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Mapping of repeated DNA sequences in plant chromosomes by PRINS and C-PRINS

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Abstract The primed in situ DNA labelling (PRINS) procedure was optimised for the rapid physical mapping of several types of repetitive DNA sequences on the mitotic chromosomes of *Vicia faba*, *Pisum sativum* and *Secale cereale*. A localization of the highly repeated *Fok*I sequence on *V. faba* chromosomes was achieved after a 7-min total reaction time. In addition, we report a procedure for direct cycling-PRINS (C-PRINS), a variation of PRINS which involves a sequence of thermal cycles analogous to the polymerase chain reaction. Compared to PRINS, C-PRINS was more sensitive. Further work is needed to improve the sensitivity of the reaction to allow for the reliable detection of low-copy DNA sequences.

Key words PRINS · C-PRINS · Ribosomal RNA genes · Telomeres · *Fok*I sequence · Repetitive DNA · Plant chromosomes

Introduction

Physical localization of DNA sequences by in situ hybridization (ISH) using radioactivelly labelled probes was first reported by Gall and Pardue (1969) and John et al. (1969). During the eighties, procedures for non-isotopic labelling of nucleic acids were developed and their many applications to plant and animal chromosome analysis were recently reviewed (Heslop-Harrison 1991; Trask et al. 1993; Jiang and Gill 1994; Leitch et al. 1994). As pointed out by Jiang and Gill (1994), the localization of DNA sequences shorter than 10 kb on plant chromosomes using ISH or FISH-(fluorescent in situ hybridization) is still very difficult. Instead, FISH-mapping of large DNA clones containing the targeted low-copy sequence is a preferred approach (Jiang et al. 1995).

An alternative approach for the physical mapping of DNA sequences, termed PRimed IN Situ DNA labelling (PRINS), was developed by Koch et al. (1989). PRINS is based on sequence-specific annealing of unlabelled probe (oligonucleotide primer or cloned probe) to target DNA in situ and its amplification with a DNA polymerase in the presence of nucleotides, of which at least one is labelled. Depending on the label, the newly sythesized DNA may be detected directly or indirectly. "Direct" procedures are based on the use flurochrome-labelled nucleotides while "indirect" methods are based on incorporation of hapten-labelled nucleotides and their detection using a fluorochrome-labelled antibody (Macas et al. 1995).

The cycling-PRINS (C-PRINS) technique involves a sequence of thermal cycles analogous to the polymerase chain reaction (Gosden et al. 1991; Terkelsen et al. 1993 a, b). C-PRINS was used to detect DNA sequences shorter than 2 kb on human chromosomes (Gosden and Lawson 1994 a,b). Because of its speed and sensitivity, the number of applications of PRINS and C-PRINS in human cytogenetics is rapidly increasing (Koch et al. 1995 a, b; Therkelsen et al. 1995; Pellestor et al. 1996).

Following Abbo et al. (1993), we applied PRINS to detect DNA sequences on pea and field bean chromosomes both in situ and in suspension (Macas et al. 1995). Chromosome labelling by PRINS was found invaluable for sorting pure chromosome fractions using flow cytometry (Pich et al. 1995). More recently, Thomas et al. (1996) used PRINS for the labelling of

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telomeres in cereals, while and Mukai and Appels (1996) applied indirect C-PRINS to detect ribosomal RNA genes on rye chromosomes.

Here we report on further improvements of the protocol for PRINS and the development of a procedure for direct C-PRINS, as compared to indirect C-PRINS.

Materials and methods

Plant material

Seeds of garden pea (*Pisum sativum* L., cv Ctirad, 2n = 14) and field bean (*Vicia faba* L., cv Inovec, 2n = 12) were obtained from commercial suppliers. Seeds of a rye (*Secale cereale* L.) strain carrying B-chromosomes were obtained from Prof. Neil Jones (Aberystwyth, U.K.).

Isolated chromosomes

The preparation of chromosome suspensions of *V. faba*, *P. sativum* and *S. cereale* has been described previously (Doležel et al. 1992, 1995; Lucretti and Doležel 1995). Immediately after isolation, the suspensions were purified using a sucrose gradient to remove cytoplasmic remnants, larger chromosome clumps, and nuclei. The layer containing chromosomes was removed and diluted in cold PCR buffer to a final concentration of 5–10% (w/v) sucrose. Ten microliters of suspension were dropped onto a clean slide and/or coverslip and left to dry at room temperature. After drying, the slides were stored at −20°C.

