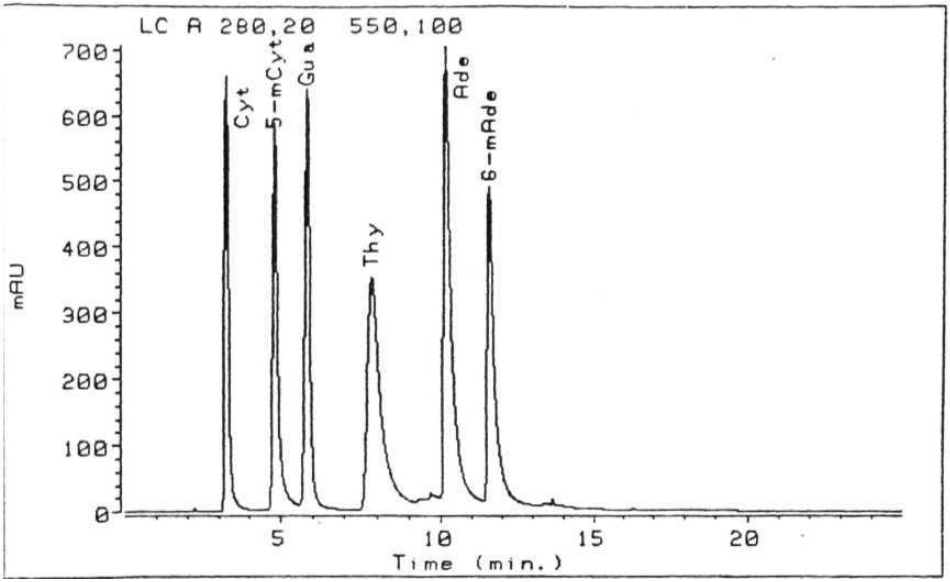

LC A 280,20 550,100
Cyt
5-mCyt
Gua
Thy
Ade
6-mAde
mAU
Time (min.)

Fig.3A

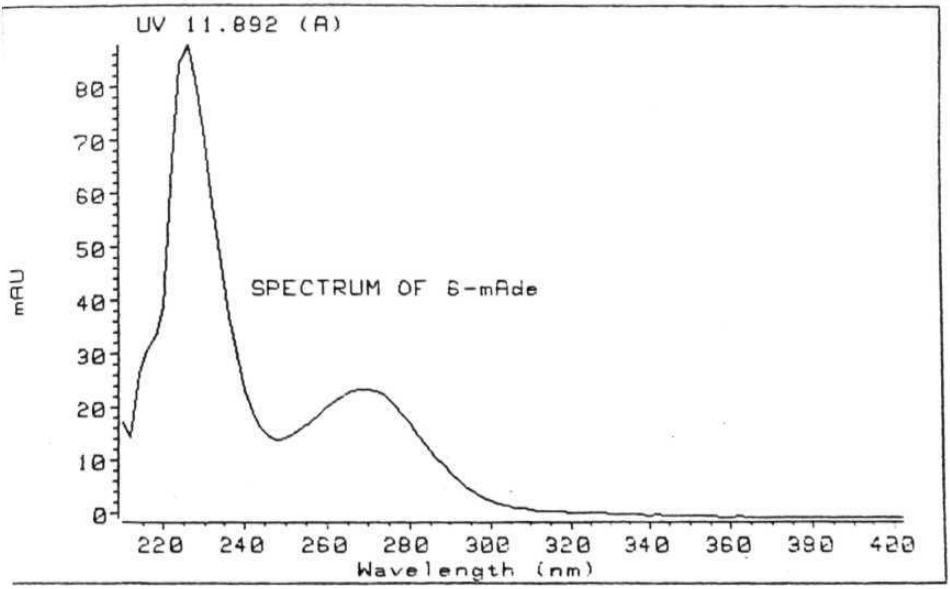

UV 11.892 (A)
SPECTRUM OF 6-mAde
mAU
Wavelength (nm)

Fig. 3B

3 Results & Discussion

3.1 Restriction fragment length variations at rDNA loci in barley (*Hordeum vulgare* L.) hybrids with *H. bulbosum*

Southern blot hybridization of *Eco RV* digests (Fig. 4) with barley rDNA probe (pHV 294) revealed a single fragment in all *bulbosum* accessions (lanes 3 - 6, 14 & 15), a 2.9 Kb fragment in *H. vulgare* accessions (lanes 12 & 13), F_1 interspecific hybrids (lanes 10, 11, 16-19) and the five derivatives (lanes 1, 2, 7-9). *H. vulgare* accessions (lanes 12 & 13) also had 7 Kb and 6.1 Kb fragments which were common to all the hybrids and one of the doubled haploid derivatives (lane 8) while the 6.1 Kb fragment is absent in one of the doubled haploid derivative (lane 7) and the three amphiploid derivatives (lanes 1, 2 & 9).

Autoradiograms of *Sac* I Southern blots hybridized to barley rDNA probe resulted in 3.9 Kb fragment common to all the parental accessions, interspecific hybrids and their derivatives (Fig. 4, lanes 1-19). In addition 6 Kb and 5.1 Kb fragments were present in *vulgare* accessions (lanes 12 & 13), interspecific hybrids (lanes 10, 11, 16-19) and in one of the doubled haploid derivative (lane 8) while the other derivatives (lanes 1, 2, 7 & 9) lacked the 5.1 Kb fragment. *H. bulbosum* accessions had 4.7 Kb and / or 4.5 Kb fragments (lanes 3-6, 14 & 15).

Tandem repeat units of barley rDNA are characterized by the presence of a unique *Eco RI* site giving rise to locus specific repeat unit lengths of 9.9 Kb and 9 Kb (Subrahmanyam *et al.*, 1994) in *vulgare* accessions (Fig. 4, Lanes 12 & 13). Southern blot hybridization of *bulbosum* accessions displayed one 8.6 / 8.4 / or 7.8 Kb repeats or a combination of these repeats (lanes 3-6, 14 & 15). While the interspecific hybrids (lanes 10, 11, 16-19) carried both parental rDNA repeats, the derivatives (lanes 1,2, 7-9) lacked *bulbosum* specific rDNA repeats. Among the derivatives, one doubled haploid derivative (lane 7) and an amphiploid derivative (lane 9) did not carry the 9 Kb

Figure 4. Southern blot hybridization of genomic DNA from parental species and interspecific hybrids / derivatives of *Hordeum* digested with *Eco RV* / *Sac* I / *Eco R*\ or *Xba* I and probed with barley rDNA clone (pHV294).

Lanes 1) VB252; 2) VB251; 3) BB2122; 4) BB353; 5) B397; 6) B395; 7) VDH3; 8) VDH2; 9) VB19; 10) VB2457; 11) VB7885; 12) CDH; M) λ *Hind* III; 13) Betzes; 14) B112; 15) B113; 16)VBI; 17) VBIII; 18)VBIV and 19) VBII

Note: The samples VB7885 (11) and VBIV(18) were absent in *Xba*I digests.

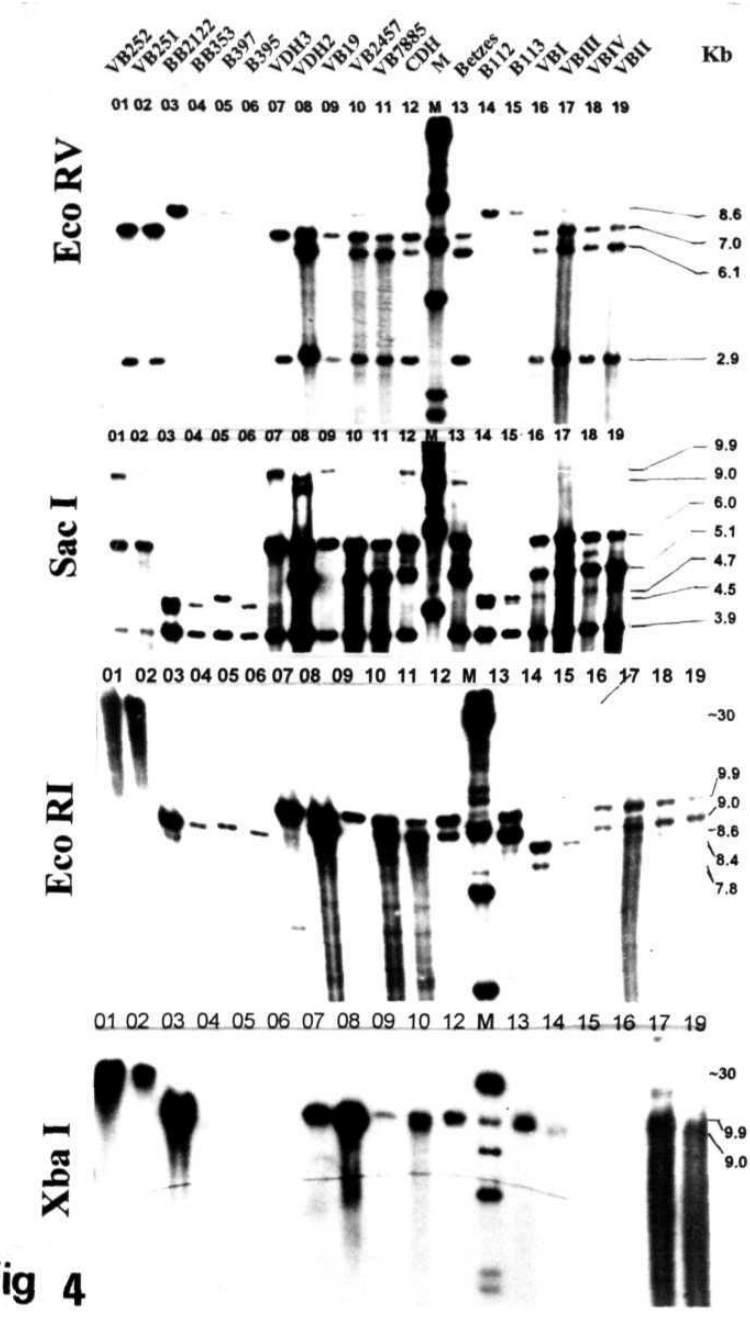

VB252
VB251
BB2122
BB353
B397
B395
VDH3
VDH2
VB19
VB2457
VB7885
CDH
M
Betzes
B112
B113
VBI
VBIII
VBIV
VBII
Kb
01 02 03 04 05 06 07 08 09 10 11 12 M 13 14 15 16 17 18 19
Eco RV
8.6
7.0
6.1
2.9
Sac I
9.9
9.0
6.0
5.1
4.7
4.5
3.9
Eco RI
~30
9.9
9.0
8.6
8.4
7.8
Xba I
~30
9.9
9.0

Fig 4

repeat of *vulgare* parent. Two derivatives of the same lineage displayed high molecular size fraction following *Eco RI* digestion (Fig. 4 lanes 1 & 2).

Southern blot hybridization of *Xba* I digests was less informative as to the sizes of the fragments among the hybrids and derivatives, two of the derivatives (lanes 1 & 2) displayed high molecular weight (~30 Kb) fraction only.

Autoradiograms of rDNA in *Bgl* II digests (Fig. 5) displayed 1.4 Kb fragment in all the lanes in addition to 9.9, 9.0, 8.5 and 7.6 Kb fragments in *vulgare* accessions (lanes 12 & 13) and interspecific hybrids (lanes 10, 12, 16-19). Accessions of *bulbosum* carried 8.6 and 7.2 or 8.4 and 7 Kb fragments or combinations. Interspecific hybrids (lanes 10, 11, 16-19) had fragments of parental origin , while four of the derivatives (lanes 1, 2, 7 & 9) lacked 9 and 7.6 Kb *vulgare* specific fragments. The two amphiploid derivatives of same lineage had a high molecular fraction as well (lanes 1 & 2).

Kpn I generated rDNA fragments resolved by autoradiography (Fig. 5) include 9.9 Kb and 9 Kb fragments in *vulgare* accessions (lanes 12 & 13), 8.6 and / or 8.4 Kb fragments in *bulbosum* accessions (lanes 3-6, 14 & 15). F_1 interspecific hybrids (lanes 10, 11 & 16-19) displayed rDNA repeat specific fragments. The derivatives lacked the *bulbosum* specific rDNA repeats. Furthermore, four of the derivatives (lanes 1, 2, 7 & 9) also lacked one of the *vulgare* specific 9 Kb rDNA repeat.

Southern blot hybridization of *Hind* III generated fragments include ~8.5 Kb band in three *bulbosum* accessions (lanes 3, 14 & 15) while high molecular weight fractions in other *bulbosum* accessions (lanes 4-6) and in all hybrids and derivatives. The interspecific hybrids (lanes 10, 17 & 19) also displayed *bulbosum* repeat unit specific fragments (Fig. 5).

Figure 5. Southern blot hybridization of genomic DNA from parental species and interspecific hybrids / derivatives of *Hordeum* digested with *Bgl* II / *Kpn* I or *Hindm* and probed with barley rDNA clone (pHV294).

Lanes 1) VB252; 2) VB251; 3) BB2122; 4) BB353; 5) B397; 6) B395; 7) VDH3; 8) VDH2; 9) VB19; 10) VB2457; 11) VB7885; 12) CDH; M) λ*Hind* III; 13)Betzes; 14) B112; 15) **B113;** 16)VBI; 17) VBIII; 18) VBIV and 19) VBII

(Bottom panel) Southern blot hybridization of genomic DNA from different *bulbosum* accessions digested with *Eco R*\ (Lanes 1-9) or *Hind* III (Lanes 11-19) and probed with barley rDNA clone (pHV294).

