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Chromosome assignment of four photosynthesis-related genes and their variability in wheat species

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Abstract Copy numbers of four photosynthesis-related genes, *PhyA*, *Ppc*, *RbcS* and *Lhcb1*1*, in wheat genomes were estimated by slot-blot analysis, and these genes were assigned to the chromosome arms of common wheat by Southern hybridization of DNA from an aneuploid series of the cultivar Chinese Spring. The copy number of *PhyA* was estimated to be one locus per haploid genome, and this gene was assigned to chromosomes 4AL, 4BS and 4DS. The *Ppc* gene showed a low copy number of small multigenes, and was located on the short arm of homoeologous group 3 chromosomes and the long arm of chromosomes of homoeologous group 7. *RbcS* consisted of a multigene family, with approximately 100 copies in the common wheat genome, and was located on the short arm of group 2 chromosomes and the long arm of group 5 chromosomes. *Lhcb1*1* also consisted of a multigene family with about 50 copies in common wheat. Only a limited number of restriction fragments (approximately 15%) were used to determine the locations of members of this family on the long arm of group 1 chromosomes owing to the multiplicity of DNA bands. The variability of hybridized bands with the four genes was less in polyploids, but was more in the case of multigene families. RFLP analysis of polyploid wheats and their presumed ancestors was carried out with probes of the oat *PhyA* gene, the maize *Ppc* gene, the wheat *RbcS* gene and the wheat *Lhcb1*1* gene. The RFLP patterns of common wheat most closely resembled those of *T. dicoccum* (Emmer wheat), *T. urartu* (A genome), *Ae. speltoides* (S genome) and *Ae. squarrosa* (D genome). Diversification of genes in the wheat complex appear to have occurred mainly at the diploid level. Based on RFLP patterns, B and S genomes were clustered into two major groups. The fragment numbers per genome were reduced in proportion to the increase of ploidy level for all four genes, suggesting that some mechanism(s) might operate to restrict, and so keep to a minimum, the gene numbers in the polyploid genomes. However, the *RbcS* genes, located on 2BS, were more conserved (double dosage), indicating that the above mechanism(s) does not operate equally on individual genes.

Key words Photosynthesis-related genes · Copy numbers · Chromosome assignments · RFLP · Origin of polyploid wheats

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Introduction

Photosynthesis requires a co-ordinated system of gene products encoded by nuclear and chloroplast DNAs, and these products are synthesized in response to environmental conditions. Signals perceived by photoreceptor(s) are transmitted to activate some light-inducible genes, most of which are photosynthesis-related (Gilmartin et al. 1990; Quail et al. 1991). Thus, an analysis of the structure of the photosynthesis-related genes and their chromosome distribution are key steps necessary to understand and manipulate the photosynthetic system at the molecular level. The information obtained would find application not only in conventional breeding programs, but also in restriction fragment length polymorphism (RFLP) mapping and gene manipulation in transgenic plants with the aim of modifying photosynthesis.

In the present study, four photosynthesis-related genes were chosen: *PhyA*, for phytochrome Type I (Quail 1991); *Ppc*, for phosphoenolpyruvate carboxylase (PEPC); *RbcS*, for ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco); and *Lhcb1*1* (Jansson et al. 1992), for the chlorophyll a/b-binding protein (CAB). Phytochrome is a regulatory photoreceptor molecule that functions interconvertibly between its inactive (Pf) and active (Pfr) forms by sequential irradiation with red and far-red light. Pfr formation (signal perception)

initiates a transduction process (Quail et al. 1991). Southern hybridization and sequence analyses suggested that there are five types of phytochrome genes in *Arabidopsis thaliana*, namely *PhyA*, *PhyB*, *PhyC*, *PhyD* and *PhyE*, which diverged approximately 100–300 million years ago (Quail 1991). *PhyA* encodes Type I phytochrome which is abundant in etiolated tissue, is rapidly degraded as Pfr in the cell, and has been extensively characterized (Furuya 1989). *PhyA* is known as a low copy gene in the genome of *Arabidopsis* (Chang et al. 1988). PEPC plays a key role in the photosynthetic fixation of carbon dioxide in C4 plants (Andreo et al. 1987) and its expression is induced by light (Sheen and Bogorad 1987). In addition to the photosynthetic pathway, PEPC is involved in various other physiological processes, even in C3 plants like rice and wheat (Matsuoka and Yamamoto 1989; Kawamura et al. 1990). Reflecting these functions, various isoforms of PEPC have been reported. In maize, at least three isoforms were implicated (Ting and Osmond 1973), which were subsequently shown to form a small multigene family (Cretin et al. 1991). Rubisco (ribulose-1, 5-bisphosphate carboxylase/oxygenase) is known as a key enzyme for photosynthesis and photorespiration (Miziorko and Lorimer 1983), and is the most abundant enzyme in nature (Ellis 1979). The Rubisco holoenzyme is a hexadecamer, eight subunits of which are encoded by chloroplast DNA (large subunits) and eight by nuclear DNA (small subunits) (McIntosh et al. 1980). While the large subunit of Rubisco is encoded by one species of chloroplast DNA molecule, the nuclear gene for the small subunit of Rubisco consists of a multigene family divided into subclasses depending on the expression pattern in plant tissues and the developmental stage (Sugita et al. 1987). The chlorophyll a/b-binding (CAB) proteins consist of light-harvesting antenna complexes (Lhc) for both photosystem I (PSI) and photosystem II (PSII). At present, four genes responsible for PSI, and six for PSII, have been reported (Jansson et al. 1992). Although genes for CAB consist of multigene families (Green et al. 1991), DNA sequences of the genes belonging to different classes of *Lhc* have diverged so extensively that they do not usually cross-hybridize (Pichersky et al. 1991).

As part of our study on the co-ordinated regulation of photosynthesis-related gene expression, we estimated the copy number of the above genes in wheat genomes by slot-blot analysis and the number of restriction fragments digested with four enzymes, and determined their chromosomal locations using aneuploids of common wheat. As far as possible we employed the exon sequences as probes to detect active genes. We also carried out RFLP analysis of these genes in polyploid wheats and their presumed ancestors to estimate the variability of these genes among wheat species.

Materials and methods

Plant materials

The following euploid and aneuploid lines of common wheat, *Triticum aestivum* cv Chinese Spring (CS) (2n = 6x = 42), were used as plant material: nullisomic-tetrasomic (NT) lines lacking a pair of chromosomes and replaced by an extra-pair of their homoeologues (except for 2A and 4B), and ditelosomics (DT) deficient for a pair of chromosome arms. Some DT lines (2AL, 4BL, 5AS, 5BS and 5DS) are maintained as monotelo-ditelosomics because of their viability and DT lines were then produced by self-pollination. Additionally, five hexaploid, four tetraploid, and ten diploid wheat species (Table 1) were also employed in the present investigation. All lines are maintained in our institute.

