Detection of rRNA and phaseolin genes on polytene chromosomes of *Phaseolus coccineus* L. by fluorescence in situ hybridization after pepsin pretreatment

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This is the first report of fluorescence in situ hybridization (FISH) on plant polytene chromosomes. Different protease pretreatments have been tested in order to improve fluorescence in situ hybridization FISH on polytene chromosomes of a plant, *Phaseolus coccineus,* with the aim to enable the detection of low-copy genes. The structural preservation of the chromosomes and the distinctness of the FISH signals were comparatively analysed with a probe for the ribosomal RNA genes after digestion with pepsin and trypsin. The pepsin pretreatment resulted in a general loosening of chromatin with good conservation of chromosome morphology and an increased number and density of signal points. The six nucleolus organizers exhibited significant differences in condensation. The pretreatment with pepsin enabled the detection of the low-copy genes encoding the seed storage protein phaseolin.

Keywords: plant, Leguminosae, ribosomal RNA genes, seed storage protein genes, protease.

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Ceci est le premier compte rendu sur l'emploi de la technique fluorescence d'Hybridation in situ (FISH) sur les chromosomes polytènes d'une plante. Plusieurs prétraitements protéasiques ont été compares afin d'améliorer la technique FISH sur le chromosomes polytènes d'une plante, *Phaseolus coccineus*, avec comme objectif de premettre la detection de genes presents en faibles copies. A l'aide d'une sonde détectant les genes d'ARNs ribosomiques, la preservation structurelle des chromosomes et la netteté des signaux ont été compares après digestion à la pepsine ou à la trypsine. Le prétraitment à la pepsine a résulté en un relâchement general de la chromatine tout en maintenant la morphologie des chromosomes, et en une augmentation du nombre et de la densité des signaux. Les six regions organisatrices des nucléoles montrent des differences significatives en terme de condensation. Le prétaitement à la pepsine a permis de détecter des genes en faibles copies codant pour une protéine de reserve, la phaséoline.

Mots clés: plante, Leguminosae, genes d'ARNs ribosomiques, proteins de reserve, protease.

[Traduit par la rèdaction]

Introduction

Hybridization with labeled probes and their subsequent detection in cytological preparations (in situ hybridization, ISH) is a powerful technique in physical mapping of genes and other DNA sequences. This technique has continuously been improved, and fluorescence in situ hybridization (FISH), which approached the sensitivity of isotopic ISH (Trask 1991), is now the most common form of non-isotopic ISH. The particular advantage of FISH is that several sequences can be detected simultaneously using different fluorochromes, and hence the order and relative position of genes and sequences can be determined for physical maps. The localization of low-copy genes is already state of the art in human cytogenetics (Wiegant et al. 1993). In plants, however, FISH was almost exclusively used for detection of repetitive sequences, while low-copy genes could be detected only in a few cases (Leitch and Heslop-Harrison 1993; Lehfer et al. 1993). On polytene chromosomes, as from the embryo suspensor of *Phaseolus coccineus* and *P. vulgaris*, the detection of low-copy genes should be fairly simple, owing to the high level of polyploidy up to 4096 and 8192C, respectively (Nagl 1962; Brady 1973). Nevertheless, so far only isotopic ISHs have been reported to localize the low-copy genes (Talbot et al. 1984) for phaseolin (Schumann et al. 1990) and for the polygalacturonase-inhibiting protein (Frediani et al. 1993).

Although ISH on plant chromosomes has its own specific problems, for example, the cell wall and the cytoplasmatic debris (Gustafson and Dillé, 1992), the degree of chromatin condensation is a general feature that can limit the accessibility of DNA for hybridization and detection. It is evident that probes labeled with haptens (e.g. biotin or digoxigenin) and their detection systems require better accessibility than isotopically labeled probes, because haptens must be detected by large molecules like avidin or antibodies. Improved accessibility of the target DNA should be obtainable by digestion of chromosomal proteins like histones and non-histone proteins, which determine the condensation of chromatin.

