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Optimization of PRINS and C-PRINS for detection of telomeric sequences in *Vicia faba*

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Abstract

Primed *in situ* labelling (PRINS) of nucleic acids was developed as an alternative to traditionally used fluorescence *in situ* hybridization (FISH). Compared to FISH, PRINS is faster and does not require preparation of labelled probes. Nevertheless, the number of applications for physical mapping of DNA sequences on plant chromosomes remains low. This is due to the fact that there are a number of factors which influence the specificity and sensitivity of the reaction. The purpose of this work was to analyse the effect of some of them, including the age of slides, type of *Taq* DNA polymerase, number and concentration of primers, the presence and concentration of bovine serum albumine and $MgCl_2$ in the reaction mixture. Furthermore, the effect of various pre-treatments on signal intensity and non-specific fluorescence was studied. A consensus *Arabidopsis*-type telomeric sequence and *Vicia faba* mitotic chromosomes were used as a model system. We have found that the age of slides was critical and that under optimal conditions it was possible to achieve relatively high signal to noise ratio.

Additional key words: field bean, fluorescence *in situ* hybridization, primed *in situ* labelling, telomeres.

Introduction

Increased interest in physical mapping of DNA sequences stimulated a significant improvement of *in situ* hybridization techniques. The use of fluorescence of fluorochrome-labelled probes led to simplification of the procedures avoiding the use of radioactive materials. Fluorescent *in situ* hybridization (FISH) was shown sensitive enough to permit localization of single copy sequences (Leitch *et al.* 1994,

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Jiang and Gill 1994). The main disadvantage of FISH is that it is time consuming and requires the preparation of labelled probes.

An alternative approach for 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 oligonucleotide primer under stringent conditions to the DNA of denaturated chromosomes. The primer serves as a initiation site for *in situ* chain elongation in the presence of nucleotides, of which at least one is labelled. Depending on the label, the newly synthesized DNA may be detected directly or indirectly. "Direct" procedures are based on the use fluorochrome-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).

For localization of highly repetitive DNA sequences, one cycle of PRINS reaction with a single primer usually results in a strong and specific signal (Kubaláková *et al.* 1997a). Localization of less abundant DNA sequences may be achieved after cycling-PRINS (C-PRINS) where labelled copies are accumulated at the site of their synthesis (Gosden and Hanratty 1993, Terkelsen *et al.* 1993). Furthermore, compared to the reaction with a single primer, the use of primers for both DNA strands results in more than linear increase of signal intensity. C-PRINS was shown to be suitable for detection of DNA sequences as short as 2 kb on human chromosomes (Gosden and Lawson 1994a, Shi *et al.* 1996).

The number of applications of PRINS and C-PRINS in human cytogenetics is rapidly increasing (Charlieu and Pellestor 1997, Pellestor 1997). On the other hand, five years after the first report (Abbo *et al.* 1993) the progress in the application of PRINS for physical mapping in plants remains slow. Nevertheless, we have shown that PRINS may be used to detect repetitive DNA sequences on chromosomes in pea, field bean and in cereals (Macas *et al.* 1995, Kubaláková *et al.* 1997a,b,c). Thomas *et al.* (1996) used PRINS for labelling of telomeres in cereals while Mukai and Appels (1996) applied indirect C-PRINS to detect ribosomal RNA genes on rye chromosomes. Shi *et al.* (1996) detected a single-copy DNA sequence on soybean chromosomes using indirect C-PRINS with two primers. Furthermore, chromosome labelling in suspension was found invaluable for sorting pure chromosome fractions using flow cytometry (Pich *et al.* 1995).

With the aim to increase the usefulness of the technique, we have analysed some factors which influence the sensitivity and specificity of PRINS for localization of DNA sequences on plant chromosomes. We have used field bean chromosomes and a consensus *Arabidopsis*-type telomeric sequences as a model system.

Materials and methods

Plants: Seeds of field bean (*Vicia faba* L. ssp. *faba* var. *equina*, 2n = 12) cv. Inovec obtained from Dr. M. Vavák (Horná Streda, Slovakia) were germinated at 25 °C in darkness and seedlings with about 2-cm roots were used for cell cycle synchronization of root tips.

Preparation of slides: Preparation of chromosome suspensions from synchronized root tip meristems was performed as described previously (Doležel *et al.* 1992). The chromosome suspension was purified on a sucrose gradient and dropped onto a slide and/or coverslip and left to dry at room temperature. Permanent squash preparations were prepared from synchronised root tips after enzyme maceration as described previously (Kubaláková *et al.* 1997). After drying, the slides were stored at room temperature or at -20 °C.