Lanes 1)* BB3032; 2)B395; 3) B397; 4) B112; 5) B113; 6) BB134; 7) BB353; 8) BB2122; 9) 3024; M) λ *Hind* III; 10) B395; 11) B397; 12) B112; 13) **B113;** 14)BB134; 15)BB353; 16) BB2122; 17) BB3024 and 18) BB3032

•Please note the shift of the sample BB3032 (1)

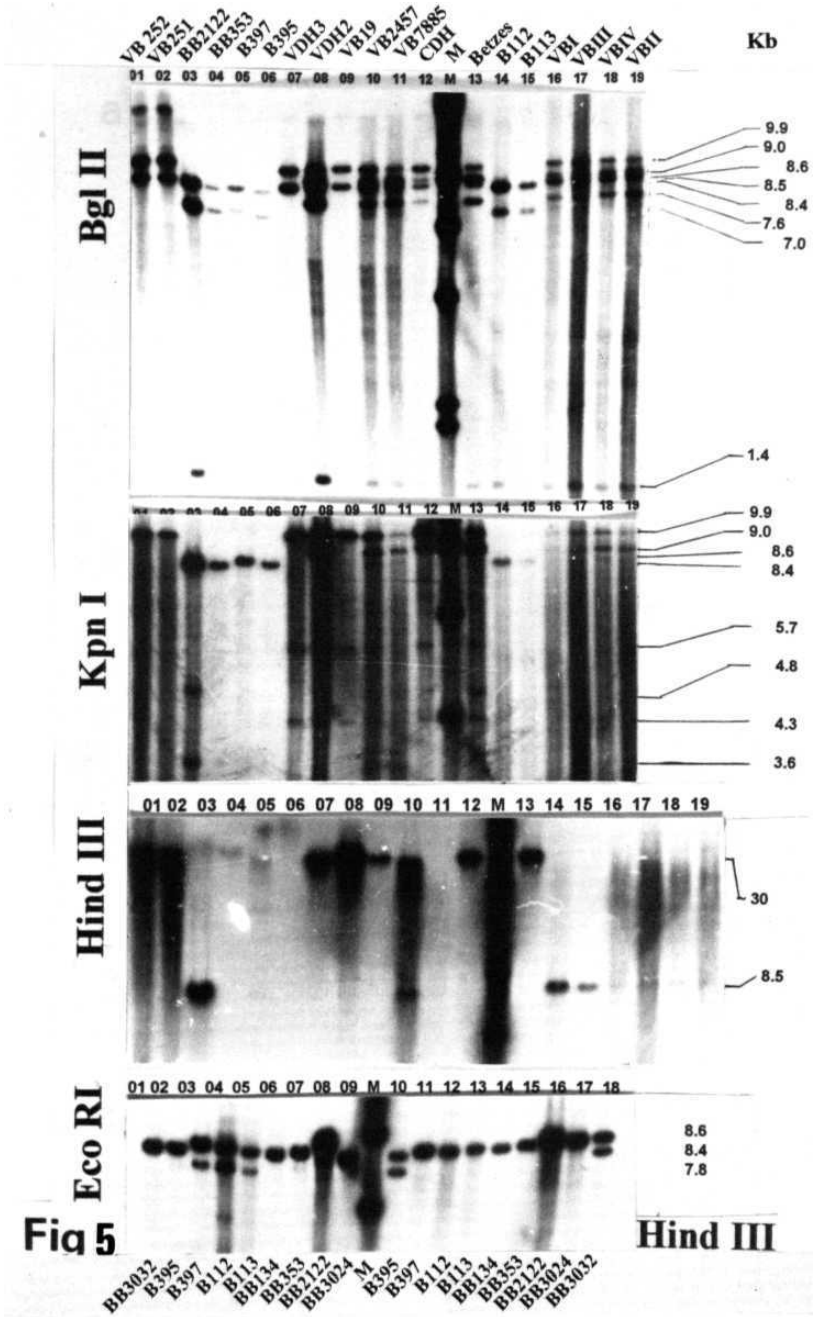

VB 252
VB251
BB2122
BB353
B397
B395
VDH3
VDH2
VB19
VB2457
VB7885
CDH
M
Betzes
B112
B113
VBI
VBIII
VBIV
VBII
Kb
01 02 03 04 05 06 07 08 09 10 11 12 M 13 14 15 16 17 18 19
Bgl II
9.9
9.0
8.6
8.5
8.4
7.6
7.0
1.4
Kpn I
9.9
9.0
8.6
8.4
5.7
4.8
4.3
3.6
Hind III
01 02 03 04 05 06 07 08 09 10 11 12 M 13 14 15 16 17 18 19
30
8.5
Eco RI
01 02 03 04 05 06 07 08 09 M 10 11 12 13 14 15 16 17 18
8.6
8.4
7.8
Hind III
BB3032
B395
B397
B112
B113
BB134
BB353
BB2122
BB3024
M
B395
B397
B112
B113
BB134
BB353
BB2122
BB3024
BB3032

Fig 5

Restriction fragment length polymorphism at the rDNA locus of *H. bulbosum* revealed by *Eco R*\ and *Hind* III digestions are presented in Fig. 5 (bottom panel). Lanes 2-9 and 10-17, 1 & 18 represent duplications of the same accessions. Presence of 8.6 or 8.4 or 7.8 Kb rDNA repeat unit lengths in each accessions or a combination of these is significant.

Restriction fragment length variation within each rDNA repeat unit following digestion's with isoschizomers *Mbo* I and *Sau* 3 *Al* are presented in Fig. 6. *Vulgare* specific *Mbo* I rDNA fragments were 2.8 Kb and 1.9 Kb which could be seen in lanes 12 and 13 while the *bulbosum* specific *Mbo* I fragment is of 1.4 Kb in length. All these fragments were found in two hybrids (lanes 17,19) and in one of the derivatives (lane 8). Four of the hybrids (lanes 10,11,16,18) displayed both the *vulgare* specific (2.8 and 1.9 Kb) *Mbo* I fragments, while four of the derivatives (lanes 1, 2, 7, 9) lacked the 1.9 Kb *vulgare* specific rDNA fragment.

Sau 3 *Al* generated rDNA fragments included additional 3.2 Kb and 2.2 Kb fragments in *vulgare* accessions (Fig. 6, lanes 12, 13). In addition two or three <1 Kb fragments appeared in two hybrids (lanes 17, 19) and one of the doubled haploid derivatives (lane 8) and 2.4, 1.4, 1 and 0.5 Kb fragments in *bulbosum* (lane 3). *Vulgare* specific and *bulbosum* specific *Sau* 3 *Al* fragments are present in hybrids (lanes 10, 11, 16-19) and in one of the derivatives (lane 8). While 1.9 Kb fragment is missing in four derivatives (lanes 1, 2, 7, 9), 2.2 Kb is also missing in two derivatives (lanes 2, 9).

Msp I generated rDNA fragments of *H. vulgare* ranged from 5.1 - 1.2 Kb in length (Fig. 7, lanes 12 & 13) but the number of fragments and their relative distribution are different. The *vulgare*-specific fragments were also present in the hybrids (lanes 10, 11, 16 - 19). Three hybrids (lanes 10, 17, 19) carried a wider range and smaller fragments. Two of the derivatives of identical lineage (lanes 1 and 2) had high moleci Jar

Figure 6. Southern blot hybridization of genomic DNA from parental species and interspecific hybrids / derivatives of *Hordeum* digested with *Mbo* I or *Sau* 3 *A*\ and probed with barley rDNA clone (pHV294).

Lanes 1) VB252; 2) VB251; 3) BB2122; 4) BB353; 5) B397; 6) B395; 7) VDH3; 8) VDH2; 9) VB19; 10) VB2457; 11) VB7885; 12) CDH; M) *X Hind* III + *Eco R*I double digest; 13)Betzes; 14) B112; 15) B113; 16) VBI; 17) VBIII; 18) VBIV and 19) VBII

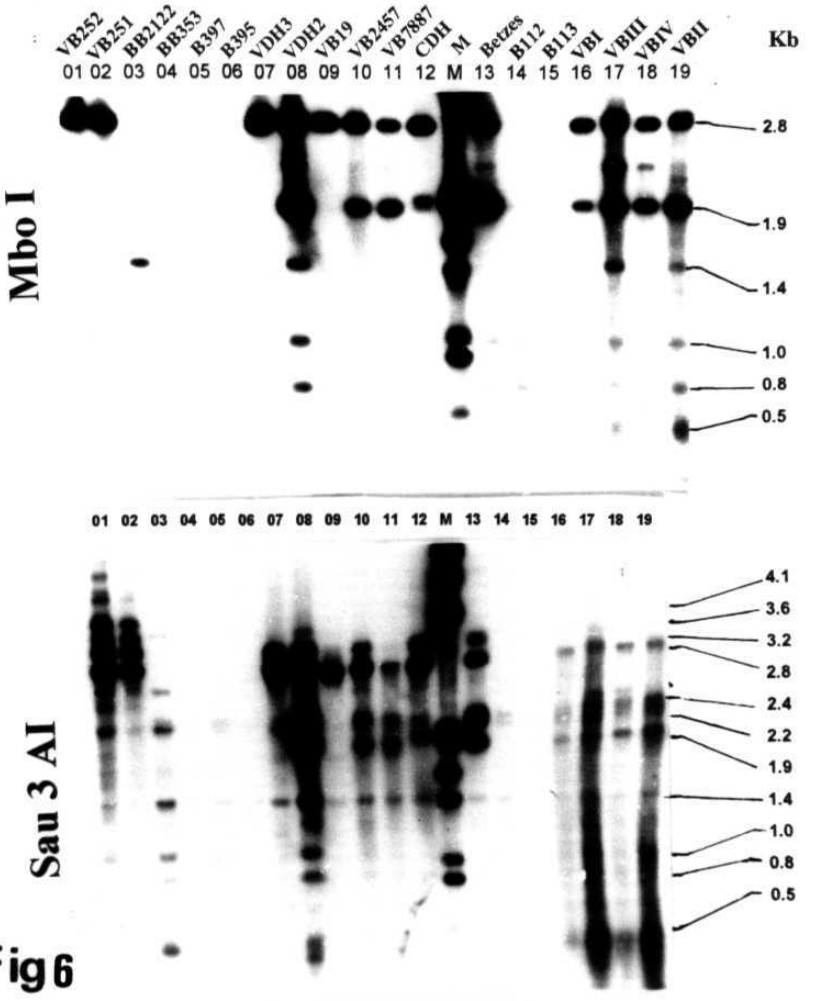

VB252
VB251
BB2122
BB353
B397
B395
VDH3
VDH2
VB19
VB2457
VB7887
CDH
M
Betzes
B112
B113
VBI
VBIII
VBIV
VBII
Kb
01 02 03 04 05 06 07 08 09 10 11 12 M 13 14 15 16 17 18 19
Mbo I
2.8
1.9
1.4
1.0
0.8
0.5
01 02 03 04 05 06 07 08 09 10 11 12 M 13 14 15 16 17 18 19
Sau 3 AI
4.1
3.6
3.2
2.8
2.4
2.2
1.9
1.4
1.0
0.8
0.5

Fig 6

size (~20 Kb) fraction, while one of the double haploid derivatives (lane 7) and an amphiploid derivative (lane 9) were characterized by the absence of less than 2.2 Kb *Msp* I fragments.

Southern blot hybridization *of Hue* III digests displayed a narrow size range of fragments in the parental accessions, for instance 0.66 Kb and 0.44 Kb in the *vulgare* accessions (Fig. 7, lanes 12, 13) and 1.27-1.49 Kb fragments in *bulbosum* accessions (lanes 3-6, 14 & 15). One of the hybrids displayed 0.66 and 0.44 Kb *vulgare* specific fragments, while another hybrid carried 0.44 Kb fragment only. Among the derivatives, two (lanes 1 & 2) displayed high molecular size fraction, one lacked 0.44 Kb *vulgare* specific fragments (lane 9).

Variation in chromosome number

Root-tip cells of the progeny from *vulgare-bulbosum* crosses were routinely checked. Majority showed haploid chromosome number (Fig. 8e) consistent with earlier results (Subrahmanyam and Kasha, 1973a). However, variation in chromosome number was noticed in some of the root-tips from the plants with hybrid morphology . Cells containing 14 chromosomes displayed only two with secondary constriction (Fig. 8a). Cells with less than 14 chromosomes exhibiting one satellite chromosome (Fig. 8b) and cells with less than 7 chromosomes (Fig. 8d, e) and a single satellite chromosome were also found. This was not reported in earlier studies. Presence of a single satellite chromosome in cells containing 9 (Fig. 8b), 6 (Fig. 8d) and 5 chromosomes (Fig. 8e) is indicative of loss of one of the *vulgare* satellite chromosome. The presence of cells with less than 7 chromosomes in the sectors of hybrids and absence of progeny with exclusively 6 or 5 chromosomes is note worthy.

Proportion of modified bases

Overall contents of modified nucleo-bases i.e. 5methyl-cytosine (^{5m}C) and 6me-

Figure 7. Southern blot hybridization ofgenomic DNA from parental species and interspecific hybrids / derivatives of *Hordeum* digested with *Msp* I or *Hae* III and probed with barley rDNA clone (pHV294).

Lanes 1) VB252; 2) VB251; 3) BB2122; 4) BB353; 5) B397; 6) B395; 7) VDH3; 8) VDH2; 9) VB19; 10) VB2457; 11) VB7885; 12) CDH; M) λ *Hind* III; 13)Betzes; 14) B112; **15) B113;** 16)VBI; 17) VBIII; 18) VBIV and 19) VBII

Note: The samples VB7885 **(11),** VBIII (17) and VBIV (18) were absent in *Hae* HI digest.