Table 1 Plant materials used for RFLP detection of photosynthesis-related genes in *Triticum* and *Aegilops*

Code no.	Plant source	Genome formula[a]	Collection no. in KIBR[b]
1	*T. monococcum* Early mutant	A (Amo)	KT 3-5
2	*T. urartu* var. nigrum	A (Aur)	KT 2-1
3	*T. boeoticum* ssp. boeoticum	A (Abo)	KT 1-1
4	*Ae. bicornis* var. typica	S^b (S^b)	KT 119-1
5	*Ae. searsii*	S^s (S^s)	KT 125-2
6	*Ae. sharonensis* var. typica	S^l (S^{sh})	KT 118-1
7	*Ae. longissima* var. typica	S^l (S^l)	KT 117-1
8	*Ae. speltoides* var. typica	S (S)	KT 115-6
9	*Ae. aucheri* var. typica	S (Sau)	KT 116-1
10	*Ae. squarrosa* var. typica	D (Dsq)	KT 120-1
11	*T. timopheevi* var. typicum	AG (AtiGti)	KT 5-1
12	*T. dicoccoides* var. kotschyanum	AB (AdsBds)	KT 6-1
13	*T. durum* var. aestivum	AB (AduBdu)	KT 9-10
14	*T. dicoccum* var. farrum	AB (AdmBdm)	KT 7-2
15	*T. aestivum* cv Chinese Spring	ABD(AaeBaeDae)	KT 20-3
16	*T. sphaerococcum* var. rotundatum	ABD(AsmBsmDsm)	KT 22-1
17	*T. spelta* var. duhamerianum	ABD (AsaBsaDsa)	KT 19-1
18	*T. compactum* var. humboldtii	ABD (AcoBcoDco)	KT 21-1
19	*T. macha* var. sub-letshchumicum	ABD (AmaBmaDma)	KT 18-1

[a] After Kihara (1954), with abbreviations given in parentheses
[b] KIBR, Kihara Institute for Biological Research, Yokohama City University

DNA isolation and Southern hybridization

Total DNAs were extracted from wheat leaves according to the cetyltrimethylammonium bromide (CTAB) method discussed by Murray and Thompson (1980). Ten micrograms of the DNAs were digested with the restriction endonucleases *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and/or *Xba*I according to the conditions specified by the manufacturer (Takara Shuzo Co. Ltd). Digested DNAs were fractionated on an 0.85% agarose gel in TAE buffer (Maniatis et al. 1982).

Fractionated DNAs were transferred to Hybond N+ (Amersham Co. Ltd.) membrane. Hybridization was carried out at 68 °C for 24 h in a solution containing 5 × SSC, 0.1% SDS, 0.5% (w/v) dry skim milk, 50 μg/ml of sonicated herring sperm DNA, along with the labelled probe. The membranes were washed in 2 × SSC −0.1% SDS at room temperature, and then in 0.2 × SSC −0.1% SDS at 68 °C, twice in each for 15 min.

DNA probes

Four photosynthesis-related genes, namely *PhyA* (gene for phytochrome A), *Ppc* (gene for phosphoenolpyruvate carboxylase), *RbcS* (gene for small subunit of ribulose 1,5-bisphosphate carboxylase), and *Lhcb1*1* of photosystem II (gene for chlorophyll a/b-binding protein), were investigated in the present study. Specific probes were made by the polymerase chain reaction (PCR) method of cloned DNAs in plasmid or by digestion of the plasmid DNA with the appropriate restriction enzymes. Oligonucleotide primers complementary to the third exon of the oat *PhyA* gene (5′-CCAGTTATCTTGGTTGTGAATG-3′ and 5′-TGCTATCTTGATCCAAGTCTGC-3′), to the second exon of the wheat *RbcS* gene (5′-GTGTGGCCAATTGAGGGCATCA-3′ and 5′-GGATTCCTCGCAGCCTGGTGGC-3′), and to the exon of the wheat *Lhcb1*1* gene (5′-CGCAAGACTGCGGCCGAAGGCC A-3′ and 5′-GGCACGAAGTTGGTGGCAATGAG-3′) were synthesized according to the published data ((Broglie et al. 1983; Hershey et al. 1985; Lamppa et al. 1985). Target sequences were amplified in a thermal cycler (Perkin-Elmer Cetus Co. Ltd.) as follows: 1 min of denaturation at 94 °C, 2 min of primer-template annealing at 60 °C, and 3 min of DNA chain elongation at 72 °C. The reaction mixture was composed of 5 ng each of plasmid DNAs containing the oat *PhyA*, the wheat *RbcS* or the wheat *Lhcb1*1* gene, 1 μM each of 5′ and 3′ primers, 0.2 mM each of dNTP, 10 mM Tris-HCl at pH8.3, 1.5 mM $MgCl_2$, 50 mM KCl, and 2.5 units of *Taq* polymerase (Takara Shuzo Co. Ltd.). DNAs were amplified for 30 cycles. The probes specific for the eighth exon of the maize *Ppc* gene (Izui et al. 1986) were produced by the double digestion of pM52/SE3 with *Eco*RI and *Sph*I. After amplifications with PCR or digestions with restriction endonucleases, target DNAs were separated by agarose-gel electrophoresis and extracted from agarose (Heery et al. 1990) for labelling with [α^{32}P] dCTP by the random primer extension method (Feinberg and Vogelstein 1983).

Slot-blot analysis

A proportional concentration series of each plasmid DNA and total DNAs of the wheat species. *T. monococcum* Early mutant (2n = 2x = 14), *T. durum* cv coerulescence (2n = 4x = 28), and *T. aestivum* cv Chinese Spring (2n = 6x = 42), were spotted onto nylon membrane, Hybond N+, by use of a slot-blot apparatus (Bio Dot SE, BioRad Co. Ltd.). Hybridization with radioactive probes, washing and detection of the hybridization products were done as described above. Based on the comparison of signal intensities between the plasmid DNA harboring each gene and the wheat DNAs, the copy number per haploid genome of each gene was calculated. The known genome sizes (Bennet et al. 1982) of *T. monococcum* (0.7×10^{10} bp), *T. durum* (1.1×10^{10} bp) and *T. aestivum* cv CS (1.45×10^{10} bp), and the inserted sizes of genes (7.6 kbp for *PhyA*, 6.0 kbp for *Ppc*, 8.6 kbp for *RbcS*, and 4.4 kbp for *Lhcb1*1*; see references listed above) were used for the calculations.

Estimation for variability of RFLPs among wheat species

DNA probes were hybridized to 19 wheat species digested with the restriction endonucleases *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII and/or *Xba*I. Restriction fragment patterns were compared among these species to estimate variabilities for the four genes and to calculate fragment similarities.

Results

Copy number of photosynthesis-related genes in the wheat genome

The copy numbers of the photosynthesis-related genes in wheat genomes of *T. monococcum* Early mutant (2n = 14; genome formula AA), *T. durum* cv coerulescens (2n = 28; AABB), and *T. aestivum* cv Chinese Spring (2n = 42; AABBDD) were estimated by comparison of hybridization intensities between a series of dilutions of total wheat DNAs and that of the plasmid DNAs harboring each gene. The copy numbers were calculated by the data obtained from four independent experiments. Hybridization profiles of dilution series of DNAs with the four genes employed are shown in Fig. 1. For example, the signal intensity of 0.5 μg of Chinese Spring (CS) DNA was similar to that of 30.5 pg of pWS4.3 which harbors the *RbcS* gene (Broglie et al. 1983). As calculated from the genome size of CS and the inserted

