Detection of ribosomal RNA genes in soybean, *Glycine max* (L.) Merr., by *in situ* hybridization[1,2]

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Corresponding Editor: R. L. Phillips

Received July 27, 1988

Accepted May 9, 1989

SKORUPSKA, H., ALBERTSEN, M. C., LANGHOLZ, K. D., and PALMER, R. G. 1989. Detection of ribosomal RNA genes in soybean, *Glycine max* (L.) Merr., by *in situ* hybridization. Genome, **32**: 1091–1095.

A biotinylated maize rRNA probe was hybridized to soybean nuclei. Hybridization was detected by using a streptavidin horseradish peroxidase biotin system. The procedure used enabled detection of heterologous complementary 18S and 25S rRNA coding genes in soybean. In diploid cultivars 'Hark' and 'Lincoln' a single pair of satellited chromosomes was present and two binding sites were detected at interphase. In plants trisomic for the satellited chromosome, three sites were observed, and in tetraploid nuclei, four sites were seen. The *in situ* hybridization results indicated that, for ribosomal RNA genes, *Glycine max* behaves as a diploid. We discuss the possibility of loss of a pair of satellited chromosomes in the evolution of soybean.

Key words: biotin-labeled probe, rRNA genes, ploidy, *Glycine max*.

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Une sonde d'ARN$_r$ de maïs biotinylée a été hybridée à des noyaux de soya. L'hybridation a été décelée par l'utilisation d'un système de biotine et de streptavidine peroxydase du raifort. La méthode utilisée a permis de détecter les gènes hétérologues complémentaires qui codent l'ARN$_r$ des sous-unités 18S et 25S chez le soya. Les cultivars diploïdes ‹Hark› et ‹Lincoln› ne possèdent qu'une paire de chromosomes satellites et deux sites de liaisons ont été décelés à l'interphase. Chez les plantes qui sont trisomiques pour les chromosomes satellites, trois sites ont été observés, alors que quatre sites ont été observés dans les noyaux tétraploïdes. Les résultats des hybridations *in situ* indiquent que, en ce qui concerne les gènes de l'ARN des ribosomes, le *Glycine max* se comporte comme une plante diploïde. La possibilité qu'il y ait eu une perte d'une paire de chromosomes satellites au cours de l'évolution du soya fait l'objet d'une discussion.

Mots clés : sonde marquée à la biotine, gènes de l'ARN$_r$, ploïdie, *Glycine max*.

[Traduit par la revue]

Introduction

Nonisotopic *in situ* hybridization has been used to map highly repeated DNA sequences in diverse organisms, ranging from *Drosophila* (Langer-Safer et al. 1982) to wheat (Rayburn and Gill 1985*a*). Biotin-labeled probes have been shown to give higher resolution but lower sensitivity when compared with ^{3}H autoradiography (Mitchell et al. 1986). Sensitivities have been improving gradually, with published reports of detection as low as 1 kilobase (kb) on human chromosomes (Garson et al. 1987). In plants, this technique was applied by Rayburn and Gill (1985*a*). They mapped, with a biotin-labeled probe, specific DNA sequences on common wheat chromosomes and other cereal species (Rayburn and Gill 1985*a*, 1985*b*, 1986*a*, 1986*b*, 1987). Albertsen and Langholz (1987) used a biotin-labeled knob sequence and ribosomal RNA (rRNA) probes to characterize somatic maize chromosomes.

The tandemly repeated multigene family encoding 18S and 25S ribosomal RNAs has been highly conserved during evolution. In soybean (*Glycine max* (L.) Merr.), the rRNA gene can be characterized genetically by total cistronic length of the gene unit of 7.8 kb and by a constant repeat length (Varsanyi-Breiner et al. 1979; Doyle and Beachy 1985). Friedrich et al. (1979) and Eckenrode et al. (1985) found 1000–1600 copies of rRNA genes per diploid nucleus. The complete rRNA gene unit of maize is 9 kb long, has 3 300 – 23 000 copies per diploid nucleus, and exhibits repeat-length heterogeneity (Buescher et al. 1984; Toloczyki and Feix 1986).