Squash preparations

Cell-cycle synchronization and metaphase accumulation was performed as described by Gualberti et al. (1996). Synchronized root tips about 1 cm in length were excised and fixed in 3:1 ethanol: acetic acid at 4°C overnight. After maceration in 2% (w/v) pectinase at 37°C for 60 min and in 1% (w/v) cellulase at 37°C for 30 min, the root tips were squashed in 45% acetic acid. The slides were made permanent using the dry ice method (Conger and Fairchild 1953) and stored desiccated at −20°C.

Primed in situ DNA labelling (PRINS)

For labelling of chromosomes dried on coverslips, small pieces (approximately 2 × 5 mm) were cut from the coverslip and immersed in a 45-µl reaction mixture in PCR tubes (Macas et al. 1995). The reactions with isolated chromosomes dried on slides, or with squash preparations, were performed using SlideSeal or SureSeal reaction chambers (Hybaid Ltd, UK). Thermal cycler PTC-100 (MJ Research, Inc., Watertown, USA) with a standard heating block or with a Slide Griddle™ plate was used for all reactions.

Slides without any pre-treatment were used directly for the reaction. Alternatively, three different pre-treatments were tested. (1) The slides were washed in ethanol series (70%, 90%, 100%) and air dried before other treatments. (2) The slides were incubated with 0.1 µg/µl RNase (Sigma) in reaction buffer (1 mM Tris-HCl, 1.5 mM $NaCl_2$, pH 7.5) at 37°C for 60 min. (3) The slides were incubated with 5 U of T4 DNA ligase (Boehringer Mannheim) in 50 µl of ligation buffer (66 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM dATP, pH 7.5) for 60 min at room temperature. After RNase or ligase treatment the slides were rinsed in PCR buffer and used for reactions.

For direct PRINS, the reaction mix consisted of 0.1 mM dATP, dCTP, dGTP, and either 0.008 mM fluorescein-12-dUTP alone or together with 0.008 mM fluorescein-15-dATP or Cy3-dUTP, 0.017 mM dTTP (dATP), 2–7.5 mM $MgCl_2$, 0.01% BSA, and 3 U/40 µl of *Taq* polymerase (Stratagene) in 1 × PCR buffer.

For indirect PRINS, the nucleotide mixture was replaced by a DIG DNA labelling mix (Boehringer Mannheim) consisting of: 0.1 mM dATP, dCTP and dGTP, 0.065 mM dTTP, 0.035 mM DIG-12-dUTP. All concentrations were calculated for respective reaction volumes and an additional 12.5% of H_2O was added to compensate for evaporation during high-temperature incubation.

Synthetic oligonucleotides specific for the *P. sativum* rDNA large spacer, the *V. faba* 5s rDNA, the *V. faba Fok*I repeat element, the consensus *Arabidopsis* telomeric sequence, and for the terminal heterochromatic segment of a rye B-chromosome were used as primers (Table 1) in concentrations ranging from 0.5 to 4 µM. The temperature profile of the reaction varied according to the primer and target sequences. It consisted of denaturation at 94–95°C for 1–5 min, primer annealing at 45–60°C for 1–15 min, and extension at 70–72°C for 3–60 min.

Cycling PRINS (C-PRINS)

The preparation of slides and the reaction mixture were the same as described above. For C-PRINS, the program was as follows: the first

Table 1 Oligonucleotide primers used in the present study

Origin	Name	Sequence	References
Repeat element of *Vicia faba*	*Fok*I	5′ CAT TAT GGA AGG TAG TCT GTT GTC GAG 3′	Kato et al. (1984)
Consensus telomeric sequence	Tel	$(AGGGTTT)_3$	Richards and Ausubel (1988)
Large 45*s* rDNA spacer of *Pisum sativum*	RS	5′ CGA AAT GCT CCG AAA CTT TGC AGA TC 3′	Kato et al. (1990)
	45*s* rev	5′ TGT GTA GGC CAA GGT TTT GTC 3′	
	45*s* for	5′ GCT TTA GCA GCA ACT GGG TC 3′	
Vicia faba 5*s* rDNA	R5	5′ ACC TCC TGG GAA GTC CTT GTG TTG 3′	Vandenberghe et al. (1984)
Repetitive family D1100 derived from the B chromosome of *Secale cereale*	AH11	5′ GCT TGC ACC GGC TTC GTC CCG 3′	Sandery et al. (1990)
	AH12	5′ GTG TAC TTG AGA CAC GCA AGC 3′	
	AH13	5′ CCA CGT TGC GGG CGC GTC CGC 3′	Houben et al. (1996)
	AH14	5′ CGC GTC GGT AAT TTG GTC TCG 3′	Wilkes et al. (1995)
	A	5′ GCC TAT TTG GGA AAC TCT TTT G 3′	
	B	5′ TTC GAT TCC TCC TTG TTG TAT TC 3′	