Chromosome labelling: Small pieces were cut from the coverslip and immersed in 0.045 cm^3 reaction mixture in PCR tubes (Macas *et al.* 1995). To prevent evaporation of reagents during the reaction with preparations on microscope slides, *SureSeal* frames or *SlideSeal* (*Hybaid*, Teddington, UK) were used. Also the addition to the reaction mixture of the *SelfSeal* reagent (*MJ Research, Inc.*, Watertown, USA) which automatically creates an evaporation-limiting barrier around the periphery of a cover glass was tested. Thermal cycler *PTC-100* (*MJ Research, Inc.*, Watertown, USA) equipped with a standard heating block or with the *Slide Griddle*™ plate was used for all reactions.

Pretreatments: Slides without any pretreatment were used directly for the reaction. Alternatively, four different pretreatments were tested: *1*) the slides were washed in cold ethanol series (70, 90, 100 %) and air dried before other treatments; *2*) the slides were incubated with 0.01 or 0.1 g dm^{-3} RNase (*Sigma*, St. Louis, USA) 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 5U T4 DNA ligase (*Boehringer*, Mannheim, Germany) in 0.05 cm^3 ligation buffer (0.66 M Tris-HCl, 50 mM $MgCl_2$, 10 mM dithiothreitol, 10 mM dATP, pH 7.5) for 60 min at room temperature; *4*) the slides were incubated in 1 or 5 mg dm^{-3} proteinase K in proteinase buffer (20 mM Tris-HCl, 2 mM $CaCl_2$) for 30 min in 37 °C. After these pretreatment, the slides were rinsed in PCR buffer and used for reactions.

PRINS: For direct PRINS the reaction mix consisted of 0.1 - 0.2 mM dATP, dCTP, dGTP, different ratios of fluorescein-12-dUTP : dTTP or fluorescein-15-dATP : dATP , 1.25 - 7.5 mM $MgCl_2$, 3U *Taq* DNA polymerase (*Stratagene*, La Jolla, USA, *Promega*, Madison, USA or *Boehringer*, Mannheim, Germany) in 0.04 cm^3 1 × PCR buffer. The use of *TaqStart*™ antibody (*Clorrtech*, Palo Alto, USA) was tested to achieve "hot start" with *Promega Taq* DNA polymerase. For indirect PRINS, the nucleotide mixture was replaced by a DIG DNA labelling mixture (*Boehringer*, Mannheim, Germany) consisting of 0.1 mM dATP, dCTP and dGTP, 0.065 mM dTTP, and 0.035 mM DIG-12-dUTP. The primers used was a consensus *Arabidopsis*-type telomeric sequence 5′-$(AGGGTTT)_3$-3′ and a staggered complementary sequence 5′-$(CCCTAAA)_3$-3. PRINS reaction consisted of denaturation at 90 - 95 °C for 1 - 5 min, primer annealing at 50 - 60 °C for 15 min, and extension at 70 - 72 °C for time, which was equal to the sum of annealing periods in cycling reactions. For C-PRINS, the program was as follows: 1 min at 90 - 95 °C, 1 min at 50 - 60 °C, and 3 min at 70 °C followed by up to 28 cycles.

Signal detection and chromosome staining: Both PRINS and C-PRINS reactions were stopped in a Stop buffer (0.5 M NaCl, 0.05 M EDTA, pH 8.0) for 1.5 - 5 min at 70 °C, followed by a washing in 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 mg cm^{-3} in Blocking buffer) at 37 °C. The slides were counterstained with propidium iodide (PI) and mounted in *Vectashield* antifade solution (*Vector Laboratories*, Burlingame, USA).

Fluorescence microscopy: The slides were examined with an *Olympus BX 60* epifluorescence microscope (Tokyo, Japan) equipped with the filter sets for PI (*U-MWG*) and FITC (*U-NIBA*). The images of PI and FITC fluorescence were acquired separately with a black and white CCD camera. The camera was connected to a PC running the *ISIS* software (*Metasystems*, Belmont, USA). The images corresponding to PI and FITC were superimposed after contrast and background optimisation.

