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BRIEF COMMUNICATION

A combined PRINS-FISH technique for simultaneous localisation of DNA sequences on plant chromosomes

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Abstract

A novel approach for simultaneous localization of two DNA sequences on plant chromosomes is described. The approach is based on a combined use of primed *in situ* DNA labelling (PRINS) with fluorescent *in situ* hybridization (FISH). Traditionally, this has been done using FISH with two probes labelled by two different marker molecules. Compared to this method, the combined PRINS-FISH procedure is faster. Furthermore, because one of the DNA sequences is localized by PRINS with specific primers, only one labelled probe is needed.

Additional key words: FokI repetitive sequence, physical mapping, 45S rRNA genes.

Two methods are currently available for physical mapping of DNA sequences on chromosomes *in situ*. Fluorescent *in situ* hybridization (FISH) procedure is based on annealing of labelled probe on DNA of denatured chromosomes or interphase nuclei (Heslop-Harrison 1991). Primed *in situ* DNA labelling (PRINS) is an alternative to FISH where unlabelled oligonucleotides hybridized to the target sequences serve as primers for chain elongation *in situ* catalyzed by a DNA-polymerase in the presence of labelled nucleotides (Koch *et al.* 1989). In many cases, it is useful to detect two

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different DNA sequences simultaneously. Traditionally, this has been done using double FISH. Here we report on a novel approach based on a combined use of PRINS and FISH.

Seeds of *Vicia faba* L., line ACB (2n = 12) with reconstructed karyotype were kindly provided by Dr. I. Schubert (IPK, Gatersleben, Germany). Cell cycle synchronization and preparation of chromosome suspensions was performed as described previously by Doležel *et al.* (1992). Permanent squash preparations were prepared from synchronised root tips after enzyme maceration as described previously (Kubaláková *et al.* 1997). Slides were stored at -20 °C and used for the reactions.

PRINS reaction was performed with a primer specific for the FokI repetitive sequence which has been first described by Kato *et al.* (1984). The reaction mixture consisted of 2 μM primer (5′CAT TAT GGA AGG TAG TCT GTT GTC GAG 3′), 0.1 mM dATP, dCTP, dGTP, 0.008 mM fluorescein-12-dUTP (*Boehringer*, Mannheim, Germany), 0.017 mM dTTP, 4 mM $MgCl_2$, 3U of Taq polymerase (*Boehringer*, Mannheim, Germany) per 0.04 cm^3. The reaction was performed using 0.02 cm^3 *SureSeal* frames (Hybaid, UK) to prevent evaporation. Thermal cycler PTC-100 (*MJ Research*, Watertown, USA) with a *Slide Griddle* plate was used with a following program: 1 min at 94 °C, 15 min at 60 °C and 45 min at 70 °C. The reaction was stopped by washing the slides for 1.5 min in stop buffer (50 mM NaCl, 50 mM EDTA, pH 8.0) at 70 °C. The slides were then passed through an ice-cold (-20 °C) ethanol series (50, 90, 100 %), air-dried and immediately used for FISH.

A 3.7 kb fragment of VER17 plasmid containing parts the 18S, 5.8S and 25S rRNA coding regions and internal transcribed spacers of *V. faba* (Yakura and Tanifuji 1983) was labelled by random priming with biotin-dUTP and used as a probe for FISH. The hybridization mixture contained 50 % formamide, 20 % dextran sulfate, 250 ng mm^{-3} sheared calf thymus DNA, and 2 ng mm^{-3} labelled probe in 2 × SSC. The mixture was denatured at 76 °C for 15 min before applying to slides and hybridization was performed at 37 °C overnight. After hybridization, the coverslips were removed and the slides were placed in 2 × SSC for 5 min. They were then given a stringent wash, for 10 min in 50 % formamide in 2 × SSC, followed by a wash in 2 × SSC for 10 min, all at 42 °C. Sites of biotin-labelled probe hybridization were detected using avidin-Cy3 and the signal was amplified using biotinylated-anti-avidin and avidin-Cy3 (both *Vector Laboratories*, Burlingame, USA).