Detection of ribosomal genes by *in situ* hybridization methods could provide an important insight into the evolution and origin of soybean. Our objective was to detect ribosomal RNA gene sequences in soybean mitotic cells by using biotin-labeled maize ribosomal DNA.

[1]Mention of a trademark or proprietary product does not imply its approval to the exclusion of other products that also may be suitable.

[2]Joint contribution: Agricultural Research Service, U.S. Department of Agriculture, and Journal Paper J-13070 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA 50011, U.S.A. Project 2763.

Printed in Canada / Imprimé au Canada

Materials and methods

Genetic stocks

Two diploid ($2n = 2x = 40$) soybean cultivars, 'Hark' and 'Lincoln', a colchicine-induced autotetraploid ($2n = 4x = 80$) cultivar, 'Lincoln' (Porter and Weiss 1948), and trisomic S ($2n = 41$) 'Hodgson' (Sadanaga and Grindeland 1984) were used for *in situ* hybridization procedures. The diploid, tetraploid, and trisomic S genotypes had, respectively, two, four, and three satellited chromosomes.

In situ *hybridization procedure*

Methods for soybean *in situ* hybridization were based on procedures given by Rayburn and Gill (1985*a*) for wheat, as modified by Albertsen and Langholz (1987) for *in situ* hybridization in maize.

Tissue preparation technique

Root tips were obtained from seedlings growing on germination paper in a growth chamber at 28°C. Root tips were harvested when the root was 6–8 cm long and were pretreated with CMD solution (0.007% cyclohexamide, 0.2% monobromonaphthalene, and 0.05% dimethylsulfoxide). After pretreatment, root tips were fixed overnight in glacial acetic acid – ethanol (1:3) and then incubated at 13°C overnight in 45% acetic acid – 2N HCl (9:1) solution. Cell walls were digested in prechilled 0.5% pectinase for 3.5 h at 4°C. Root tips were stored in 70% ethanol at 4°C. Squashes in 45% acetic acid were prepared using acid-cleaned, gelatin-subbed slides. Prepared slides were stored at −70°C.

Nick translation procedure

A nick translation kit was obtained from ENZO Biochem. Inc., New York (catalogue No. EBP-803-8). The plasmid pZmr1 hybridizing specifically to maize ribosomal genes was labeled with biotin 11 dUTP (Albertsen and Langholz 1987). Label incorporation was accomplished at 19°C for 2.5 h. The biotinylated DNA probe was purified by a Sephadex G-50 spin column.

Hybridization

The hybridization mixture was prepared with deionized formamide (50 μL), 50% dextran sulfate (20 μL), 20× SSC (10 μL), 10 μL/mL calf thymus DNA (4 μL), and 20 μg/mL of DNA probe. The mixture was heated for 10 min at 100°C and was chilled on ice for at least 5 min.

Slides air dried for 1 h were denatured in 70% formamide in 2× SSC at 60°C for 3.5 min and rapidly dehydrated in a cold ethanol series (70%, 95%, 100%) at −20°C. Twenty microlitres of the hybridization mixture was added to each slide. Slides were incubated for 3 h at 37°C.

Detection of hybridization

After incubation, slides were washed successively in 2× SSC buffers, the first time for 5 min at 45°C, the second time for 10 min at 50°C, and the third time for 5 min at room temperature. Finally, the slides were washed for 2 min in 0.1% Triton X-100 in PBS buffer and in PBS buffer at room temperature for 5 min. After washing, the tissue was exposed (at 37°C for 30 min) to biotin-binding streptavidin with 0.6% horseradish peroxidase. Detection kit, Detech-HRP, was obtained from ENZO Biochem. Inc., New York (catalogue No. EBP-820-1). Biotinylated DNA was visualized by the formation of a precipitate with the chromogen 3-amino-9-ethylcarbozole (AEC). This final detection solution (3 mL sodium acetate, 75 μL 1% H_2O, 60 μL AEC) was applied to the slides (500 μL/slide) and incubated in darkness for 30 min at room temperature.