Results and discussion

Plant telomeric sequences were localised using PRINS for the first time by Thomas *et al.* (1996). Although the authors used a non-cycling PRINS with a Klenow enzyme (instead of now almost exclusively used *Taq* DNA polymerase), they were able to detect telomeric sequences on chromosomes in some cereals, grasses and clover. In our experiments with *V. faba*, discrete paired signals were observed at termini of all chromosomes (Fig. 1*A*). In addition to terminal signals, interstitial signals were observed in short arms of acrocentric chromosomes 2 and 5 (Fig. 1*B*). This is in agreement with the observation made after FISH with biotin-labelled synthetic plant telomere repeats (Schubert 1992). In contrast to this work, signals at centromere of chromosome 1 were never observed in our study. With the aim to further increase the intensity of specific labelling and to decrease non-specific labelling of chromosomes, some factors and reaction conditions were analysed (Table 1).

In contrast to a non-cycling PRINS, cycling PRINS (C-PRINS) has been reported to be more sensitive (Terkelsen *et al.* 1993). We have observed signals at chromosome termini after C-PRINS with only 5 reaction cycles, the reaction resulted in strong signals after 10 cycles. Further increase in the number of cycles did not increase the signal intensity and negatively influenced specificity of the reaction. Generation of increasing amounts of labelled DNA is also suggested by the shape around chromosome as described Terkelsen *et al.* (1993). In contrast to our observation, Gosden and Lawson (1994) described a gradual increase in the signal intensity up to 20 or 30 cycles of PRINS reaction on human chromosomes. Interestingly, also Shi *et al.* (1996) observed a specific labelling of DNA small single-copy sequences on soybean chromosomes after 30 cycles of PRINS. Optimal

number of reaction cycles depends on length and number of copies of sequences which we identified.

The use of a primer pair for both DNA strands produced significantly stronger signals compared to a reaction with one primer especially in case of C-PRINS. This observation proved that labelled products are not only made from newly synthesized complementary strands, but also from the labelled DNA synthesized in previous cycles of PRINS reaction. This confirms the observation of Terkelsen *et al.* (1993) made with human chromosomes. The author estimated a 20 % increase in signal intensity per one cycle.

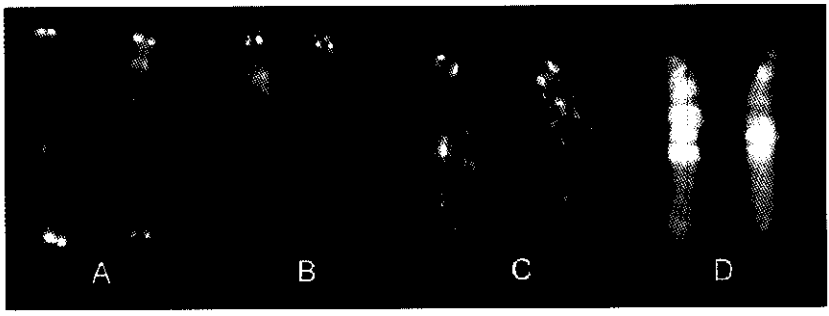

Fig. 1. Examples of *Vicia faba* chromosomes after PRINS and C-PRINS with telomeric-specific primers. *A* - clear signals were observed at termini of all chromosomes; *B* - in addition to terminal signals, interstitial signals were observed in short arm of acrocentric chromosomes II and V; *C* - indirect PRINS employing digoxigene-labelled nucleotides which were detected using an anti-DIG-fluorescein; this modification resulted in a formation of a non-specific ring around chromosomes; *D* - non-specific labelling of chromosomes (either due to a too high temperature during denaturation or due to unsufficient ageing of chromosome preparations). The localisation of non-specific labelling corresponds to the known distribution of repetitive DNA sequence *FokI* on field bean chromosomes.

We have also tested indirect PRINS, employing digoxigenin-labelled nucleotides which were detected using anti-DIG-fluorescein. This variation of the procedure resulted in higher signal intensity, but at the same time resulted in an increased level of non-specific labelling of chromosomes and in formation of a ring of non-specific fluorescence around chromosomes. This effect was especially pronounced with C-PRINS (Fig. 1*C*). Compared to direct or indirect PRINS and to indirect C-PRINS, the direct C-PRINS reaction reproducibly resulted in specific labelling and in the best signal to background ratio. Further increase in the signal intensity without increasing non-specific labelling was achieved after using two labelled nucleotides (fluorescein-12-dUTP and fluorescein-15-dATP) in a reaction mixture. Also the increase in primer concentration up to 2 μM led to the improvement of signal intensity. Further increase in primer concentration had no visible effect.

The intensity of the signals and the extent of non-specific labelling depended critically on the type and age of preparations. Compared to squash preparations, more specific labelling was achieved with isolated chromosomes. In any case, telomeric sequences were not detected on fresh preparations, best results were obtained with

Table 1. Factors which affect signal intensity and non-specific background on chromosomes.

