MOLECULAR CHARACTERIZATION OF THE RIBULOSE BISPHOSPHATE CARBOXYLASE/OXYGENASE MULTIGENE FAMILY IN HEXAPLOID WHEAT

A Dissertation

by

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ABSTRACT

Molecular Characterization of the Ribulose Bisphosphate Carboxylase/Oxygenase Multigene Family in Hexapolid Wheat.

(May 1992)

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Ribulose bisphosphate carboxylase/oxygenase (rubisco, EC 4.1.1.39) catalyzes the first step in the Calvin pentose phosphate cycle. It is composed of eight large subunits and eight small subunits. The large subunits are encoded by chloroplast genes (*rbc*L) and the small subunits are encoded by a nuclear multigene family (*rbc*S). In this research, the number of *rbc*S genes in hexaploid wheat *Triticum aestivum* var. Chinese Spring was estimated, their chromosome-arm locations were determined, two major gene subfamilies were characterized and evolutionary relationships among the family members were described. The gene locations were determined by Southern blot hybridizations of DNA extracted from aneuploid derivatives of wheat variety Chinese Spring. Several Southern blot hybridization experiments were performed in which a 530 bp piece of DNA from exon II of a *rbc*S gene from wheat (inserted into the pTaySs 3.2) was used as a probe to determine the chromosome locations of the *rbc*S genes. The results indicate that *rbc*S genes are located in the long arms of the homoeologous group 5 chromosomes (5AL, 5BL and 5DL) and

in the short arms of the homoeologous group 2 chromosomes (2AS, 2BS and 2DS). The *rbc*S gene family members in hexaploid wheat were divided into more divergent gene subfamilies by using two different 3' end specific probes, designated 3' end probe I and 3' end probe II, in Southern blot hybridization analysis of hexaploid wheat aneuploids. It was found that the *rbc*S multigene family consists of at least two divergent gene subfamilies. One of them is a small subfamily, *rbc*S-1, consisting of about three copies per polyhaploid genome. The members of this subfamily are located in 5AL, 5BL and 5DL. The second subfamily, *rbc*S-2, is large, consisting of about nine copies per polyhaploid genome. The members of this subfamily are distributed between the chromosomes of homoeologous groups 2 and 5. Because of the fact that these two subfamilies do not include every *rbc*S gene, it is suggested that there exists at least one other subfamily that has not yet been identified. The number of *rbc*S gene copies at different stringency conditions was estimated by genomic reconstruction analysis and by calculating the numbers and sizes of the DNA fragments produced from Southern blot hybridization experiments. The estimated number of *rbc*S genes in hexaploid wheat is 16-18 per polyhaploid genome.

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I. INTRODUCTION

The cultivated wheats *Triticum aestivum* ($2n = 6x = 42$, genomes A, B and D) and *Triticum turgidum* ($2n = 4x = 28$, genomes A and B) are the most important source of carbohydrate in the majority of countries in the temperate zone (Leonard and Martin, 1963) and they provide above 20% of the food calories consumed by the people of the world (Reitz, 1967). Also, they rank first among the food grains consumed directly by humans and their production leads all crops, including rice, maize and potatoes (Briggle and Curtis, 1987). Moreover, a significant amount of wheat is used for animal feed (Briggle and Curtis, 1987). For the reasons mentioned above and others, it is important for plant breeders and geneticists to study the organization and function of the genetic material of wheat with the aim of improving the productivity of this important crop.

One of the most important enzymes in any green plant, including wheat, is ribulose bisphosphate carboxylase/oxygenase (rubisco, EC 4.1.1.39), the most abundant enzyme on earth. Rubisco is a bifunctional enzyme that catalyzes the primary reaction of carbon dioxide fixation in chloroplasts, the first step in the Calvin pentose-phosphate cycle (Jensen and Bahr, 1977).

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The basic reaction is as follows:

CO_2 + D-ribulose1,5-bisphosphate $\xrightarrow{Mg++}$ 2(3-phospho-D-glycerate)

At the same time, the enzyme catalyzes the primary event in photorespiration. In this reaction, oxygen takes the place of carbon dioxide as a substrate. Carbon dioxide and oxygen, therefore, compete for the same active site on the enzyme; the rates of the two reactions depend on the relative concentrations of the two gases. The Km of the activated enzyme with carbon dioxide as the substrate is about 20 uM, whereas with oxygen as the substrate the Km is about 200 uM. Carbon dioxide is the preferred substrate. In short, rubisco catalyzes the primary step in photorespiration and it also catalyzes the primary step in photosynthetic CO_2 fixation. When the enzyme is involved in photorespiration or oxygenation, the rate of photosynthesis or CO_2 fixation is substantially decreased (Lorimer, 1981). Consequently, acquiring the ability to manipulate rubisco genes may lead in the future to the ability to manipulate the steps in CO_2 fixation which in turn may lead to increases in crop productivity.

In most photosynthetic organisms, including wheat, the rubisco holoenzyme is a hetero-16-mer composed of eight identical large subunits, each with a molecular weight of 50-60 kilodaltons, and eight small subunits, each with a molecular weight of 12-20 kilodaltons (McIntosh *et al.*, 1980). The large subunits are encoded by a single chloroplast gene (Coen *et al.*,

1977) and are synthesized inside the organelle (Blair and Ellis, 1973), whereas the small subunits are encoded by a nuclear multigene family (Kawashima and Wildman, 1972) and are synthesized on free cytoplasmic ribosomes in the form of a precursor containing a transient peptide (Schmidt and Mishkind, 1986). The small subunit precursors are then transported into the chloroplast where they are processed to the mature form (Mishkind *et al.,* 1985).

There is conflicting evidence regarding the degree of importance of the amount of rubisco enzyme in limiting the rate of photosynthesis (Foyer *et al.*, 1982). But the genetic manipulation of major crops, including wheat, to increase the amount of rubisco enzyme in leaves and to improve the efficiency at which CO_2 competes with O_2 for reaction with the enzyme to improve the rate of photosynthesis are major goals of agricultural research. The location of the genes encoding for the rubisco enzyme is of fundamental importance for its genetic manipulation. The chloroplast gene coding for the large subunit for wheat rubisco has already been cloned and analyzed (Bowman *et al.*, 1981). Localization and characterization of the nuclear genes encoding wheat small subunit would lay the background for molecular genetic manipulation of this enzyme in the future.

The central focus of this study is the genetic and molecular characterization of the small subunit multigene family of hexaploid wheat. The specific objectives are to: (1) determine the approximate number of members of the rubisco multigene family;

(2) characterize rubisco gene subfamilies at the molecular level; (3) determine chromosome and chromosome-arm locations of rubisco gene subfamilies; and (4) infer possible evolutionary relationships among rubisco multigene family members. The rubisco small subunit genes continue to serve as a model for study of the nuclear gene families that are common in plant genomes. Research into their organization, structure, evolution and expression should play an important role in elucidating the basic issues of plant biology (Caroline *et al.*, 1989).

II. LITERATURE REVIEW

Rubisco is the major protein in the stroma of chloroplasts, comprising more than 16% of the protein of plants. It is the major soluble protein in plants and is the most abundant protein in nature (Ellis, 1979). In most photosynthetic organisms, rubisco is a hetero-16-mer, composed of eight identical large subunits, each with a molecular weight of 50-60 kilodaltons, and eight small subunits (SSU), each with a molecular weight of 12-20 kilodaltons (McIntosh *et al.*, 1980). Considerable evidence has accumulated to establish that the large subunit contains the binding sites for the sugarphosphate, carbon dioxide and a metal ion along with the catalytic site (Miziorko and Lorimer, 1983). The large subunit is catalytically active in the absence of the small subunit; the role of the small subunit in catalysis is unknown (Miziorko and Lorimer, 1983). The large subunits are encoded by a single chloroplast gene, designated *rbc*L, which is present as a single copy per chloroplast DNA molecule in chloroplasts. It has been mapped and sequenced in the chloroplast genome of several plants (McIntosh *et al.*, 1980, Zurawski *et al.*, 1981, Shinozaki and Sugiura, 1982, Whitfeld and Bottomley, 1983, Sasaki *et al.*, 1985 and Dyer, 1985).

In contrast to the large subunit, the small subunit is encoded by a nuclear gene family, the members of which are designated *rbc*S. A small subunit mRNA corresponding to *rbc*S is translated in the cytoplasm into a precursor protein containing an amino-terminal extension, named the transit peptide, that mediates the

transport of the polypeptide into the chloroplast and which is cleaved off during or shortly after transport (Schmidt and Mishkind, 1986). Mishkind *et al.* (1985) have shown that this process occurs in two steps. The mature small subunit polypeptide then assembles in the chloroplast stroma with the large subunit polypeptide to form the holoenzyme (Berry *et. al.*, 1990). Other evidence (Ostrem *et al.*, 1989) suggests that the processing of the small subunit involves a single proteolytic cleavage, the specificity of which is determined by a conserved region at the carboxyl-terminal end of the transit peptide, and that the processing occurs during or immediately following transit across the chloroplast envelope. While the large subunit bears the sites for activation and catalysis, the role of the small subunit is not fully understood despite its requirement for full enzyme activity.

In all species studied to date, all of the *rbc*S genes have demonstrated common characteristics: they are regulated by light (Gallagher *et al.*, 1985), exhibit a classic phytochrome-linked response (Fluhr and Chua, 1986c, in pea, and Berry-Lowe and Meagher, 1985, in soybean) and are expressed in an organ-specific fashion, being most abundant in biosynthetic organs, such as leaves and pericarps (Coruzzi *et al.*, 1984; Nagy *et al.*, 1985; Fluhr *et al.*, 1986b).

The regulation of the small subunit nuclear genes has been studied in great detail. Fluhr *et al.* (1986a) discovered a 280 base pair (bp) long enhancer-like element upstream of *rbc*S-3A (a member of the pea *rbc*S gene family) which regulated light

induction of the gene, its phytochrome response, and its leaf specificity in transgenic plants. They also suggested that the enhancer-like element contained one or two light-response elements. Aoyagi *et al.* (1988) demonstrated that this *rbc*S-3A enhancer-like element possessed all of the necessary DNA sequences for expression in the correct cell types in transgenic tobacco.

Analysis of the promoter region of the pea *rbc*S-3A gene identified a 58 bp sequence (containing conserved sequence elements, termed boxes I, II and III) that contains two regulatory elements that decrease transcription in the dark (Kuhlemeier *et al.*, 1987a). Positive light responsive elements which overlap with these boxes were found by Kuhlemeier *et al.* (1987b) who concluded that additional positive light responsive elements were located both further upstream and downstream in the promoter. A factor that interacts with boxes II and III in the pea *rbc*S-3A promoter has also been identified (Green *et al.*, 1987).

Berry *et al.* (1985, 1986) examined *rbc*S gene expression in amaranth and concluded that the expression of the *rbc*S genes is controlled at both the transcriptional and translational level. Berry *et al.* (1990) further showed that the regulation of expression of these genes in amaranth seedlings is, in part, at the level of translational initiation. Other studies have shown that the *rbc*S genes in soybean and petunia are regulated not only at the transcription level but also on the level of RNA turnover (Shirley and Meagher, 1990; Thompson and Meagher, 1990)

In all dicotyledon and monocotyledon species studied, several copies of *rbc*S have been found in the nuclear genome. This suggests that the small subunit polypeptides are encoded by a multigene family, i. e., are a set of functionally related genes. The evidence for this is reviewed below.

Tumer *et al.* (1986) isolated five *rbc*S genes from petunia and found that each is expressed differentially in leaves. Dean *et al.* (1987) found eight nuclear *rbcS* genes in petunia and divided them into three subfamilies based on the sequence homology among the genes. One subfamily contained six genes, five of which were linked within 22 kilobases of each other. The other two subfamilies each contained a single gene. Dean *et al.* (1987) also found that (i) the amino acid sequence of the transit peptide coding region of the different petunia *rbc*S genes appear to be more diverged than the mature *rbc*S coding region, (ii) the genes of the same subfamily encode the same mature *rbc*S polypeptide and (iii) the coding region of seven of the eight petunia *rbc*S genes is interrupted by two introns. The position of these two introns is conserved in all the higher-plant *rbc*S genes studied to date, although one of the petunia *rbc*S genes contains an additional intron (Dean *et al.*, 1987).

Sugita *et al.* (1987) cloned and sequenced five *rbc*S genes from tomato. Two of these, designated *rbc*S-1 and *rbc*S-2, are present as single genes at individual loci, while the other three genes, designated *rbc*S-3A, *rbc*S-3B and *rbc*S-3C, are organized in a

tandem array within 10 kilobases of one another. The *rbc*S-2 gene contains three introns; whereas all of the other members of the tomato *rbc*S gene family contain two introns in the same positions as the first and second introns in the petunia *rbc*S genes. The additional intron in the *rbc*S-2 gene is located in the same position as the third intron of the petunia *rbc*S gene. The coding sequence of *rbc*S-1 differs by 14% from that of *rbc*S-2 and by 13.3% from that of *rbc*S-3. The exon and intron sequences of *rbc*S-3A are identical to those of *rbc*S-3C, and differ by 1.9% from those of *rbc*S-3B. Nucleotide sequence analysis suggests that the five *rbc*S genes encode four different precursors and three different mature polypeptides. The nucleotide sequences 5' and 3' to the coding region of the five tomato *rbc*S genes shows some nucleotide-sequence homology, but far less than that seen in petunia genes.

Polans *et al.* (1985) determined that the entire *rbc*S multigene family in the pea consists of at least five members, is clustered on chromosome five within a region of less than four map units and segregates as a single Mendelian unit. They suggested that the tight linkage between these genes may reflect relatively recent duplication events that have not undergone rearrangement. Fluhr *et al.* (1986b) concluded that the number of individual *rbc*S genes in pea cannot be much greater than five. They compared the nucleotide sequences of these five genes and determined that the genes could not be divided into subfamilies based on nucleotide sequence homology. They also found that there was little nucleotide sequence divergence between the coding regions of the

five *rbc*S genes and that the five genes encode identical mature small subunits. Finally, all of the pea *rbc*S genes have two introns, located at the same positions as those in the petunia and tomato (Polan *et al.*, 1985; Cashmore, 1983; Coruzzi *et al.*, 1984; Fluhr *et al.,* 1986b)

Berry-Lowe *et al.* (1982) isolated one copy of the soybean multigene family, designated SRS1, and determined its nucleotide sequence. Grandbastien *et al.* (1986) found that the soybean multigene family contained more than six members. They isolated a second member of the family, designated SRS4, from a genomic library, determined its sequence, and compared it to the sequence of SRS1. Both genes had two introns located at the same position as in other dicotyledons plants. Grandbastien *et al.* (1986) also found that the organization of the three exons encoding the SRS4 precursor protein is identical to most other small subunit genes in dicots. SRS1 and SRS4 appear to be very closely related: the three exons are 93%-96% homologous in nucleotide sequence, the polypeptide sequences in the three exons encoding the 178 amino acid precursor polypeptide are nearly identical, the two introns are about 75% homologous and the flanking regions are more than 85% homologous. Grandbastien *et al.* (1986) proposed that the SRS1 and SRS4 genes may be alloalleles or homoeologous alleles. Finally, the expression patterns of SRS1 and SRS4 are very similar (Shirley *et al.,* 1990)

Pinck *et al.* (1986), on the basis of the Southern analysis, suggested that there were at least seven *rbc*S genes in the nuclear

DNA of *Nicotiana sylvestris* (one of the progenitors of the cultivated tobacco, *Nicotiana tabacum*). Poulsen *et al.* (1986) isolated an *rbc*S gene (*rbc*S-8B) from *Nicotiana plumbaginifolia*, a diploid species, and determined its complete nucleotide sequence. The structure of *rbc*S-8B is remarkably similar to that of NtSS23, one of the two unlinked *rbc*S genes of *N. tabacum* characterized by Mazur and Chui (1985). Both *rbc*S genes contain three introns located in identical positions with respect to amino acid coding regions. O'Neal *et al.* (1987) isolated three additional *rbc*S genes, designated TSSU3-1, TSSU3-2 and TSSU3-8, from *N. tabacum* and sequenced TSSU3-2 and TSSU3-8. These three genes are not closely linked. One gene, TSSU3-8, contains sequences which are highly homologous to the NtSS23, suggesting that NtSS23 and TSSU3-8 represent genes whose similarities arose from gene duplication and/or gene conversion.