In this paper, we describe the detection of ribosomal RNA genes and the low-copy genes coding for phaseolin on polytene chromosomes of *Phaseolus coccineus* by fluorescence in situ hybridization after pretreatment with pepsin. To our knowledge it is the first report about FISH on plant polytene chromosomes.

Materials and Methods

Polytene chromosome preparation

Plants of *Phaseolus coccineus* cv. Hammond's Dwarf Scarlet (2n = 22) were grown in the Botanical Garden of the University of Kaiserslautern. Immature pods with seeds, in which the cotyledons filled 25-50% of the embryo sac, were harvested and kept sealed in a plastic bag for 4-7 days at 4°C. Seeds were then fixed with ethanol - acetic acid (3:1) for 1 week at 4°C and stored in 70% ethanol at -20°C. The suspensors were dissected from the seeds, preincubated for 5-10 min in sucrose-MES buffer [6% sucrose in 25 mM MES (2(N-morpholino]ethanesul-

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fonic acid) pH 5.5), macerated in a 5% mixture of pectinase (Serva, Heidelberg) and pectinase 5S (Serva, Heidelberg) in sucrose-MES buffer for 3 h at 37°C, and postfixed in propionic acid - lactic acid (1:1) for 3 h. Single cells from the suspensor were squashed in 45% acetic acid onto slides coated with poly-L-lysine (1 mg/ml in distilled water). After removing the cover slips, preparations were dehydrated in 100% ethanol and air dried.

Pretreatment of chromosomes

Chromosome preparations were incubated alternatively in a solution of 1 mg/mL porcine pepsin (Serva, Heidelberg) in 0.01 N HCl, or merely in 0.01 N HCl for 4.5 h at 37°C. They were then rinsed in 0.01 N HCl and BT buffer (0.1 M sodium hydrogen carbonate, pH 8.3; 0.05% Tween-20) for 3 min each, dehydrated, and air dried. Pepsin concentrations of 50 µg/mL and 2 mg/mL were also tested. To investigate the effect of the second protease, trypsin, preparations were incubated in 3.5 mg/mL trypsin (Trypsin 1:250, Difco) in 0.9% NaCl for 9 h and rinsed in 0.9% NaCl and BT buffer.

DNA probes

pTA250 contains an 8.8-kb EcoRI fragment with one repeat unit coding for the 18S, 5.8S and 25S ribosomal RNA (rDNA) of *Triticum aestivum* (Gerlach and Bedbrook 1979). AG-pPVPh3.0 contains a genomic 3-kb EcoRI-BamHI fragment with an 1.8-kb sequence encoding the ß-phaseolin subunit and an 1.2-kb untranslated 3' flanking sequence of *Phaseolus vulgaris* cv. Tendergreen (Slightom et al. 1983; Talbot et al. 1984). For hybridization, the inserts of the two clones were used as probes. The labeling was performed with biotin-16-dUTP by nick translation (Rigby et al. 1977), according to the manufacturer's specifications (Boehringer Mannheim).

In situ hybridization

Preparations were incubated in 100 µg/mL DNase-free RNase A for 30 min at 37°C, washed twice in PBS (0.13 M NaCl, 0.007 M NaH_2PO_4, 0.03 M Na_2HPO_4, pH 7.4) for 30 min at 60°C and 10 min at room temperature, dehydrated and air dried. The hybridization solution contained 50% formamide, 5% dextran sulphate, 2x SSC (0.3 M sodium chloride, 0.03 M sodium citrate) and 1 µg/µL sheared salmon sperm DNA. Then the biotinylated DNA probe was added to a final concentration of 5 µg/mL, denaturated for 10 min at 82°C, immediately transferred on ice, and chilled for 5 min. Chromosomal DNA was denaturated with 70% formamide in 2x SSC for 3 min at 70°C, dehydrated in 70%, 85%, 100% ethanol at -20°C for 5 min each, and air dried. Ten microlitres of hybridization mixture were applied onto each slide, covered with a siliconized glass cover slip, sealed with rubber cement, and placed into a humid chamber for hybridization at 37°C overnight. For posthybridization washes, slides were rinsed 3x 3 min in 2x SSC and then 3x 2 min in BT buffer at 42°C.