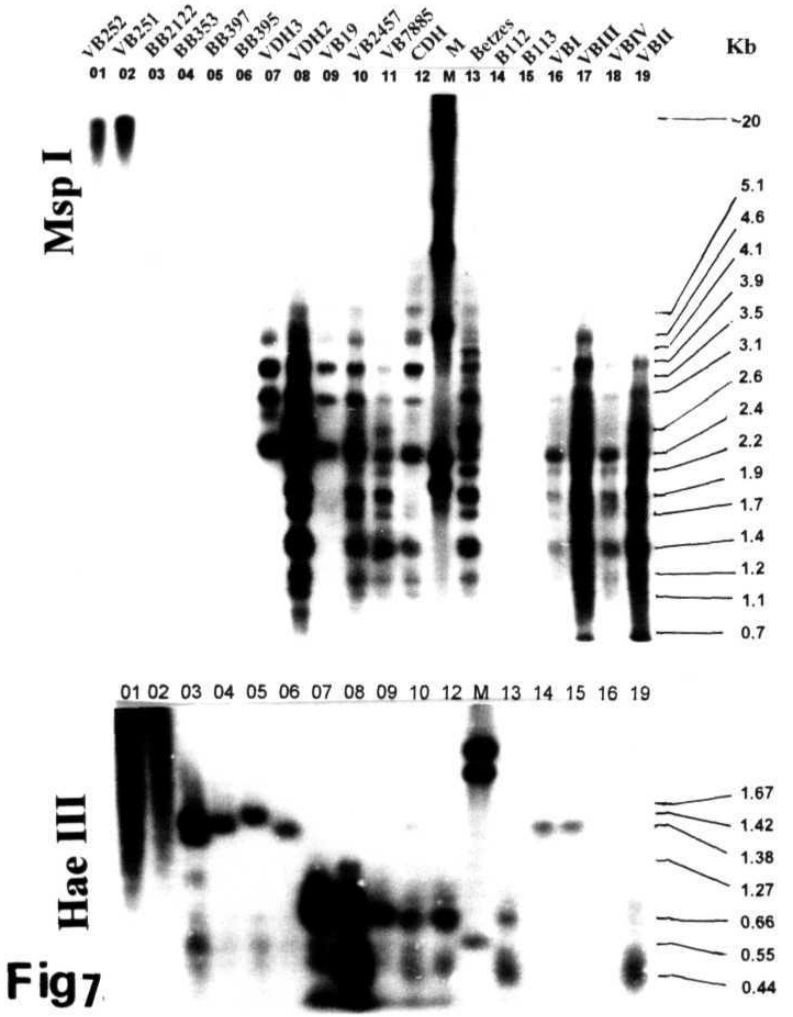
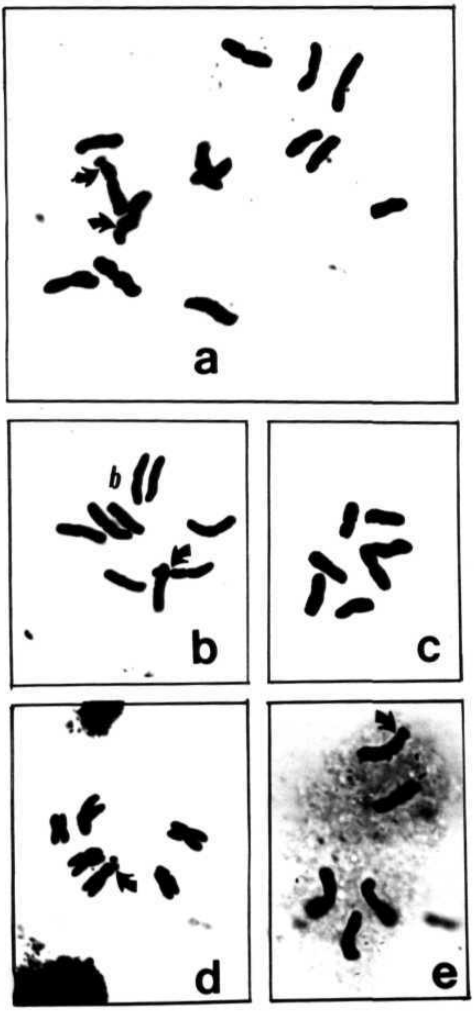

VB252
VB251
BB2122
BB353
BB397
BB395
VDH3
VDH2
VB19
VB2457
VB7885
CDH
M
Betzes
B112
B113
VBI
VBIII
VBIV
VBII
Kb
01 02 03 04 05 06 07 08 09 10 11 12 M 13 14 15 16 17 18 19
Msp I
~20
5.1
4.6
4.1
3.9
3.5
3.1
2.6
2.4
2.2
1.9
1.7
1.4
1.2
1.1
0.7
01 02 03 04 05 06 07 08 09 10 12 M 13 14 15 16 19
Hae III
1.67
1.42
1.38
1.27
0.66
0.55
0.44

Fig7

Figure 8. Cytology: Root-tips cells of *H. vulgare-bulbosum* hybrid with: a) 14 chromosomes; b) 9 Chromosomes; c) 7 chromosomes; d) 6 Chromosomes and e) 5 Chromosomes.



8

thyl-adenine (^{6m}A) in hybrids ranged from ~22 - 24% and 4-12% (Table 4) respectively. From the data among interspecific hybrids the overall extent of ^{5m}C was narrow but statistically significant, whereas a 3 to 4-fold variation in ^{6m}A occurred among the *vulgare-bulbosum* hybrids compared to their parental species. Though the extent of cytosine methylation is similar, its distribution in the genome could be different.

Species - specific rDNA repeat units: Composition of Hybrids:

The presence of *bulbosum*-specific full length 8.6 Kb rDNA fragment, *vulgare*-specific 9.9 and 9.0 Kb fragments in the F_1 interspecific hybrids (VB7885, VB2457, VBI, VBII, VBIII, VBIV (Figs. 4 & 5, lanes 10, 11, 16-19) confirms their hybrid nature for the rDNA loci. Interspecific hybrid VB7887 displayed full length *bulbosum* specific 8.6 Kb rDNA repeat in *Eco R*\ and *Eco RV* digestions following hybridization with barley rDNA probe (data not included).

Number of rDNA repeat units in *bulbosum*

The rDNA repeat unit length in *H. bulbosum* was reported as 8.5 Kb (Molnar *et al.*, 1989) and *bulbosum genome* contains one NOR as demonstrated by silver staining (Molnar *et al.*, 1989, Linde-Laursen *et al.*, 1990). Among the *bulbosum* accessions used in the present study, two diploid lines (112 and 113) and one tetraploid line (2122) had two rDNA repeat lengths *i.e.* 8.6 and 7.8 Kb suggesting that these *bulbosum* accessions are heterozygous for their rDNA repeat unit lengths at the rDNA locus. However, 7.8 Kb fraction was very low compared to 8.6 Kb fraction in *Eco R*\ digests (Fig. 4) indicating differences in the copy number in the homologues. The band intensity of 3.9 Kb *Sac* I fraction was found to be higher compared to either 4.7 or 4.5 Kb fragments, suggesting that 8.6 or 8.4 Kb repeat units gave 4.7 or 4.5 Kb fragments in addition to 3.9 Kb fragments each and 7.8 Kb repeat unit gave rise to two 3.9 Kb fragments one of which is similar to the above. Thus, it appears that all these *bulbosum* accessions possess two rDNA repeat lengths.

Table. 4 Extent of methylation in parents and interspecifichybrids

Parent/Hybrid	%5mC	%6mA
BB 112	24.32 ± 0.26	12.24 + 0.08
BB 113	24.37 ± 0.39	9.74 ± 0.22
HV 6478	23.05 ± 0.03	9.34 ±0.15
VB I	24.61 ± 0.01	11.75 + 0.05
VB III	24.85 ± 0.05	3.53 ± 0.02
VB IV	25.80 ± 0.05	8.36 ± 0.05
VB7882	23.94 ± 0.02	11.06 ±0.04
VB 7885	23.81 ± 0.01	4.02 ± 0.01
VB7887	22.20 ± 0.05	9.62 ± 0.05
VB 2457	23.85 ± 0.07	7.91 ± 0.01

Variation at individual NOR loci

The presence of two rDNA repeat unit lengths in diploid *bulbosum* accessions, three different repeat unit lengths (8.6, 8.4 and 7.8 Kb) in tetraploid accessions indicate differences in the levels of heterozygosity at the rDNA locus at the disomic and tetrasomic levels.

The presence of more than one repeat unit length might have arisen due to the non-homogenization of variation at homologous sites contrary to the proposal that variation at a single locus (rDNA) reaches homogeneity in a single generation, while only beneficial variations were fixed and others were either diluted or lost in subsequent generations (Dover *et ah,* 1993). *H. bulbosum* is self-incompatible species (Lundquist, 1962) and in the absence of any selection pressure it may maintain its heterozygosity.

Loss of species - specific rDNA repeat units :

Intensities and ratios of 9/ 9.9 Kb in hybrids (VB2457, VBI and VBIII) deviated from *vulgare* parent (Fig. 4, Table. 5). Hybrids VB7885, VBI and VBIII showed less intense in their 9 Kb rDNA specific repeat compared to 9.9 Kb repeat in other hybrids *viz.* VB2457, VBIV, VBII and *vulgare* parent as evident from deviation in 9/ 9.9 Kb ratios suggesting loss of some of 9 Kb rDNA specific repeats in these hybrids (Fig. 4, Table 5). Ratio of *vulgare / bulbosum* rDNA in the hybrids suggests the loss of part of 9 Kb *vulgare* specific rDNA repeats in the hybrids. Hybrid 7887 gave rise 9/ 9.9 ratio as 2.3 with *Eco. RV* digestion indicating extensive loss of 9 Kb rDNA specific repeats (data not included).

Composition of derivatives

Absence of *vulgare*-specific 9 Kb rDNA repeat unit / fragments (Table 6A & 6B) generated by *Eco RI, Xba* I (Fig. 4), *Bgl* II and *Kpn* I (Fig. 5), 9 Kb repeat unit specific fragments 6.1 Kb *Eco RV,* 5.1 Kb *Sac* I (Fig. 4), 7.6 Kb *Bgl* II (Fig. 5). 1.9 Kb

Table 5. Ratio of rDNA repeat units and proportion of modification in *Hordeum* interspecific hybrids / derivatives.

EcoR V Fragments	VB252	VB251	BB2122	BB353	B397	B395	VDH3	VDH2	VB19	VB2457	VB7885	CDH	Betzes	B112	B113	VBI	VBIII	VBIV	VBII
8.6 Kb			+		+					+	+			+	+	+	+	+	+
8.4 kb				+		+													
*9 / 9.9 #	*	*					*	1.40	*	0.53↓	1.60	0.64↓	1.60			0.72↓	0.70↓	1.3	1.5
V / B										5.50	6.70					6.90	5.60	6.0	6.8

9.0/9.9 Kb represented by their respective fragments (6.1Kb/ 7.0Kb); *) 6.1 Kb Not detectable;

Sac I Fragments	VB252	VB251	BB2122	BB353	B397	B395	VDH3	VDH2	VB19	VB2457	VB7885	CDH	Betzes	B112	B113	VBI	VBIII	VBIV	VBII
HMW %	25.0	25.0					17.0	21.0	19.0			14.0	16.0						
*9 / 9.9 #	*	*					*	1.4	*	0.67↓	1.67	0.8↓	1.7			0.8↓	0.8↓	1.2	1.5
V / B										6.0	6.0					5.0	6.0	6.0	5.0
4.5/4.7 Kb			0.24											0.25	0.4				

9/9.9 Kb represented by 5.1 / 6.0 fragments; *) 5.1 Kb fragment not detectable; 4.5Kb and 4.7Kb represent 8.4 and 8.6Kb of bulbosum rDNA repeats

Eco R I Fragments	VB252	VB251	BB2122	BB353	B397	B395	VDH3	VDH2	VB19	VB2457	VB7885	CDH	Betzes	B112	B113	VBI	VBIII	VBIV	VBII
30.0 Kb	++	++																	
*9/9.9 Kb							*	1.4	*	0.67↓	1.7	0.64↓	1.5			0.7↓	0.9↓	1.3	1.5
V/B										4.5	4.9					4.3	4.46	4.35	4.6
7.8/8.6 Kb			0.18		0.24									0.4	0.4				
7.8/8.4 Kb				0.19															

Kpn I Fragments	VB252	VB251	BB2122	BB353	B397	B395	VDH3	VDH2	VB19	VB2457	VB7885	CDH	Betzes	B112	B113	VBI	VBIII	VBIV	VBII
*9/9.9 Kb	*	*					*	1.36	*	0.50↓	1.50	0.68↓	1.75			0.70↓	0.56↓	1.0	1.4
V/B										3.30	3.26						4.2	3.4	3.4

+) present; HMW %) 9.9+9.0 / 9.9+9.0+6.0+5.1+3.9; #) Ratio of their corresponding repeat specific fragments;
***) 9.0Kbrepeat unit/ specific fragment not detectable; V / B - *vulgare* to *bulbosum* rDNA**

Table 6A. Distribution of rDNA repeat specific restriction fragments in interspecific hybrids between *Hordeum vulgare* and *Hordeum bulbosum* and their derivatives

Enzyme / sequence;	*Eco. R*V GA^TA^TC				*Sac* I GAGC^TC					*Eco. R*I GA^A^TTC^					*Kpn* I GGTAC^C^				*Hind* III A^A^GC^TT	
Fragments (Kb);	8.6	7.0	6.1	2.9	6.0	5.1	4.7	4.5	3.9	30	9.9	9.0	8.6	8.4	7.8	9.9	9.0	8.6	8.4	30	8.5
Parents / hybrids																					
Betzes		+	+	+	+	+			+		+	+				+	+			+	
CDH		+	+	+	+	+			+		+	+				+	+			+	
BB2122	+						+	+	+				+		+			+			+
BB353	+							+	+					+					+		
B112	+						+	+	+				+		+			+			+
B113	+						+	+	+				+		+			+			+
B395	+							+	+					+					+		
B397	+						+		+				+					+			
VB2457	+	+	+	+	+	+	+	+	+		+	+	+		+	+	+			+	+
VB7885	+	+	+	+	+	+	+	+	+		+	+	+		+	+	+			+	
VBI	+	+	+	+	+	+		+	+		+	+	+			+	+			+	+
VBII	+	+	+	+	+	+		+	+		+	+	+			+	+			+	+
VBIII	+	+	+	+	+	+		+	+		+	+	+			+	+			+	+
VBIV	+	+	+	+	+	+		+	+		+	+	+			+	+			+	+
Derivatives																					
VB251		+		+	+				+	+										+	
VB252		+		+	+				+	+						+				+	
VB19		+		+	+				+		+					+				+	
VDH3		+		+	+				+		+					+				+	
VDH2		+	+	+	+	+			+		+	+				+	+			+	