Wolter *et al.* (1988) isolated and characterized five *rbc*S genes from potato (*Solanum tuberosum*). One of these was isolated as a cDNA clone, whereas the other four were isolated in two genomic clones, representing two different loci (designated 1 and 2) containing one and three genes, respectively. The three genes at locus 2 are arranged in tandem array in a 10-kb region; whereas the single gene, locus 1, is separated from locus 2 by at least 12 kb. The introns and exons of the three genes of locus 2 are highly conserved with respect to size and position. The two introns are located in the same relative position as in other dicots. The gene at locus 1 contains three introns that occur at the same place as do

the three introns in the *rbc*S gene of petunia and tomato. Comparison of the transit peptide encoded by the potato cDNA and its homologue in tomato disclosed only one amino acid difference. This may suggest an important and specific function for these particular polypeptides (Wolter *et al.,* 1988).

Krebbers *et al.* (1988) found that the *rbc*S multigene family of the weed (*Arabidobsis thaliana*) consists of four genes divided into two subfamilies. Subfamily B consists of three genes that reside in tandem in an 8 kb stretch of the same chromosome; subfamily A contains only one gene which is completely unlinked to genes of subfamily B. The first three genes are greater than 95% homologous in DNA sequence and encode polypeptides that are identical in length and nearly identical in amino acid sequence. The genes of subfamily B are more similar to each other in nucleotide sequence than any are to the single gene of subfamily A. All four genes are interrupted by two introns whose placement within the coding region of the genes is conserved and similar to that observed in other dicots. The introns of the genes of subfamily B are similar in length and nucleotide sequence, but show no similarity to the introns of the A gene. Comparison of the DNA sequence within the immediate 5' and 3' flanking sequences among the four genes revealed only limited regions of homology. All four genes are expressed.

Hutchison *et al.* (1990) obtained one genomic clone from the larch (*Larix laricina*) which contained the *rbc*S coding region plus upstream and downstream regions. The gene contained two introns.

The deduced amino acid sequence of the product of this gene was similar to rubicso proteins from other plants and to the proteins from monocots.

Silverthorne *et al.* (1990) studied rbcS genes in the monocot duckweed (*Lemna gibba*) and estimated that the family contained 12-14 members. Six of the genes were isolated and five were partially sequenced. The transit peptide nucleotide sequences and the five sequenced genes were conserved, but less so than the mature polypeptide coding sequence. Each of the *rbcS* genes examined contained only one intron. The position of the single intron corresponded to the position of the second intron in dicots. The intron sequences among the five genes differed in length and sequence.

One genomic *rbc*S clone and three distinct *rbc*S cDNA clones have been isolated from maize (*Zea mays*) The transcripts of these three genes account for approximately of 90% of the *rbc*S mRNA in maize leaves (Sheen and Bogorad, 1986). Lebrun *et. al.* (1987) isolated and sequenced a genomic clone, and discovered that the coding region was interrupted by an unique intron of 163 bp.

Jellings *et. al.* (1983) determined the amounts of rubisco per cell in aneuploids of the wheat cultivar Chinese Spring. They also identified homoeologous chromosome group 4 as the site of major control of rubisco levels. Broglie *et al.* (1983) isolated and sequenced a cDNA clone containing a full length *rbc*S wheat gene, designated pW9. They also observed more than ten fragments in

Southern blots analysis of hexaploid wheat nuclear DNA using pW9 as a probe. These fragments ranged in size from approximately two to 23 kilobases and each fragment was of sufficient size to contain the entire small subunit gene. Smith *et al.* (1983) isolated and sequenced three wheat cDNA clones that contained *rbc*S genes and concluded that the clones were probably derived from three different mRNAs. This supports the suggestion of Broglie *et al.* (1983) that the *rbc*S small subunit in wheat is encoded by a multigene family. Murray *et al.* (1984) isolated five other genomic *rbc*S clones in wheat and sequenced the coding region of one of them. They found a high degree of homology between this clone and the clones isolated previously by Broglie *et al.* (1983) and Smith *et al.* (1983). Chao *et. al.* (1989) found a very complex pattern of 30-40 bands when genomic DNA from hexaploid wheat was digested with *Hin*dIII and probed with the *rbc*S cDNA clone isolated by Broglie *et al.* (1983). However, when genomic DNA from wheat-barley addition lines were probed, roughly 12 *rbc*S genes were detected per haploid genome. The various rbcS genes appeared to be located in the chromosomes of at least two different chromosome groups. When Chao *et al.* (1989) compared the *rbc*S fragment patterns in barley with those in the wheat-barley addition lines, they concluded that there were at least four *rbc*S genes in each of the group 2 chromosomes and at least one in each of the group 7 chromosomes, leaving roughly six *rbc*S genes unaccounted for. Based on examination of group 2 aneuploids of Chinese Spring, Chao *et al.* (1989) were also able to locate *rbc*S fragments to the short arms of the group 2 chromosomes.

In a recent study, Galili *et al.* (1991) digested total genomic DNA from nullisomic-tetrasomic and ditelosomic derivatives of the hexaploid cultivar Chinese Spring with *Hin*dIII, which does not cut within the coding sequence of wheat *rbc*S nor within its intron (Galili *et al.*, 1991). They probed the DNA with a cDNA *rbc*S clone obtained from Smith *et al.* (1983) and inferred the chromosomal locations of *rbc*S genes based on the presence or absence of bands and on changes in the relative intensities of bands. The hybridization pattern consisted of 14 bands, containing at least 21 different types of DNA fragments which were allocated to two homoeologous chromosome groups. Fifteen fragments were assigned to the shorts arm of the group 2 chromosomes (four to 2AS, seven to 2BS and four to 2DS) and six fragments were assigned to the long arms of the group 5 chromosomes (two each to arms 5AL, 5BL and 5DL). Galili *et al.* (1991) concluded that the *rbc*S genes of common wheat are arranged in each genome in at least two clusters: a large cluster in the short arms of the chromosomes of group 2 (*rbc*S-2A, *rbc*S-2B and *rbc*S-2D) and a smaller cluster in the long arms of the chromosomes of group 5 (*rbc*S-5A, *rbc*S-5B and *rbc*S-5D).

There are several other species in which *rbc*S cDNA clones have been partially characterized. These include spinach (Tittgen *et al.*, 1986), cucumber (Greenland *et al.*, 1987), *Silense pratensis* (Smeekens *et al.*, 1986), *Amaranthus hypochondriacus* (Berry *et al.*, 1985), *Flaveria trinervia* (Adams *et al.*, 1987), *Helianthus annuus* (Waksman and Freyssinet, 1987), *Sinapis alba* (Oelmuller *et al.*,

1986) and rice (Matsuoka *et al.*, 1988).

Studies on the nucleotide sequence divergence of *rbc*S genes have indicated that the mechanism of concerted evolution may have homogenized *rbc*S sequence in some species (Pichersky *et al.*, 1986; Dean *et al.*, 1987; O'Neal *et al.*, 1987). Meagher *et al.* (1989) examined nucleotide sequences encoding the mature portion of 31 *rbc*S genes from 17 genera of plants, green algae and cyanobacteria, and suggested that gene conversion was the primary mechanism for *rbc*S gene sequence homogenization within species.

III. MATERIALS AND METHODS

1) Materials

Triticum aestivum cv. Chinese Spring and aneuploid derivatives thereof, including compensating nullisomic-tetrasomic and ditelosomic lines, were used as plant materials in all experiments. *Lophopyrum elongatum* is a diploid Triticeae species ($2n$ = 14 genome E). The following *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* substitution and additional lines were also used: 1E, 2E(2A), 2E(2B), 2E(2D), 3E(3D), 4E, 5E(5A), 5E(5B), 5E(5D), 6E and 7E. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines.

Five *rbc*S genomic clones in Charon 32 phage (and one plasmid subclone) obtained from Dr. Michael G. Murray (Senior Research Scientist at Agrigenetics in Madison, Wisconsin) served as the *rbc*S probe source. Dr. Murray and his group isolated these five clones from a genomic DNA library of *T. aestivum* cv. Yamhill (personal communication). The pTaySs 3.2 subclone is a 3.2 kilobase *Bam* H1 fragment.

2) Methods

a) Probe preparation

Phage DNA was extracted from pTaYSs7.3, one of the five clones isolated by M. G. Murray, using the method of Maniatis *et al.* (1982). An *Eco*R1/*Bam*H1 genomic fragment (which includes the coding region, 3' end and 5' end of the *rbcS* gene) was subcloned into a Bluescript plasmid (pTaY*rbcS* 5.8) and a restriction map was determined (Figure 1). The plasmid pTaYSs3.2, supplied by Dr. Murray, contains the coding region of the gene, the 3' flanking region, and the 5' flanking region (Figure 1). The DNA of both plasmids was extracted using the methods of Maniatis *et al.* (1982). The DNA was then digested according to the manufacturer's instructions (i) with *Sal*I to cut out exon II of the gene in the pTaYSs 3.2 clone, (ii) with *Sal*I and *Bam*HI to cut out the 3' end of the gene in the pTaYSs 3.2 clone and (iii) with *Dra*I and *Eco*RI to cut out the 3' end of the gene in pTaY*rbc*S 5.8. The restricted DNA was electrophoresed in 1% agarose gels and three fragments were extracted. One fragment of 1 kb was from pTaY*rbc*S5.8 and included the 3' end of the gene. This fragment (probe) is referred to hereafter as the 3' end probe I. Two fragments were from pTaYSs3.2. The first contained a 530 bp of the second exon of the coding region and is referred to hereafter as the exon probe. The second contained 988 bp, including the 3' end of the gene, and is referred to hereafter as the 3' end probe II. These three fragments (probes) were labelled with P^{32} according to

Feinberg and Vogelsein (1984).

b) Slot blot hybridization

Rubisco gene copy number was determined by slot blot hybridization using a slot blot apparatus. The slots were pretreated by filling each with 400 ul of preboiled 100 ug/ml herring sperm DNA (DNA carrier), waiting for 30 minutes, and then rinsing each slot with 1M ammonium acetate. The slot blot apparatus was assembled with prewetted Gene Screen Plus nylon membrane between the plates. Six different samples were prepared for each experiment as follows:

i) Sample number 1 contained five ug of hexaploid wheat genomic DNA which had been extracted from the leaves of *T. aestivum* cv. Chinese Spring, plus 250 ng of herring sperm DNA as a DNA carrier. DNA extractions from lyophilized leaf tissue were carried out using the Saghai-Maroof *et al.* (1984) modification of the method of Murray and Thompson (1980).

ii) Sample number 2 contained 250 ng of Herring sperm DNA.

iii) Sample number 3 contained a specific amount of linear pTaYSs 3.2 plasmid equal to five copies per polyhaploid genome of a wheat *rbc*S gene, plus 250 ng of herring sperm DNA.

iv - vi) Samples number 4 to number 6 contained linear pTaYSs 3.2 plasmid equal to 10, 20 and 40 copies per polyhaploid genome of a wheat *rbc*S gene, respectively, plus 250 ng of herring sperm DNA.

In one experiment, seven samples were prepared instead of six. These samples were prepared as described above except that samples number 3 to number 7 contained 1, 2, 4, 8 and 16 copies of a wheat *rbc*S gene per polyhaploid genome.

All samples were denatured by adding 1/40 volume of 4M NaOH and boiling for 10 minutes, and then renatured by adding 1/4 volume of 5M ammonium acetate. All samples were applied immediately to the pretreated slots and left overnight. The next day, slots were rinsed with 1M ammonium acetate, the apparatus was disassembled, the nylon membrane was rinsed with 2XSSC, and DNA was crosslinked to the membrane by irradiating the wet membrane at a distance of 8 cm for 2 minutes with a 302 nm wavelength UV device (UV dosage = 7.0 $mW/cm^{2)}$. The membranes were prehybridized overnight in 1% BSA (bovine serum albumin) fraction V, 1mM EDTA, 0.5M $NaHPO_4$ pH 7.2 and 7% SDS (Na dodecyl SO_4), and hybridized the following day in the same solution after the addition of P^{32}-labelled exon probe, hybridization was for 16 hours. Membranes were then washed for 1 hour in 0.5% BSA fraction V, 1mM EDTA, 40mM $NaHPO_4$, pH 7.2 and 5% SDS, washed again for another 1 hour in 1mM EDTA, 40mM $NaHPO_4$, pH 7.2, and 1% SDS, and then exposed to X-ray film for 2 hours at -80 ^oC. The temperatures used for both hybridization and washing were 45, 50, 55, 60, 65 and 70 ^oC. The intensity of each band was measured using a model 620 Bio-Rad video densitometer; intensity differences between slots were compared using IBM 1-D analyst software (Bio-Rad version 2.01).

c) Genomic DNA hybridization

Genomic DNA was extracted from leaves of *T. aestivum* cv. Chinese Spring, from 21 compensating nullisomic-tetrasomic strains chosen so that nullisomy for each chromosome is present once among the lines, and from *T. aestivum* cv. Chinese Spring-*Lophopyrum elongatum* addition and substitution lines. The DNA extractions from lyophilized leaf tissue were carried out using the Saghai-Maroof *et al.* (1984) modification of the method of Murray and Thompson (1980). DNA was completely digested with different restriction enzymes according to the manufacturer's instructions and electrophoresed in 1% agarose gels. HindIII-digested lambda DNA was used as a control to estimate fragment sizes. DNA was blotted onto Zeta Probe nylon membranes (Southern, 1975) and prehybridized in 4X SSPE (20X SSPE stock is composed of 3.6M NaCl, 0.2 M Na_2HPO_4 and 0.02 M EDTA), 4X Denhardt's (100X Denhardt's stock is composed of 2% BSA, 2% polyvinylpyrolidone and 2% Ficoll), 0.5% SDS, and 0.2 mg/ml herring sperm carrier DNA. Blots were hybridized in the same solution following the addition of 10% dextran sulfate and P^{32}-labelled probe. The blots were washed once in 2X SSC (20X SSC stock is composed of 3M NaCl and 0.3 M Na citrate) and 0.5% SDS for 15 minutes at room temperature and twice in 0.1X SSC and 0.1% SDS for 30 minutes at 65°C. The blots were exposed to X-ray film for several days at -80°C.

In each compensating nullisomic-tetrasomic strain, and in the substitution and addition lines, a pair of chromosomes is missing

or added. Consequently, fragments from missing or added chromosomes should be missing or added from specific blots. Accordingly, the presence or absence of *rbc*S fragments was used to assign missing or added fragments to a specific chromosome. The same methods used to locate genes to specific chromosomes using nullisomic strains, substitution lines, and addition lines, were used to locate genes to arm positions using ditelosomic strains of homoelogous groups 2 and 5. The ditelosomic lines of group 2 used were 2AS, 2BS, 2DS and 2DL; and of group 5 were 5AL, 5BL and 5DL.

IV. RESULTS

Members of a gene family can be defined as those DNA segments which retain sufficient nucleotide sequence similarity to be recognizable under a particular set of stringency conditions used in a DNA-DNA hybridization experiment. The strategy used to provide an initial description of the *rbc*S multigene family of hexaploid wheat was essentially based on the principles of restriction fragment length polymorphism (RFLP) analysis.

1) Chromosome and chromosome-arm locations of *rbc*S genes in hexaploid wheat

The nucleotide sequences of the coding regions of gene family members are more similar to each other than are the nucleotide sequences of flanking regions and introns (Literature Review). In order to locate the hexaploid wheat *rbc*S genes to chromosomes and chromosome-arms, several Southern blot hybridizations were performed in which the 530 bp piece of DNA from exon II of the *rbc*S gene from wheat, inserted into pTaySs 3.2 (the exon probe), was used as the radioactive probe. Because of the high specificity of the probe, it was expected that all *rbc*S-related sequences in the hexaploid wheat genome would be detected with similar efficiency under the hybridization conditions. Firstly, the *rbc*S homoeologous group location was detected, secondly the chromosome location was determined and thirdly, the chromosome arm location was identified.

a) Homoeologous group location of *rbc*S genes

The chromosomes of hexaploid wheat and other triticeae species are divided into seven homoeologous groups. Seven addition and substitution lines of *T. aestivum* var. Chinese Spring-*L. elongatum* were chosen so that either a chromosome addition or substitution was present for each group. Chosen were 1E, 4E, 6E and 7E (additions), and 2E(2A), 3E(3D), and 5E(5D) (substitutions). These lines allowed independent examination of each homoeologous chromosome group to determine the homoeologous group location of the *rbc*S multigene family in *L. elongatum.* Genomic DNA from Chinese Spring as well as from the substitution and addition lines was digested with the restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII and *Xba*I. Autoradiograms produced from different Southern blot hybridization experiments were examined to identify the missing or added *rbc*S fragments in each line examined.