Staining

After final incubation and washing in phosphate-buffered saline (PBS), the slides were stained with 3% Leishman stain for 3–5 min until the desired intensity was reached.

Results

Repeated DNA sequences can be revealed by *in situ* hybridization on interphase chromosomes as well as on metaphase chromosomes (Rayburn and Gill 1987). We observed distinct hybridization signals in interphase soybean mitotic cells by using a maize rRNA gene probe (Figs. 1–4). Utilizing interphase nuclei in soybean facilitated quick assessment of rRNA gene sequences inasmuch as the number of good chromosome preparations was severely limited by the hybridization procedure. Two hybridization sites were detected in the nuclei of the diploid cultivars 'Hark' and 'Lincoln' (Fig. 1). These hybridization sites were distinct, well defined, and similar in size. We did not observe any secondary binding sites in the soybean interphase nuclei that we examined. Other observations provided evidence of the additive response of hybridization with the number of satellited chromosomes. In trisomic S, which is aneuploid for the satellited chromosome, three hybridization sites were identified (Figs. 2 and 3). Figure 3 shows the expected close association with the nucleolus. Autotetraploid cultivar 'Lincoln' had four sites for rRNA genes in the nuclei (Fig. 4).

Discussion

Previous hybridization analysis at the nucleic acid level with a rRNA gene specific probe revealed considerable similarity of the rRNA gene unit between maize and soybean. Rivin et al. (1983) reported successful hybridization of the pGmr1 rRNA gene probe from soybean with the rRNA gene from maize. Nucleotide sequence comparison of soybean and maize 18S rRNA genes (1807 and 1905 base pairs (bp), respectively) showed 93.5% homology, with only 129 nucleotide replacements between the two genes scattered through the sequences (Eckenrode et al. 1985). The maximum number of sites observed in interphase nuclei agreed with the number of satellited chromosomes in diploid, trisomic S, and autotetraploid soybeans. This observation indicates that the satellited pair of chromosomes in *G. max* contain the rRNA genes. The number of binding sites for rRNA genes observed at the cellular level was not suppressed by the presence of additional homologous chromosomes at the trisomic or tetraploid level.

Although we did not quantify our hybridization sites, they seemed to represent sites of very strong hybridizations. This was expected, considering the high reiteration of rRNA genes and the overall conservation of rRNA gene sequences from organism to organism. We did not observe any other hybridizations, suggesting that other significant numbers of rRNA genes do not exist. Some nonisotopic *in situ* hybridization techniques have been described that give sensitivities of 1 (Garson et al. 1987) and 1.3 kb (Bhatt et al. 1988) in animal systems and of 17 kb in plant systems (Ambros et al. 1986). Other laboratories working on plants soon will achieve sensitivities that will rival those of animal systems. We do not know the lower sensitivity limit of our hybridization procedure, but because of our probe length of 9 kb and the repeated nature of the target site, a relatively small number of rRNA gene sequences seems likely to be detectable.

In contrast to some Leguminosae species, soybean has a high chromosome number that is suggestive of a polyploid evolution. According to Lackey (1980, 1981), soybean probably is derived from a diploid ancestor that has (had) $x = 11$ chromosomes. Most genera of the legume tribe Phaseolae are $2n = 22$. It is unlikely that a true diploid could lose its satellited chromosomes and still produce viable gametes. However, chromosome loss after polyploidization would eliminate