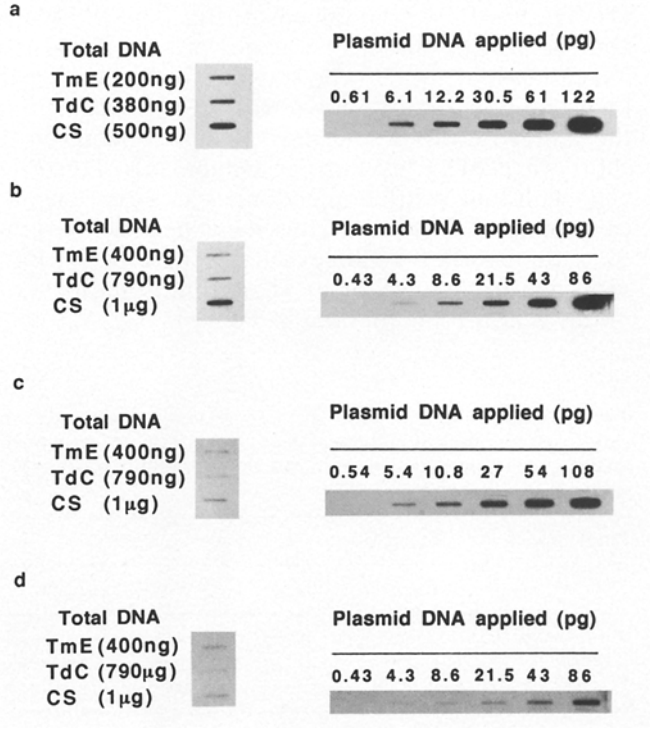

Fig. 1a–d Slot-blot analysis for the estimation of the copy numbers of genes in the genomes; (**a**) *RbcS*, (**b**) *Lhcb1*1*, (**c**) *PhyA* and (**d**) *Ppc*. *Tme* stands for *Triticum monococcum* Early mutant; *TdC* for *T. durum* var. coerulescens; and *CS* for *T. aestivum* cv Chinese Spring

size of the gene, the copy number of *RbcS* in CS was estimated as approximately 100 per haploid genome. Simultaneously, the copy numbers of the four genes in the three wheat genomes were estimated as presented in Table 2. It is noteworthy that the copy numbers of *PhyA* and *Ppc* were underestimated, since these probes are heterologous for wheat.

Chromosome assignment of photosynthesis-related genes

Chromosome locations of photosynthesis-related genes were determined by the use of aneuploid lines of common wheat, namely, ditelosomic lines and nulli-tetrasomic lines. The ditelosomics are defined as a series of lines in which a chromosome arm is deleted. The gene locations on chromosome arms are thus determined directly due to the deletion of DNA bands in comparison with the control DNA. The nulli-tetrasomics are defined as a series of lines that lack a pair of chromosomes that are replaced by an extra pair of their homoeologous chromosomes. Chromosome locations of the genes are thus determined by a lack of hybridization signal(s) for nullisomics, and an increased intensity of that for tetrasomics, against a pure line.

For determination of the location of the *PhyA* gene, total DNAs of aneuploid lines as well as CS were digested with *Bam*HI, *Dra*I, *Eco*RI and *Xba*I. Three bands were detectable for CS after Southern hybridization with the *PhyA* probe. These bands were shown to be located on homoeologous group 4 chromosomes. For example, *Dra*I digestion of CS (Fig. 2) produced three bands of 9.4 kbp, 7.7 kbp and 5.3 kbp. Of these three bands, the 9.4-kbp fragment was missing in DT4DL, the 7.7-kbp fragment was lacking in DT4AS, and the 5.3-kbp fragment was not present in DT4BL, indicating that the 7.7-kbp fragment is located on the long arm of chromosome 4A, the 5.3-kbp fragment on the short arm of 4B, and the 9.4-kbp fragment on the short arm of 4D. Chromosome assignments of the *PhyA* gene with four restriction endonucleases are schematically depicted in Fig. 6A. Thus, it can be concluded that the common wheat genome contains three loci for *PhyA* gene (one locus per genome). The chromosome locations of this gene are summarised in Table 3.

Table 2 Copy number of photosynthesis-related genes in wheat species (Copy numbers of individual genes were estimated by slot-blot analysis, values presented here indicate the copy numbers per haploid genome)

Plant species	Gene			
	RbcS	*Lhcb1*1*	*PhyA*	*Ppc*
T. monococcum	25–50	10–20	< 5	5–10
T. durum	*ca.* 50	10–20	< 5	5–10
T. aestivum	*ca.* 100	35–70	*ca.* 5	10–25

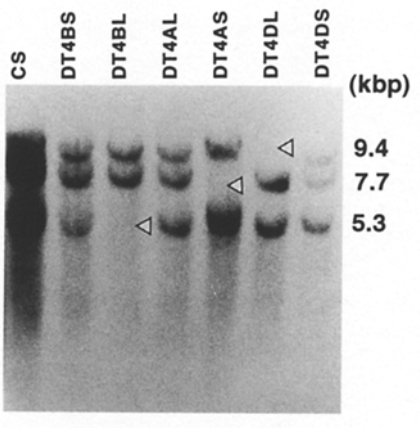

Fig. 2 Southern hybridization patterns of ditelosomic lines probed with *PhyA* after digestion with *Dra*I. *Triangles* indicate missing fragments in each line

The chromosome locations of the *Ppc* gene were also determined by the use of aneuploids of common wheat. *Eco*RI digestion of common wheat DNA produced six fragments as shown in Fig. 3 (17.8 kbp, 16.1 kbp, 9.3 kbp, 8.1 kbp, 7.4 kbp and 5.2 kbp). By using ditelosomics, we were able to assign these fragments to the following chromosome arms: 17.8-kbp to 3BS; 16.1 kbp to 3AS; 9.3 kbp to 7AL; 8.1 kbp to 7BL; 7.4 kbp to 3DS; and 5.2 kbp to 7DL. Southern hybridization data of nulli-tetrasomics confirmed the assignment (Fig. 3). *Hin*dIII digestion of aneuploids gave the same chromosome assignments of the gene, although the bands from 3AS and 7BL co-migrated to the same position. Consequently, there are six loci for this gene in common wheat; two loci being assigned to each genome (Fig. 6 B). The designation of these genes is listed in Table 3.

The restriction enzymes *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I produced 15–17 fragments after Southern hybridization of common wheat DNA probed with the *RbcS* gene. By utilizing aneuploid lines, we determined the locations of about half of the fragments. A typical example is shown in Fig. 4, and hybridization patterns are idiogrammed in Fig. 6 C. The location of the other bands was not determined because of their loss in all aneuploid lines due to multiplicity. The *rbcS* genes were located on the short arms of homoeologous group 2, and the long arms of homoeologous group 5, chromosomes. The fragments patterns and locations corresponded to those reported by Galili et al. (1992). In addition, two more bands were detectable, one of which was assigned to 2AS (0.6 kbp). As judged from the hybridization pattern with four restriction enzymes (Fig. 6C) and the data from Galili et al. (1992), we conclude that three subfamilies of the *RbcS* gene can be assigned to the short arms of homoeologous group 2 chromosomes and one subfamily to the long arms of homoeologous group 5 chromosomes. These genes are designated as shown in Table 3.

Seventeen to 21 fragments of common wheat DNA digested with three restriction enzymes, *Dra*I, *Hin*dIII

Table 3 Designation of RFLP loci of photosynthesis-related genes and their chromosome locations in common wheat (? chromosome locations were not determined)