Table 6B. Distribution of rDNA repeat specific restriction fragments in interspecific hybrids between *Hordeum vulgare* and *Hordeum bulbosum* and their derivatives

Enzyme / sequence;;	*Bgl* II AGATC^T							*Mbo* I GA^TC			*Sau* 3 *A*I GATC^					*Msp* I C^CGG			*Hae* III GGC^C			
Fragments (Kb);	20	9.9	9.0	8.5	7.6	7.2	14	2.8	2.3	1.9	3.6	3.2	2.8	2.2	1.9	20	9.9	9.0	1.4	2	1.38	0.66	0.44
Parents / hybrids																							
Betzes		+	+	+	+		+	+		+		+	+	+	+		+	+				+	+
CDH		+	+	+	+		+	+		+		+	+	+	+		+	+				+	+
BB2122				+		+	+				+								+		+		
BB353				+		+	+														+		
B112				+		+	+														+		
B113				+		+	+														+		
B395				+		+	+												+				
B397				+		+	+																
VB2457		+	+	+	+		+	+		+		+	+	+	+		+	+			+	+	+
VB7885		+	+	+	+		+	+		+			+	+	+		+	+				-	-
VBI		+	+	+	+		+	+		+			+	+	+		+	+				+	+
VBII		+	+	+	+		+	+	+	+			+	+	+		+	+	+			+	+
VBIII		+	+	+	+		+	+	+	+		+	+	+	+		+	+				-	-
VBIV		+	+	+	+		+	+		+			+	+	+		+	+				-	-
Derivatives																							
VB251	+	+		+			+	+			+	+	+			+					+		
VB252	+	+		+			+	+			+	+	+			+					+		
VB19		+		+			+	+					+				+					+	
VDH3		+		+			+	+				+	+				+					+	
VDH2		+	+	+	+		+	+		+		+	+	+	+		+	+				+	+

Hae III fragments represent their rDNA repeat units; *Msp* I rDNA specific fragments represented by their repeat units

Mbo I, 2.2 and 1.9 *SauA*I (Fig. 6), 2.2, 1.9, 1.7, 1.4, 1.1 and 0.7 Kb *Msp* I, G.44 Kb *Hae* III (Fig. 7) in the hybrid derivatives VB252, 251, VDH3 and VB19 indicate elimination of the *vulgare*-specific 9 Kb rDNA repeat. VB19 and VDH3 did not contain any *bulbosum* specific rDNA. Full length *bulbosum* specific rDNA repeats/ fragments generated by *Eco RV, Sac* I (Fig. 4), *Bgl* II, *Kpn* I and *Hind* III (Fig. 5) are absent in VB251 and VB252 but *bulbosum* specific 3.4, 2.8 and 2.1 Kb *Sau* 3 *A*I fragments (Fig. 6) and 1.27 Kb *Hae* III fragment (Fig. 7) were present. *Bulbosum* specific full length rDNA repeat/ repeat specific fragments were absent in VB19 and VDH3 derivatives which lost one of the *vulgare*-specific 9 Kb rDNA repeat/ repeat-specific fragments.

Hybrid derivatives (VB251, VB252, VDH3, VDH2 and VB19) were progeny derived from the same cross combination. Any ensuing consequences that arise later might have been initiated among the different hybrid zygotes and interaction of the parental genomes in the hybrid, and of these derivatives with the environment. Inadequately protected sequences are destined to be eliminated under the influence of elimination factors in the hybrids. Our results demonstrate the presence of *bulbosum*-specific 2.4 Kb (rDNA) fragment in hybrids VBIII and VB1V. *De novo* methylation is a slow process, presence of *bulbosum* genome or part thereof may trigger elimination / degradation of the *bulbosum* specific DNA as evident from the absence *of bulbosum* specific rDNA fragments in VDH3 and VB19. The absence of *vulgare* specific 9 Kb rDNA repeat or 9 Kb repeat specific fragments is likely the consequence of loss of inadequately protected *vulgare*-specific sequences. The absence of a second chromosome with the secondary constriction in the root-tip cells in some of the hybrid sectors from the same cross is consistent with this proposal that occasionally *vulgare* chromosomes are eliminated in *vulgare-bulbosum* hybrids. Alternative elimination in crosses between *H. vulgare* and *H. marinum* may represent a similar mechanism. It has been shown that elimination of *bulbosum* genome in first zygotic division or in the subsequent 3 to 4 zygotic cell divisions or may get prolonged for few days gradually leading

to haploid formation or may lead to chimeric situation resulting in variation in the chromosome number in different root-tip cells or in different tillers of the same plant or different progeny of the same interspecific or intergeneric hybrids (Subrahmanyam and Kasha, 1973a, Laurie and Bennett, 1989).

De novo methylation:

Hybrids (VB7885, VB2457, VBI, VBII, VB1II, VBIV) possessed *bulbosum* genic material (Fig. 4 - *Eco RV, Eco R*\, *Sac* I, Fig. 5. - *Hind* III). Hybrids VB7885 and VB2457 have not shown any high molecular weight range fragments, suggesting no modifications, whereas VBIII and VBII showed new 2.6 Kb fragments unlike the parents suggesting modifications in these hybrids. Differences in the methylations among the hybrids suggest that *de novo* methylation may occur any time after hybridization and differential methylation of species specific sequences leads to differential susceptibility to degradation / elimination of genome specific sequences. *De novo* methylation may function as a defense / protection mechanism (Bestor, 1990; Barlow, 1993; Doerfler, 1991). Introduction of homologous sequences as artificial constructs triggered modifications (methylation) of the resident genes or both (*trans*-inactivation) leading to the loss of function (Finnegan and McEiroy, 1994;Flavell, 1994; Matzke *et al.*, 1996; Hobbs *et al.*, 1993; Wassenegger *et al.*, 1994). Site of integration and copy number influenced the methylation *{de novo)* status of the construct (Meyer, 1995). Linked duplications in *Neurospora* undergone 100% methylation whereas unlinked to 50% in a single generation (Butler and Metzlenberg, 1990). Homology as little as 90 to 370 bp of the promoter region triggered such modification in the constructs (Vauchert, 1993; Hobbs *et al.*, 1990, 1993; Meyer, 1995; Matzke and Matzke, 1995).

In wheat-rye intergeneric hybrids, treatment of 9-day old embryo with azacytidine (inhibitor of DNA methyl transferase) restored expression of rye NOR, however, treatment prior to day 9 did not restore the expression, indicating *de novo* methy-

lation of rye NOR occurs at day 9 leading to repression (Neves *et al.*, 1995). It has been shown that methylation (imprinting) pattern will be erased in gametogenesis and reestablished after fertilization, during development (eraser-resetting) in plants (Neves *et al.*, 1995) and animals (mammales) (Jaenisch, 1997; Kevrne *et al,* 1996; Razin and Kafri, 1994; Surani *et al,* 1990).

Bacteria are known to possess restriction / modification system (Kuhlein and Arber, 1972) for protection against invading phages. Occasionally phages overcome host restriction by various mechanisms (Bickle and Kruger, 1993). Methylation of such sequences in bacteria functioning as a part of restriction-modification system aiding successful defense against invading sequences in selective-silencing of such sequences but maintaining and perpetuating its own DNA is already established (Arber, 1974; Bickle and Kruger, 1993).

Extent of modification

Methylations leading to high molecular weight (30 Kb) fraction in *Eco RI*, *Xba* I digests of VB252 and VB251, *Hind* III digests in all hybrids/derivatives, *vulgare* accessions represented as single repeat or dimers due to methylation of the recognition sites of *Sac* I and *Bgl* II in the rDNA repeat units (Fig. 4 and 5). Relative proportions of rDNA in high molecular weight and single repeat fractions (Table 5 & 7) is a reflection of the extent of methylation at the restriction sites within the rDNA repeat units in the derivatives. Methylation of rDNA resulting in high molecular weight in higher plants was reported (Ellis *et al.*, 1989; Olmedilla *et al.*, 1984; Moore *et al.*, 1993; Montero *et al,* 1992).

ddm (decrease in DNA methylation) mutants in *Arabidopsis* showed similar amounts of modified cytosine and plants varied in their morphological features indicating that differences in the distribution of methylated bases in plants a; individual genes

affect their expression leading to phenotypic differences (Kakutani *et al.*, 1996; Finnegan *et al.*, 1996; Ronemus *et al.*, 1996; Vongs *et al.*, 1993; Richards, 1997). Integration of ^{6m}A containing reporter gene into genome lead to rearrangements, instability and subsequent.loss of the gene in barley (Rogers and Rogers, 1992). However ^{6m}A as a part of GATC sequences near the promoter increased the *α amylase*, CaMV35S, maize *Adh* 1 and maize *ubiquitin* gene promoters strength as reporter gene constructs in transient barley aleurone system (Rogers and Rogers, 1995). Critical cytosine methylation in promoters regulating gene expression is known in plants (Thompson and Flavell, 1988; Finnegan and Dennis, 1993; Jupe and Zimmer, 1993; Torres-Ruiz and Hemleben, 1994) and animals (Cedar and Razin, 1990; Surani *et al.*, 1990).

Differential methylation

Differences in the ratios of 9.0/9.9 and 7.6/8.5 indicate differential methylation of the two rDNA repeats vis *a vis* at the respective loci (Table 7). Hybrids had 10 to 15% deviation in their 8.5 Kb rDNA fraction, 10 to 20% in their 7.6 Kb fraction as compared to *H. vulgare* indicating that hybrids have less methylation at their *Rrn* loci than their parental species (Table 7).

Proportions of individual repeat/repeat specific fragments represent the distribution of methylated sites. Hybrid derivatives VB252 and VB251 displayed 30 Kb rDNA fraction in *Eco R*\ and *Xba* I digests indicating methylation of alternate sites or two consecutive sites in the repeat 9.9 Kb result in ~ 20Kb or ~30 Kb fragments respectively. Proportion of these fragments represents the number of sites methylated in that fraction.

The isoschizomers *Mbo* I and *Sau* 3 *Al* recognize the sequence GATC but the former is sensitive to adenine methylation, whereas the latter is sensitive to cytosine methylation in the . ite. Differences in the fragments generated by the enzyme pair are

Table 7. Differential modification and ratio of rDNA repeats in *Hordeum* interspecific hybrids/derivatives.

Bgl II Fragments	VB252	VB251	BB2122	BB353	B397	B395	VDH3	VDH2	VB19	VB2457	VB7885	CDH	Betzes	B112	B113	VBI	VBIII	VBIV	VBII
HMW (%)	20.0	19.0																	
I (%)	50.0	50.0					60.0	60.0	56.0	60.7	56.0	48.3	46.0			63.0	64.0	59.0	57.0
II (%)	*	*					*	54.0	*	57.0	54.0	57.0	41.0			66.0	60.0	62.0	57.0
III (%)			62.0		49.0									51.6	54.5				
IV (%)				64.0		62.0													
9.0/9.9kb								1.34		0.70↓	1.70	0.5↓	1.50			0.57↓	0.74↓	1.10	1.40
7.6/8.5kb								1.01		0.65	1.60	0.65	1.49			0.58	0.67	0.78	1.16

Relative proprtion of rDNA fragments: I) 8.5 +1.4 / 9.9+8.5+1.4; II) 7.6+1.4 / 9.0+7.6+1.4; III) 7.2+1.4 / 8.6+7.2+1.4;

IV) 7.0+1.4 / 8.4+7.0+1.4; *) 9.0 Kb component absent; HMW) 20 / 20+9.9+8.5+1.4; I & II) sensitive monomer rDNA fraction

interpreted as methylation of the base in the recognition sequence resulting in high molecular weight fraction. *Mbo* I generated either 2.8 Kb fragment representing 9.9 Kb or 1.9 Kb representing 9.0 Kb repeat or both in *vulgare* accessions, interspecific hybrids and derivatives, whereas *Sau* 3 *A]* generated 3.2 and 2.8 (9.9 Kb specific), 2.2 and 1.9 Kb (9.0 Kb specific) fragments. In addition high molecular weight (3.6 and 4.1 Kb) fraction appeared in VB252 and VB251 indicating cytosine methylation. The presence of lower molecular weight fraction in *Sau* 3 *A* indicates adenine methylation (Fig. 6). Proportion of individual bands in high molecular weight fraction in *Sau* 3 *A* (Table 8) represents cytosine methylation, while the absence of low molecular weight fraction in the *Mbo* I digests represents adenine methylation.

The ratio of 2.8/3.2 and 1.9/2.2 (Table. 8) represents methylation pattern at independent *Rrn* loci . Low percentage of 3.2 or 2.2 indicates less of cytosine methylation at individual *Rrn* loci. High molecular weight fraction in VB252, VB251 indicates cytosine methylation. Deviation in the ratio of repeat specific fragments generated by *Mbo* I to that of *Sau* 3 *A* indicates differential methylation. Low or undetectable level of 3.2 Kb fragments indicates less cytosine methylation *Rrn* 1 locus as in VB7885 and VB19. Though CDH possessed more 9.9 Kb fraction only 40% is methylated. VB19 probably had lost part of 9.9 Kb repeat and might have contributed to the low or undetectable level of 3.2 Kb fraction which in turn relates to methylation status. In VB19, VB7885 and VBII major proportion of 9.9 Kb repeat resulted in 2.8 Kb fraction. Low or undetectable level of 3.2 Kb in VB19 may be due to the loss of part of 9.9 Kb repeats at *Rrn* 1. High ratios of 2.8/3.2, and 1.9/2.2 are indicative of low methylation levels (Table 8).