The results of a Southern blot hybridization in which DNA from the seven addition and substitution lines was digested with *Bam*HI are shown in Figure 2. The substitution lines 2E(2A) and 5E(5A) each lack two bands, line 2E(2A) has two additional bands and line 5E(5A) has one additional band. An autoradiogram of the same seven lines, but digested with *Bgl*II, is shown in Figure 3. Line 5E(5A) shows one additional band and line 2E(2A) shows two additional bands. Southern blots of the same seven substitution and addition lines digested with *Eco*RI, *Hin*dIII and *Xba*I are shown in Figures 4-6, respectively. As in the digestions with *Bam*HI and

*Bgl*II (Figures 2-3), additional or missing bands were observed only in lines 2E(2A) and 5E(5A).

b) Chromosome location of *rbc*S genes

Each chromosome of hexaploid wheat cv. Chinese Spring compensates for the absence of another chromosome in other genomes in nullisomic-tetrasomic combinations. As examples, chromosome 1D compensates for the absence of 1A and 1B; chromosome 1B compensates for the absence of 1A and 1D; and chromosome 1A compensates for the absence of 1D and 1B. Twenty one compensating nullisomic-tetrasomic strains in which nullisomy for each chromosome is present once among the lines were analyzed. In this way the chromosome location of the *rbc*S genes was determined based on missing bands from Southern blots of these aneuploid lines.

A Southern blot containing DNA from the 21 compensating nullisomic-tetrasomic lines digested with *Dra*I and probed with the exon probe is shown in Figure 7. A single band is absent in nullisomic 2A tetrasomic 2D (N2AT2D), N2BT2D, N2DT2B and N5AT5D, whereas two bands are absent in N5BT5D and N5DT5B. A Southern blot containing DNA from the 21 compensating nullisomic-tetrasomic lines digested with *Sac*I and probed with the exon probe is shown in Figure 8. Two bands are absent in N5BT5D and one band is absent in N5DT5B. A Southern blot was prepared by digesting genomic DNA from the three *T. aestivum* var. Chinese Spring-*L. elongatum* homoeologous group 5 substitution

lines with *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Hin*dIII, *Sac*I and *Xba*I and probing the blot with the exon probe. The resulting autoradiogram (Figure 9) revealed the absence of one or two bands from each of the group 5 substitution lines, regardless of the restriction enzyme used for DNA digestion.

c) Chromosome-arm location of *rbc*S genes in hexaploid wheat

The above results indicated that *rbc*S gene family members were located on homoeologous group 2 and group 5 chromosomes. To further localize the *rbc*S genes, experiments using group 2 and 5 ditelosomic lines were carried out. In ditelosomic lines, one of the two arms of a chromosome is absent. The absence of a particular fragment from a Southern blot of a ditelosomic line implies that the fragment is located in the missing chromosome arm. Genomic DNA from the available ditelosomic lines of group 5, namely lines 5AL, 5BL and 5DL, was digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Sac*I and *Xba*I and probed with the exon probe. The resulting autoradiogram (Figure 10) indicates that all homoeologous group 5 bands are present. Genomic DNA from the available ditelosomic lines of group 2, namely lines 2AS, 2BS, 2DS and 2DL, was digested with *Bam*HI, *Dra*I and *Hin*dIII and probed with the exon probe. The results (data not shown) revealed missing bands only in line 2DL.

The results obtained from the hybridization of different wheat lines with the exon probe are summarized in Table 1. This table shows that the number of fragments hybridized to wheat DNA digested with *Bam*HI is 19, *Bgl*II is 20, *Dra*I is 13, *Eco*RI is 13,

*Hin*dIII is 14, *Sac*I is 14 and *Xba*I is 19. The average number of fragments hybridized to the exon probe is 16. These results indicated that *Dra*I, *Eco*RI, *Hin*dIII and *Sac*I digests produce fewer fragments detected with the exon probe than *Bam*HI, *Bgl*II and *Xba*I digests.

The exon probe experiments can be summarized as follows: genomic DNA from seven addition and substitution lines of Chinese Spring-*L. elongatum*, representing the seven chromosomes of *L. elongatum*, the 21 possible nullisomic-tetrasomic lines and all available groups 2 and 5 ditelosomic lines, were digested with different restriction enzymes and hybridized with the exon probe. The results showed that the *rbc*S gene family members hybridized with the exon probe are locating in 2AS, 2BS, 2DS, 5AL, 5BL and 5DL. Digestion with some enzymes show hybridization with more fragments than other enzymes and the average number of fragments was 16.

2) *Rbc*S gene subfamilies

As noted in the Literature Review, coding sequences of members of every *rbc*S gene family thus far studied are highly conserved, whereas the 3' flanking regions of these members are clearly divergent. Thus, the 3' end regions can be used as gene-specific probes in the analysis of the *rbc*S multigene family in hexaploid wheat. Southern blot hybridizations were performed using two gene-specific probes, derived from 3' ends of two *rbc*S gene clones. Restriction maps of these two probes revealed that

they are different (Figure 1). It was expected that each probe would hybridize to members of different rbcS gene subfamilies, i. e., that the *rbc*S gene family in hexaploid wheat could be divided into different subfamilies by using both 3' end specific probes in Southern hybridizations.

a) *Rbc*S-1 gene subfamily detected by *rbc*S 3' end probe 1

Hybridization results (data not shown) with 3' end probe I demonstrated the occurrence of a small number of *rbc*S genes, designated subfamily *rbc*S-1, which hybridize to the 3' end probe I in hexaploid wheat. The 3' end probe I was then used to determine copy number, homoeologous group location, chromosome location and chromosome arm location of the *rbc*S-1 subfamily members.

i) Estimating copy number and homoeologous group location of the *rbc*S-1 subfamily in hexaploid wheat

DNA of the seven *T. aestivum* var. Chinese Spring-*L. elongatum* addition and substitution lines used previously was digested with *Bam*HI and hybridized with the 3' end probe I. The results, shown in Figure 11, indicate that four fragments hybridized to 3' end probe I and one of these was missing in the 5E(5D) substitution line. Results obtained with the same lines following digestion with *Bgl*II and hybridization to 3' end probe I are shown in Figure 12. Four fragments hybridized to 3' end probe I, and two of these were missing in the 5E(5D) substitution line. Results obtained from the same seven addition and substitution lines digested with *Eco*RI, *Hin*dIII and *Xba*I and hybridized with 3' end probe I are shown in

Figures 13, 14 and 15, respectively. With *Eco*RI digestion, two fragments hybridized to 3' end probe I and one of these was absent from 5E(5D) (Figure 13). Similarly, with *Hin*dIII, two fragments hybridized to 3' end probe I and one of these was absent from the 5E(5D) line (Figure 14). With *Xba*I, three fragments hybridized to 3' end probe I and one of these was absent from the 5E(5D) line (Figure 15).

ii) Chromosome locations of the *rbc*S-1 subfamily in hexaploid wheat

The above results indicated that the *rbc*S genes of Chinese Spring are located in homoeologous groups 2 and 5. DNA from all possible group 2 and 5 chromosomes of Chinese Spring-*L. elongatum* substitution lines, namely, lines 2E(2A), 2E(2B), 2E(2D), 5E(5A), 5E(5B) and 5E(5D), as well as Chinese Spring, was digested with *Bam*HI, *Hin*dIII and *Dra*I and hybridized with 3' end probe I.

With *Bam*HI digestion, 3.0, 3.4, 6.6 and 7.4 kb fragments hybridized to 3' end probe I (Figure 16). Three of the four fragments were absent from group 5 substitution lines: the 7.4 kb fragment was absent in the 5E(5D) line, the 6.6 kb fragment was absent in the 5E(5A) line and the 3.0 kb fragment was absent in the 5E(5D) line. The 3.4 kb fragment was present in all six lines. With *Hin*dIII digestion (Figure 17), 11.3 and 5.1 kb fragments hybridized to 3' end probe I. The 5.1 kb fragment was absent in the 5E(5D) line, whereas the 11.3 kb fragment was present in all six lines. With *Dra*I, 3.3, 4.2 and 14.1 kb fragments hybridized to 3' end probe

I (Figure 18). The 3.3kb fragment was absent in the 5E(5B) line, the 4.2 kb fragment was absent in the 5E(5D) line and the 14.1 fragment was absent in the 5E(5A) line. When *Bgl*II, *Eco*RI and *Xba*I were used in similar Southern blot hybridizations, the average number of the fragments detected was three (data not shown).

iii) Chromosome-arm location of the *rbc*S-1 subfamily

DNA of the 5AL, 5BL and 5DL ditelosomic lines of group 5 was digested with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Sac*I and *Xba*I and probed with 3' end probe I to determine whether the members of the *rbc*S-1 subfamily reside on a single chromosome-arm or all three chromosome-arms of homoeologous group 5. As shown in Figure 19, all of the fragments which hybridized to 3' end probe I are present on all ditelosomics of homoeologous group 5 chromosomes. The results obtained from hybridization of different wheat lines with 3' end probe I are summarized in Table 2. This table shows that the number of fragments hybridized to wheat DNA digested with *Bam*HI is four, *Bgl*II is four, *Dra*I is three, *Eco*RI is two, *Hin*dIII is two and *Xba*I is three. The average number of fragments hybridized to 3' end probe I is three. These results indicated that the *rbc*S-1 subfamily members are located on the long arms of the three chromosomes of homoeologous group 5.

b) *Rbc*S-2 subfamily detected by *rbc*S 3' end probe II

DNA from the seven *T. aestivum* var. Chinese Spring-*L. elongatum* substitution-addition lines used previously was singly digested with *Bam*HI and *Eco*RI and probed with 3' end probe II in order to search for a second *rbc*S gene subfamily. With *Bam*HI, 10 fragments hybridized to 3' end probe II and one of them was absent from the 5E(5D) substitution line (Figure 20). With *Eco*RI, nine fragments hybridized to the 3' end probe II (Figure 21). DNA from the 5E(5A), 5E(5B) and 5E(5D) substitution lines was also digested with *Bam*HI, *Bgl*II, *Hind*III and *Eco*RI and hybridized to the 3' end probe II. The results obtained in these experiments are summarized in Table 3. The number of fragments that hybridized to *Bam*HI digest was 10, *Bgl*II was 10, *Eco*RI was seven and *Hind*III was nine. These results indicate that the *rbc*S-2 is large and distributed among group 2 and 5 chromosomes.

3) Estimation of the number of *rbc*S genes in hexaploid wheat

a) Genomic reconstruction analysis

To estimate the approximate number of *rbc*S genes in hexaploid wheat var. Chinese Spring, blots of (i) samples of DNA containing different known quantities of *rbc*S isolated from a plasmid and (ii) a sample of hexaploid wheat Chinese Spring DNA containing an unknown number of *rbc*S genes, were probed with the exon probe under various conditions of stringency. Five *u*g of Chinese Spring DNA and different amounts of *Hind*III digested plasmid pTaYSs 3.4,

which contains a full-length *rbc*S gene, were present on each blot. In preliminary experiments, blots with a wide range in the number of *rbc*S genes were tested. Subsequent experiments were performed with blots containing a smaller range of *rbc*S genes. The number of known *rbc*S genes per polyhaploid genome was calculated as follows:

$$\text{One gene per polyhaploid genome} = \left(0.53 / (16 \times 10^6)\right) \times 5$$

where (0.53) is the length in kilobases of the probe used, (16×10^6) is the number of DNA kilobases in a polyhaploid of the hexaploid wheat genome (Bennett, 1972) and (5) is the amount in micrograms of the Chinese Spring genomic DNA used in these experiments.

The estimated number of *rbc*S genes depends on the stringency of the hybridization conditions. The estimated copy number decreases under high stringency conditions and the number increases under low stringency conditions.

The slot blot hybridizations were performed at temperatures of 70, 65, 60, 55, 50 and 45 ^{o}C, using a 0.5 M sodium phosphate buffer. Higher stringency conditions represent washing where the temperature of the wash was the same as the hybridization temperatures, but the sodium phosphate concentration was reduced to 0.04 M. The melting temperature of the probe-genomic *rbc*S duplex was determined by the salt concentration in the washing solution, since the stringency conditions of the wash were higher than the stringency conditions of the hybridization.

The melting temperature (Tm) of the probe-genomic *rbc*S duplex was calculated as follows:

Tm = 81.5 + 16.6 x log (sodium concentration) + 0.409 (G+C) - (600/L).

where G+C is the percentage of guanine and cytosine in the probe (48.8% for the exon probe) and L is the length of the probe in base pairs (530 bp for the exon probe). The sodium concentration was 0.04, giving a Tm = 75 ^{o}C.

Autoradiograms of the slot blots obtained under different stringency conditions are shown in Figure 22. The number of *rbc*S genes in hexaploid wheat was estimated by comparing the signal level of slots containing the standards with that of the slots containing wheat genomic DNA. The measured areas of the standards versus the unknown are shown in Table 4. At each stringency condition, the area for the standard was blotted against the standard copy number used. The slope and the Y axis intercept of the standard curve were calculated, and the copy number of the unknown was estimated. The estimated number of *rbc*S gene copies per polyhaploid genome was then plotted against different stringency conditions (Figure 23). As shown in Figure 23, the estimated number of *rbc*S copies per polyhaploid genome increases from zero to ten by decreasing the experiment temperature from 70 ^{o}C (stringency condition of Tm - 5) to 65 ^{o}C (stringency condition of Tm - 10). The estimated copy number increases from 10 to 15 by decreasing the temperature of the experiment from 65

°C (stringency condition of Tm - 10) to 60 °C (stringency condition of Tm - 15). The estimated copy number remains the same when the experiment temperature is decreased from 60 °C (stringency condition of Tm - 15) to 50 °C (stringency condition of Tm - 25). Finally, beyond the point of the optimum temperature (Tm - 25), the estimated number of copies increases sharply.

b) Southern blot hybridization analysis

In order to estimate the number of *rbc*S copies by Southern hybridization analysis, the number and molecular weights of the fragments present in all genomic blots were tabulated. The average number of detectable fragments that hybridized to the exon probe is 16 (Table 1). Comparisons of the hybridization patterns produced by the exon probe with those produced by 3' end probe I and 3' end probe II reveals that several fragments present in all aneuploid lines when the exon probe was used are absent when the two 3' end probes were used. Such bands putatively arise from hybridization of the exon probe with two *rbc*S fragments of the same size and whose structural genes have similar DNA sequences, but whose 3' end flanking regions differ. The fragments identified in this way were the 3.0 kb and 7.4 kb fragments in the *Bam*HI digests, the 2.8 kb, 7.0 and 9.8 kb fragments in the *Bgl*II digests, the 14.1 kb fragment in the *Dra*I digests, the 3.4 kb fragment in the *Eco*RI digests, the 3.5 kb, 5.1 kb and 11.3 kb fragments in the *Hind*III digests, and the 6.4 kb and 7.4 kb fragments in the *Xba*I digests. Taking all of these additional fragments into consideration, the average number of fragments

becomes 18 (Table 4).

V. DISCUSSION

In this research, several fundamental questions concerning the molecular basis and genetics of the *rbc*S multigene family in hexaploid wheat were addressed. These questions include the chromosome and chromosome-arm locations of wheat *rbc*S genes in hexaploid wheat, the approximate number of members of this multigene family and whether the *rbc*S multigene family is comprised of more than one subfamily.

1) Chromosome and chromosome-arm locations of *rbc*S genes in hexaploid wheat

There are various procedures by which genes can be localized to specific chromosomes and chromosome-arms in hexaploid wheat. The procedures of aneuploid analysis are the most accurate of these techniques. In these analyses, observations and measurements are made on variation in characteristics among strains that are aneuploid for each chromosome or chromosome arm and a disomic strain. Fortunately, recent recombinant DNA technology has provided the means to detect genetic variability at the DNA level in any organism. One of the most important and useful of these methods is the Southern blot technique. By hybridizing these Southern blots with a specific probe for a certain gene, a band or a group of bands can be observed on the autoradiograms that are produced. The disomic wheat variety Chinese Spring and its aneuploid derivatives were used as the source of genetic variability for gene localization and the Southern blot hybridization technique

was used as a tool to detect variation in *rbc*S genes. Most of the chromosomal determinations of gene location that have been conducted in hexaploid wheat are based on this variety and its aneuploid derivatives. The genomic DNA was extracted from Chinese Spring as well as from Chinese spring-*L. elongatum* substitution and addition lines, compensating nullisomic-tetrasomic lines and ditelosomic lines. The *rbc*S exon probe was used to detect genetic variability among the lines in order to determine the chromosome and chromosome-arm locations of the *rbc*S genes in hexaploid wheat.