Gene for	Gene symbol	Chromosomal location	Fragment size (kbp)
Phytochrome A (Type I protein)	*XPhyA-4A-Dra*I	4AL	7.7
	*XPhyA-4B-Dra*I	4BS	5.3
	*XPhyA-4D-Dra*I	4DS	9.4
Phosphoenolpyruvate carboxylase (PEPC)	*XPpc-7A-Eco*RI	7AL	9.3
	*XPpc-7B-Eco*RI	7BL	8.1
	*XPpc-7D-Eco*RI	7DL	5.2
	*XPpc-3A-Eco*RI	3AS	16.1
	*XPpc-3B-Eco*RI	3BS	17.8
	*XPpc-3D-Eco*RI	3DS	7.4
Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco)	*XRbcS-2A-Hin*dIII	2AS	8.7
	*XRbcS-2A-Hin*dIII	2AS	3.5
	*XRbcS-2A-Hin*dIII	2AS	3.2
	*XRbcS-2A-Hin*dIII	2AS	1.9
	*XRbcS-2A-Hin*dIII	2AS	0.6[b]
	*XRbcS-2B-Hin*dIII	2BS	12.9[a]
	*XRbcS-2B-Hin*dIII	2BS	12.1[a]
	*XRbcS-2B-Hin*dIII	2BS	8.7
	*XRbcS-2B-Hin*dIII	2BS	4.7[a]
	*XRbcS-2B-Hin*dIII	2BS	3.5
	*XRbcS-2B-Hin*dIII	2BS	3.2
	*XRbcS-2B-Hin*dIII	2BS	1.9
	*XRbcS-2B-Hin*dIII	2BS	1.3[a]
	*XRbcS-2D-Hin*dIII	2DS	9.8[a]
	*XRbcS-2D-Hin*dIII	2DS	7.3
	*XRbcS-2D-Hin*dIII	2DS	2.5[a]
	*XRbcS-2D-Hin*dIII	2DS	1.9
	*XRbcS-5A-Hin*dIII	5AL	11.0
	*XRbcS-5A-Hin*dIII	5AL	5.8
	*XRbcS-5B-Hin*dIII	5BL	9.8
	*XRbcS-5B-Hin*dIII	5BL	5.8
	*XRbcS-5D-Hin*dIII	5DL	11.0[a]
	*XRbcS-5D-Hin*dIII	5DL	7.3
	?	?	1.0[b]
Light-harvesting antenna complex for photosystem II (CAB)	*XLhcb1-1A-Hin*dIII	1AL	5.9
	*XLhcb1-1B-Hin*dIII	1BL	8.8
	*XLhcb1-1D-Hin*dIII	1DL	7.3

[a] Signal intensities were distinguishable between aneuoploids and the pure line of common wheat in the present investigation. The other chromosome locations of the *RbcS* gene were cited from Galili et al. 1992

[b] The DNA fragments newly observed in the present investigation

and *Xba*I, were found after Southern hybridization with the *Lhcb1*1* gene probe. By using aneuploid lines, we determined the chromosome locations of three bands. The other bands were not missing in any of the aneuploid lines, mainly because of band multiplicity (Figs. 5 and 6D). Fragments of 8.8 kbp, 7.3 kbp and 5.9 kbp produced by *Hin*dIII digestion were assigned to 1BL, 1DL and 1AL, respectively (Table 3).

RFLP analysis of photosynthesis-related genes in wheat species

Restriction fragment length polymorphisms (RFLPs) of the four photosynthesis-related genes in polyploid wheats and their presumed ancestral species (Table 1) have been analyzed.

With the *PhyA* gene probe, 12 different fragments in *T. aestivum* cv Chinese Spring were detected by Southern hybridization of fragments obtained with four restriction enzymes, *Bam*HI, *Dra*I, *Eco*RI and *Xba*I. The chromosome arm locations of all these fragments were determined in common wheat. A typical hybridization profile of restriction fragments obtained by digestion with *Xba*I is shown in Fig. 7A as an example. No RFLPs of the *PhyA* gene were found among hexaploid wheats. The tetraploid wheats, except for Timopheevi, showed an identical pattern to the hexaploid wheats concerning the bands produced by the A and B genomes. *T. timopheevi* revealed an A genome pattern identical to that of the other polyploid wheats, but showed different fragment patterns from those of Emmer wheats (B genome). In comparison to polyploid species, diploid species showed great variation in fragment patterns.

All fragments found in Chinese Spring digested with the two enzymes *Eco*RI and *Hin*dIII after hybridization with the *Ppc* gene probe were allocated to the chromosome arms of homoeologous groups 3 and 7. Little RFLP was found in either hexaploids or tetraploids (Fig. 7B). On the other hand, large variability of the restriction fragments was revealed among the diploid groups.

Of 61 bands that hybridized with the *RbcS* gene probe after digestion with four restriction enzymes,

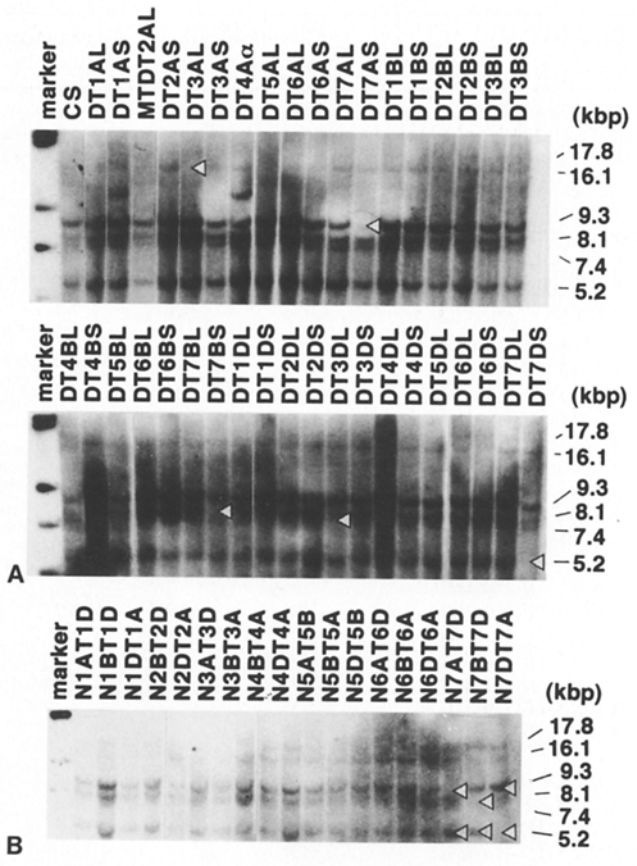

Fig. 3A, B Southern hybridization patterns of aneuploid lines probed with *Ppc* after digestion with *Eco*RI. Ditelosomic lines (**A**) and nullitetrasomic lines (**B**). *Triangles* indicate missing and increasing bands

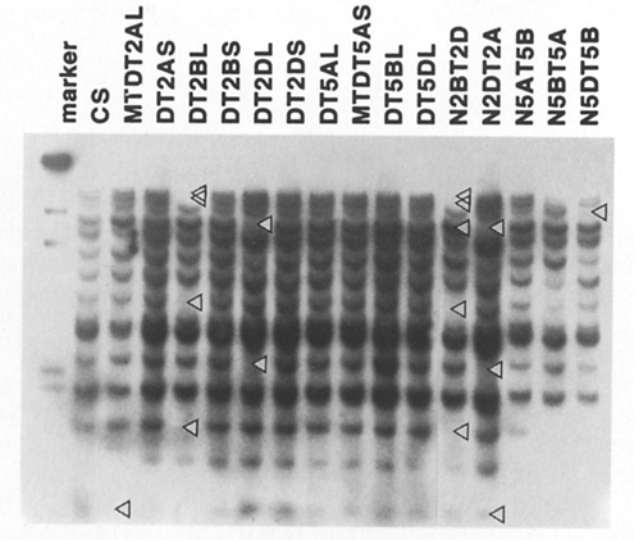

Fig. 4 Southern hybridization patterns of aneuploid lines probed with *RbcS* after digestion with *Eco*RV. Ditelosomics (*DT*) and nullitetrasomics (*NT*) of homoeologous groups 2 and 5 were used