Isoschizomers *Hpa* II and *Msp* I recognize CCGG and *Hpa* II is sensitive to methylation of cytosines whereas *Msp* I is sensitive to external cytosine and insensitive to methylation of internal cytosine i the sequence. *Hpa* II generated high molecular

weight indicating extensive methylation of the sites. *Msp* I generated high molecular weight (~20 Kb) fraction in VB252, VB251 and discrete fragments in VDH3, VDH2, VB19, VB2457, VB7885, CDH, Betzes, VBI, VBIII, VBIV and VBII. Fragments specific to 9 Kb rDNA repeat were absent in VDH3 and VB19 as inferred from the absence of the repeat specific 2.2 Kb *Msp* I fragments consistent with the results obtained using other enzymes. *Msp* I generated high molecular weight fraction in VB252 and VB251 is indicative of the extensive cytosine methylation at the CCGG sequences.

Comparison of relative distribution of signal intensities among the fragments within each lane (hybrid) with those of the parental species (Table 9) provides an insight into the extent and distribution of ^{5m}C at the CCGG sites of rDNA repeats (Loci). Decrease in intensity of low molecular weight fragments and proportionate increase in high molecular weight fraction in that lane indicates methylation of the external cytosine (CCGG) of the internal sites within that fragment. 3.1 Kb and 3.5 Kb fragments in hybrids (VBI,VBIII and VBII), 1.7 and 1.9 Kb (VB7885 VB2457, VBI) and 2.8, 2.6 Kb are an indication of such pattern (Table 9). Differences in the proportion of the intensity between the lanes represent extent of methylation. The fragments generated in the *Msp* I are higher than expected from their base composition *i.e.* 400 bp indicating methylation of some the sites. *Hpa* II generated high molecular weight range and *Msp* I only discrete bands, therefore internal sites in that fragment represent possible methylation of both cytosines in the site. 20-30% of external cytosine modification of *Msp* I sites was reported in wheat (Jeddeloh and Richards, 1996).

Conversely low proportion at high molecular weight fraction and higher proportion at low molecular weight fraction indicates that demethylation of some of the internal sites in the high molecular weight fragments. 2.4 and 2.6 Kb in hybrids (VB2457, VB7885, VBI, VBII, VBIII and VBIV, Table 8). Specific demethylation of *Hpa* II sites were correlated with expression of rRNA genes in wheat (Flavell *et al*., 1988). The new

Table 8. Extent of modification and ratio of rDNA repeat fragments 1.9+ 2.2(9.0Kb) / 2.8+3.2(9.9Kb) in *Hordeum* Interspecific hybrids/derivatives.

Mbo I Fragments	VB252	VB251	BB2122	BB353	B397	B395	VDH3	VDH2	VB19	VB2457	VB7885	CDH	Betzes	B112	B113	VBI	VBIII	VBIV	VBII
2.5 kb	+							+		+	+		+			+	+	+	+
2.35 kb	+	+	+					+		+	+					+			
1.4 kb			+					+						+			+		+
*1.9/2.8	*	*					*	1.4	*	0.73↓	1.7	0.6↓	1.6			0.73↓	0.66↓	0.78↓	1.3

+) present; *) 1.9Kb not detectable; 2.8 represents 9.9Kb;1.9Kb represents 9.0 Kb

Sau3 A I Fragments	VB252	VB251	BB2122	BB353	B397	B395	VDH3	VDH2	VB19	VB2457	VB7885	CDH	Betzes	B112	B113	VBI	VBIII	VBIV	VBII
4.1 Kb	25	19																	
3.6 Kb	14	14	+																
3..2 Kb	30	33					37	40	*	36	18	40	40			25	24	23	*
3.0 Kb							14												
2.2 Kb								63		55	65	46	62			48	39	48	47
2.8/3.2							0.9	1.2	*	1.7	*	1.7	1.5			3.0	3.1	3.3	*
1.9/2.2								0.6		0.8	0.5	1.2↑	0.6			1.0	1.5	1.1	1.1
**9.0/9.9	**	**					**	1.6	**	0.9↓	2.1↑	0.4↓	1.9↑						

4.1Kb, 3.6Kb, 3.2Kb, 3.0Kb and 2.2Kb fragments presented as %; *) 3.2Kb component not detectable; 3.2(Kb) and 2.8(Kb) represent 9.9(Kb); 2.2Kb and 1.9Kb represent 9.0Kb; **) components of 9.0Kb not detectable

Table 9. Proportion/distribution of different *Msp* I rDNA fragments in *Hordeum* interspecific hybrids/derivatives.

Msp I Fragments	VB252	VB251	BB2122	BB353	B397	B395	VDH3	VDH2	VB19	VB2457	VB7885	CDH	Betzes	B112	B113	VBI	VBIII	VBIV	VBII
20.0 kb	*	*																	
5.1 kb													2.9						
4.6 kb							5.0	4.6		6.0	2.3	8.0	3.7						
4.1 kb							8.7	7.0	15.8	6.3	4.4	14.6	3.9			7.1	9.6	5.6	6.1
3.5 kb			7.3				25.0	11.1	22.6	11.0	6.0	17.1	9.7			14.1	13.8	10.1	13.9
3.1 kb			5.3				23.0	9.4	18.3	10.0	6.8	7.2	7.3			5.6	8.0	7.9	6.5
3.0 kb							6.2												
2.8 kb								7.4								5.1	8.2	4.7	4.9
2.6 kb			26.0					4.7		5.0	9.1		11.6			4.0	6.6	5.0	9.8
2.4 kb							32.4	5.4	34.4	14.0	12.6	21.3	6.6			22.5	15.1	15.8	14.6
2.2 kb								+		6.3	7.9		6.3			+	+	+	+
2.17 kb								6.7						13.4	23.0	4.1		9.7	4.6
1.9 kb			35.0					8.8		14.9	16.8		11.5	16.3	14.2	12.4	11.4	10.0	6.0
1.7 kb								6.6		7.8	7.8	7.7	5.3	16.0		5.8	9.6	8.2	5.8
1.4 kb								13.9		10.3	14.1	12.7	15.0	28.0	23.0	9.2	9.6	13.2	2.9
1.2 kb			7.0					6.3		6.0	6.0	7.6	5.1	29.0	16.0	5.8	4.8	3.1	6.0
1.0 kb			7.5					4.7		3.4	3.3	4.4	2.3			3.8	3.1	6.4	4.4
0.9 kb			5.6					3.1		2.5									

*) HMW ; +) detectable

bands which appeared in individual lanes represent methylation/ demethylation of the sites in that fragment or demethylation in other lanes giving higher proportion of low molecular weight fraction or methylation of both cytosines in the site resulting in increase in high molecular weight fraction in other lanes for instance 2.8 Kb in VDH2 (Table 9).

Symmetrical and asymmetrical methylation

The dinucleotide sequence CpG and trinucleotide sequence CpXpG arc considered as symmetrical sites recognized by maintenance methylase which methylates during DNA replication. The extent of methylation is high in plant genomes (up to 4%) whereas it ranges between 0.5 and 1% in animal genomes. The proportion of cytosine residues methylated range from 20 to 30% in plants (Cheung *et al.,* 1992) and 1 to 8% in animals (Antiquerra and Bird, 1993). Methylation sequences include CpG dinucleotide and trinucleotide CpXpG sequences in plants (Antiqerra and Bird, 1993; Bird, 1993; Gardiner-Garden *et al.*, 1992; Hepburn *et al.,* 1987) and only CpG in animals (Gruenbaum *et al,* 1981; Hergerberg, 1991; Meehan *et al,* 1992).

The similarity in restriction fragment pattern in hybrids and parent indicates imprinting, *i.e.* transfer of parent-specific methylation pattern. The new bands in hybrids are indicative of demethylation of the external methyl cytosine in that fraction. The appearance of new bands in hybrids were reported in *Zea mexicana* X *Tripsacum* hybrid (Lin et al., 1986). The CpG islands are distributed in close proximity (200 bp apart) as indicated in *Msp* I restriction profiles of the rDNA (Fig. 7). The average distribution of these islands is about 125 to 250 bp in plants and nearly 550 bp in animals (Antequera and Bird 1993, Gardiner-Garden *et al.,* 1992). Such islands in the active genes are resistant to *de novo* methylation (Bestor *et al,* 1992). It is known that symmetrical methylations are involved in gene regulation and imprinting in the development of mammales (Surani *et al.,* 1990; Keverne *et al.,* 1996). Up to 90% of *Hpa* II

sites are methylated in rice (Cheung *et al.,* 1992). Over 80% modification of CpG sequences in animals was reported (Antiquera and Bird, 1993). The loss or decrease of DNA methyl transferase function, (in turn methylation) resulted in embryonic lethality and death suggesting that specific methylations are required for normal embryonic development (Li *et al.,* 1993).

The extent of cytosine asymmetrical methylation as indicated in *Sau* 3 *Al* (Figs. 6, 7, and Tables 8, 9) was limited as compared to symmetrical methylation pattern. Asymmetrical methylation was extensive in the derivatives VB252 and VB251 compared to *H. vulgare*. The significance of asymmetrical methylation was reported in maize *Al trans*-gene in *Petunia* (Meyer *et al.,* 1994) and *Neurospora* (Selker *et al.,* 1993).

Hae III recognizes GGCC a reverse sequence of *Msp* I sequence CCGG. The probability of their distribution should be equal but *Hae* III generated 0.66 Kb fragment specific to 9.9 Kb repeat and 0.44 Kb fragment specific to 9 Kb repeat. This enzyme generated low molecular weight fragments compared to *Msp* I in the corresponding *Rrn* loci indicating lesser cytosine methylation in GGCC sequences compared to those generated by *Msp* I. There was no discernible variation in the distribution of *Hae* III fragments whereas as distribution of *Msp* I sites and the proportion of methylation varied (Table 9, Fig. 7). However VB252 and VB251 had a complicated pattern in *Hae* III digests and high molecular weight fraction in *Msp* I digests indicating extensive methylation of these sites.

Hybrid root-tip cells exhibiting 2 SAT chromosomes instead of 3 reflects nucleolar dominance in interspecific hybrids of barley which is consistent with earlier reports (Lange and Jochemsen, 1976; Subrahmanyam and Azad, 1978b; Jessop and Subrahmanyam, 1984).

Bulbosum rDNA exhibiting variation in repeat length in different accessions with *Eco RI* and *Hind III* reflects non homogenization of their variation at individual NOR since *bulbosum* is self incompatible: in the absence of any selection such variation may be maintained. Present investigation revealed 2 rDNA repeat lengths contrary to one reported earlier (Molnar *et al.*, 1989). In *bulbosum* (B2122) which is considered as autotetraploid, 3 different rDNA repeat lengths were evident. *Vulgare* is self pollinating and maintains homogeneity at individual loci, whereas *bulbosum* has evolved self incompatibility promoting / facilitating variation. Homogenization of any deleterious effect(s) may ultimately lead to the extinction of the species

Self pollination of synthetic *Brassica* polyploids showed extensive rearrangements in the genome including gain and / or loss of restriction fragments. The fragments that were not present in F_2 appeared in F_5 which were parent specific (Song *et al.*, 1995). These changes could have arisen due to genetic instabilities including chromosome rearrangements, point mutation, gene conversion, DNA methylation and others yet to be detected. DNA methylation could also contribute to genomic changes. Rapid genomic changes could accelerate evolutionary process among progenies of newly found polyploids (Song *et al*, 1995) Genome reorganization can occur quickly in polyploids and hybrids (Parkonny *et al.*, 1994). Polyploids exhibit gene silencing eventually leading to extensively diploidized polyploid genomes at functional level, gene diversification resulting in regulatory or functional divergence of duplicated genes, (Soltis and Soltis, 1993,1995).

Restriction modification of prokaryotes acting as evolutionary vesteiges in eukaryotes operating as defense mechanism was opined (Bestor, 1990; Dorfler, 1991). Methylation leading to gene silencing in eukaryotes functions similar to that of restriction-modification in prokaryotes was drawn (Matzke and Matzke, 1996; Dawson, 1996). Recombination enzymes in eukaryotes were functional equivalent of prokaryotic re-

striction enzymes has been proposed (Kasuno *et al.*, 1995). Restriction-modification is not reported in eukaryotes owing to its larger genome size and other technical difficulties in assaying these enzymes. Hybrids displayed differential methylation at rDNA loci. Sequences inadequately protected may be lost as a consequence of restriction. Other changes also may be occurring which are not having any phenotypic effect or severe effect leading to loss of such phenotypes. DNA methyl transferase from plants was shown to methylate cytosine moities in CpG and CpXpG sequences *in vitro* (Jeddeloh and Richards, 1996). Such methylation may lead to protection from endonucleases activity thereby leading to stability. Plant genomes exhibit various proportions of modified bases in their genomes. DNA methylation in plants has been implicated in several processes in addition to gene regulation, inhibition of genome rearrangements by inactivating transposable elements and repetitive sequences and protects genome integrity (Foss *et ah,* 1993; Fedoroff, 1996; Freedman and Pukkila, 1993).

A set of hybrids (VBIII and VBIV) exhibited *de novo* methylation. *De novo* methylation may reflect the ability of the genome defense mechanism (Bestor, 1990; Doerfler, 1991; Finnegan and McEiroy, 1994; Yoder *et al.*, 1997). It may play an important role in intragenomic parasites such as transposable elements and repeated sequences thereby protecting the integrity of the genome (Yoder *et a!.*, 1997).

DNA hypomethylation of transposable elements , which are known to incorporate differential DNA methylation as an epigenetic regulation mechanism, leads to abnormal expression (Fedoroff, 1996). *De novo* methylation appears to be a slow process that targets specific genes for silencing and can provide selective advantage for cell growth in culture or tumour growth in animals. Genome imprinting has also been proposed to serve as a surveillance mechanism for chromosome loss and cancer preven tion. Imprinting is related to fortuitous extension of methylation responses reflecting

host defense mechanisms against invading of foreign DNA (Barlow 1993; Yoder *et al.*, 1997). Host defense against invading foreign DNA might be another selective force (Barlow 1993; Yoder *et al*, 1997).