Immunocytochemical detection

The detection was performed according to Pinkel et al. (1986) with minor modifications described as follows. Preparations were blocked with 5% bovine serum albumine (BSA, Sigma, A-7030) in BT buffer for 5 min and then incubated in 28 µg/mL FITC-conjugated ExtrAvidin® (Sigma) in BT buffer with 5% BSA for 60 min at 37°C. Thereafter, slides were washed 3x 5 min in BT buffer at 42°C. For signal amplification, preparations were blocked with 5% goat serum (Vector Labs) for 5 min and subsequently incubated in 10 µg/mL biotinylated anti-avidin antibody (Vector Labs) in BT buffer for 35 min at 37°C. After washing, a further detection step followed with a modified incubation in 56 µg/mL FITC-conjugated ExtrAvidin® for 30 min.

Fluorescence microscopy

To reduce bleaching of the fluorescence signals, preparations were mounted in an antifade solution, to which 2µg/mL DAPI (4',6 diamidino-2-phenylindole) and 2 µg/mL propidium iodide were added to counterstain chromosomes. The antifade solution consisted of one part Tris-HCl, pH 7.4, and nine parts glycerol with 2.3% 1,4-diazo-bicylo-(2,2,2)-octane (DABCO, Sigma) and 0.02% sodium azide. Slides were examined with an epifluorescence microscope (Axioskop, Zeiss) using filter set 02 (excitation 365 nm, emission 420 nm LP) for DAPI, and 09 (excitation 450-490 nm, emission 520 nm LP) for FITC and propidium iodide. Photographs were taken on Agfa APX 100 film.