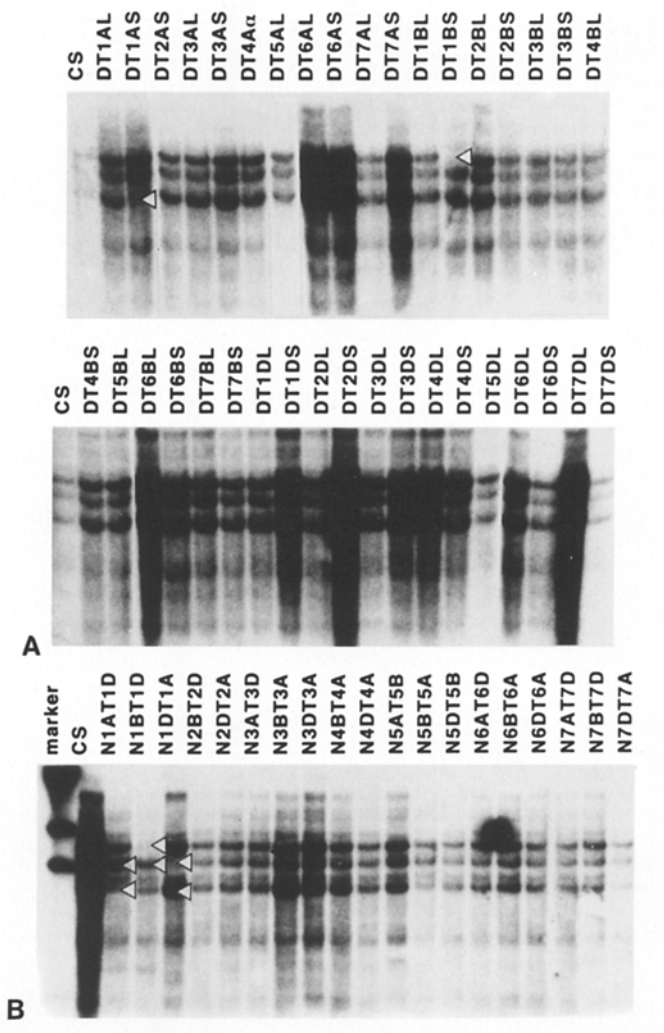

Fig. 5A, B Southern hybridization patterns of aneuploid lines probed with *Lhcb1*1* after digestion with *Hin*dIII. *Triangles* indicate missing and increasing bands

*Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I, 34 bands (55.7%) were found to be localized in Chinese Spring chromosomes. The genes are located on the short arms of homoeologous group 2 and the long arms of homoeologous group 5 chromosomes. RFLPs of the gene among wheat species are presented in Fig. 7 C. The *RbcS* gene is a multigene family with approximately 100 copies in hexaploid wheat. Wide variations of RFLPs for *RbcS* were found among wheat species, even for polyploid wheats (92.4% fragment similarity in hexaploid wheats). Two variant fragments in comparison to Chinese Spring were detected among hexaploid wheats; one is in 2BS of *T. spelta*, *T. compactum* and *T. macha*, and the other is in 2DS of *T. sphaerococcum*. All other fragments were identical with each other. Tetraploid wheats revealed more variations than hexaploid wheats. Great variability of RFLPs among diploid species was found.

Only 14.5% of the fragments (8 of 55 bands) that hybridized with the *Lhcb1*1* gene in Chinese Spring, digested with the three restriction enzymes, *Dra*I, *Hin*dIII and *Xba*I, were assigned to chromosome locations. RFLPs of the genes among wheat species are shown in

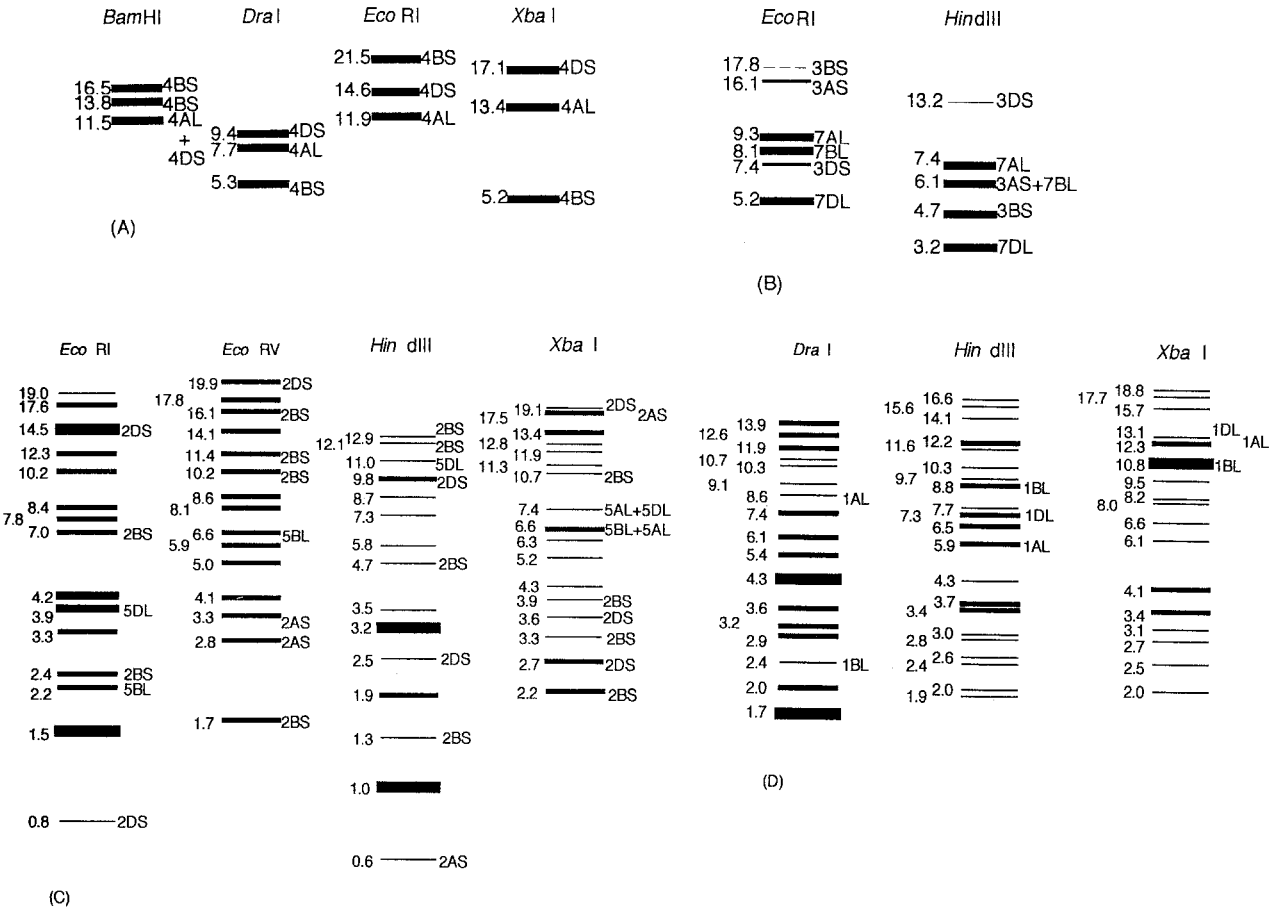

Fig. 7 D. RFLPs of polyploids were conservative except for the genes located in 1AL of *T. macha* and the G genome of *T. timopheevi*. More variations in RFLP were found in tetraploids than hexaploids. About a half of the fragments produced from *T. timopheevi* commonly shared similarity with those from Emmer wheat, suggesting a similarity of the A genome and a difference of the G genome between them. Great variations in RFLP were again observed among diploid species.