Southern hybridization using *T. aestivum cv. Chinese Spring-L. elongatum* addition and substitution lines demonstrated that, regardless of the restriction enzyme used, homoeologous group 2 and 5 aneuploids show either the addition or absence of *rbc*S fragments. This pattern of hybridization indicated that *rbc*S genes are located in homoeologous group 2 and 5 in *T. aestivum* and *L. elongatum*. Study of compensating nullisomic-tetrasomic lines and group 5 substitution lines disclosed the absence of some *rbc*S fragments from each of the group 2 and group 5 lines. This indicated that *rbc*S genes are located in all three chromosomes of homoeologous group 2 (2A, 2B and 2D) and in all three chromosomes of homoeologous group 5 (5A, 5B and 5D). The study of group 2 and group 5 ditelosomic lines indicated that some *rbc*S genes are located in the long arms of the homoeologous group 5 chromosomes (5AL, 5BL and 5DL) and that other *rbc*S genes are located in the short arms of the homoeologous group 2

chromosomes (2AS, 2BS, and 2DS).

These results are in agreement with those of Galili *et al.* (1991) who concluded that *rbc*S genes of hexaploid wheat are located in the short arms of group 2 chromosomes and the long arms of the group 5 chromosomes. Chao *et al.* (1989) found that some *rbc*S genes are located in the short arms of group 2 chromosomes. However, they also detected an extra *rbc*S fragment in the 7H wheat-barley chromosome addition lines. This discrepancy may be due to the fact that Chao *et al.* (1989) used the PW9 clone of Broglie *et al.* (1983) to probe total genomic DNA. PW9 is a cDNA clone which contains the sequences of the transit peptide of the small subunit. Galili *et al.* used the pTS512 cDNA clone of Smith *et al.* (1983), which contains only the sequences of the mature peptide. In this study, the probe used was a part of exon II which does not contain the sequences of the transit peptide. It is possible that the extra fragment visualized by Chao *et al.* (1989) was due to hybridization between the transit peptide sequences of the PW9 clone and homologous sequences in chromosome 7 of barley. It should be noted that Chao *et al.* (1989) could not confirm the existence of group 7 fragments in wheat using nullisomic-tetrasomic lines.

By quantitative measurements of rubisco, Jelling *et al.* (1983), inferred that genes controlling the quantity of rubisco protein are located in homoeologous group 4. Our results do not contradict these findings since these investigators may have detected regulatory genes that control the level of rubisco in the cell.

to be located in the short arms of chromosomes 2A, 2B, and 2D. The number of *rbc*S genes in this subfamily was approximately nine.

In conclusion, the *rbc*S multigene family consists of at least two divergent gene subfamilies, *rbc*S-1 and *rbc*S-2. Since the three members of the *rbc*S-1 subfamily are located in three chromosomes, then they must comprise one locus in each group 5 chromosome. The number of *rbc*S gene copies in the *rbc*S-2 gene subfamily is approximately nine and they are located in homoeologous groups 2 and 5. These two subfamilies do not include every member of the *rbc*S multigene family, thus another subfamily or subfamilies must exist.

3) Copy number of *rbc*S genes in hexaploid wheat

The number of *rbc*S genes in hexaploid wheat was estimated quantitatively using genomic reconstruction procedures and the exon probe. Experiments conducted at different stringency conditions demonstrated that the estimated number of gene copies increased from ten at high stringency conditions (Tm -10) to 15 at low stringency conditions (Tm - 15). Further lowering the stringency conditions to Tm -25 did not change the estimated number of *rbc*S genes. In other words, over the range of 15 ^{o}C, the *rbc*S gene copy number estimate did not change. However, under stringency conditions of Tm - 30, the estimate of gene copy number increased to 91. Thus, all *rbc*S genes must have some sequence similarities with the conservative exon probe used for hybridization at Tm -15. Since the probe used study was part of

the exon of the *rbc*S gene, it is likely that it hybridizes with all *rbc*S members under stringency condition of Tm - 15 to Tm - 25. At very low stringency conditions (Tm - 30), the probe may hybridize with sequences which are not part of the gene family. These results indicate that the approximate number of *rbc*S genes per haploid genome is 16.

The number of *rbc*S genes in hexaploid wheat was also estimated from analyses of genomic Southern blots. The results of Southern analyses are not as accurate as the results of the genomic reconstruction analysis because of the possibility that restriction enzyme sites exist within *rbc*S genes, giving two bands on Southern blots. In other words, study of Southern blots may overestimate the number of *rbc*S genes. The estimated number presented here is the average of the results obtained from Southern blot experiments using six different restriction enzymes. It is gratifying to note that the Southern blot analyses confirm the results which were obtained by the genomic reconstruction experiments in that they indicate that there are 18 *rbc*S genes per polyhaploid genome. Thus, it can be concluded that the number of *rbc*S genes copies in the hexaploid wheat is approximately 16-18 per polyhaploid genome.

These results differ from those of Chao *et al.* (1989) who estimated that there were about 33 genes per polyhaploid genome in hexaploid wheat. The difference in estimates could be due to the different probes used Chao *et al.*, 1989, used a cDNA probe which contained the transit polypeptide sequences) or to the

hybridization conditions, since changes in stringency will alter estimates of the number of gene copies. Chao *et al.* (1989) also based their estimate of *rbc*S copies on fragments produced from digestions with only one enzyme. This could have resulted in an overestimation of *rbc*S gene number since it is possible that more than one site for the enzyme used by Chao *et al.* occurred in *rbc*S genes.

Galili *et al.* (1991) estimated the minimum number of *rbc*S gene copies, from Southern experiments, in common wheat to be 21 per polyhaploid genome. Dean *et al.* (1989), after surveying most of the published results about the number of *rbc*S genes in higher plants, suggested that the estimated range for a diploid species would be from two to eight or nine. This would be from six to 24 or 27 for a hexaploid species such as wheat.

VI. CONCLUSION

The results of this research indicate that the ribulose bisphosphate carboxylase/oxygenase enzyme of hexaploid wheat is encoded by a multigene family. The estimated number of gene copies in this multigene family is approximately 16-18 per polyhaploid genome. The genes are located in the long arms of the chromosomes of homoelogous group 5 (5AL, 5BL and 5DL) and in the short arms of the chromosomes of homoelogous group 2 (2AL, 2BL and 2DL). The family consists of at least three subfamilies, two of which have been characterized. The first subfamily, located in 5A, 5B and 5D, consists of three members per polyhaploid genome. The second subfamily consists of approximately nine members which are distributed among the chromosomes of groups 2 and 5.

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APPENDIX A

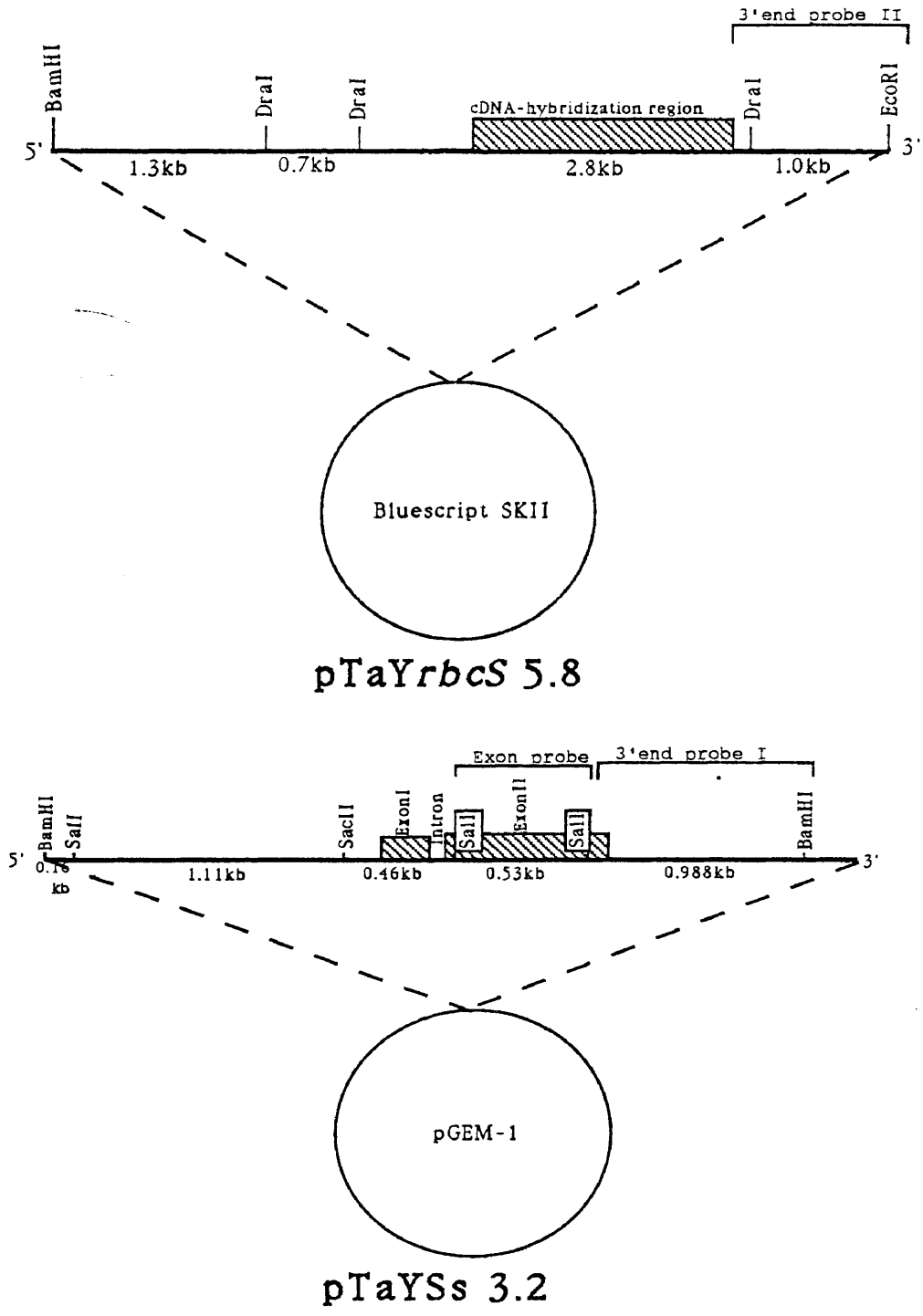


Figure 1. Restriction maps of two different wheat *rbc*S genes. Distances between restriction enzyme sites are in kilobases.

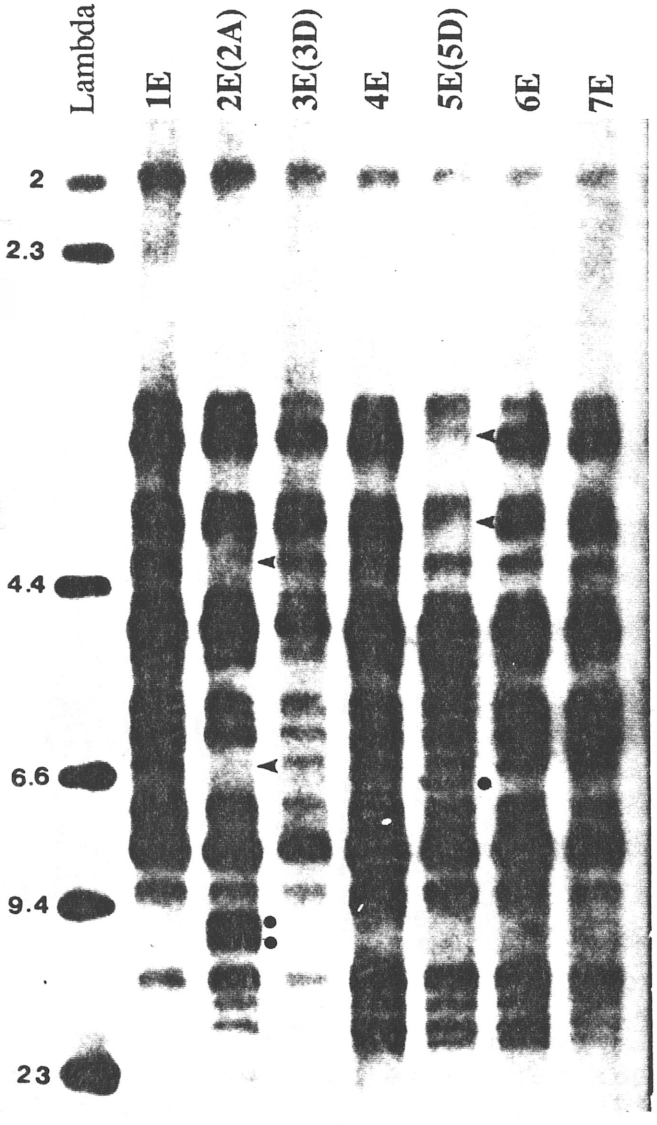


Figure 2. Southern blot of *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* addition and substitution lines. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines. Genomic DNA was digested with *Bam*HI and hybridized with the *rbcS* exon probe. The first lane from the left contains molecular weight markers. The arrows indicate missing bands and the dots indicate added bands.

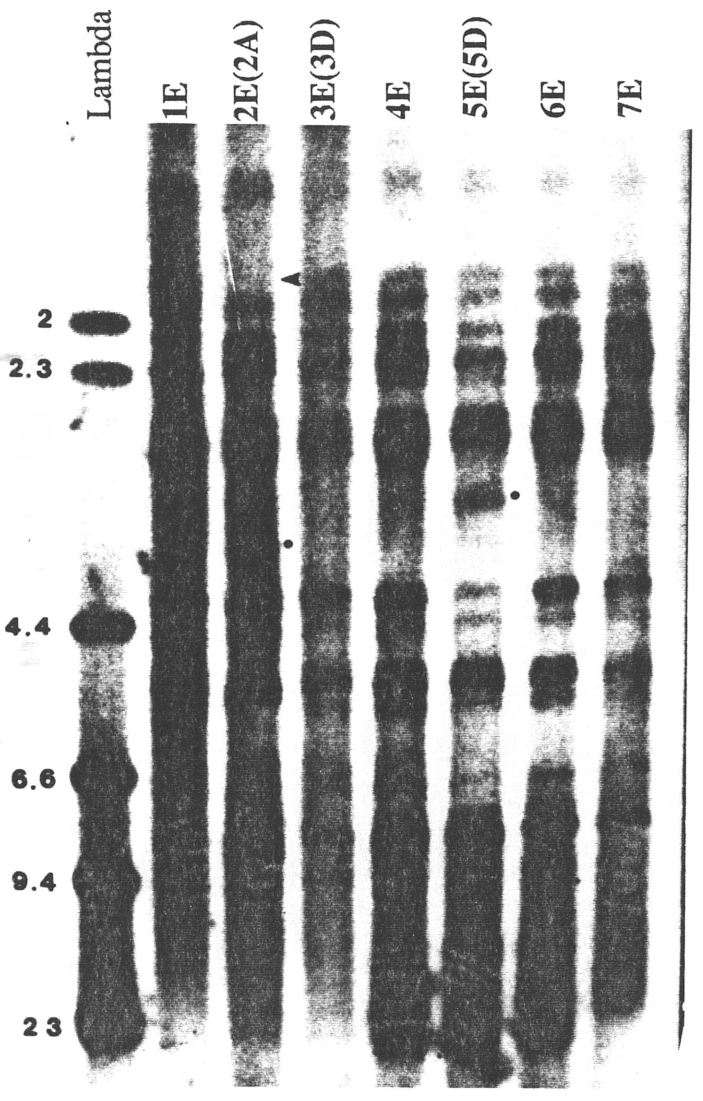


Figure 3. Southern blot of *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* addition and substitution lines. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines. Genomic DNA was digested with *Bgl*II and hybridized with the *exon* probe. The first lane from the left contains molecular weight markers. The arrow indicates a missing band and the dots indicate added bands.

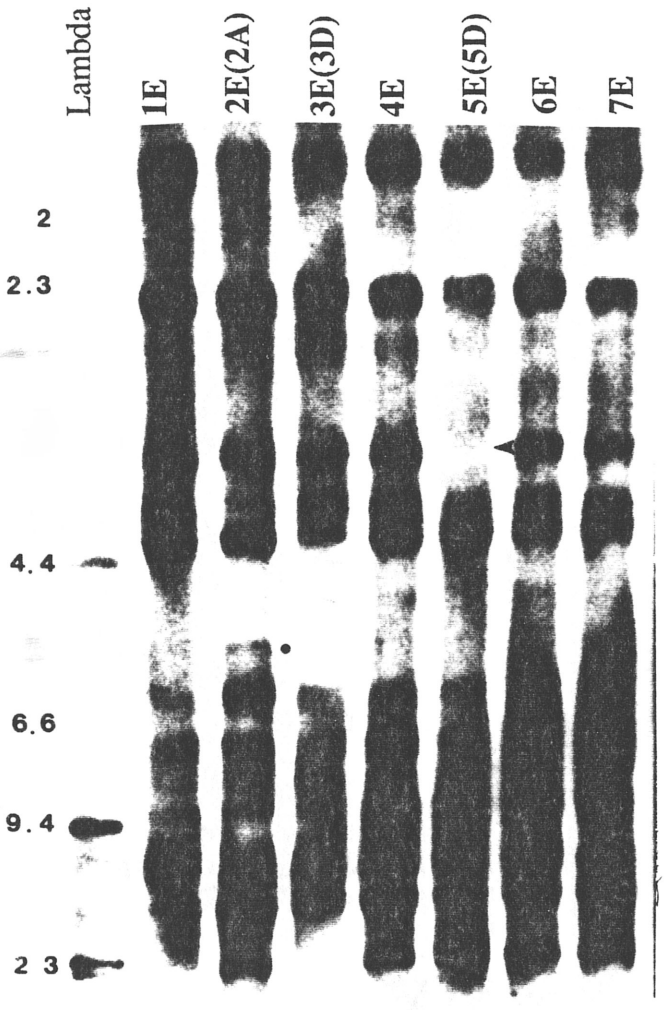


Figure 4. Southern blot of *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* addition and substitution lines. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines. Genomic DNA was digested with *Eco*RI and hybridized with the exon probe. The first lane from the left contains molecular weight markers. The arrow indicates a missing band and the dot indicates an extra band.