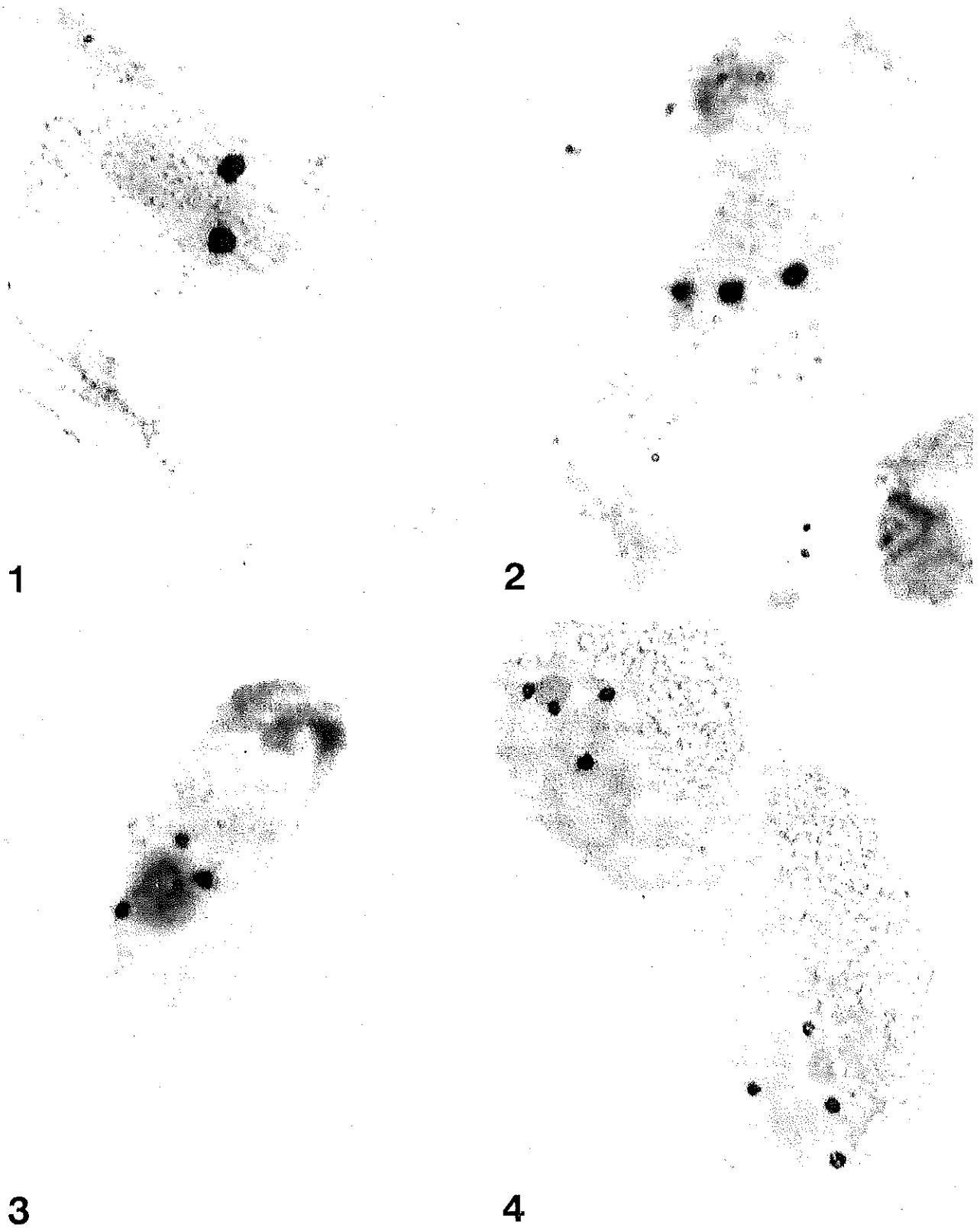

FIG. 1. Hybridization sites in somatic cell of diploid cultivar 'Lincoln'. FIGS. 2 and 3. Hybridization sites in somatic cells of trisomic S. FIG. 4. Hybridization sites in somatic cell of tetraploid cultivar 'Lincoln'.

specific genes and could be an explanation for our results. Appels et al. (1980) showed that rRNA gene elimination from the A genome of hexaploid wheat occurred after polyploidization. In soybean, we could hypothesize this occurring either after hybridization of two Phaseolae species or after an autopolyploidization event.