Since all fragments that hybridized to the *PhyA* and *Ppc* gene probes were assigned to chromosomes, fragment similarity was calculated in a total of six restriction enzyme-probe combinations so as to estimate the phylogenetic relationships of these genes among 33 genome types of 19 wheat species (Table 2). Total numbers of hybridized fragments and fragment similarities are presented in Table 4. Based on the fragment similarities, a dendrogram was constructed by the UPGMA method, as depicted in Fig. 8. The 33 genome types of the 19 species clustered into three major groups: the A genome group, including *T. monococcum*, *T. boeoticum*, *T. urartu* and the A genomes of polyploid wheats; the B genome group, including the B and G genomes of polyploid wheats and *Ae. speltoides*; and a third group including the S and D genomes, with the exception of the S genome of *Ae. speltoides*. Fragment variability of polyploid wheats was lower in comparison to that of diploids. No RFLPs within the A and D genomes of polyploids were found, while some variations among B genomes were detected, indicating the variability of fragment pattern in the B genome of polyploids. The fragment patterns of the A genome of polyploids most closely resembled those of *T. urartu* (Fig. 8), suggesting that *T. urartu* is the most plausible A genome donor of polyploid wheats. The fragment patterns of the B genome in hexaploid wheats, except *T. spelta*, were identical to those of the B genome in Emmer wheat, and similar to those of *Ae. speltoides*. Those of the G genome in *T. timopheevi* were relatively distant from the B genome group (Fig. 8). It is noteworthy that great variation for RFLPs were found in S genome group so that this group was divided into two major clusters. Additionally, the fragment patterns of *Ae. aucheri*, which was collected in Turkey (KT116-1 as collection number of KIBR), differed markedly from those of other S genome group members.

Fig. 6A–D Ideograms and chromosome assignments of Southern hybridization profiles of *Triticum aestivum* cv Chinese Spring (CS) probed with the (**A**) *PhyA*, (**B**) *Ppc*, (**C**) *RbcS* and (**D**) *Lhcb1*1* genes. Molecular sizes of DNA fragments are given on the left side, and chromosome locations of individual fragments are indicated on the right side

Based on the Southern hybridization patterns with photosynthesis-related genes, the average number of DNA fragments per genome at three ploidy levels were calculated (Table 5). It should be emphasized that the

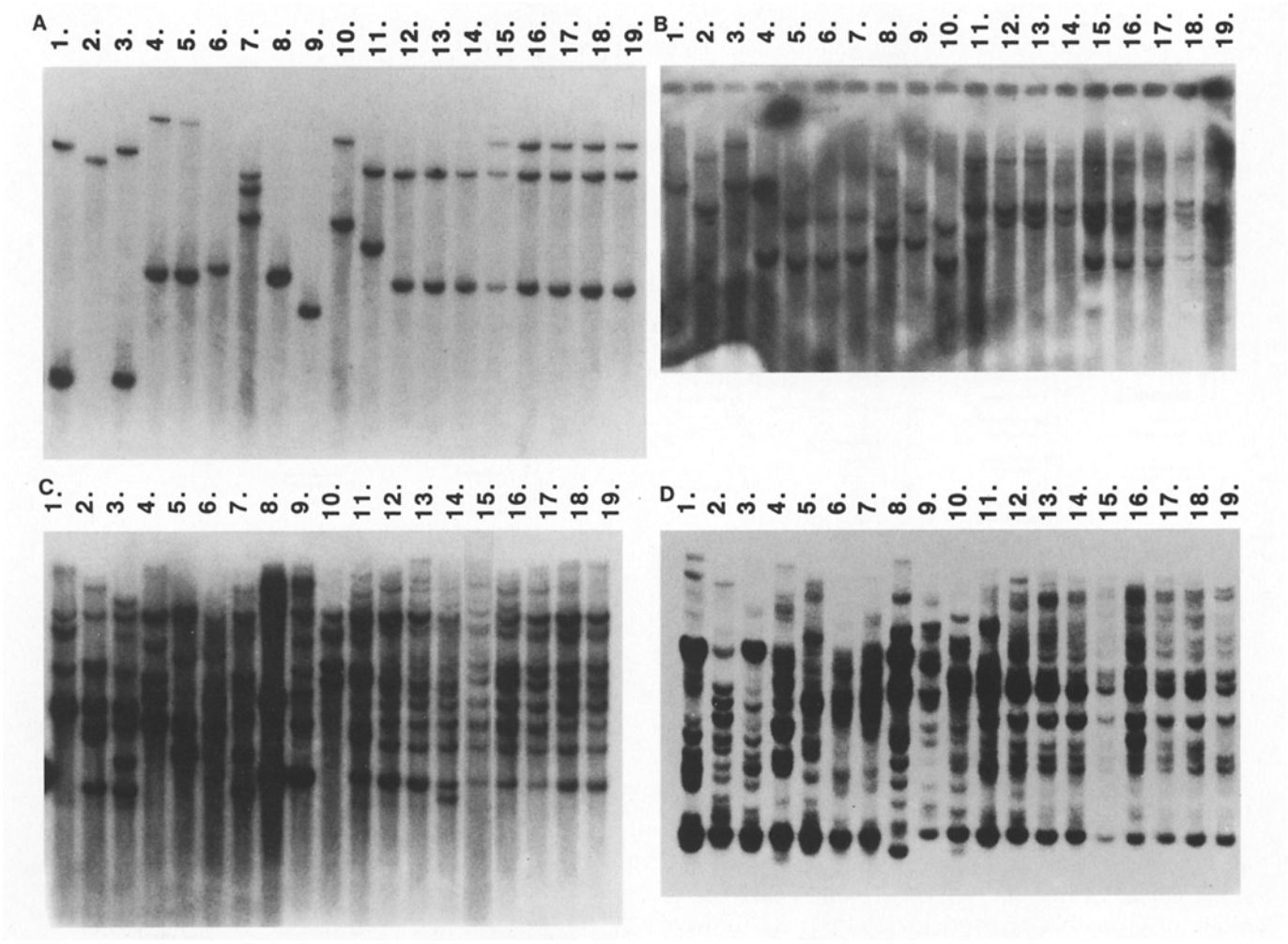

Fig. 7A–D RFLPs of polyploid wheat and their presumed ancestors probed with the (**A**) *PhyA*, (**B**) *Ppc*, (**C**) *RbcS*, and (**D**) *Lhcb1*1* genes. For code numbers of wheat species, see Table 1

average number of fragments that hybridized with photosynthesis-related genes per genome decreased in proportion to the increase in ploidy level, i.e., diploids > tetraploids > hexaploids. A reduction in the number of hybridized DNA fragments with an increased ploidy level in wheats was found for all genes examined. Furthermore, variability for numbers of hybridized fragments was greater in diploids than in polyploids. The numbers of hybridized fragments of S genome plants were especially different from one species to another: those of *Ae. sharonensis* (S^l genome) were the least variable among diploids for every gene, compared to polyploids, while those of *Ae. bicornis* were the most variable among S genome species. It is striking that the numbers of hybridized fragments of the A genome were much greater than those of most of the S genome, involving every gene comparison in the case of *Ae. bicornis*. On the average, fragment numbers per genome of *T. urartu*, *Ae. speltoides* and *Ae. squarrosa*, which are the most plausible candidates for the A, B and D genome donors of polyploid wheats, were almost double in every comparison to the genes of hexaploids. Thus, the fragment patterns of polyploid wheats suggest restricted diversity during polyploidization. But, there was an interesting exception.: *RbcS* genes located on 2BS were both more retained and more variable than other genes in polyploids, maintaining the same order of fragment numbers as those in diploids (Table 3).

Discussion

We have examined copy numbers and determined chromosome locations of four photosynthesis-related genes, *PhyA*, *Ppc*, *RbcS* and *Lhcb1*1*, in the wheat genome. These genes were chosen depending on their copy number (a single or low copy number of *PhyA3*, a small multigene family in the case of *Ppc* and a multigene family for both *RbcS* and *Lhcb1*1*) and their functional characteristics. We used probes specific for the conserved region of the coding sequence of the individual genes after a homology search so as not to underestimate the copy number of the genes, and hybridized to the wheat genomic DNAs under stringent conditions so as to minimize the hybridization signals produced by pseudogenes.