Conclusions

Bulbosum specific fragments are present among interspecific hybrid(s) / derivatives

Loss of *vulgare* specific r-DNA repeat and *vulgare* chromosome in some of the hybrid(s) / derivatives

Presence of high molecular size rDNA repeat *of vulgare* NOR 6

Differential amplification / methylation of r-DNA repeats among hybrid(s) / derivaties

Symmetrical and asymmetrical modifications of *vulgare* r-DNA repeats occur (possibly *via* integration or recombination of *bulbosum* r-DNA)

Hybrid specific *de novo* methylation

Presence of two rDNA repeats in *bulbosum* is indicative of maintenance of heterozygosity at NOR locus for the two parental gametic rDNA repeat lengths since *bulbosum* genome has a single NOR

3.2 Repeat specific amplification and methylation at rDNA loci in barley addition lines and segmental aneuploids

Restriction fragment length variation at specific *Rrn* loci was studied in a set of seven barley addition lines along with the background parent (*Triticum aestivum* var *Chinese spring)* and the donor (*Hordeum vulgare var Betzes)* of the addition chromosomes. Seven different homozygous duplication lines (Fig. 9) with the background genotype as diploid control were chosen to assess the effect of differing segments and to identify specific segment(s) within the "12-16" region which modulates rDNA amounts and/or methylation.

Southern blots of restriction digests of DNA from different aneuploids hybridized with a barley rDNA probe (pHV 294) are presented in (Figs. 10, 11, 12). A 9.5 Kb *Eco RI* fragment was common to wheat (lane 1) and the addition lines 1H, 2H, 3H, 4H, 5H, 6H and 7H (lanes 2-8), and extra 9.9 Kb fragment in 6H (lane 7) and 9 Kb fragment in 5H (lane 6) while the barley parent displayed 9.9 Kb and 9 Kb fragments representing NOR 6 *(Rrn* 1) and NOR 7 *(Rrn 2)* respectively. Duplications D31, 32, 35 and 36 showed high molecular size (> 20 Kb) rDNA fraction while D33, 34 and D8 had the monomer (9.9 and 9 Kb) equivalents of rDNA repeats as in their diploid control.

Xba I digests gave high molecular weight (30 and 20 Kb) rDNA fraction in duplications D32, D35, D36 in addition to the monomeric components which were common to the other duplications.

Eco RV generated monomeric (9.5 Kb rDNA repeat) fraction in wheat (Fig. 10, lane 1) and addition lines (Fig. 10, lanes 2-8). Barley (lane 9) displayed 7,6.1 and

2.9 Kb fragments of wliich 6.1 and 2.9 Kb fragments were common to the addition line

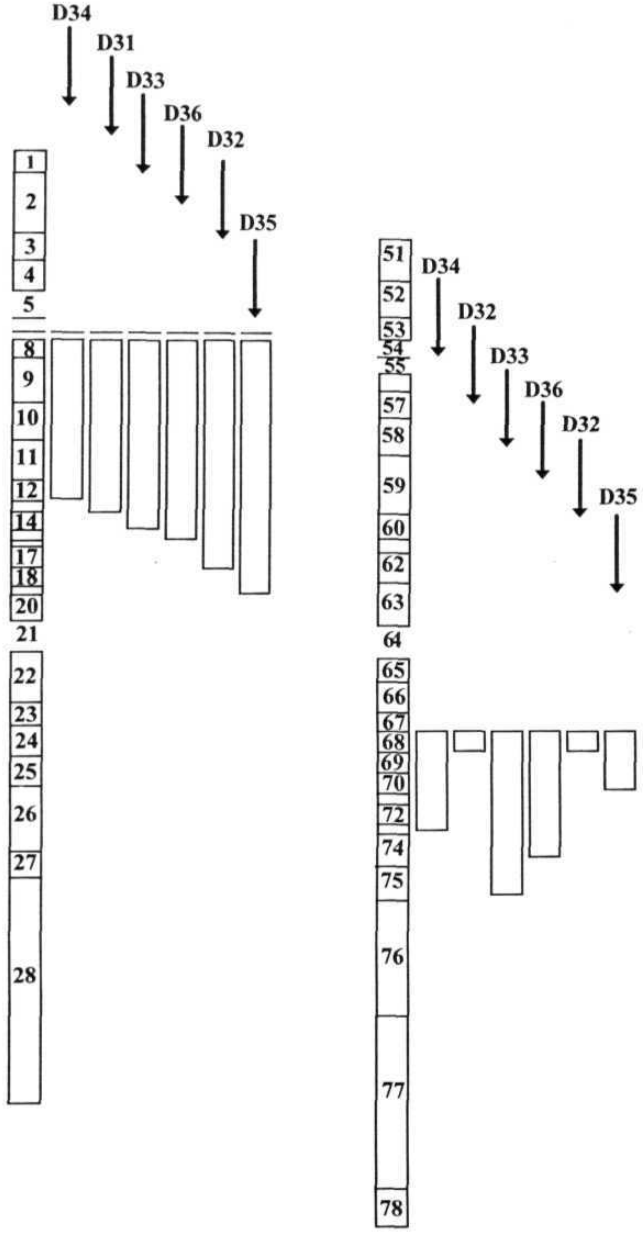


Figure 9. Idiogram of chromosomes 6 and 7 of standard karyotype and segmental duplications limited to chromosome arms involved in the segmental duplications (12-19).

5H while 7 and 2.9 Kb fragments were common to 6H. All the duplications showed three fragments as in the diploid control (lane 17).

Sac I digest gave a common 3.9 Kb fragment in all addition lines and segmental aneuploids as in their background genotypes. Wheat and the addition lines displayed a faint 5.6 Kb fragment (Fig. 10 lanes 1-8). Addition line 5H had an additional 5.1 Kb fragment while 6H had an additional 6 Kb fragment both of which are present in their donor genotype (lane 9). All the duplications and their background genotype possessed 6 Kb and 5.1 Kb *Sac* I fragments. Duplications D31, D32, D35, D36 and D8 displayed monomeric equivalents of the 9.9 and 9.0 Kb fragments.

Bgl II digests displayed a common 1.4 Kb fragment in all the addition lines and segmental aneuploids. Furthermore 9.5 and 8.1 Kb fragments were exclusive to addition lines (Fig. 11 lanes 2-8) and their background wheat (Fig. 11 lane 1). Addition line 5H (lane 6) contained 9 and 7.6 Kb fragments whereas 6H had 9.9 and 8.5 Kb fragments (lane 7). Segmental aneuploids and their diploid control (lane 10 - 17) displayed 9.9. 9.0, 8.5 and 7.6 Kb fragments. Duplications D31, D32, D35, D36 & D8 in addition had high molecular weight fractions.

Isoschizomers *Mbo* I and *Sau* 3 AI profiles revealed differences in the GATC bordered fragments within each rDNA repeat unit and between different rDNA loci. A 1.4 Kb *Mbo* I fragment was common to wheat and addition lines only whereas 2.8 Kb and 1.9 Kb *Mbo* I fragments were common to all segmental aneuploids and their diploid controls (Fig. 11 lanes 9 -17). Addition line 5H had 1.9 Kb fragment (lane 6, NOR 7 specific)) whereas 6H had 2.8 Kb fragment (lane 7). *Sau* 3 *AI* digests of DNA from addition lines and wheat did not differ from *Mbo* I digests. Significantly all segmental aneuploids had additional 3.2 and 2.2 Kb *Sau* 3 *A* I fragments (lanes 10 - 17) as in their diploid controls (lane 17). *Sau* 3 *Al* also generated a common 1.4 Kb fragment similar to

Figure 10. Southern blot hybridization of genomic DNA from barley addition lines and segmental aneuploids digested with *Eco R*\ / *Xba* I / *Eco RV* or *Sac* I and probed with barley rDNAclone (pHV294).

Lanes 1) CS; 2) 1HS; 3) 2H; 4) 3H; 5) 4H; 6) 5H; 7) 6H; 8) 7H; M)* *X Hind* III; 9) Betzes; 10)D31; 11)D32; 12)D33; 13)D34; 14)D35; 15) D36; 16) D8; 17) Bonus

*Please note the shift of marker lane in *Eco RV* and *Sac* I digests.

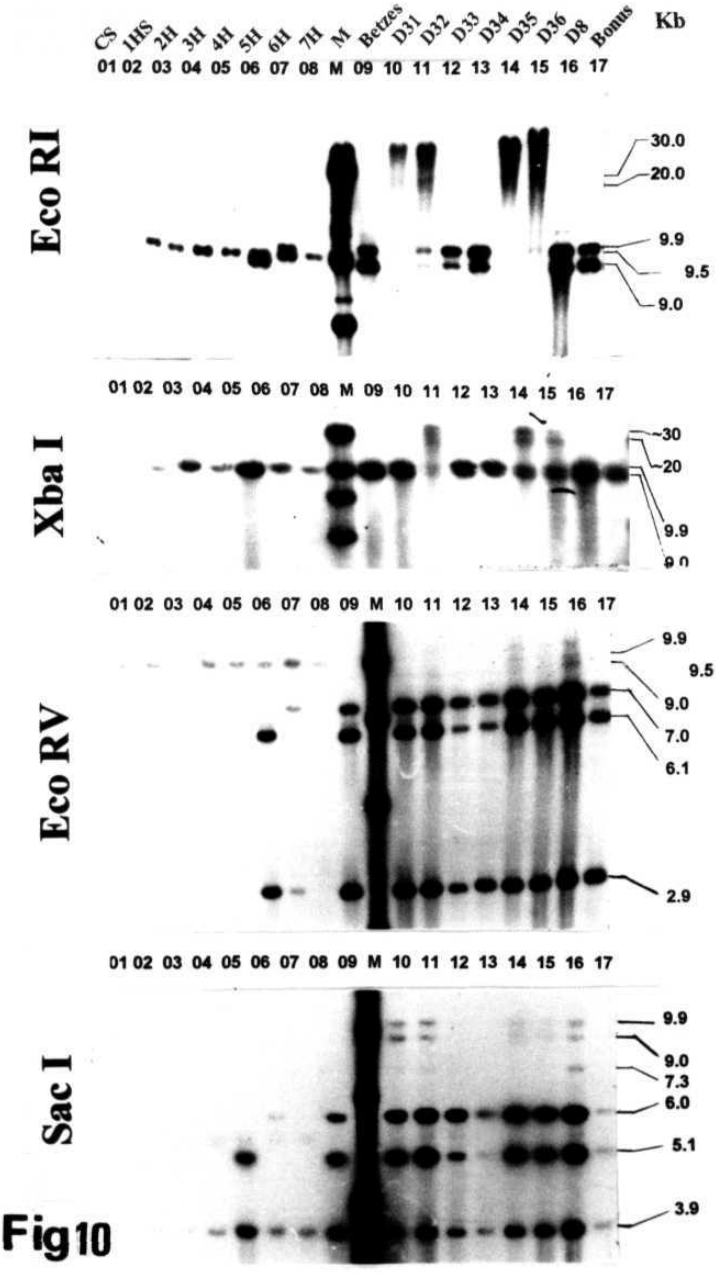

CS 1HS 2H 3H 4H 5H 6H 7H M Betzes D31 D32 D33 D34 D35 D36 D8 Bonus Kb
01 02 03 04 05 06 07 08 M 09 10 11 12 13 14 15 16 17
Eco RI
30.0
20.0
9.9
9.5
9.0
Xba I
30
20
9.9
Eco RV
9.9
9.5
9.0
7.0
6.1
2.9
Sac I
9.9
9.0
7.3
6.0
5.1
3.9

Fig10

Figure 11. Southern blot hybridization of genomic DNA from barley addition lines and segmental aneuploids digested with *Bgl* II / *Mbo* I or *Sau* 3 *A*\ probed with barley rDNA clone (pHV294).

Lanes 1) CS; 2) 1HS; 3) 2H; 4) 3H; 5) 4H; 6) 5H; 7) 6H; 8) 7H; M)* *X Hind* III; 9) Betzes; 10) D31; 11) D32; 12) D33; 13)D34; 14)D35;15) D36; 16) D8; 17) Bonus

Please note the shift of the samples D34 (13); D35 (14) and D36 (15) in *Mbo* I and *Sau* 3 *A*\ digests.

*λ *Hind* III and *Eco* RI double digests in *Mbo* I and *Sau* 3 *A*\ Southern blots.

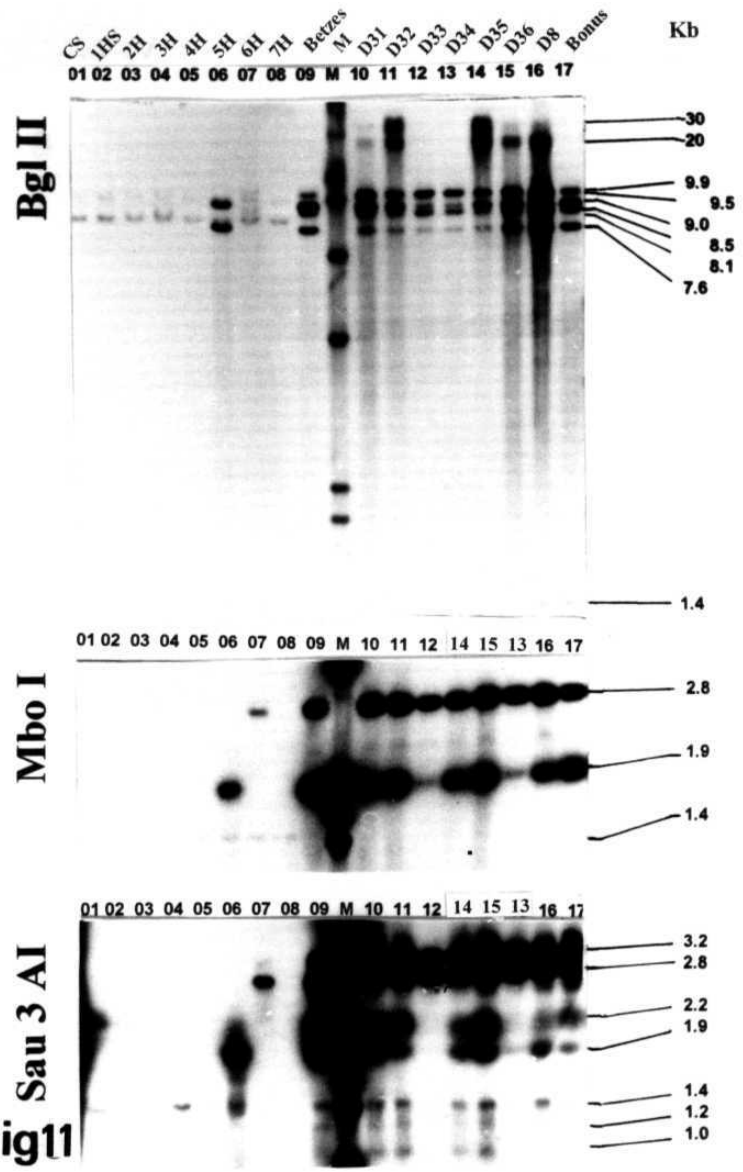

CS
1HS
2H
3H
4H
5H
6H
7H
Betzes
M
D31
D32
D33
D34
D35
D36
D8
Bonus
Kb
01 02 03 04 05 06 07 08 09 M 10 11 12 13 14 15 16 17
Bgl II
30
20
9.9
9.5
9.0
8.5
8.1
7.6
1.4
01 02 03 04 05 06 07 08 09 M 10 11 12 14 15 13 16 17
Mbo I
2.8
1.9
1.4
01 02 03 04 05 06 07 08 09 M 10 11 12 14 15 13 16 17
Sau 3 AI
3.2
2.8
2.2
1.9
1.4
1.2
1.0

Fig11

that of addition lines or *Mbo* I digests of addition lines. Duplications D31, D32, D35, D36 in addition displayed low molecular weight (< 1.2 Kb) *Sau* 3 *A*\ fragments.