Results and discussion

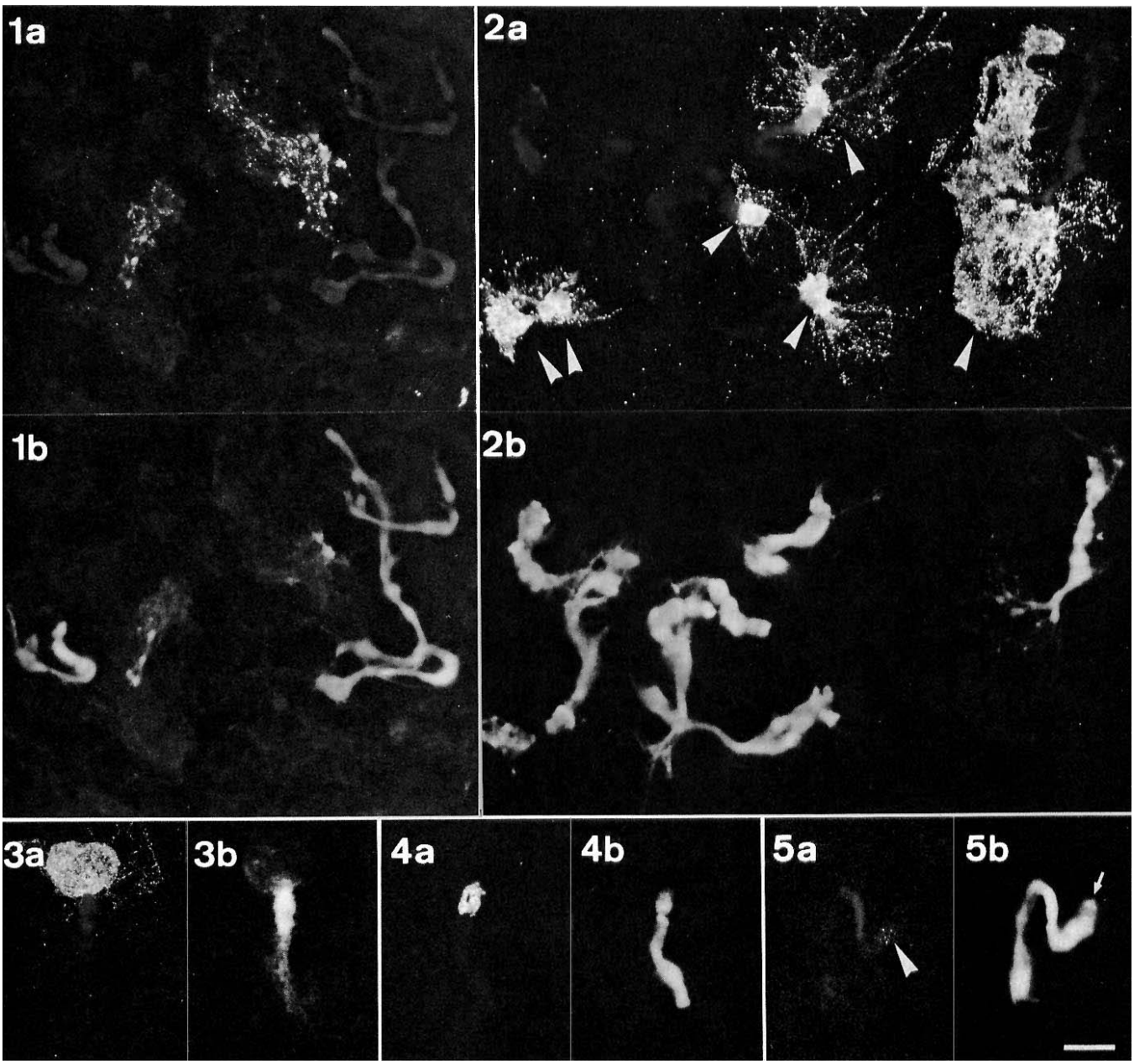
Effects of digestion

Preparations of the polytene chromosomes of *Phaseolus* were digested with either pepsin or trypsin as a pretreatment for fluorescence in situ hybridization (FISH). The effects of the proteases were studied with regard to chromosome morphology and the intensity of hybridization signals using an rDNA probe as a test system. The aim was to develop a technique to detect low-copy genes, here the phaseolin genes, by FISH.

The only visible change in morphology after incubation with pepsin was a loosening of chromatin. The intensity of hybridization signals was enhanced by the increased density and number of fluorescent spots per locus (Figs. 1*a* and 2*a*). The reduction of cytoplasmatic debris improved the detection in addition, since signals stood out more clearly against the black background than against the diffuse shimmering fluorescence of the debris in the untreated slides. Higher and lower pepsin concentrations were not found to improve the hybridization signals. Some enhancement of the hybridization signals was also observed in comparison with the untreated slides when preparations were merely incubated with HCl. The improvement by HCl did not, however, reach the level of pepsin treatment (results not shown). A reasonable explanation for loosening of chromatin and enhancement of the hybridization signals after pepsin pretreatment might be a certain disintegration of the higher order structure of chromatin by extraction of histone proteins with HCl (Comings and Avelino 1974, Zhang and Yang 1990), and the digestion of remaining nonhistone proteins by pepsin (Jeppesen et al. 1978). The result might be an increased accessibility of the target DNA for both the probe and the molecules of the detection system, and hence can be considered to be critical for in situ hybridization. No change in chromosome morphology and no improvement of hybridization signals could be observed after incubation with trypsin. This observation is noteworthy, since the applied concentrations and incubation times were not only those generally used in human cytogenetics for chromosome banding but were also extended to a 10 times higher concentration and a 540 times longer incubation time.

rDNA sites

Hybridization signals of the rDNA probe were observed on 6 of the 22 polytene chromosomes, which correspond to the diploid complement of *Phaseolus coccineus* (Fig. 2*a*). The number of six rDNA loci is in agreement with previous reports of six nucleolus organizing chromosomes on the basis of isotopic rRNA hybridization (Avanzi et al. 1972) and silver staining − Giemsa C-banding (Schweizer and Ambros 1979). Comparing the chromosomes displaying hybridization sites with those of the karyotype presented by Schumann et al. (1990), it is evident that the rDNA loci are situated on chromosomes 1, 5 and 11. There is no