			Interval	Optimal for signal intensity	Optimal for background
Non cycling				indirect labelling	direct labelling
Number of cycles	direct labelling		1 - 30	10	1
	indirect labelling		1 - 10	1	1
Age of slides	time at RT [d]		1 - 54	30	15 - 30
	time at -20 °C [d]		1 - 120	30	30
Temperature	denaturation [°C]		90 - 95	*	90
	annealing [°C]		50 - 60	55	50
Number of primers			1	+	n.d.
			2	+++	n.d.
Concentration	primers [μM]		1 - 4	2	n.d.
	dATP, dCTP, dGTP [μM]		100 - 200	n.d.	n.d.
Ratio	dTTP/fl.-dUTP		1/2 - 1/4	1/2	1/4
	fl.-dUTP [μM]***		8	+	n.d.
	fl.-dUTP + fl. -dATP [μM]***		8 + 8	++	n.d.
Taq DNA polymerase	*Stratagene*	$MgCl_2$ [mM]	1.50 - 7.5	4	n.d.
		BSA [%]	0.01 - 0.1	0	0.1
	Promega	$MgCl_2$ [mM]	1.25 -.4.5	2.5	n.d.
	Boehringer	$MgCl_2$ [mM]	1.25 - 4.5	2.75	n.d.
		BSA [%]	0.01 - 0.1	0 - 0.01	0.1
Type *Taq* DNA polymerase	*Stratagene*			+++	+
	Promega			+++	++
	Boehringer			++	+**
RNase [g dm^{-3}]			0, 0.1, 0.01	0	0.1 +++
Ligase [U mm^{-3}]			0.1	0	+++
Proteinase K [μg cm^{-3}]			1-5	1	5 ++
TaqStart™ antibody			half volume	0	half volume
ddNTP [μg]	*Taq* polymerase		100	0	+++
	Klenow enzyme		100	0	+++
Dehydration	ethanol			none	+++
Type of reaction	Eppendorf tube			+++	+
	SlideSeal			+	++
	SureSeal			++	++
	SelfSeal			++	++
Chromosomes dried on slide				+++	+
Root-tip squashes				+	+++

*- depends on age of slide; ** - depends on BSA concentration; *** - 8 μM fluorescein-dUTP + 17 μM dTTP or 8 μM fluorescein d-UTP + 17 μM dTTP + 8 μM fluorescein-dATP + 17 μM dATP; n.d. - no difference; fl. - fluorescein.

3 - 4 weeks-old slides, especially when primer for only one DNA strand was used. C-PRINS with a pair of primers gave relatively strong signals already on 1-week-old preparations. It is interesting to note that the strongest background was always found on fresh slides (less than 1-week-old) and that with ageing non-specific chromosome

labelling was decreasing. This observation does not agree with that of Gosden and Lawson (1995) who found that the non-specific labelling of human chromosomes was increasing with the age of slides. The authors recommended ligase treatment to decrease the background on older slides and speculated that the background was due to 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. Unfortunately, also the signal intensity was reduced after both treatments.

Denaturation temperature was found very critical, especially when freshly made chromosome preparations were used. A higher than optimal denaturation temperature resulted in non-specific labelling which corresponded to the known distribution of repetitive DNA sequence *FokI* (Yakura and Tanifuji 1983) on *V. faba* chromosomes (Fig. 1*D*). It may be speculated that this was due to a special structure of *FokI* chromatin, however, the exact nature of this effect is not clear. To preserve chromosome morphology on fresh (1- to 2-d-old) preparations, Gosden and Lawson (1994) 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. Similarly, the addition of BSA to the reaction mixture decreased non-specific labelling of chromosomes as well as the signal intensity. This could explain our observation that *Promega* and *Stratagene Taq* DNA polymerases gave better results than *Taq* DNA polymerase from *Boehringer* whose PCR buffer contains BSA. The use of the *TaqStart*™ antibody from *Clontech* permitted to perform a "hot start" reaction with the *Promega Taq* polymerase. However, also this modification of the procedure resulted not only in reduced non-specific labelling but also in decreased signal intensity.

To conclude, this study lead to the identification of various factors which influence the specificity and sensitivity of the PRINS reaction with plant chromosomes. Due to its simplicity and speed, PRINS is an attractive alternative to FISH. However, in contrast to FISH, PRINS appears to be very sensitive to chromatin structure. The results obtained in this study as well as those obtained with PRINS in other plant species (Kubaláková *et al.* 1997a,b,c) indicate that the procedure must be optimised for each object.