cycle consisted of 4 min at 94–95°C, 5 min at 45–60°C, 10 min at 70°C, followed by up to 28 cycles of 1 min at 94–95°C, 1 min at 45–60°C, and 3 min at 70°C. In the final cycle, the extension was prolonged to 10 min at 70°C.

Signal detection and chromosome staining

Both PRINS and C-PRINS reactions were terminated in a stop buffer (0.5 M NaCl, 0.05 M EDTA, pH 8.0) for 1.5–5 min at 70°C, and a wash buffer (0.1 M maleic acid, 0.15 M NaCl, 0.05% Tween-20, pH 7.5) at room temperature, for 5 min. Fluorescein-dUTP-labelled samples were then immediately counterstained whereas DIG-labelled ones were incubated for 20 min in a blocking buffer (0.1 M maleic acid, 0.15 M NaCl, 0.5% blocking reagent, pH 7.5) at 37°C and then for 30 min in anti-DIG-fluorescein solution (20 μg/ml in blocking buffer) at 37°C. Non-specific bound antibody was removed by washing three times in wash buffer at room temperature. Then, the slides were incubated in wash buffer containing propidium iodide (0.2 μg/ml) or 4′,6-diamidino-2-phenylindole (DAPI, 0.2 μg/ml) for 15 min to counterstain the chromosomal DNA. Coverslips were mounted in Vectashield antifade solution (Vector Laboratories, Inc., Burlingame, USA).

Fluorescence microscopy

The preparations were observed using a BX60 Olympus fluorescence microscope. Filter sets WB, NIBA and a dual-band filter (FITC/PI) were used for the visualization of fluorescein or PI/fluorescein fluorescence. Photographs were taken on Kodak Ektapress Multi film (100–1000 ASA). Alternatively, the slides were analysed using the ISIS System (MetaSystems, Belmont, USA).

Results and discussion

PRINS with a single primer specific for the *Fok*I repetitive sequence resulted in strong signals on all acrocentric *V. faba* chromosomes (Fig. 1 A). The position of bands corresponded to their known genomic position (Fuchs et al. 1994). Strong signals were obtained even after "instant" PRINS lasting only for 7 min (95°C for 1 min, 60°C for 1 min and 70°C for 5 min) as reported by Gosden and Lawson (1995). Thus PRINS may be used for the rapid localization of highly repeated DNA sequences on plant chromosomes. Specific signals were also observed with all other primers (See Table 1) using the standard program (see Materials and methods). However, the intensity of the label was rather low. It was possible to increase the signal intensity without increasing the background by using two labelled nucleotides. Also increasing the primer concentration up 4 μM led to an improvement of signal intensity.

To further increase the intensity of specific labelling, we tested indirect PRINS, employing digoxigenin-labelled nucleotides. In agreement with our previous work (Macas et al. 1995), this variation of the procedure resulted in higher signal intensity. At the same time, it resulted in an increased level of non-specific labelling of chromosomes, cytoplasm, and cytoplasmic remnants.

With a standard PRINS procedure the reaction is performed only once. However, the sensitivity of the method may be improved by increasing the number of reaction cycles (Therkelsen et al. 1993 a, b). Repeated PRINS is now usually referred to as cycling-PRINS (C-PRINS), (Gosden and Lawson 1994 b). In order to test the effect of repeated reactions on plant chromosomes, we performed C-PRINS using the Tel primer (See Table 1) with isolated *V. faba* and *P. sativum* chromosomes. A signal was observed after five reaction cycles, and a strong signal was observed after ten cycles. In *P. sativum*, clear signals were visible exclusively at the termini of all chromosomes (Fig. 1 E) which agrees with previous studies using FISH (Rawlins et al. 1991). In addition to terminal signals, interstitial signals were observed in the short arms of acrocentric chromosomes 2 and 5 in *V. faba* (Fig. 1 B). This is in agreement with the observation made after FISH with biotin-labelled synthetic plant telomere repeats (Schubert 1992). However, contrary to Schubert (1992), a signal at the centromere of chromosome 1 was never observed after C-PRINS. Strong signals were also evident after C-PRINS with the R5 primer specific for 5*s* rDNA, on a major site of 5*s* localization on a short arm of chromosome 5*s* of *P. sativum* (Fig. 1 C), as discussed by Simpson et al. (1990).