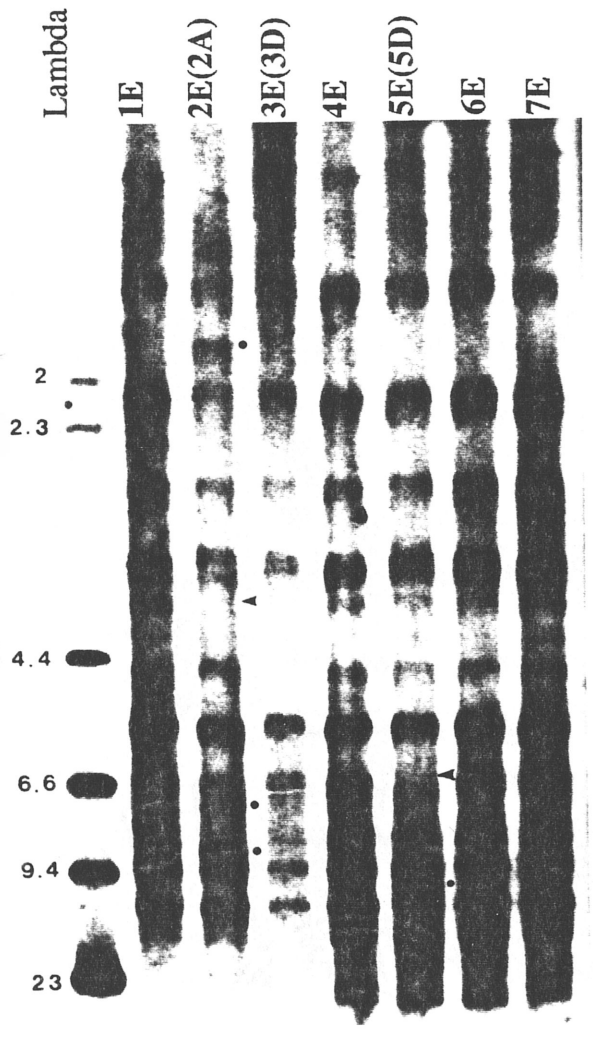


Figure 5. Southern blot of *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* addition and substitution lines. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines. Genomic DNA was digested with *Hind*III and hybridized with the exon probe. The first lane from the left contains molecular weight markers. The arrows indicate missing bands and the dots indicate added bands.

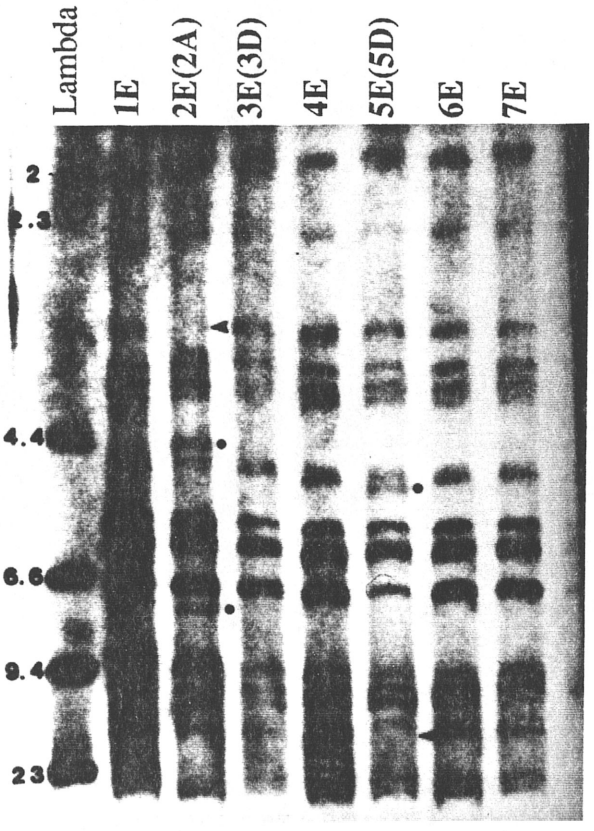


Figure 6. Southern blot of *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* addition and substitution lines. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines. Genomic DNA was digested with *Xba*I and hybridized with the exon probe. The first lane from the left contains molecular weight markers. The arrows indicate missing bands and the dots indicate extra bands.

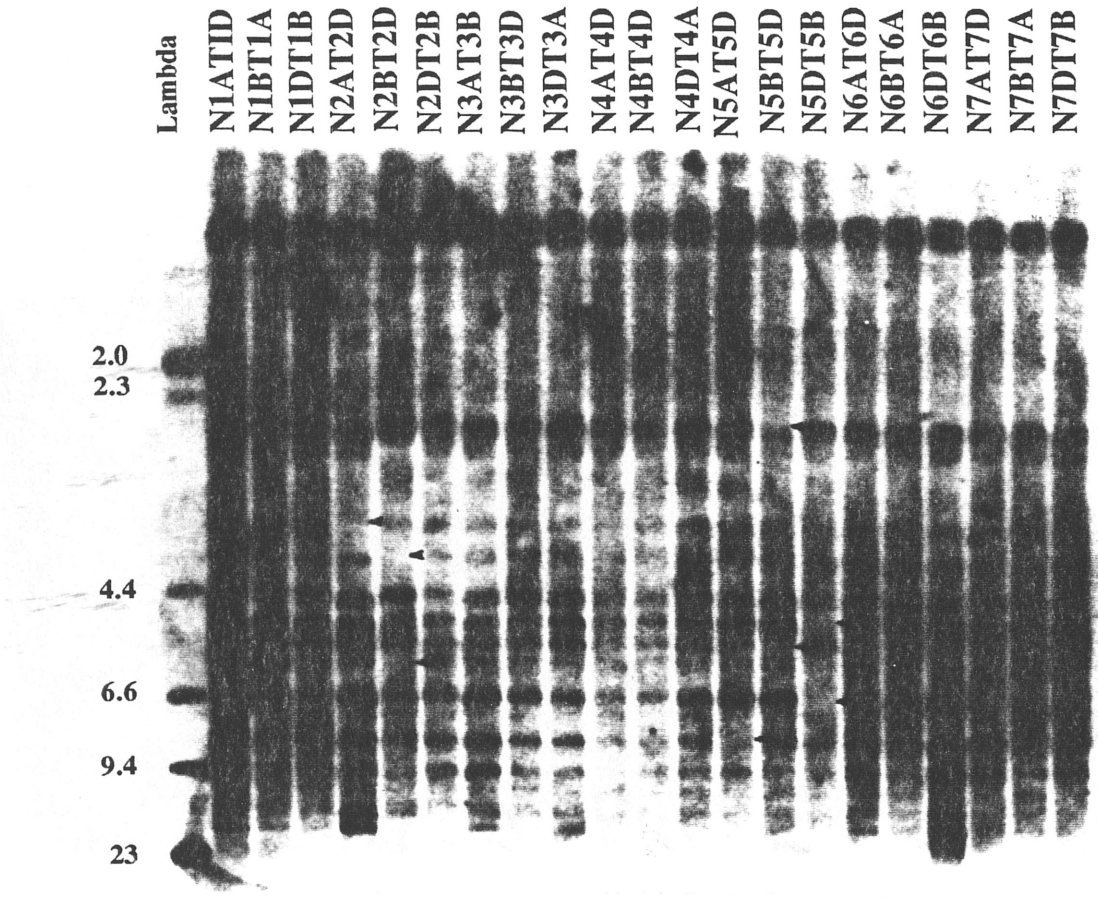


Figure 7. Southern blot of *Triticum aestivum* cv. Chinese Spring compensating nullisomic-tetrasomic lines. The first lane from the left contains the molecular weight marker. DNA was digested with *Dra*I and hybridized with the *rbc*S exon probe. Arrows indicate missing bands.

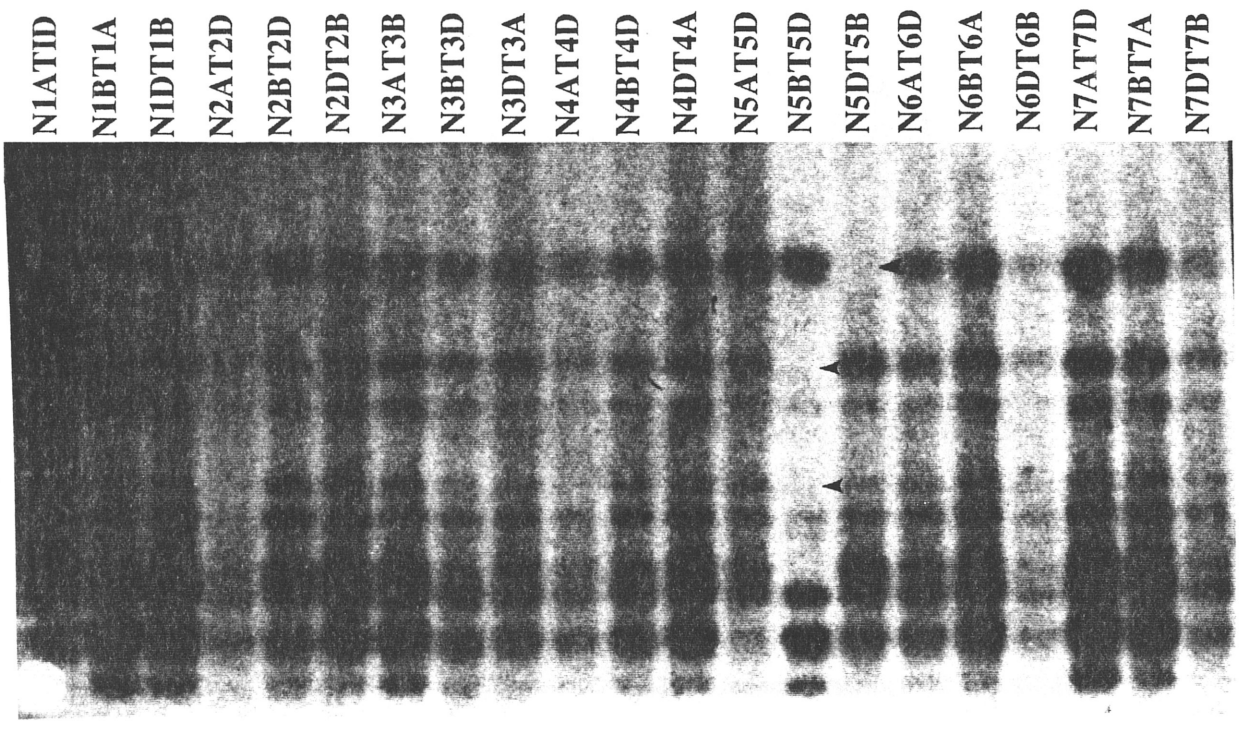


Figure 8. Southern blot of *Triticum aestivum* cv. Chinese Spring compensating nullisomic-tetrasomic lines. Arrows indicate missing bands.

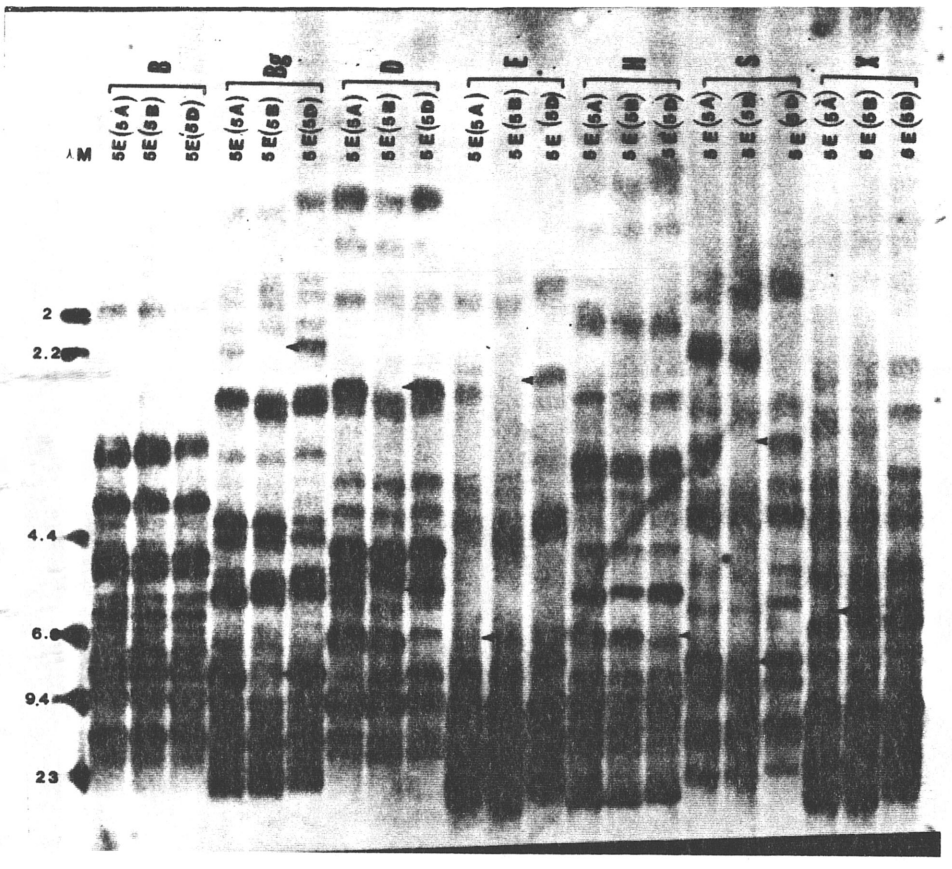


Figure 9. Southern blot of *Triticum aestivum* var. Chinese Spring-*lophopyrum elongatum* group 5 sustitution lines. The first lane from the left contains lambda DNA molecular weight markers. Genomic DNA was digested with the following restriction enzymes: *Bam*HI (B), *Bgl*II (Bg), *Dra*I (D), *Eco*RI (E), *Hind*III (H), *Sac*I (S) and *Xba*I (X); and hybridized with the *rbc*S exon probe. Arrows indicate some of the missing bands.

