

## PRINS AND C-PRINS: PROMISING TOOLS FOR THE PHYSICAL MAPPING OF THE LUPIN GENOME

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**Abstract:** Two molecular cytogenetics methods, PRINS (primed *in situ* DNA labeling) and C-PRINS (cycling PRINS), were optimized for the physical mapping of several types of DNA sequences on the mitotic chromosomes of the narrow-leaved lupin (*Lupinus angustifolius* L.). The fragment of the *FokI* element from *Vicia faba* was localised by indirect PRINS reaction. Two other sequences, fragments of the coding sequences of *L. luteus* and of *L. angustifolius*, were localised by indirect C-PRINS. These techniques are faster and more sensitive than FISH, and they allowed the mapping of short DNA fragments. The data obtained shows that both types of PRINS are valuable tools for chromosome identification in lupin.

**Key words:** PRINS, C-PRINS, Physical mapping, Chromosomes, *Lupinus angustifolius* L.

### INTRODUCTION

The identification of individual lupin chromosomes by traditional methods of cytology is not possible due to their high numbers, size gradient, and similar morphology [1, 2]. Hence, cytogenetics methods have been applied to physically map the *Lupinus* genome. Thus far, fluorescence *in situ* hybridisation (FISH) was mainly applied to the search for chromosome markers in lupins [3-6]. FISH is an efficient and widely used method for the identification of a number of

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Abbreviations used: BAC – bacterial artificial chromosome; C-PRINS – cycling PRINS; DAPI – 4',6'-diamidino-2-phenylindole; FISH – fluorescence *in situ* hybridization; PCR – polymerase chain reaction; PRINS – primed *in situ* DNA labeling

DNA sequence loci and for their physical location in plant genomes, but is time-consuming and requires labeled probes. Due to the limited sensitivity of FISH, probes have to be homologous to large fragments of the analysed genome or to tandem-repeated sequences. The PRINS procedure is an alternative method for detecting DNA sequences on chromosomes, and it has several advantages over FISH. It is more sensitive, as it can localise shorter DNA sequences, and the reaction does not require labeled probes. Moreover, a minimum of sequence information is sufficient to design and synthesize oligonucleotide primers. The entire procedure is also faster.

Both PRINS, first described by Koch [8], and its variant C-PRINS (cycling PRINS) [9] are based on the amplification of DNA fragments using sequence-specific primers and target DNA *in situ*. The procedure is carried out on a microscope slide in the presence of a DNA polymerase and nucleotides, of which at least one is labeled. However, while the PRINS reaction takes place during one thermal cycle, C-PRINS involves a sequence of thermal cycles analogous to the polymerase chain reaction (PCR), which provides an increased amount of amplified labeled product and correspondingly increases the sensitivity of the technique [9]. In both cases, the newly synthesized DNA can be detected directly (fluorochrome-labeled nucleotides) or indirectly (the nucleotides being labeled with biotin or digoxigenin, and the signals detected using fluorochrome-conjugated antibodies).

PRINS and C-PRINS found important applications in cytogenetic research dealing not only with the human genome [9-11], but also with plant genomes. Abbo [12] used digested plasmid DNA carrying wheat rDNA as primers for PRINS in rye, while Mukai and Appels [13] used C-PRINS for the physical mapping of the 45S rRNA and 5S rRNA genes in rye. In subsequent years, other studies on plants were performed [14-16]. In this paper, we report on the optimization of the PRINS and C-PRINS protocols for the rapid physical localisation of some DNA sequences on mitotic chromosomes of the narrow-leaved lupin *L. angustifolius*.

## MATERIAL AND METHODS

The narrow-leaved lupin (*L. angustifolius* L.,  $2n = 40$ ) is a self-pollinating plant of agricultural importance, belonging to the Malacospermae (smooth-seeded) group of the *Lupinus* genus. Seeds of *L. angustifolius* cv. Sonet used for this study were obtained from the Polish *Lupinus* Gene Bank in the Wiatrowo Plant Breeding Station.

To obtain preparations with sufficient metaphases, cell cycle synchronization was applied. That procedure, described by Doležel for several plant species [17], was modified for *Lupinus*. Seeds were imbibed for 12 h in distilled water at 25°C and then germinated on moistened filter paper (24 h, 25°C). They were then transferred to an aerated solution of 3 mM hydroxyurea (18 h, 25°C), and placed in a HU-free aerated Hoagland solution (4 h, 25°C). To accumulate cells in

metaphase, seeds were transferred to a 2.5- $\mu$ M oryzalin solution (2 h, 25°C). Permanent squash preparations were made from root meristems according to Schwarzacher *et al.* [7] and Naganowska and Zielińska [3]. The slides were stored at -20°C.

Before performing the PRINS and C-PRINS reactions, the slides were dried overnight at 37°C. Frame-Seal Chambers (MJ Research, Inc.) were stuck to the slides. The first steps in the routine FISH procedure (RNase treatment, post-fixation, pepsin digestion and heat denaturation) turned out to be unnecessary.

Three DNA sequences were chosen for localisation on the mitotic chromosomes of *L. angustifolius*:

1) a fragment of the *FokI* element from *Vicia faba* [16], 5'-CATTATGGAAGGTAGTCTGTTGTCGAG-3', synthesized by GENSET SA;

2) the coding sequence from the cDNA library of the yellow lupin (*L. luteus*) [18], Rev: 5'GACTACATTGAATCATGAAGA(ACG)AGGGACGAG3', For: 5'AGAAGACATGCATTGCCATGGTTC3', with the primers designed using the Oligo 4.1 computer program;

3) the marker sequence for the anthracnose resistance gene of *L. angustifolius* [19], AntjM2P1 Rev: 5'TCATCTCTAAATCCTATCTCAG3', AntjM2P2 For: 5'GTACCTGATGACAATTAGTCAC3'.