References

Abbo, S., Miller, T.E., King, I.P.: Primed-induced *in situ* hybridization to plant chromosomes. - Genome **36**: 815-817, 1993.

Doležel, J., Čihalíková, J., Lucretti, S.: A high yield procedure for isolation of metaphase chromosomes from root tips of *Vicia faba*. - Planta **188**: 93-98, 1992.

Charlieu, J.P., Pellestor, F.: Chromosome-specific PRINS. - In: Gosden, J.R. (ed.): Methods in Molecular Biology. Vol. 71. PRINS and *In Situ* PCR Protocols. Pp. 7-12. Humana Press, Totowa 1997.

Gosden, J., Hanratty, D.: PCR *in situ*: a rapid alternative to *in situ* hybridization for mapping short, low copy number sequences without isotopes. - Biotechniques **15**: 78-80, 1993.

Gosden, J., Lawson, D.: Rapid chromosome identification by oligonucleotide-primed *in situ* DNA synthesis (PRINS). - Human mol. Genet. **3**: 931-936, 1994.

Gosden, J., Lawson, D.: Instant PRINS: a rapid method for chromosome identification by detecting repeated sequences *in situ*. - Cytogenet. Cell Genet. **68**: 57-60, 1995.

Jiang, J., Gill, B.S.: Non-isotopic *in situ* hybridization and plant genome mapping the first ten years. - Genome **37**: 717-725, 1994.

Koch, J.E., Kolvraa, S., Petersen, K.B., Gregersen, N., Bolund, L.: Oligonucleotide-priming methods for the chromosome-specific labelling of alpha satellite DNA *in situ*. - Chromosoma **98**: 259-265, 1989.

Kubaláková, M., Čihalíková, J., Doležel, J.: Localization of GAA microsatellite on rye chromosomes by direct cycling-PRINS (C-PRINS). - In: Maluszynska, J. (ed.): Abstract of the Symposium Molecular Biology for Agriculture. P. 147. Institute of Plant Molecular Biology, České Budějovice 1997c.

Kubaláková, M., Čihalíková, J., Doležel, J.: Localization of GAA microsatellite on barley chromosomes by direct cycling-PRINS (C-PRINS). - In: Abstract of Spring Symposium on Plant Cytogenetic. P. 26. Silesian University, Ciesyn 1997b.

Kubaláková, M., Macas, J., Doležel, J.: Mapping of repeated DNA sequences in plant chromosomes by PRINS and C-PRINS. - Theor. appl. Genet. **94**: 758-763, 1997a.

Leitch, A.R., Schwarzacher, T., Jackson, D., Leich, I.J.: *In Situ* Hybridization: a Practical Guide. - BIOS Scientific Publishers, Oxford 1994.

Macas, J., Doležel, J., Gualberti, G., Pich, U., Schubert, I., Lucretti, S.: Primer-induced labelling of pea and field bean chromosomes *in situ* and in suspension. - BioTechniques **19**: 402-408, 1995.

Pellestor, F.: PRINS' technique: A new strategy for *in situ* chromosome detection. - Ann. Pathol. **17**: 9-10, 1997.

Pich, U., Meister, A., Macas, I., Doležel, J., Lucretti, S., Schubert, I.: Primed *in situ* labelling facilitates flow sorting of similar sized chromosomes. - Plant J. **7**: 1039-1044, 1995.

Mukai, Y., Appels, R. : Direct chromosome mapping of plant genes by *in situ* polymerase chain reaction (*in situ* PCR). - Chromosome Res. **4**: 401-403, 1996.

Schubert, I.: Telomeric polymorphism in *Vicia faba*. - Biol. Zentralbl. **111**: 164-168, 1992.

Shi, L., Zhu, T., Morgante, M., Rafalski, J.A., Keim, P.: Soybean chromosome painting: A strategy for somatic cytogenetics. - J. Hered. **87**: 308-313, 1996.

Terkelsen, C., Koch, J., Kolvraa, S., Hindkjaer, J., Pedersen, S., Bolund, L.: Repeated primer *in situ* labelling: formation and labelling of specific DNA sequences in chromosomes and nuclei. - Cytogenet. Cell Genet. **63**: 235-237, 1993.

Thomas, H.M., Williams, K., Harper, J.A.: Labelling telomeres of cereals, grasses and clover by primed *in situ* DNA labelling. - Chromosome Res. **4**: 182-184, 1996.

Yakura, K., Tanifuji, S.: Molecular cloning and restriction fragment analysis of *EcoRI*-fragment of *Vicia faba* rDNA. - Plant Cell Physiol. **24**: 1327-1330, 1983.