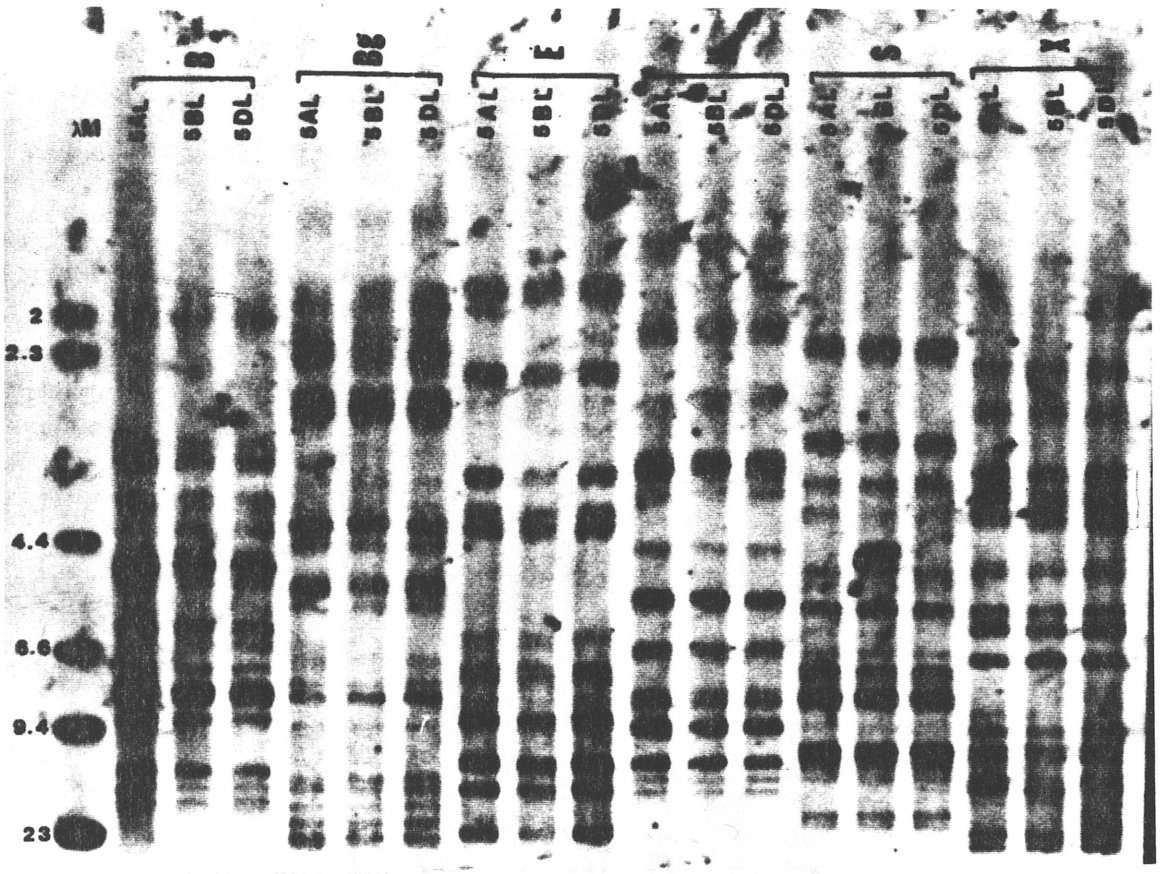


Figure 10. Southern blot of group 5 ditelosomic lines of *Triticum aestivum* var. Chinese Spring. 5AL, 5BL and 5DL designate lines ditelosomic for the long arms of chromosomes 5A, 5B, and 5D, respectively. Genomic DNA was digested with the following restriction enzymes: *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Sac*I (S) and *Xba*I (X), and hybridized with the *rbc*S exon probe. The first lane from the left contains lambda DNA molecular weight markers.

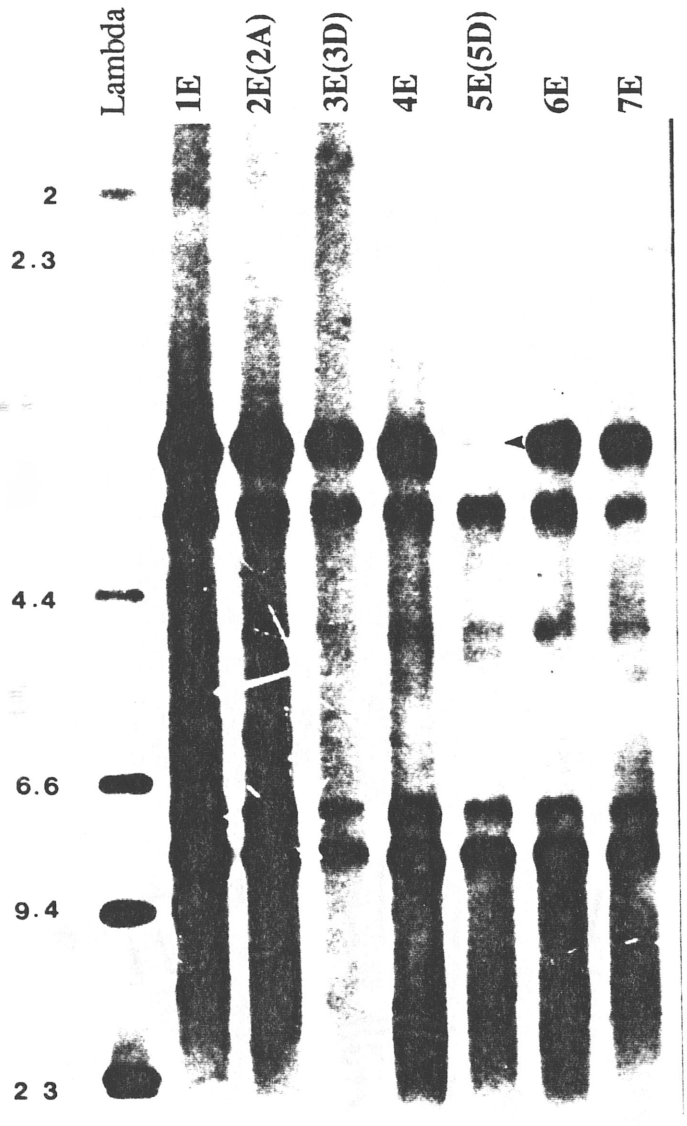


Figure 11. Southern blot of *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* addition and substitution lines. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines. Genomic DNA was digested with *Bam*HI and hybridized with the *rbc*S 3' end probe I. The first lane from the left contains molecular weight markers. The Arrow indicates a missing band.

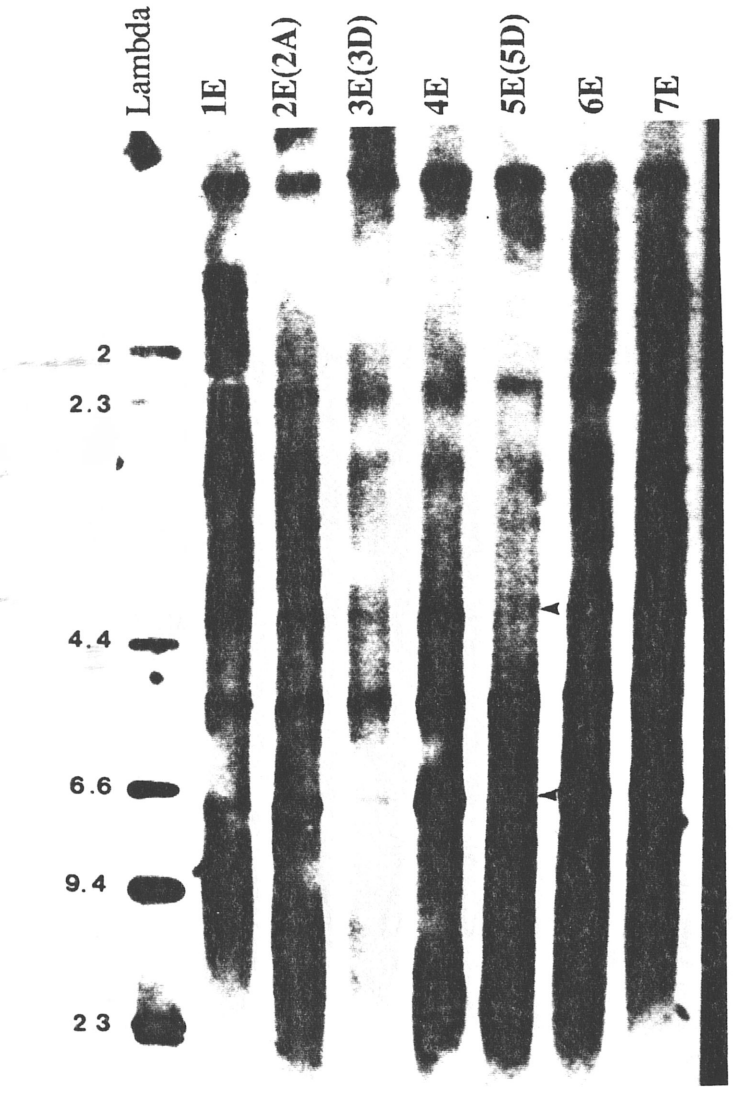


Figure 12. Southern blot of *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* addition and substitution lines. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines. Genomic DNA was digested with *BgL*II and hybridized with the *rbc*S 3' end probe I. The first lane from the left contains molecular weight markers. The arrows indicate the missing bands.

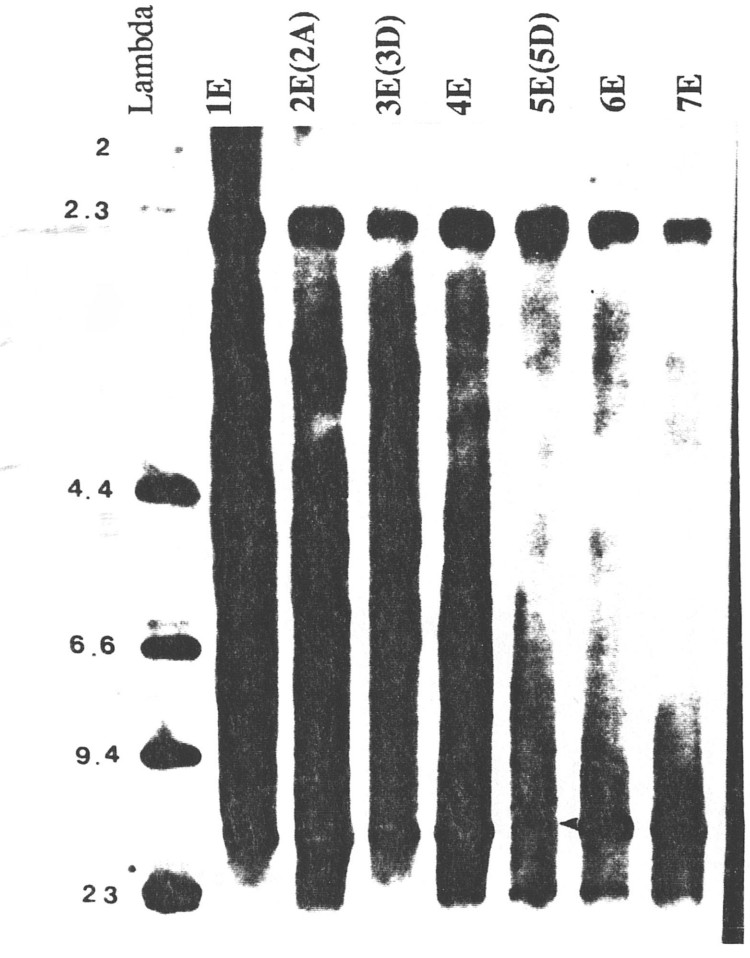


Figure 13. Southern blot of *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* addition and substitution lines. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines. Genomic DNA was digested with *Eco*RI and hybridized with the *rbc*S 3' end probe I. The first lane from the left contains molecular weight markers. The arrow indicates a missing band.

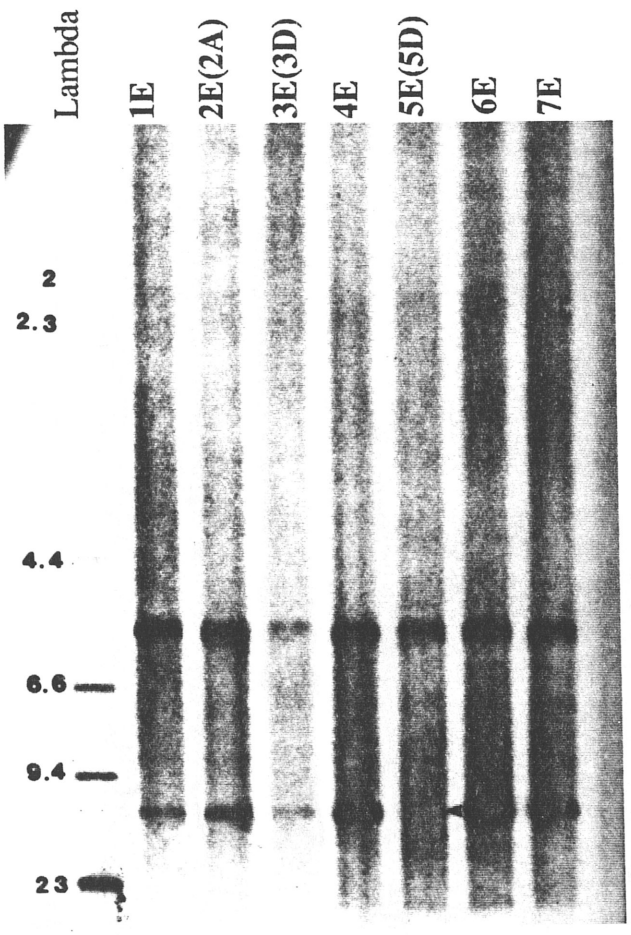


Figure 14. Southern blot of *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* addition and substitution lines. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines. Genomic DNA was digested with *Hind*III and hybridized with the *rbc*S 3' end probe I. The first lane from the left contains molecular weight markers. The arrow indicates a missing band.

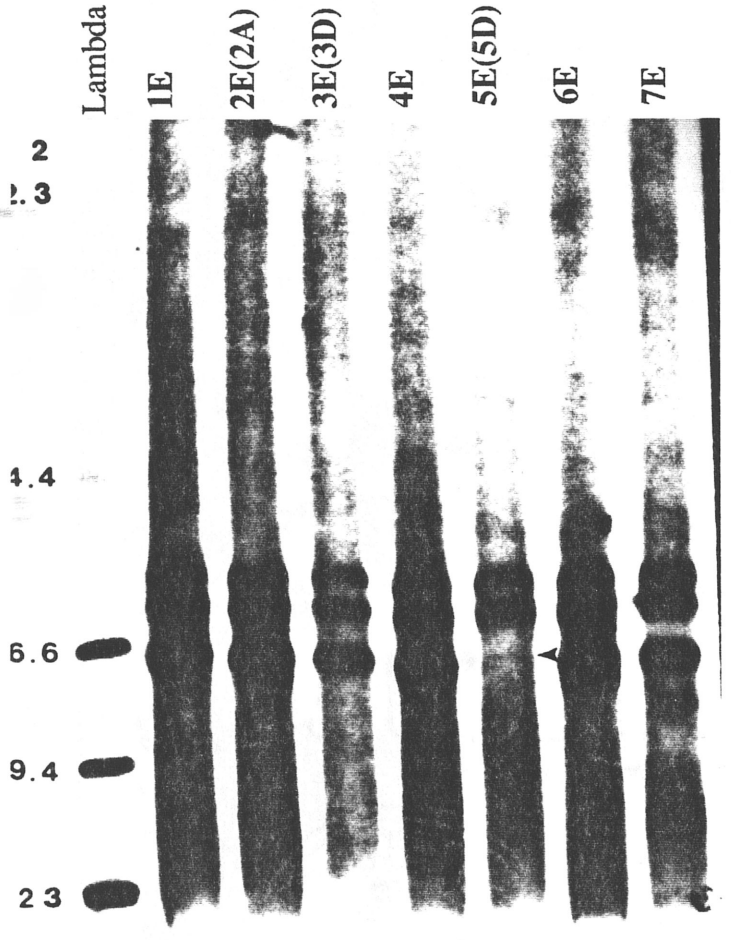


Figure 15. Southern blot of *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* addition and substitution lines. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines. Genomic DNA was digested with *Xba*I and hybridized with the *rbc*S 3' end probe I. The first lane from the left contains molecular weight markers. The arrow indicates a missing band.