The chromosomes and nuclei were counterstained with DAPI (200 ng cm^{-3} in 4 × SSC containing 0.2 % Tween 20) and the slides were mounted in *Vectashield* antifade solution (Vector Laboratories, USA). The slides were examined with *Olympus BX 60* epifluorescence microscope (Tokyo, Japan) equipped with the following filter sets: *U-MWU* (DAPI fluorescence), *U-NIBA* (narrow band, FITC fluorescence), *U-MWG* (CY3 fluorescence). The images of DAPI, FITC and CY3 fluorescence were acquired separately with a black and white CCD camera. The camera was connected to a PC running the *ISIS* image processing software (*Metasystems*, Belmont, Germany). The images corresponding to DAPI, FITC and CY3 were superimposed after contrast and background optimization.

The combined PRINS-FISH procedure described here resulted in clear and strong signals in the NOR of the acrocentric chromosome pair III and in a number of signals corresponding to FokI sequences (Fig. 1*A*). The position of all FokI bands corresponded to their known genomic position (Fuchs *et al.* 1994). This result indicates that there was no negative interference between both methods applied sequentially. Interestingly, non-specific background in cytoplasm was considerably lower compared to a PRINS reaction performed alone. This favourable effect was most likely due to prolonged incubation in hybridization mixture and stringent washes after FISH. Clear signals were observed also on interphase nuclei (Fig. 1*B*).

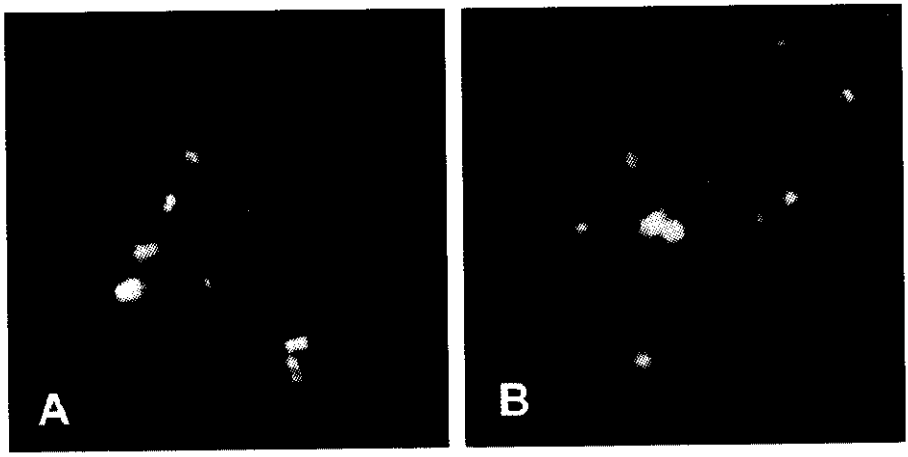

Fig. 1. Localization of 45S rRNA genes and FokI repetitive sequences using combined PRINS-FISH procedure in *Vicia faba*, line ACB. FokI repeat was detected after PRINS using a specific primer and incorporation of fluorescein-dUTP (*yellow*), rRNA genes were detected using FISH with a biotin-labelled probe, detected by avidin-Cy3 (*red*). Chromatin was counterstained with DAPI (blue). *A* - metaphase chromosomes, *B* - interphase nuclei.

A standard FISH procedure usually involves a pre-treatment with RNase and post-fixation in ethanol : acetic acid or formaldehyde. Subsequently, chromosomal DNA must be denatured. In a combined PRINS-FISH procedure described here, these steps are omitted. Denaturation of DNA performed during the PRINS reaction is sufficient also for FISH. However, to achieve optimal results, FISH should not be started later than 2 h after 'post-PRINS' dehydration through the ethanol series. A similar observation was made by Hindkjaer *et al.* (1995a,b) with their PRINS-painting procedure for human chromosomes.

A combined PRINS-FISH procedure has been developed for the first time for plants. The method allows co-localization of two repetitive DNA sequences on chromosomes or interphase nuclei. We expect that it will find a number of applications in physical mapping of plant genomes.

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