Another explanation for only two rRNA hybridization sites in soybean is that the additional rRNA sequences brought in by a polyploidization event were reduced or "decompensated" to a level characteristic for soybeans. Tartof (1973) described rDNA compensation in *Drosophila* as a process that increased rRNA genes in those *Drosophila* stocks that lacked a nucleolar organizer region (NOR) from one parent. However, Phillips et al. (1974) showed that this compensation did not occur in maize. In *Crepis capillaris* and *C. dioscorides* hybrids, suppression of the secondary constriction in the chromosome contributed by *C. dioscorides* does occur. The rRNA genes are not eliminated, however, but undergo transcriptional suppression (Doerschug et al. 1976). *In situ* hybridization with an rRNA gene probe should result in hybridization sites where these genes are located, even though the genes are not being transcribed.

After polyploidization, there is a strong tendency to evolve into a diploid state by chromosome rearrangement and by sequence diversification (Leipold and Schmidtke 1982). In the process of phyletic derivation, many polyploid species express diploid-like behavior, which can assure their evolutionary success (Jackson 1982). Genetically controlled diploid-like pairing patterns were documented for amphiploids, including oats (Thomas and Al-Ansari 1988) and wheat (Ceoloni and Feldman 1987). Singh and Hymowitz (1985) suggested the presence of genetic pairing control in amphiploids of the genus *Glycine* Willd., subgenus *Glycine*.

There is also a natural tendency in polyploids to revert to a diploid level of gene expression (Ferris et al. 1979). Loss of duplicate gene expression is common in polyploid plants, although the evidence in most cases is circumstantial (Haufler 1987). For most genetic markers, soybean displays disomic inheritance. Loss of genes or transcriptional suppression could be the reason for this phenomenon. Pachytene chromosome analysis has revealed that 35.84% of the soybean genome is heterochromatic; often entire arms are heterochromatic (Singh and Hymowitz 1988). Actively transcribed genes are seldom located in heterochromatic regions.

Data on reassociation kinetics of soybean genomic DNA were consistent with a polyploid origin of soybean (Gurley et al. 1979). In addition, restriction fragment length polymorphism (RFLP) analyses conducted on certain segregating F_2 populations revealed that many of the probes detected more than one locus (Keim et al. 1989). These duplicated RFLP markers could be remnants of polyploidy in soybean.

If soybean truly is a diploidized polyploid, our observations of the number of sites for 18S and 25S rRNA coding genes indicate that there has been a loss of rRNA genes during diploidization. These results are consistent with results obtained for other species that have undergone diploidization. In hexaploid wheat *T. aestivum* (AABBDD), for example, satellited chromosomes 1B and 6B possess approximately 90% of the total rRNA genes and form major nucleoli (Endo and Gill 1984; Jordan et al. 1982). Other secondary constrictions on chromosomes 5D and 1A can be observed only in metaphase under favorable conditions in some varieties (Bhowal 1972). The 5D and 1A chromosomes carry very few rRNA genes and are considered to have weak nucleolar organizers (Jordan et al. 1982; Cermeno et al. 1984). Previous *in situ* hybridization of labeled rRNA to metaphase chromosomes provided strong evidence that the diploid wheat *T. monococcum*, designated as the AA genome, possesses two pairs of nucleolar organizer chromosomes. These were identified on chromosomes 1A and 5A (Gerlach et al. 1980; Miller et al. 1983). The absence of a second nucleolar organizer in the A genome of hexaploid wheat suggests that rRNA genes have been lost from chromosome 5A (Gerlach et al. 1980). Similar phenomena of the loss of sets of NOR genes occurred in the evolution of *Avena sativa* (Rajhathy and Thomas 1974; Jellen et al. 1988). Further studies are required on rRNA genes in *G. max*, *G. soja*, and wild perennial *Glycine* species to determine if diploid-like behavior is associated with gene elimination during soybean evolution.

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