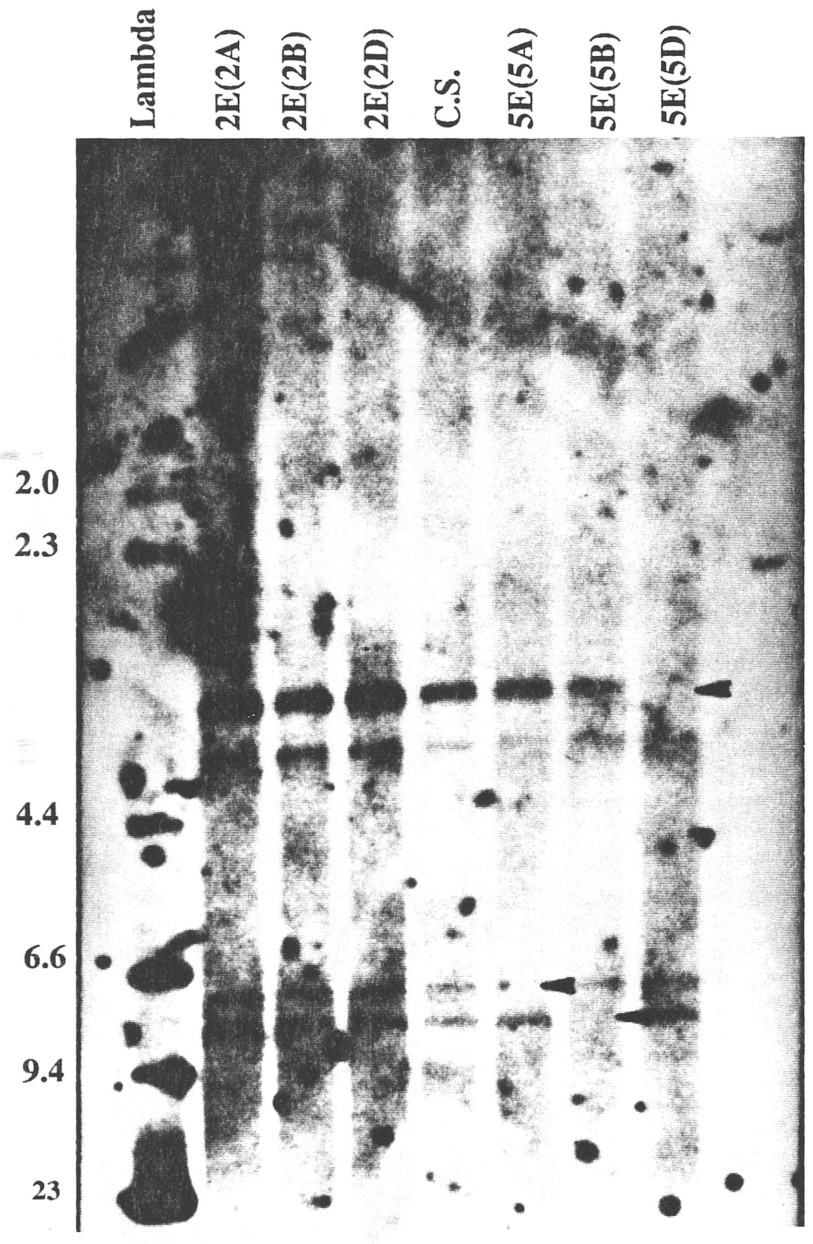


Figure 16. Southern blot of *Triticum aestivum* var.Chinese Spring-*Lophopyrum elongatum* groups 2 and 5 substitution lines. Genomic DNA was digested with *BamHI* and hybridized with *rbc*S 3' end probe I. The first lane from the left contains molecular weight markers. Arrows indicate missing bands.

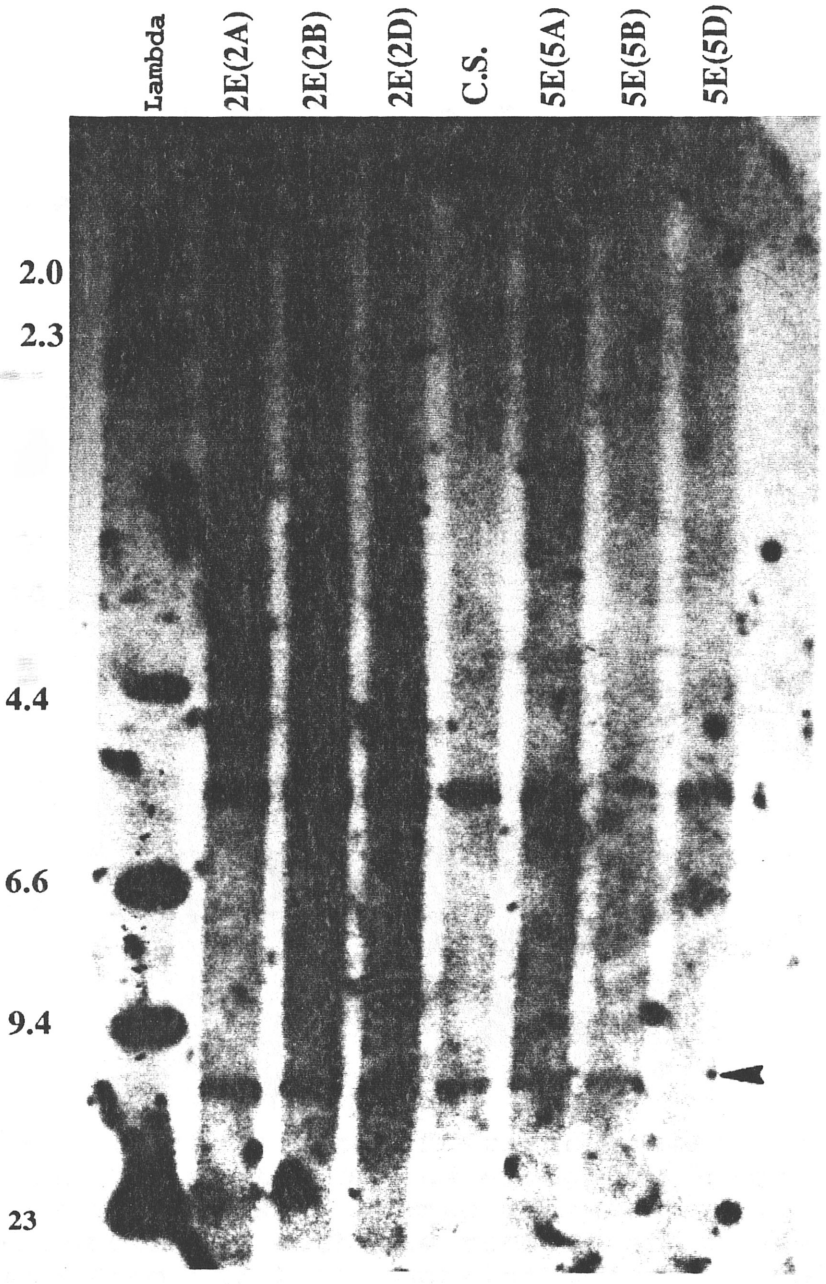


Figure 17. Southern blot of *Triticum aestivum* var.Chinese Spring-*Lophopyrum elongatum* groups 2 and 5 substitution lines. Genomic DNA was digested with *Hind*III and hybridized with the *rbc*S 3' end probe I. The first lane from the left contains molecular weight markers. The arrow indicates a missing band.

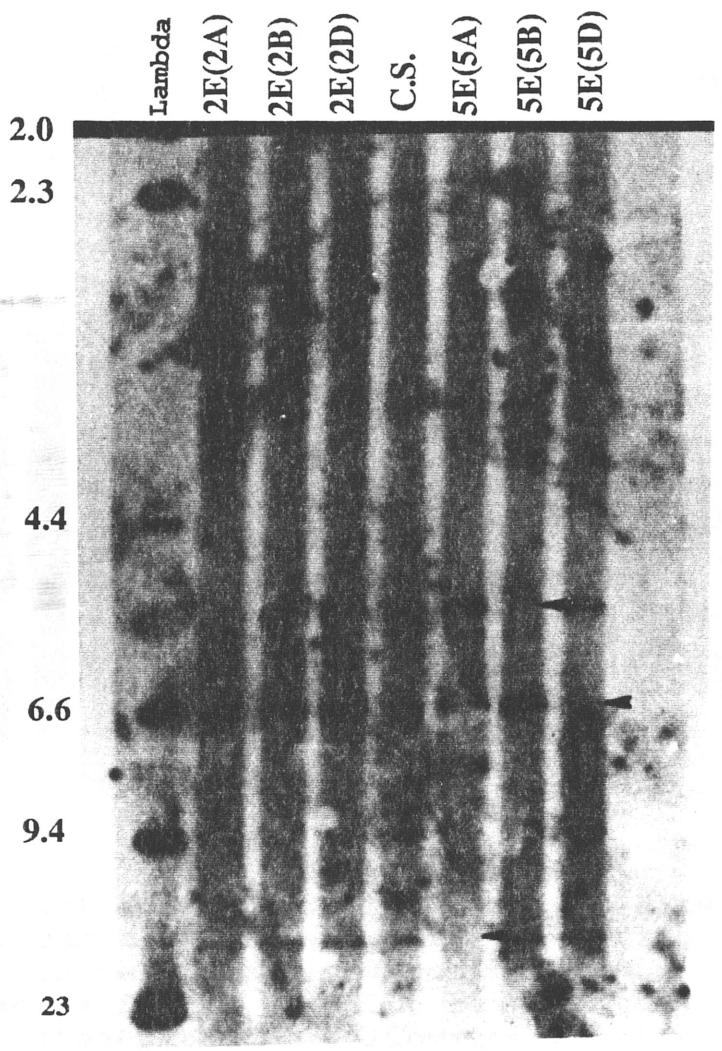
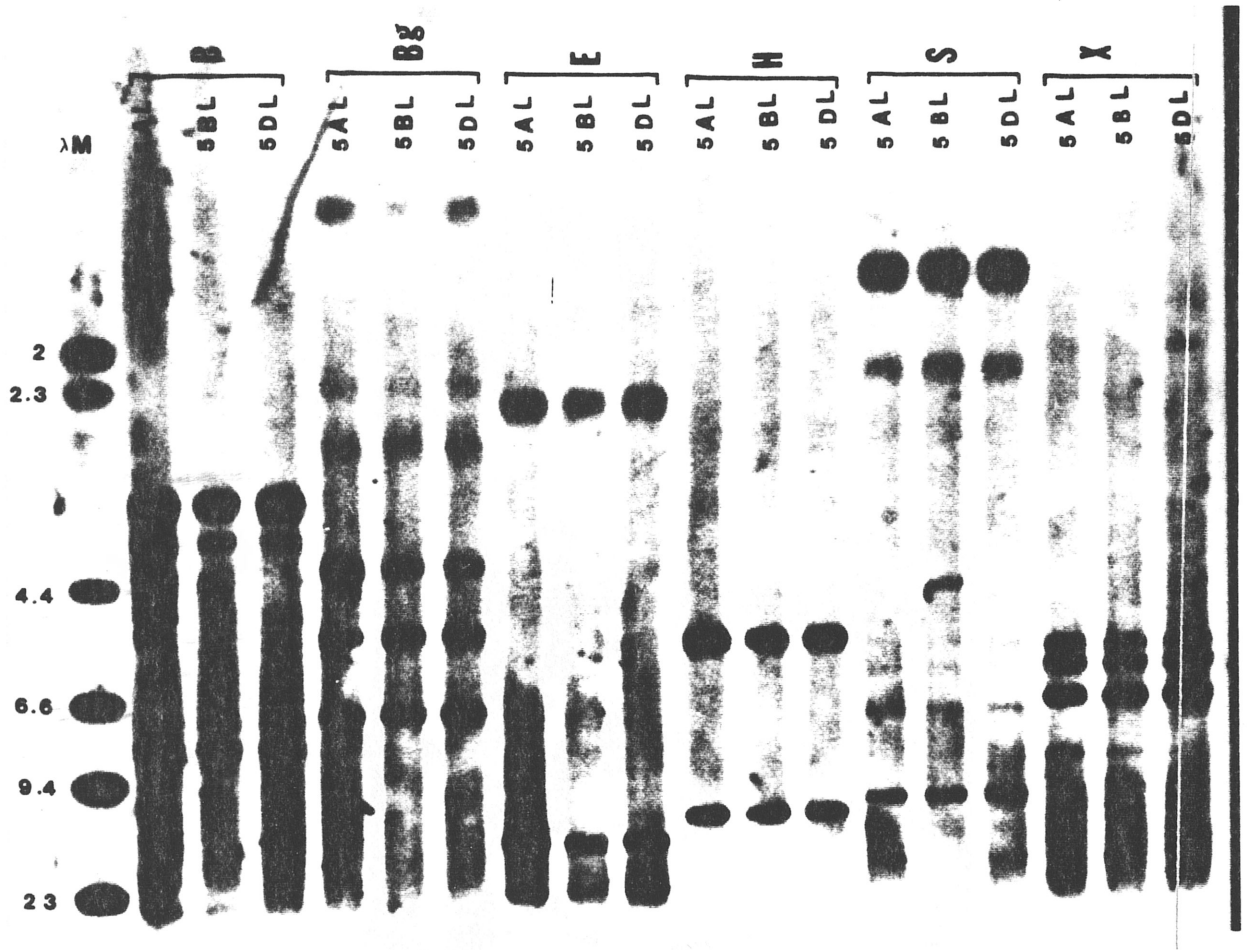


Figure 18. Southern blot of *Triticum aestivum* var.Chinese Spring-*Lophopyrum elongatum* groups 2 and 5 substitution lines. Genomic DNA was digested with *Dra*I and hybridized with the *rbc*S 3' end probe I. The first lane from the left contains molecular weight markers. Arrows indicate missing bands.



(Figure 19) Southern blot of group 5 ditelosomic lines of *Triticum aestivum* var. Chinese Spring. 5AL, 5BL and 5DL designate lines ditelosomic for the long arms of chromosomes 5A, 5B, and 5D, respectively. Genomic DNA was digestes with the following restriction enzymes: *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Sac*I (S) and *Xba*I (X), and hybridized with the *rbc*S 3' end probe I. The first lane from the left contains lambda DNA molecular weight markers.

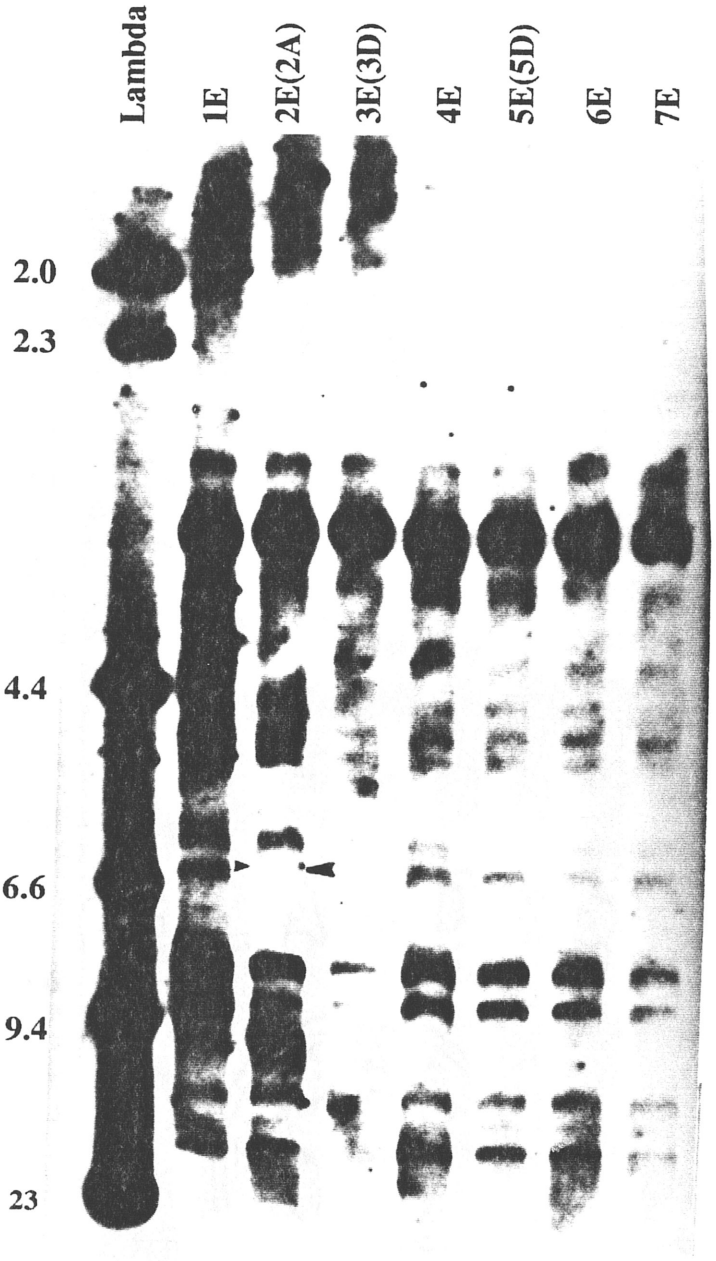


Figure 20. Southern blot of *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* substitution and addition lines. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines. Genomic DNA was digested with *Bam*HI and hybridized with *rbc*S 3' end probe II. The first lane from the left contains the molecular weight markers. The arrows indicate a missing band.