Telomeric and 5*s* rDNA sequences were detected using only one primer. We have found that further increase in signal intensity may be achieved after C-PRINS with a pair of specific primers. Figure 1 D shows the signal obtained after the reaction with a pair of primers specific for the 45*s* rDNA spacer in *P. sativum*. A similar increase in signal intensity was observed during the localization of a repetitive family, D1100, derived from a rye B-chromosome (Sandery et al. 1990). The position of signals corresponded to the known location of these sequences (Simpson et al. 1990; Wilkes et al. 1995). Further improvement of the sensitivity of C-PRINS was achieved after employing nested primers (AH11, AH12, AH13, AH14). As expected, C-PRINS with four primers specific for D1100 yielded significantly stronger signals compared to the reaction with only two primers (A, B), (Fig. 1 F). To confirm that the amplification of the signals was due to repeated cycles, and not due to prolonged incubation time, we have performed PRINS reactions with the reaction period corresponding to the sum of incubation periods in C-PRINS. In all cases, the signal intensity was much stronger after C-PRINS.

With all primers, an increase in the number of cycles over ten did not increase signal intensity (as analysed visually) and negatively influenced the specificity of the reaction. C-PRINS with 20 cycles caused strong non-specific labelling of chromosomes and a decrease of the signal/noise ratio. Theoretically, the PRINS technique should produce less background signal compared to FISH. However, our results with plant chromosomes did not confirm this expectation. Interestingly, we have