Msp I digests did not reveal discernible differences in the rDNA profiles of wheat and five addition lines except in 5H and 6H. Addition line 5H (which carries 9 Kb rDNA repeat unit of barley) had a wide range of rDNA fragments from 4.8 Kb to 0.5 Kb while addition line 6H (carrier of 9.9 Kb repeat unit of barley) had a distinct profile of fragments in the fraction between 2.4 and 4.5 Kb region. Duplications D31, D32, D36 and D 8 (Fig. 12 lanes 10, 11, 15, 16) displayed fragments expected from both repeats as in diploid control (lane 17). Duplications D33 and D34 displayed low proportion of rDNA in the lower fragment size range expected from 9.0 Kb repeat unit.

rDNA repeat unit length in addition lines

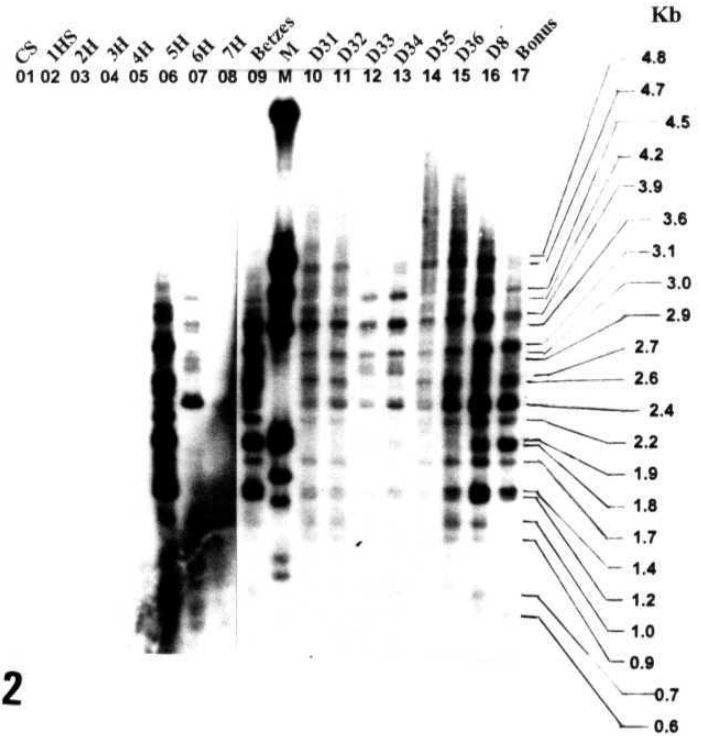
All the seven addition lines displayed wheat specific rDNA repeat unit of 9.5 Kb in *Eco RV* (Fig. 10) indicated no influence of any additional pair of barley chromosomes on rDNA repeat length. Like wise barley *Rrn* loci specific rDNA repeat unit lengths of 9 Kb and 9.9 Kb did not undergo any change (Figs. 10 and 11) in the wheat background. Addition of *Aegilops cylindrica* chromosomes induced deletions and rearrangements in common wheat (Hohmann *et al.* 1995).

Amplification

In the addition line for *Rrn* 1 (6H) the barley specific 9.9 Kb rDNA repeat was only 0.625 times as wheat specific **rDNA** unit (Table 10). The barley specific 9 Kb rDNA repeat unit was 2.1 times as wheat specific rDNA repeat in addition line for *Rrn 2* (5H) (Table 10). From the autoradiograms of *Eco Rl*, *Eco RV* and *Sac* I generated fragments (Fig. 10), signal intensity ratios between the barley rDNA repeat unit specific fragments and the wheat rDNA fragments clearly indicate higher proportion of barley specific 9 Kb rDNA repeats in addition line 5H and a low proportion of barley specific

Figure 12. Southern blot hybridization of genomic DNA from barley addition lines and segmental aneuploids digested with *Msp* I and probed with barley rDNA clone (pHV294).

Lanes 1) CS; 2) 1HS; 3) 2H; 4) 3H; 5) 4H; 6) 5H; 7) 6H; 8) 7H; M) *X Hind* III + *Eco* RI double digests; 9) Betzes; 10) D31; 11) D32; 12) D33; 13) D34; 14) D35; 15) D36; 16) D8; 17) Bonus


CS 1HS 2H 3H 4H 5H 6H 7H Betzes M D31 D32 D33 D34 D35 D36 D8 Bonus
01 02 03 04 05 06 07 08 09 M 10 11 12 13 14 15 16 17
Kb
4.8
4.7
4.5
4.2
3.9
3.6
3.1
3.0
2.9
2.7
2.6
2.4
2.2
1.9
1.8
1.7
1.4
1.2
1.0
0.9
0.7
0.6

Msp I

Fig 12

9.9 Kb repeats of rDNA in 6H. The relative abundance of 9 Kb and 9.9 Kb barley rDNA repeats in the two addition lines 5H and 6H can be estimated as the ratio of the two independent intensity ratios of barley/wheat specific rDNA fragments in the respective addition lines *i.e.* 2.1/0.625 (Table 10) which comes to ~3.3. This is two fold higher than the 9/9.9 Kb ratios in the donor barley. Thus differential amplification for rDNA repeats occurs in alien background also. 6H of barley seems to control the activity of homoeologous 6B though it had less number of copies, 5H and 6H were shown to be active in wheat background (Santos *el al*., 1984). NOR activity of 1R of rye is suppressed by the presence of wheat, *Aegilops ventricosa* and barley SAT chromosomes (Lacadena *et al*. 1984).

Methylation

Isoschizomers *Mbo* I and *Sau* 3 A1 generated the barley specific 2.8 and 1.9 Kb rDNA fragments in addition lines 6H and 5H respectively (Fig. 11) which are derived from 9.9 and 9 Kb repeat. *Sau* 3 *A*1 generated 3.2 and 2.2 Kb fragments as faint bands indicating minor proportion of cytosine methylation in GATC sites (Fig. 11). The extent of methylation in wheat and addition lines 1H, 2H, 3H, 4H and 7H is ~20% as it represented full length repeat unit fraction in *Bgl* II digests. Methylation of wheat rDNA in addition lines 5H and 6H is approximately 40% and 27% respectively (Fig. 11, Table. 11). Our study also showed that barley NOR 7 having more copies than NOR 6. Half of the NOR 7 specific rDNA in addition line 5H belong to the full length monomeric *Bgl* II fragments and the rest as the sub-monomeric fragments (Fig. 11, Table 11) while 70% of the NOR 6 specific rDNA belong to the sub-monomeric *Bgl* II fragments suggesting differential methylation of the two rDNA repeats at their *Bgl* II sites in all addition lines of barley as in wheat and barley.

All addition lines of barley showed 2.4 Kb *Msp* I fragment as in wheat and barley. Additional fragments in high molecular weight fractions in addition to 2.2 Kb

Table 10. Ratio of barley rDNA repeats 9.0 (Kb) / 9.9 (Kb) and proportion in high molecular size restriction fragments.

Eco RI Fragments	CS	1HS	2H	3H	4H	5H	6H	7 H	Betzes	D31	D32	D33	D34	D35	D36	D8	Bonus
9.5 kb	+	+	+	+	+	+	+	+									
9.0/9.5						2.1											
9.9/9.5							0.625										
9.0/9.9									1.6		0.65	0.42↓	0.52↓			0.9	1.4
HMW %										71.0	74.0			84.0	78.0		

HMW) 30.0Kb+20.0Kb / 30.0Kb+20.0Kb+9.9Kb+9.0Kb

Xba I Fragments	CS	1HS	2H	3H	4H	5H	6H	7 H	Betzes	D31	D32	D33	D34	D35	D36	D8	Bonus
9.5	+	+	+	+	+	+	+	+									
9.0,9.9Kb									+	+	+	+	+	+	+	+	+
HMW %											70.0			68.0	47.0*		

HMW) 30.0Kb+20.0Kb / 30.0Kb+20.0Kb+9.9Kb+9.0Kb;*) 20.0Kb / 20.0Kb+99Kb+9.0Kb

Sac I Fragments	CS	1HS	2H	3H	4H	5H	6H	7 H	Betzes	D31	D32	D33	D34	D35	D36	D8	Bonus
5.1/6.0									1.5	1.2	1.1	0.61↓	0.36↓	1.0	1.0	1.0	1.5
HMW %										10.9	9.3			9.0	8.6	8.2	

HMW %) 9.9Kb+9.0Kb / 9.9Kb+9.0Kb+6.0Kb+5.1Kb+3.9Kb; 5.1 - represents 9.0 Kb repeat; 6.0Kb - represents 9.9Kb repeat

Eco RV Fragments	CS	1HS	2H	3H	4H	5H	6H	7 H	Betzes	D31	D32	D33	D34	D35	D36	D8	Bonus
9.5	+	+	+	+	+	+	+	+									
6.1/7.0									1.6	1.0	1.0	0.42↓	0.44↓	1.2	1.2	1.0	1.6

+) Present; 6.1- represents 9.0Kb repeat; 7.0 - represents 9.9Kb repeat

and low molecular weight components in the addition line 5H indicates methylation of internal sites within the rDNA repeat units and the presence of over 50% of barley rDNA in the high molecular weight fraction represents the extent of cytosine methylation at the CCGG sites (Table 12). Higher proportion of 2.4 Kb fraction in 6H compared to the 5H and low percentage of rDNA in high molecular fraction in 6H suggests low level of cytosine methylation in 6H (Table 12).

Role of specific segment(s) in amplification/ de-amplification

Transposition of a specific segment (12-16) from NOR 6 to NOR 7 in barley brings about de-amplification of rDNA at the NOR 7 locus, while duplication of the same segment on both the NOR chromosomes brings about the overall de-amplification of rDNA (Subrahmanyam *et al.*, 1994). The compensation in rRNA genes occurs when an X chromosome carrying wild or partially bobbed (deleted NOR) locus is placed in X/0 males or in females in which the other X chromosome carries a complete deletion for the NOR (Procunier and Tartoff, 1978). In *Drosophila* where males and females differ in number of sex chromosomes the expression of sex linked genes is equalized by dosage compensation (Marin *et aL,* 1996). The segment "12 to 16" has contiguous *cis*-function and its relative position with respect to the two *Rrn* loci determines differential amplification. Deviation in the ratios of 9.0/9.9 Kb rDNA repeats in the duplication D8 of 0.9 from the control Betzes in the present study (Table 10) is consistent with the earlier findings (Subrahmanyam *et aL,* 1994).

Duplications D31 to D36 differ from each other for the segment duplicated. Any differences in the repeat unit / specific rDNA fragments in these duplications are due to the differences in the dosage of the segment(s) duplicated as indicated in Fig 9. D33 and D34 showed higher intensity of 9.9 Kb rDNA repeat/specific fragment in *Eco RV, Sac* I, and *Eco R*\ (Fig. 10).

Table 11. Differential modification of wheat and barley rDNA and ratio of barley rDNA repeats in segmental aneuploids.

Bgl II Fragments	CS	1HS	2H	3H	4H	5H	6H	7H	Betzes	D31	D32	D33	D34	D35	D36	D8	Bonus
I	80.0	80.0	81.0	85.0	81.0	58.0	68.0	81.0									
II										22.6	52.5			56.8	27.0	19.0	
III							73.0		34.0	32.0	37.0	43.0	40.0	30.0	31.0	52.0	43.0
IV						50.0			35.0	35.6	41.0	60.0	62.0	35.0	32.5	53.0	43.5
7.6/8.5 kb									1.7	1.3	1.4	0.5↓	0.8↓	1.6	1.2	0.9	1.4
9.0/9.9 kb									1.7	1.0	1.2	0.5↓	0.5↓	1.4	1.2	0.9	1.5

Relative proportion of rDNA fragments I) 8.1Kb+1.4Kb / 9.5Kb+8.1Kb+1.4Kb
II) 3.0Kb+20.0Kb / 30.0Kb+20.0Kb+9.9Kb+9.0Kb+8.5Kb+7.6Kb+1.4Kb III) 8.5Kb / 9.9Kb+8.5Kb
IV) 7.6Kb / 9.0Kb+7.6Kb; 8.5Kb represents 9.9 Kb; 7.6 Kb represents 9.0 Kb ; I ,II,III & IV presented in %

Mbo I Frag,ements	CS	1HS	2H	3H	4H	5H	6H	7H	Betzes	D31	D32	D33	D35	D36	D34	D8	Bonus
1.9/2.8 kb									1.5	0.9	0.87	0.22↓	0.68	0.84	0.22↓	0.48	1.4

*Sau*3 *A*I Fragments	CS	1HS	2H	3H	4H	5H	6H	7H	Betzes	D31	D32	D33	D35	D36	D34	D8	Bonus
I									55.0	44.0	47.0	61.0	46.5	42.0	58.0	55.0	53.5
II									43.0	37.0	35.0		43.0	39.0		61.0	59.0
19/2.8 kb									0.53	0.33	0.37	0.12	0.38	0.46	0.2	0.28	0.15
2.2/3.2 kb									0.9	0.43	0.48		0.44	0.53		0.19	0.3
III									0.7	0.4	0.4	0.16↓	0.4	0.46	0.11↓	0.24	0.24

3.2Kb and 2.8Kb represents 9.9Kb rDNA repeat; 2.2Kb and 1.9Kb represent 9.0Kb rDNA repeat;
I & II are in %; I;) 2.8Kb / 3.2Kb+2.8Kb: II) 1.9Kb / 2.2Kb+1.9Kb; III) 2.2Kb+1.9Kb(9.0Kb) / 3.2Kb+2.8Kb(9.9Kb)

Table 12. Proportion / distribution of different *Msp* I rDNA fragments in barley segmental aneuploids.