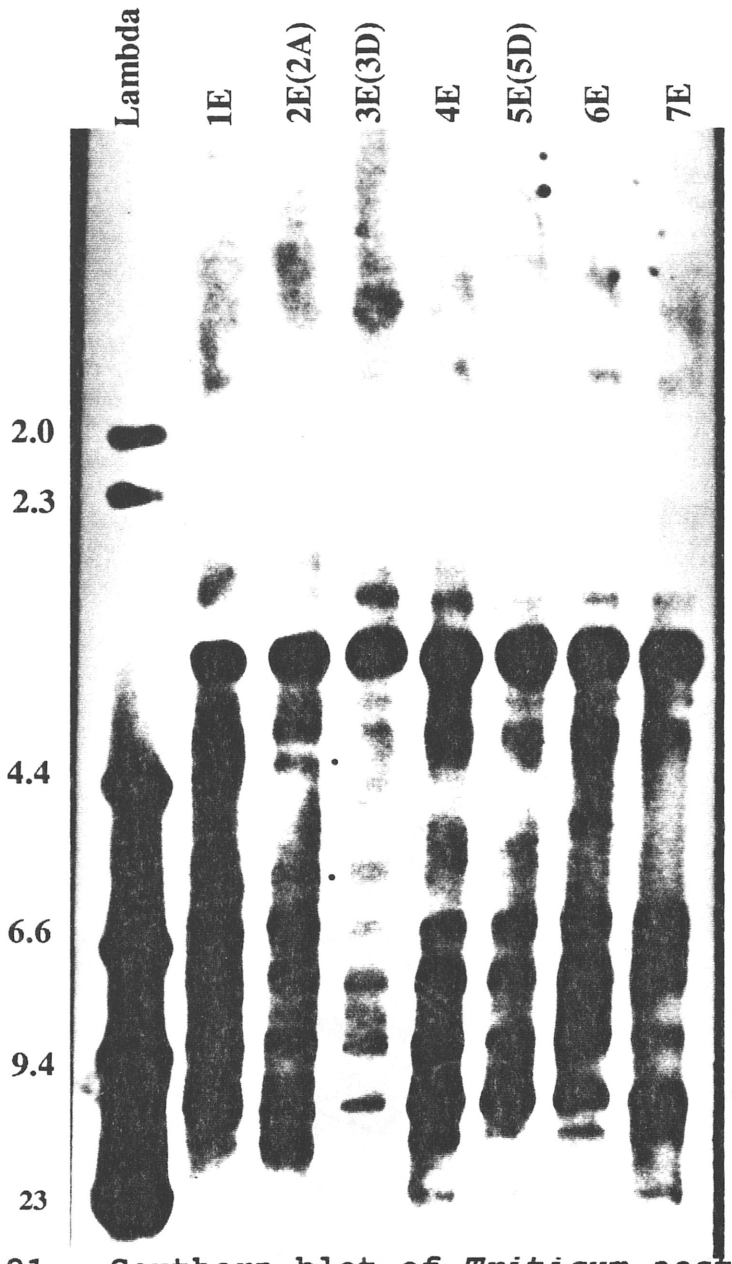


Figure 21. Southern blot of *Triticum aestivum* var. Chinese Spring-*Lophopyrum elongatum* substitution and addition lines. 1E, 4E, 6E and 7E designate addition lines and 2E(2A), 3E(3D) and 5E(5D) designate substitution lines. Genomic DNA was digested with *Eco*RI and hybridized with the *rbc*S 3' end probe II. The first lane from the left contains molecular weight markers. The dots indicate added bands.

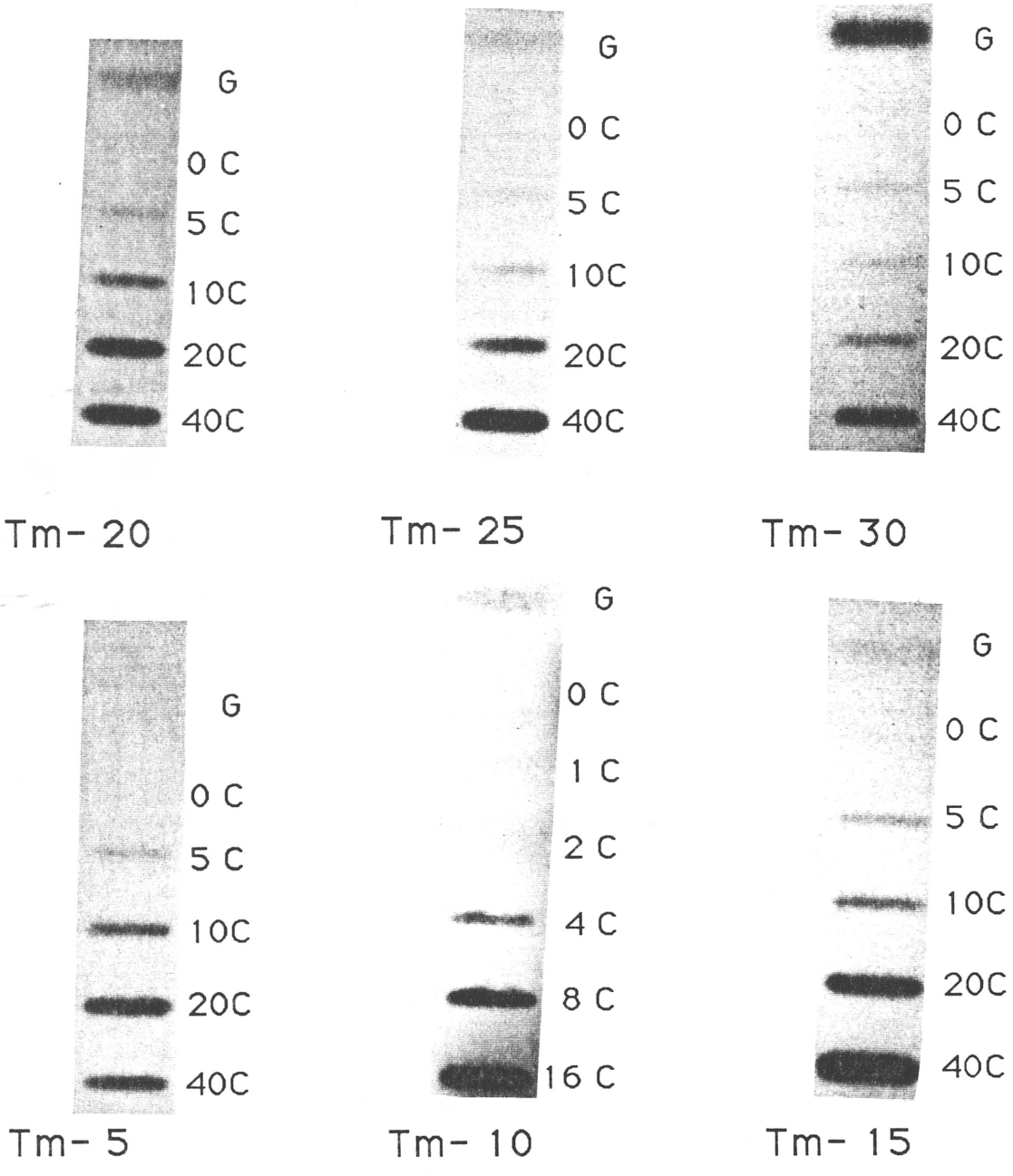


Figure 22. Results of slot blot hybridization analyses of *Triticum aestivum* var. Chinese spring *rbc*S genes carried out under different stringency conditions. Tm is the calculated melting temperature of the probe-genomic *rbc*S duplex. G = 5 *ug* of wheat genomic DNA. 0C - 40C: Zero to 40 copies per polyhaploid genome of the wheat *rbc*S gene.

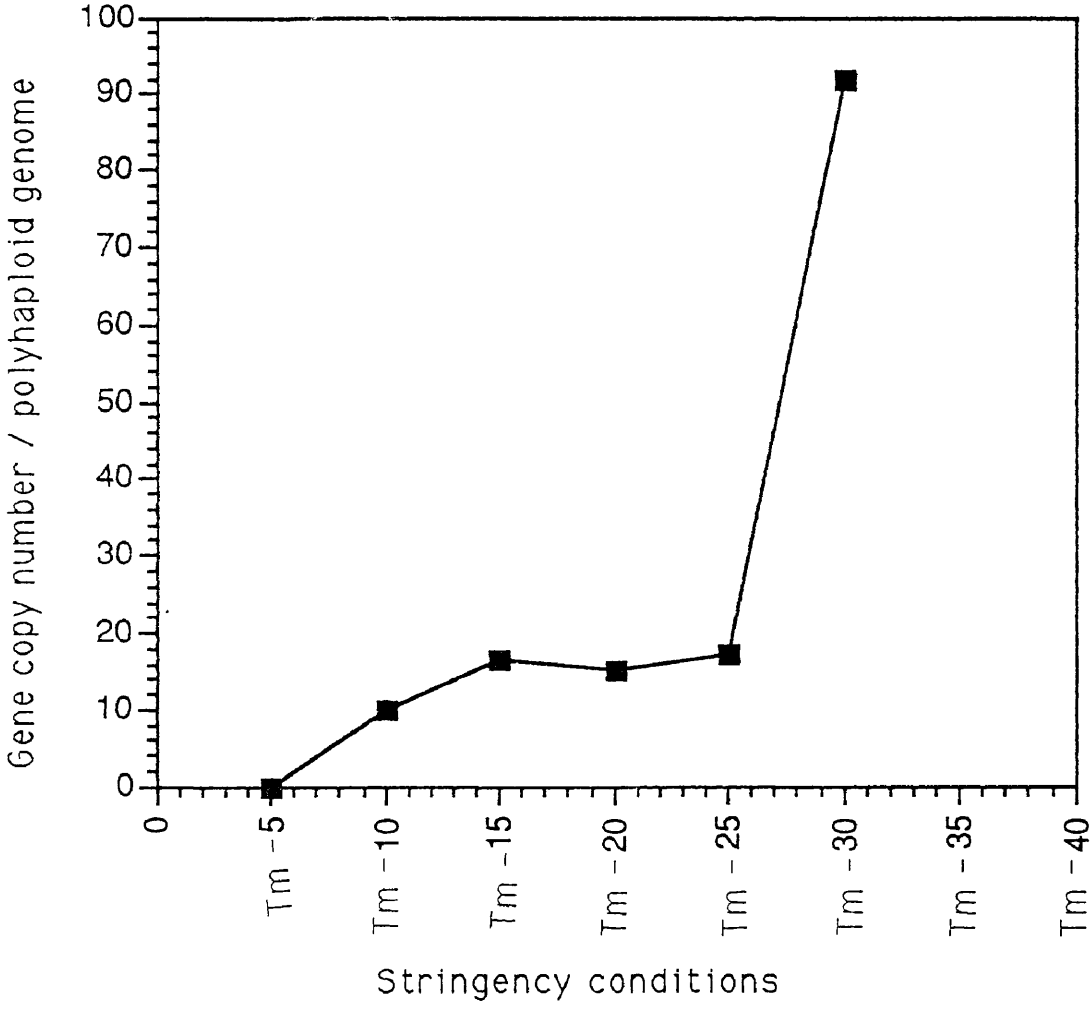


Figure 23. Estimated number of *rbc*S gene copies per polyhaploid genome in *Triticum aestivum* var. Chinese spring under different stringency conditions of hybridization. Tm is the calculated melting temperature of the probe duplex formation

APPENDIX B

Table 1. Number and sizes in kilobases of DNA bands of *Triticum aestivum* var. Chinese Spring that hybridized with the *rbc*S exon probe.

No	*Bam*HI	*Bgl*II	*Dra*I	*Eco*RI	*Hind*III	*Sac*I	*Xba*I
1	2.0	1.5	1.5	1.9	1.7	2.1	2.3
2	2.8	1.9	2.5	2.5	2.1	2.8	2.7
3	3.0	2.0	3.3	3.4	2.6	3.2	3.4
4	3.4	2.2	3.6	3.9	3.1	3.5	3.7
5	3.5	2.3	4.2	4.2	3.5	4.3	3.9
6	3.8	2.6	4.6	5.6	4.3	4.9	4.1
7	4.2	2.8	5.1	6.3	5.1	6.0	5.0
8	4.3	4.0	5.5	7.4	6.3	6.7	5.9
9	4.5	4.3	6.4	7.8	7.8	7.5	6.4
10	5.1	5.1	8.1	9.6	9.3	10.4	6.4
11	5.5	5.5	9.9	13.0	11.3	11.0	7.4
12	5.9	6.3	14.1	15.8	11.3	12.5	7.4
13	6.6	7.0	17.0	24.9	13.0	17.2	11.3
14	7.4	8.3			14.3	18.5	12.3
15	8.5	9.8					13.8
16	11.7	13.4					16.1
17	13.3	14.6					17.7
18	14.9	16.5					29.0
19	16.3	19.7					37.9
20		22.8					

Table 2. Number, sizes in kilobases and, where known, chromosome-arm locations of DNA fragments of *Triticum aestivum* var. Chinese Spring that hybridized with *rbc*S 3' end probe I.

No	*Bam*HI	*Bgl*II	*Dra*I	*Eco*RI	*Hind*III	*Xba*I
1	3.0 (5DL)	2.3(5BL)	3.3(5BL)	2.5(5BL)	5.1(5DL)	5.9(5AL)
2	3.4	4.0(5DL)	4.2(5DL)	15.8(5DL)	11.3	6.4(5BL)
3	6.6(5AL)	5.5(5BL)	14.1(5AL)			7.4(5DL)
4	7.4(5BL)	7.0(5DL)				

Table 3. Number and size in kilobases of DNA fragments of *Triticum aestivum* var. Chinese Spring that hybridized with *rbc*S 3' end probe II.

No	*Bam*HI	*Bgl*II	*Eco*RI	*Hind*III
1	3.0	2.0	24.9	14.3
2	3.0	2.6	13.0	9.3
3	4.3	2.8	9.6	7.8
4	4.5	4.3	7.8	3.5
5	5.5	5.1	7.4	4.3
6	5.9	6.3	4.2	3.1
7	7.4	7.0	3.4	2.6
8	8.5	9.8		2.1
9	11.7	19.7		1.7
10	14.9	22.8		

Table 4. Number and sizes in kilobases and, where known, chromosome-arm locations of DNA fragments of *Triticum aestivum* var. Chinese Spring that hybridized with *rbc*S exon probe, 3' end probe I and 3' end probe II.

No	*Bam*HI	*Bgl*II	*Dra*I	*Eco*RI	*Hind*III	*Xba*I
1	2.0(2BS)	1.5(2BS)	1.5	1.9	1.7	2.3(2BS)
2	2.8(2DS)	1.9(2AS)	2.5(5BL)	2.5(5BL)	2.1	2.7(2DS)
3	3.0(5DL)	2.0(2DS)	3.3(2AS)	3.4(5AL)	2.6	3.4(2AS)
4	3.0 (5BL)	2.2	3.6(2BS)	3.4(5DL)	3.1	3.7(2DS)
5	3.4	2.3(5BL)	4.2(5DL)	3.9	3.5(2AS)	3.9
6	3.5(5DL)	2.6	4.6(5DL)	4.2(5AL)	3.5(5AS)	4.1
7	3.8(2AS)	2.8(5AL)	5.1(5BL)	5.6	4.3	5.0
8	4.2	2.8	5.5(2BS)	6.3(5AL)	5.1(5BL)	5.9(5AL)
9	4.3	4.0(5DL)	6.4(5DL)	7.4(5BL)	5.1	6.4(5BL)
10	4.5(2DS)	4.3(2DS)	8.1(5AL)	7.8	6.3(5DL)	6.4
11	5.1	4.9	9.9	9.6	7.8	7.4(5DL)
12	5.5(2DS)	5.5(2AS)	14.1(5AL)	13.0(2DS)	9.3	7.4
13	5.9(2AS)	6.3	14.1	15.8	11.3(5DL)	11.3
14	6.6(5AL)	7.0	17.0	15.8(5DL)	11.3	12.3
15	7.4	7.0(5DL)		24.9	13.0	13.8
16	7.4(5BL)	8.3(5BL)			14.3	16.1
17	8.5(2AS)	9.8(2BS)				17.7(5DL)
18	11.7	9.8(5BL)				29.0
19	13.3	13.4(2BS)				37.9
20	14.9	14.6(2BS)				
21	16.3	16.5(2AS)				
22		19.7				
23		22.8(2DS)				

Table 5. Genomic reconstruction hybridization experiments conducted at different stringency conditions.

Stringency condition	Standard copy number	Area[2] for standard	Slope[3]	Y[4] interception	Area[5] for unknown	Estimated copy number[6]
Tm[1] - 30	5 10 20 40	0.848 1.638 2.020 2.799	0.038	0.2	3.707	91.8
Tm- 25	5 10 20 40	1.657 2.069 3.47 5.694	0.14	0	2.402	17.1
Tm - 20	5 10 20 40	1.86 2.422 4.172 4.644	0.175	0.4	3.058	15.2
Tm - 15	5 10 20 40	1.943 1.969 3.692 6.11	0.172	0	2.84	16.5
Tm - 10	1 2 4 8 16	1.141 1.675 2.181 3.021 4.152	0.141	1.1	2.512	10.0
Tm - 5	5 10 20	2.351 2.822 4.254			0	0

1. The calculated melting temperature of the exon probe duplex formation.

2. The measured intensity differences between different slots using a model 620 Bio-Rad video densitometer and IBM 1-D analyst software (Bio-Rad version 2.01).

3 and 4. Measured from the standard curves by plotting column 2 against column 3 for each stringency condition.

5. The measured area for the unknown.

6. Estimated copy number from the standard curve.