FIGS. 1-5. Polytene chromosomes of *Phaseolus coccineus* after fluorescence in situ hybridization (FISH) with biotinylated probes, detected by ExtrAvidin®-FITC and amplification of the signals with biotinylated anti-Avidin antibody. Chromosomes were counterstained for DNA simultaneously with propidium iodide and with DAPI (figures labeled as *a* show FITC and propidium iodide fluorescence, figures labeled as *b* show DAPI fluorescence). Fig. 1*a*. Two out of the six hybridization signals of the rDNA probe (pTA250) without pretreatment. Fig 2*a*. Six rDNA loci (arrowheads) with signal points of increased density and number after pepsin pretreatment. Figs. 3 and 4. Different patterns of hybridization signals with the rDNA probe corresponding with the state of condensation of the NORs. Fig. 3*a*. Dispersed signal points on a decondensed NOR (3*b*) and Fig. 4*a*. Compact hybridization signal on a condensed NOR (4b). Fig. 5*a*. Hybridization sites of the phaseolin clone AG-pPVPh3.0 after FISH with the 3-kb fragment, detected after three rounds of signal amplification. The hybridization site (arrowhead) is located in decondensed chromatin on the short arm. Fig. 5*b*. The blocks of telomeric heterochromatin on the short arm (arrow) suggest classification of the labeled chromosome as No. 7. Scale = 25 μm

evidence for an additional rDNA locus on chromosome 2 as described by Avanzi et al. (1972) and Tagliasacchi et al. (1993). This contradicting result might be due to difficulties in the identification of chromosomes or due to different cultivars (genotypes) used.

The nucleoloar organizing regions (NORs) of the *Phaseolus* polytene chromosomes display various functional structures, even in one and the same nucleus. They are highly decondensed and ramified (puffed) in the active state and completely condensed in the inactive state (Nagl 1970).

The distribution of hybridization signals over the NORs clearly coincide with their degree of condensation. The signal points are scattered over a puffed NOR (Figs. 3*a* and 3*b*), while they are tightly piled up over condensed NORs (Fig. 4*a* and 4*b*).

Phaseolin gene sites

The main seed storage protein, phaseolin, is encoded by a small multigene family of six to eight genes per haploid genome in *Phaseolus vulgaris* (Talbot et al. 1984) and is

therefore also very likely in the closely related species *P. coccineus*. Pretreatment with pepsin enabled, for the first time, the detection of the low-copy genes for phaseolin by FISH on polytene chromosomes of *P. coccineus*. The hybridization sites were located on 15 slides of five experiments in the subtelomeric regions of the short arms in decondensed (puffed) chromatin of two homologous chromosomes (Fig. 5*a* and 5*b*; only one of the homologous chromosomes is shown). These medium-sized, submetacentric chromosomes are characterized by the absence of intercalary heterochromatin bands in the long arm and by telomeric blocks of heterochromatin in the short arm, as clearly visible in DAPI-fluorescence (Fig. 5*b*). We now interpret this chromosome pair as No. 7, according to a new (so far unpublished) polytene karyotype of *Phaseolus coccineus* based on DAPI fluorescence microscopy after pepsin treatment. The classifiaction as chromosome 3 as made earlier (Schumann et al. 1990) on the basis of radioactive in situ hybridization must be revised. Unfortunately, there are still some ambiguities in the identification of the 22 chromosomes.

Conclusions

Pepsin pretreatment results in an enhanced intensity of hybridization signals in FISH on *Phaseolus coccineus* polytene chromosomes without damaging their morphology. This method will, therefore, help to localize other low-copy genes and DNA sequences to establish a precise karyotype by physical mapping of markers and could lead to a better correlation of the physical map with the linkage map of *Phaseolus vulgaris* (Nodari et al. 1993).

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