Table 4 No. of commonly shared bands (upper right half) and fragment similarity (lower left half) between each pair of 33 genome types of 19 wheat species in Southern hybridization patterns probed with the *PhyA* and *Ppc* genes (Total number of hybrid bands in each genome type is given in italics)

Genome type	Amo	Aur	Abo	S^b	S^s	S^{sh}	S^l	S	Sau	Dsq	A^l	Gti	Bds	Bdu	B^2	Bsa	D^3
Amo	*16*	4	10	2	2	1	2	1	1	3	3	1	3	2	3	3	2
Aur	29.6	*11*	1	1	1	1	2	0	1	2	5	1	1	1	1	1	2
Abo	69.0	8.3	*13*	2	0	1	2	1	1	1	2	1	1	1	2	1	1
S^b	13.3	8.0	14.8	*14*	1	3	3	1	0	1	0	1	2	2	2	2	0
S^s	14.8	9.1	0.0	8.0	*11*	1	3	2	0	4	1	0	3	2	2	2	3
S^{sh}	8.3	10.5	9.5	27.3	10.5	*8*	3	1	0	2	1	0	1	0	0	0	1
S^l	12.9	15.4	14.3	20.7	23.1	26.1	*15*	1	0	5	1	1	2	1	1	1	3
S	8.0	0.0	9.1	8.7	20.0	11.8	8.3	*9*	2	1	0	1	4	3	3	3	1
Sau	0.0	11.1	10.0	0.0	0.0	0.0	0.0	25.0	7	0	0	0	0	0	0	0	0
Dsq	21.4	17.4	8.0	7.7	34.8	20.0	37.0	9.5	0	*12*	1	0	3	2	2	2	8
A^l	25.0	52.6	19.0	0.0	10.5	12.5	8.7	0.0	0	10.0	*8*	0	1	0	1	1	1
Gti	7.4	9.1	8.3	8.0	0.0	0.0	7.7	10.0	0	0.0	0.0	*11*	3	3	3	3	0
Bds	24.0	10.0	9.1	17.4	30.0	11.8	16.7	44.4	0	28.6	11.8	30.0	*9*	7	8	8	1
Bdu	16.0	10.0	9.1	17.4	20.0	0.0	8.3	33.3	0	19.0	0.0	30.0	77.8	*9*	8	7	0
B^2	24.0	10.0	18.2	17.4	20.0	0.0	8.3	33.3	0	19.0	11.8	30.0	88.9	88.9	*9*	8	0
Bsa	24.0	10.0	9.1	17.4	20.0	0.0	8.3	33.3	0	19.0	11.8	30.0	88.9	77.8	88.9	*9*	0
D^3	16.0	21.1	9.5	0.0	31.6	12.5	26.1	11.8	0	80.0	12.5	0.0	11.8	0.0	0.0	0	*8*

[1] Three genome types, Amo, Aur and Abo, are included, because no RFLPs were found among them

[2] In B, Bdm, Bae, Bsm, Bco and Bma are included, because no RFLPs were detected among them

[3] All D genomes of hexaploid wheats are included in the column, because fragment patterns among them were identical with each other

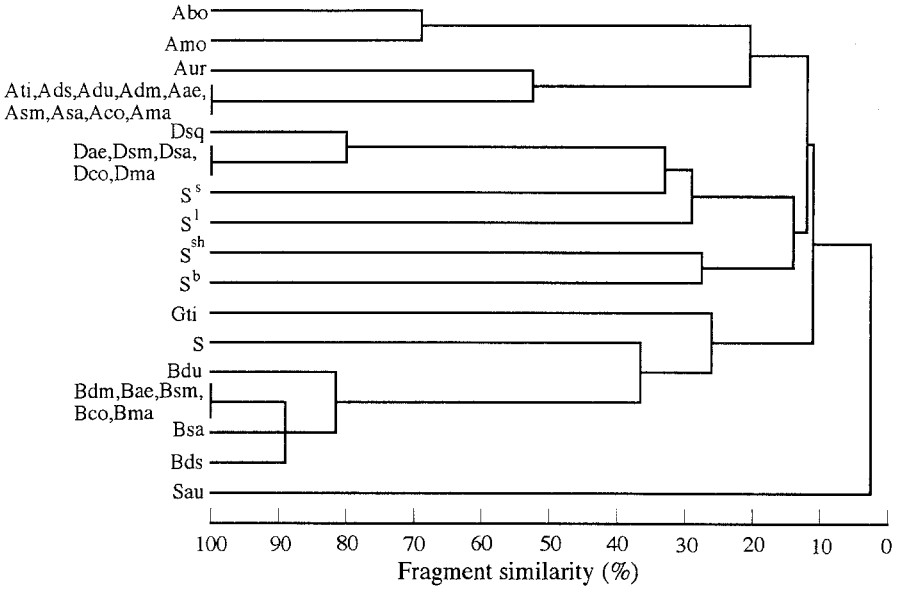

Fig. 8 A dendrogram showing phylogenetic relationships of the *PhyA* and *Ppc* genes among 33 genomes of 19 wheat species, based on fragment similarities and constructed by UPGMA. For genome abbreviations, see Table 1

The copy number of the *PhyA* gene was estimated to be one per haploid genome by slot-blot and Southern-hybridization analyses, and the gene was assigned to the arms of the homoeologous group 4 chromosomes of common wheat. This copy number is in good agreement with those reported in other plants such as oat (Hershey et al. 1985), pea (Sato 1988), rice (Kay et al. 1989), maize (Christensen and Quail 1989), and *Arabidopsis thaliana* (Sharrock and Quail 1989). It has recently become apparent that multiple phytochromes are found in plant cells and are encoded by a small family of divergent *Phy* genes. In fact, five genes, namely *PhyA*, *PhyB*, *PhyC*, *PhyD* and *PhyE*, were isolated in *Arabidopsis thaliana* (Quail 1991), three of which (*PhyA*, *PhyB* and *PhyC*) have been sequenced (Sharrock and Quail 1989) and mapped to chromosomes 1, 2, and 5, respectively (Chang et al. 1988). Molecular characterization suggests that the *PhyA* and *PhyB* genes correspond to the polypeptides previously designated as Type 1 and Type 2 phytochromes (Furuya 1989). The Type 1 phytochrome

Table 5 Average number of DNA fragments per genome at three ploidy levels (for genome formula, see Table 1, the average numbers of DNA fragments produced from each genome were calculated from the data presented in Table 4)

Gene	Diploid				Tetraploid			Hexaploid
	A	S[a]	D	Total	AB	AG	Total	ABD
PhyA	1.92	1.63	2.0	1.75	1.13	1.25	1.16	1.0
Ppc	2.83	2.08	2.0	2.30	2.0	2.5	2.13	1.80
RbcS	9.0	8.63	6.25	8.5	6.04	6.0	6.03	5.05
*Lhcb1*1*	11.6	14.1	12.3	12.4	8.17	7.83	8.1	6.1

[a] S, S^b, S^l, S^s genomes are included

is known as photoreceptor molecule which is reversibly interconvertible between inactive (Pf) and active (Pfr) forms by red and far-red light. The Pfr form initiates the signal transduction process. Expression of photosynthesis-related genes such as *RbcS*, *Cab* and *Phy* is itself controlled by these signal perception molecules (e.g., Gilmartin et al. 1990). The determination of the chromosome location of the *PhyA* gene in wheat supports the previous finding that the group 4 chromosomes are a site of major control of the levels of Rubisco (Jellings et al. 1983). At least four more genes for various types of phytochrome remain to be mapped in the wheat genome.