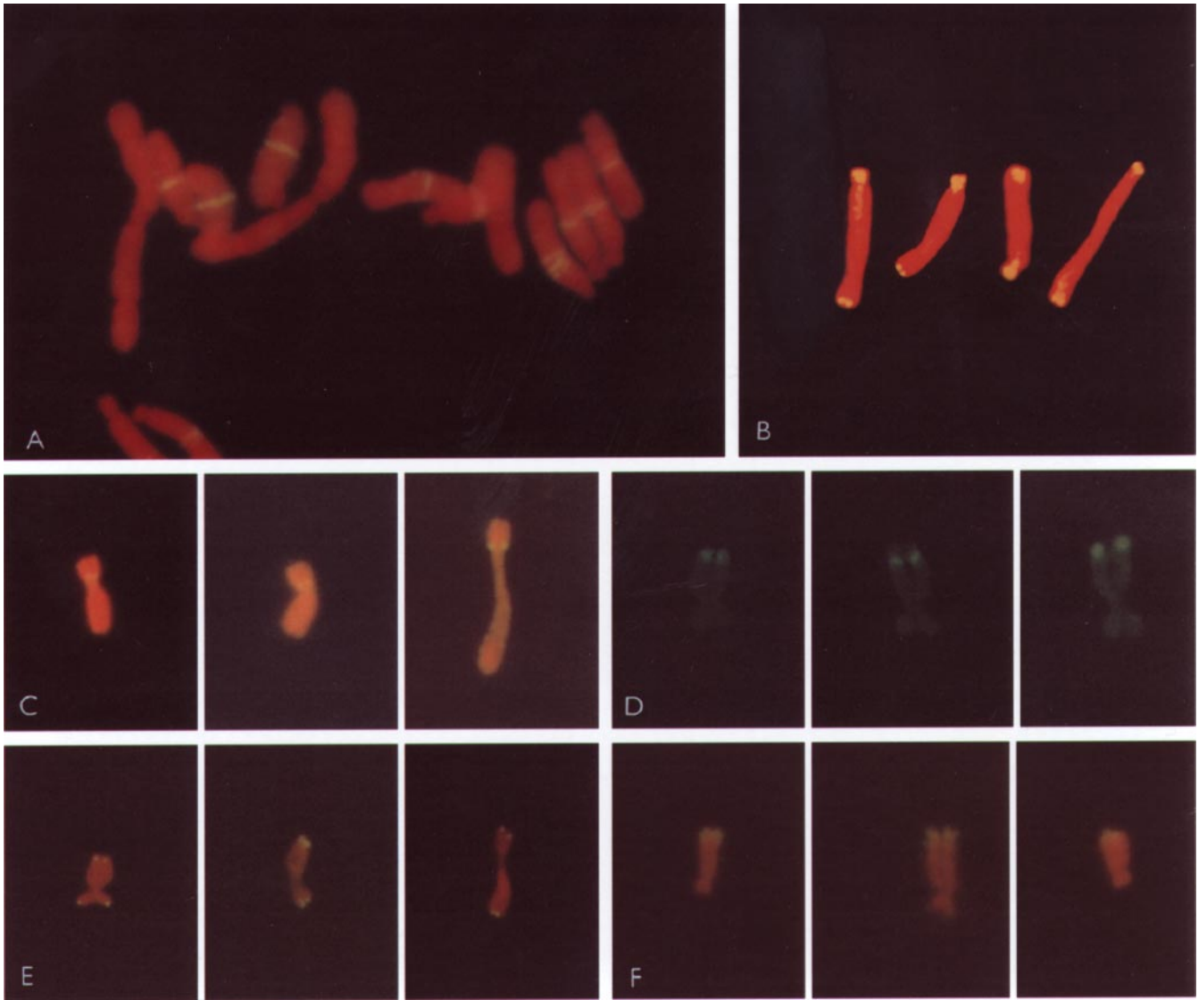

Fig. 1 A–F Localization of repetitive DNA sequences on plant chromosomes using direct PRINS (**A**) and C-PRINS (**B–F**). **A** PRINS on a squash preparation of *V. faba* performed with a primer specific for the *Fok*I repetitive sequence. **B** Localization of telomeric sequences on *V. faba* acrocentric chromosomes using a primer specific for consensus plant telomeric sequence. In addition to terminal signals, interstitial signals were observed in short arms of acrocentric chromosomes 2 and 5. **C** Visualization of 5*s* rDNA genes on *P. sativum* chromosome 5. **D** Detection of 45*s* rDNA on *P. sativum* chromosomes with a pair of primers specific for rDNA large spacer. The label was found exclusively on chromosomes no. 4 and no. 7. **E** Localization of telomeric sequences on *P. sativum* using a primer specific for consensus plant telomeric sequence. **F** Visualization of rye B-chromosome-specific repetitive sequence using nested primers specific for a repetitive family D1100. The end of the B-chromosome long arm is labelled. With the exception of **B**, which was recorded using the ISIS system, all figures are original photographs. **D** was taken using a narrow band filter specific for FITC; the remaining pictures were taken using a fluorescein/propidium iodide dual band filter

found that the background can be reduced by lowering the annealing temperature. Although the reason for this is not clear, a similar observation was made by Gosden and Lawson (1994 b).

In addition to the annealing temperature, the intensity of the signal and the non-specific background depended critically on the age of preparations. While telomeric sequences were best detected on 2–4-week-old slides, other sequences were best detected on 1-week-old slides. A similar dependence was observed for PRINS on human chromosomes (Pellestor et al. 1995). It is interesting to note that the strongest background was always found on fresh slides (less than 1-week old) whereas, that with aging, non-specific chromosome labelling decreased. This observation differs from that made by Koch et al. (1995) who found that the background for human chromosomes increased with the age of slides. They recommend ligase treatment to decrease the background on older slides and speculated that the background arises from non-specific initiation at single-strand nicks in the chromosomal DNA. Although in our experiments T4 DNA ligase treatment resulted in a decrease of the background, RNase treatment was more effective. However, the signal intensity was also reduced after both treatments. To preserve

chromosome morphology on fresh (1–2-day old) preparations, Gosden and Lawson (1994 b) recommend a passage through an ethanol series. In our hands this treatment negatively influenced chromosome morphology, decreased signal intensity, and caused additional rings of non-specific label around the chromosomes.

The procedures for PRINS and C-PRINS described here were found suitable for the rapid detection of repetitive DNA sequences on mitotic chromosomes in several plant species. In contrast to the indirect C-PRINS procedure used by Mukai and Appels (1996) the method for direct C-PRINS described here is faster and more sensitive. Work is in progress to improve the sensitivity of direct C-PRINS to allow for the reliable detection of low- or even single-copy sequences.

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