Msp I Fragments	CS	1HS	2H	3H	4H	5H	6H	7 H	Betzes	D31	D32	D33	D34	D35	D36	D8	Bonus
5.8 kb														21.0			
5.4 kb										7.1				9.0			
4.8 kb										3.0	3.9			7.8	10.0	8.9	3.8
4.7 kb												5.2	4.8	4.4			
*4.5 kb						14.0				4.6	7.8			4.0	6.9		
#4.2 kb							25.0					17.4	14.1			2.8	3.8
*3.9 kb						11.7				6.6	4.4			3.8	5.3		3.0
3.6 kb							27.0			12.0	13.2	19.3	16.2	6.5	10.0		3.5
3.4 kb																10.6	
3.1 kb						13.0				10.5	12.5	10.5	9.0	3.3	8.1	7.3	11.0
#2.9 kb							11.8					14.5	14.7			4.0	
*2.7 kb						14.0				10.1	11.1			3.5	10.6	7.3	8.4
#2.4 kb	+	+	+	+	+	+	36.4	+	+	9.5	11.0	22.5	18.1	5.8	10.0	8.1	11.9
2.2 kb						6.0				11.6	10.0			3.6	10.0	5.2	7.0
*1.9 kb						10.0						5.0	4.0			7.2	23.0
*1.7 kb						4.0				11.0	11.8			10.0	8.5	5.1	7.6
1.4 kb						15.0				12.0	13.6	5.5	14.5	3.5	13.0	11.4	12.8
1.2 kb						9.3				4.8	5.3		4.3	3.5	4.4	11.8	5.1
1.0 kb						2.0										4.0	

+) Present; *) 9.0 (Kb) specific fragments; #) 9.9 (Kb) specific fragments

The presence of more 9.9 Kb repeats in D33 and D34 (Fig. 10 lanes 12,13) and near identical copy number of 9.9 and 9.0 Kb repeats/specific fragments in other duplications (D31, D32, D35, D36 Fig. 10 and Table 10) indicate differential amplification of the two rDNA repeats, the extent of which is determined by the dosage / composition of the critical segments (Fig. 9, Tables 11 & 13).

A comparison of the ratios of 9.0/9.9 Kb rDNA repeat unit specific fragments among the duplications suggested amplification of 9.9 Kb and de-amplification of 9 Kb rDNA repeats (Fig. 10, Table 10). The intensity of 9.9 Kb was higher in D34 and D33, whereas D31 showed similar amounts in both 9.9 and 9.0 Kb repeat fractions *(Eco RV, Sac* I, Fig. 10, *Bgl* II, Fig. 11 and Tables 11 and 12). This suggests that duplication of segments 12 and 14 as in D34 and D33 (see Fig. 9) were involved in de-amplification of 9.0 Kb rDNA repeat while segment 13 may have compensatory or opposite effect to that of segment 12 and / or 14.

Methylation

High molecular weight rDNA fraction in *Eco R*\ digests, *Bgl* II generated high molecular weight (multimeric) fraction and *Sac* I generated full length monomeric (9.9 and 9.0 Kb) fraction in addition to sub-monomeric fragments in duplications D31, D32, D35 and D36 indicate that a portion of rDNA sequences in these duplications undergo methylation at *Eco R*\, *Bgl* II and *Sac* I sites. High molecular size rDNA fractions generated by *Eco R*\, *Bgl* II and *Sac* I and its absence in *Xba* I digests of D31 reflects differences in the site specific adenine and/or cytosine methylations. The absence of rDNA in the high molecular range in *Xba* I digests is an indication of lack of cytosine methylation in " AGATCT" sequences.

Duplications D31, D32, D35 and D36 had over 70 % of their rDNA in high molecular weight fractions following *Eco R*\ digests (Table 10) while *Xba* I generated

Table 13. Hybridization levels of the 9.9 and 9.0 Kb rDNA repeats in discrete segmental duplication lines.

	Segments duplicated		*EcoR* I				*Xba* I				*EcoR* V			*Sac* I					*Bgl* II							*Mbo* I		*Sau 3 A*I			
	(6)	(7)	G*A*ATT*C				T*CTAG*A				GAT*ATC			GAG*CTC					AGAT*CT							G*ATC		GAT*C			
			30	20	9.9	9.0	30	20	9.9	9.0	7.0	6.1	2.9	9.9	9.0	6.0	5.1	3.9	30	20	9.9	9.0	8.5	7.6	1.4	2.8	1.9	3.2	2.8	2.2	1.9
D31	7-13	68-69	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	++	++	+	+	+	+	+	+	+	++	+
D32	7-17	68	++	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+	++	++	+	+	+	+	+	+	+	++	+
D33	7-14	68-75			++	+			+	+	++	+	+			++	+	+			++	+	+	+	+	++	+	+	+	+	+
D34	7-12	68-73			++	+			+	+	++	+	+			++	+	+			++	+	+	+	+	++	+	+	+	+	++
D35	7-19	68-71	++	+			+	+	+	+	+	+	+	+	+	+	+	+	++	+	++	++	+	+	+	+	+	+	+	+	+
D36	7-15	68-74	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+		+	++	++	+	+	+	+	+	+	+	++	+
D8	12-16				++	+			+	+	+	+	+	+	+	+	+	+		+	++	++	+	+	+	+	+	+	+	+	++
Retzes	(Control 2x)				+	++			+	+	+	++	+			+	++	+			+	++	++	+	+	+	++	+	+	+	+

(6) - chromosome 6 (6H) specific segments , (7) - chromosome 7 (5H) specific segments

47-70% of rDNA as multimers (20 -30 Kb) in duplications D32, D35 and D36. *Sac* I generated up to 10% of rDNA as monomers of 9.9 and 9.0 Kb in D31, D32, D35, D36 and D8. These differences in the proportion of rDNA in the high molecular fractions generated by different restriction enzymes clearly demonstrates the site specific non-random methylation in the rDNA sequences among the set of duplications.

Differential methylation

Among *Bgl* II generated rDNA fractions in the diploid controls (Fig. 11, lanes 9 and 17), the proportion of the two full length monomers and the corresponding sub-monomeric fragments (Fig. 11, Table 11), there were no discernible differences. Among the duplications (D31, D32, D35, D36 and D8) which displayed marginal differences in the ratios of full length repeats. The ratio of sub-monomeric fraction of 9.9 Kb repeat was far less compared to that of 9 Kb repeat. However, the proportion of rDNA in the multimeric (high molecular weight) fraction was variable among the five duplications D31, D32, D35, D36 and D8 (Fig. 11, lanes 10, 11, 14, 15, 16& Table. 11 &13).

The 7.6 Kb fraction was 30 to 40% in D31, D32, D35 and D36 and approximately 60% in D33 and D34. This indicates that 20% more of 7.6 Kb fraction in D33 and D34 compared to duplications D31, D32, D35 and D36. *Mbo* I is sensitive to adenine methylation whereas *Sau* 3 *A*\ is sensitive to cytosine methylation. Generation of 3.2 Kb and 2.2 Kb GATC bordered fragments represent cytosine methylation in the sequence (internal sites) within the rDNA repeats, and smaller fragments in *Sau* 3 *A*1 indicates methylation of adenine residues in the site.

Differences in the ratios of 2.8/3.2 Kb and 1.9/2.2 among the duplications is a reflection of differences in the of cytosine methylation at the individual NORs (3.2 Kb fragment of 9.9 Kb and 2.2 Kb in 9.0 Kb repeat). 2.8/3.2 indicated higher proportion in

D33 and D34, whereas other duplications D31, D32, D35 and D36 showed narrow variation, suggesting that less methylation in D33 and D34. Comparison of the ratios of 1.9/2.8 Kb fragments generated by *Mbo* I and *Sau* 3A1 in each duplication revealed substantial differences in methylation of adenine or cytosine at the GATC sites at the *Rrn* loci (Tables 11& 13)

Segment-specific methylations

Proportion of each *Msp* I fragment represents the extent of methylation of the internal cytosine of the sites in the rDNA repeats. The sum of the different fragment sizes exceeds the total length of a specific rDNA repeat (in the addition lines 5H and 6H), it is conceivable that the differential methylation of CCGG sites within each rDNA repeat leads to variation in the proportion of discrete fragments in different rDNA repeats (Fig. 12, Table 12). The intensity of each band depends on the distribution of non-methylated sites bordering the fragments in question. Variation in the proportion of different fragments originating from each monomeric repeats among the duplications reflect segment-specific methylation (Table 12). The low levels of *Msp* I sub-monomeric fragments representing 9 Kb rDNA repeat in the duplications D33 and D34, indicate high levels of cytosine methylation of specific repeat representing "CpG" islands. This may be related to the regulation of expression among the rDNA loci.

The presence of sub-monomeric 4.2 Kb and 2.9 Kb fragments in D33, D34 and their absence in D31, D32, D35, and D36 indicate differences in the methylation levels of CCGG sites bordering the NOR 6 specific sub-monomeric fragments giving rise to high molecular weight fragments in the duplications D31, D32, D35 and D36 (Table 12).

Symmetrical and asymmetrical Methylation

CpG and CpXpG sequences in eukaryotic DNA are considered as symmetrical, and CpA and CpT are asymmetrical, and methylation of cytosine in these sequences are

called as symmetrical and asymmetrical respectively. *Sau* 3 *A*\ generated 3.2 (9.9) and 2.2 (9.0) Kb rDNA specific fragments that were not visible in *Mbo* I digests and deviation of 7.6 Kb / 8.5 Kb ratio (Table 11) suggests extensive methylation of the asymmetrical sites in all the segmental duplications and control. Deviation of 1.9/2.8 Kb ratio from *Mbo* I to *Sau* 3 *A*\ indicates the extent of methylation in these duplications. *Msp* I generated discrete bands in all the duplications, the bands that were absent in a particular duplication and visible in others suggests that methylation / demethylation of external cytosine in the site of *Msp* I (Fig. 12 & Table 12). The discrete bands suggests that the sites are distributed at regular spacing as it generated at regular periodicity as was reported in animals and plants (Antequera and Bird 1993).

Role of specific segments in methylation

Duplication of segments 13, 15 and 19 showed increasing amounts of rDNA in high molecular weight range suggesting duplication of specific segments increases the proportion of sites methylated. Duplication of segments 12-13 as in D31, 12-15 as in D36, 12- 19 as in D35, increase the number of methylated *Eco RI* sites in the rDNA. However, duplication of segment 13 did not show any difference at the of *Xba* I sites in D31 (Fig 10, Table 10) Duplication of segments 15, 17, 19 generated ~20 Kb rDNA (dimeric) fraction in D32, D35, D36 while duplication of segments 17 and 19 generated ~30 Kb rDNA (trimeric) fraction in addition to ~20 Kb fraction in D32 and D35 at the *Xba* I site (Fig. 10 & Tables 10 & 13).

Duplication of segments 12, 13, 14, 15, 16, 17 and 19 generated 9.9 and 9.0 Kb rDNA repeats in addition to repeat specific fragments in *Bgl* II digests, while segments 13,15,16,17 and 19 duplication resulted in ~20 Kb fraction indicating the influence of the duplicated segments on methylation at specific sites (Fig. 11, Table 11), whereas duplication of segments 17 and 19 generated trimeric (~30 Kb) fraction in addition to

dimeric (~20 Kb), full length repeat and repeat specific fragments, suggesting that duplication of segments 17 and 19 results in higher proportion of rDNA methylated and role of these segments in (Fig. 11 & Tables 11 & 13) differential methylation as evident from the differences in the *Mbo* I and *Sau* 3 *A*\ fragments. Segments 12 and 14 did not influence the extent of methylation though 9.9 Kb fraction increased when these specific segments are duplicated. Duplication of segments 13,15, 17 and 19 exhibited locus-specific segment modifications, where as duplication of segments 12 and 14 resulted in extensive methylation in few sites as indicated by fewer number of high intensity bands as in D34 and D33 (Fig. 12, Table 12)

Differential amplification of genes at different *Rrn* loci and altered methylation pattern in rDNA of structurally altered karyotypes of barley have an important bearing on the role of karyotypic changes in speciation in general, since differential amplification of different genes leads to increase and/or decrease in specific sequences and altered methylation patterns may lead to differential expression of specific genes, as evident in wheat (Flavell *et al*. 1988, Thompson and Flavell 1988) maize (Jupe and Zimmer 1993) and *Pisum* (Watson *et al*, 1987).

Conclusions

Differential amplification appear to be repeat length (1GS) specific

Ectopic homologous sequences seems to affect resident sequences in a dosage / size dependent manner as in Wheat(5H and 6B are affected but 5H affected more severely)

Symmetrical modifications were limited to addition lines (5H and 6H)

Specific segments (12 and 14) control differential amplification of rDNA, although the mechanism is not clear

Position and dose of specific segment(s) influence differential amplification and " segment - specific" methylation

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