The copy number of the *Ppc* gene was estimated by slot-blot analysis to be several in diploid wheat, 5–10 in tetraploid wheat and 10–25 in common wheat. On the other hand, Southern hybridization of wheat DNA digested with various restriction enzymes gave only a few hybridization signals per genome, indicating two loci per genome (Fig. 6B and Table 3). This estimated gene number is comparable to those numbers for rice (Matsuoka and Yamamoto 1989) and maize (Kawamura et al. 1990; Cretin et al. 1991). Hybridized fragments were assigned to the short arm of chromosome 3 and the long arm of chromosome 7 in common wheat. Previously, Chao et al. (1989) determined the location of one *Ppc* locus on the 7L chromosome of common wheat. In the present study, we assigned another locus of the gene to the chromosome 3S. Although PEPC (phosphoenolpyruvate caboxylase) is well known as a key enzyme for CO_2 fixation in C_4 photosynthesis (O'Leary 1982), it is also involved in various physiological processes even in C_3 plants (Ting and Osmond 1973; Latzko and Kelly 1983). In maize, a typical C_4 plant, at least three isoforms were suggested (Kawamura et al. 1990): (1) a C_4-form prevalent in green leaves, (2) a C_3-form prevalent in etiolated leaves, and (3) a root-form. The genes encoding these different types of PEPC have partial homologies (Kawamura et al. 1990; Cretin et al. 1991). But, it is still uncertain as to which loci of common wheat correspond to which forms of PEPC.

RbcS is known to be a multigene family in higher plants. In the present study, the copy number of the gene was estimated to be 25–50 in diploid wheats, approximately 50 in tetraploids, and approximately 100 in common wheats. Restriction endonuclease digestion with four enzymes produced 8.5 fragments, on average, in diploids, 12.1 in tetraploids, and 15.1 in hexaploids after Southern hybridization. This suggests that each fragment contains several copies of the sequence. Actually, the signal intensity of *RbcS* seems to be stronger than that of a single gene such as *PhyA* (Fig. 4). About half of the bands were assigned to a chromosome arm in the present study. Recently, Galili et al. (1992) overcame this problem by using 3'-specific probes. They were successful in classifying all 22 genes (14 fragments digested with *Hin*dIII) into four subfamilies, namely SF-1, SF-2, SF-3, and SF-4, whose numbers are similar to those reported in other plants such as pea (Fluhr et al. 1986), tomato (Sugita et al. 1987), and potato (Wolter et al. 1988). Since their fragment pattern obtained with *Hin*dIII was identical with that of ours (we found two more fragments), the gene designation is summarized in Table 3. These lines of evidence suggest that: (1) the wheat genome contains four subfamilies of the *RbcS* gene, three of which are located on chromosome 2B, (2) each subfamily contains a few genes, and (3) each gene is represented by a few copies of the sequence. The multiplicity and heterogeneity of *RbcS* indicate that *RbcS* is an active gene (Dean et al. 1987), and is differentially expressed in different tissues and in different developmental stages in response to environmental stimuli such as light (Dean et al. 1989).

The CAB gene is also known to be a multigene family. The accumulation of sequence data on this gene shows that there are at least ten distinct types of CAB (Green et al. 1991), four of which are assigned to PSI (*Lhca1–Lhca4*), and six to PSII (*Lhcb1–Lhcb6*). We used *whAB1.6* (Lamppa et al. 1985), which encodes the Type I protein for PSII, as a probe. Since hardly any homologies were found among the six types of the *Lhcb* gene (Pichersky et al. 1991), we conclude that the *Lhcb1* gene was investigated in the present study. The copy number of this gene was estimated to be 10–20 in diploid and tetraploid wheats and about 50 in hexaploid wheat. Restriction endonuclease digestions with three enzymes produced an average of 12.4 fragments in diploids, 16.1 in tetraploids, and 18.2 in hexaploids after Southern hybridization. This suggests that about ten *Lhcb1* genes are contained in the haploid genome of wheat and that some fragments overlap in polyploid wheats because of their sequence similarity. This estimation of the copy number of the *Lhcb1* gene is similar to that for other plants such as petunia (Stayton et al. 1986) and soybean (Walling et al. 1988), but is more than that of rice (Luan and Bogorad 1989). The overlapping fragments resulted in a failure to obtain precise chromosome assignments for this gene and only one gene was successfully assigned a chromosome arm location by the use of aneuploids of common wheat. This suggests less variability of the *Lhcb1*1* gene in comparison to *RbcS* and the recent diversification of the *Lhcb1* gene among the wheat group. Specific probes for non-coding regions and/or

other aneuploid series, such as barley addition lines, are required to distinguish differentiated genes.

RFLP patterns of polyploid wheats were conservative for the four photosynthesis-related genes, although more variations were observed in tetraploid wheats. As judged from the fragment similarities (Fig. 8), *T. urartu*, *Ae. sepltoides*, and *Ae. squarrosa* are the most plausible donors of the A, B and D genomes of polyploid wheats, confirming previous data (Kihara 1944; McFadden and Sears 1944; Nishikawa 1983; Dvorak et al. 1988; Galili et al. 1991). But, RFLPs corresponding to the B genome in polyploids were more variable than those for other genomes, both in terms of the number of fragments produced by Southern hybridization and the polymorphisms of those fragments. Actually, although RFLPs corresponding to the A genome of Timopheevi wheat were similar, fragment patterns of the G genome showed high divergence from those of other members of the Emmer wheat group (Fig. 8). These lines of data support the idea that the diversification of the genomes of wheat and its relatives has mainly occurred at the diploid level, and that diphyletic crosses have led to the production of allopolyploid wheat species (Kihara 1954; Tsunewaki et al. 1992). In comparison to the fragment variability of polyploid wheats, diploids revealed great variations for fragment polymorphisms. Fragment patterns produced by the S genome especially, showed extreme variability. In fact, RFLP patterns of S genome members could be divided into two groups, in which the B genome group including *Ae. speltoides* and the G genome of *T. timopheevi* constituted one major group distant from the other S genome representatives (Fig. 8). This classification of wheat species coincides the phylogenetic relationships estimated by RFLP analyses of chloroplast DNA (Ogihara and Tsunewaki 1988) and mitochondrial DNA (Terachi and Tsunewaki 1992), but does not support the classical data (Kihara 1954). Critical molecular analyses for S genome diversity using DNA probes isolated from single-copy regions as well as repetitive sequences are required to understand the differentiation of S genomes.

It is striking that not only low copy genes but also multigenes in the wheat genome show reduced copy numbers in proportion to the increase in ploidy level (Tables 2 and 5), suggesting some mechanism(s) for keeping to a minimum the number of genes involved in photosynthesis. This proposition is further supported by rDNA and protein studies of polyploid wheats, null alleles of the rDNA region (Appels et al. 1980), and isozymes such as *Est-5* (Ainsworth et al. 1987), *Gpi-1* (Chojecki and Gale 1982) and the glutenin storage protein (Thompson et al. 1983). It has also been reported that the DNA contents of polyploid wheats are not proportional to ploidy level, but are gradually reduced in relative amount along with the increase of ploidy (Bennet et al. 1982; Furuta et al. 1986). Consequently, it is likely that some mechanisms(s) for restricting the DNA content of polyploids operates on the entire genome and involves both coding and non-coding regions. But, there is an interesting exception. The number of genes for *RbcS* located on 2BS is retained at the diploid level, whereas that at other locations is restricted (Tables 3 and 5). A similar case has been reported for the structure and expression of rDNA (18*s*-5.8*s*-25*s* rRNA) (Gerlach and Bedbrook 1979; Martin et al. 1982). These findings suggest that the mechanism(s) for maintaining a minimum gene number in polyploids does not operate equally on the individual genes and that some types of gene can escape from the selection pressure(s) for restricting polyploid genome size.

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