The presence of all the sequences in the genome of the narrow-leaved lupin was ascertained by PCR using the same primers. Moreover, we checked the whole *FokI* element (59 bp) by PCR (primers designed on the basis of the *FokI* sequence [20], Rev: 5'CAAGGCTACCATCCATT3', For: 5'TCATTATGGAA GGTAGTCTG3'). The *L. angustifolius* DNA was isolated from young leaf tissue using a DNeasy Plant Mini Kit (Qiagen). 50 ng of DNA was used as a template in a 20  $\mu$ l reaction volume containing 10  $\mu$ M of each primer, 2.5 mM dNTP and 2 U Taq Polymerase (Invitrogen). Amplification was performed in an MJ PTC-200 thermal cycler for 30 cycles (after a preliminary denaturation at 91°C for 5 min). Each cycle comprised: denaturation for 1 min at 91°C, primer annealing for 1 min at an appropriate temperature gradient (for the *FokI* fragment 43-59°C, for the whole *FokI* element 40-52°C, for the cDNA 57-62°C, and for the anthracnose resistance marker 54-57°C), and sequence extension for 1 min at 72°C. The last step was a final extension for 10 min at 72°C. The PCR products were separated on a 2% agarose gel and visualized under UV light following ethidium bromide staining. A DNA Ladder (Gibco BRL) was the size marker used for electrophoresis.

For localisation on the *L. angustifolius* chromosomes, the first sequence (the *FokI* fragment) was analysed by indirect PRINS, and the remaining two (cDNA and resistance marker) by indirect C-PRINS. The reaction mixtures for PRINS and C-PRINS consisted of: 2  $\mu$ M of each primer, 0.1 mM dATP, dCTP, dGTP and 0.035 mM DIG-12-dUTP (Roche) with 0.065 mM dTTP, 3 mM MgCl<sub>2</sub> and 3 U Taq Polymerase (Invitrogen). Twenty-five microliters of mixture were put into each frame and covered with a polyester coverslip. The PRINS cycle comprised: denaturation (91°C, 5 min), primer annealing (55°C, 15 min) and

sequence extension (72°C, 30 min). For C-PRINS, the program was: preliminary denaturation (91°C, 5 min), followed by up to 20 cycles of denaturation (91°C, 1 min), primer annealing (57°C, 1 min) and sequence extension (72°C, 1 min), and final extension (72°C, 10 min). The reaction was conducted in the MJ Thermal Cycler PTC-200 with a *Slide Griddle* plate. The reaction was stopped by adding stop buffer (0.5 M NaCl, 0.05 M EDTA, pH 8.0) at 70°C for 2 min. After the removal of the stop buffer, the slides were incubated in blocking buffer (0.5% blocking reagent – Roche; 0.1 M maleic acid – Fluka; 0.15 M NaCl, pH 7.5) at 37°C for 30 min and then for 60 min in anti-DIG-fluorescein (Roche) solution (20 µg/ml). The slides were then placed in a wash buffer (0.1 M maleic acid, 0.15 M NaCl and Tween-20, pH 7.5) at room temperature for 5 min, and counterstained with DAPI in a Vectashield antifade solution (Vector). The preparations were examined with an OLYMPUS BX60 Research System Microscope. Images were acquired with a black and white CCD camera interfaced to a PC running the analySIS 3.0 software (Soft Imaging System). The results presented were based on 5-10 reliable metaphase plates.

## RESULTS AND DISCUSSION

The first landmarks on the small-sized lupin chromosomes, difficult to distinguish morphologically, were the rDNA loci (18-25S and 5S rDNA) localised by double-target fluorescence *in situ* hybridisation (FISH) [3-6]. However, in *L. angustifolius* (2n = 40), these loci could serve as markers for only several chromosome pairs [3, 6]. As the remaining chromosomes were still unidentified, it was necessary to localise other cytogenetic markers. In this study, we applied the PRINS and C-PRINS techniques to localise short DNA fragments in the *L. angustifolius* genome.

Due to a lack of information on similar investigations in lupins, for the PRINS reaction, we used sequences that had been analysed for other legumes and that had a good chance of giving positive results for *L. angustifolius*. The *FokI* repeat, considered species-specific for *Vicia faba* [20], was not detected in any other taxa. However, as far as we know, it had not been investigated in *Lupinus*. The fragment of the *FokI*-element was previously applied in the physical mapping of *V. faba* by PRINS and C-PRINS [15, 16, 20]. The next two sequences – a fragment from the cDNA *L. luteus* library and the marker sequence for the anthracnose resistance gene from *L. angustifolius* – were used as available lupin sequences.

The PCR reactions for all the sequences analysed yielded amplified products. For the *FokI* fragment, we obtained products (Fig. 1A) varying in the intensity of the bands, of a size gradient from about 300 to 1500 bp. The amplification products for the entire *FokI* unit (Fig. 1B) ranged from 400 to 1500 bp. The fragment from the cDNA *L. luteus* library gave products of 100-400 bp (Fig. 1C), and the anthracnose resistance gene marker gave one product of ca. 200 bp (Fig. 1D).

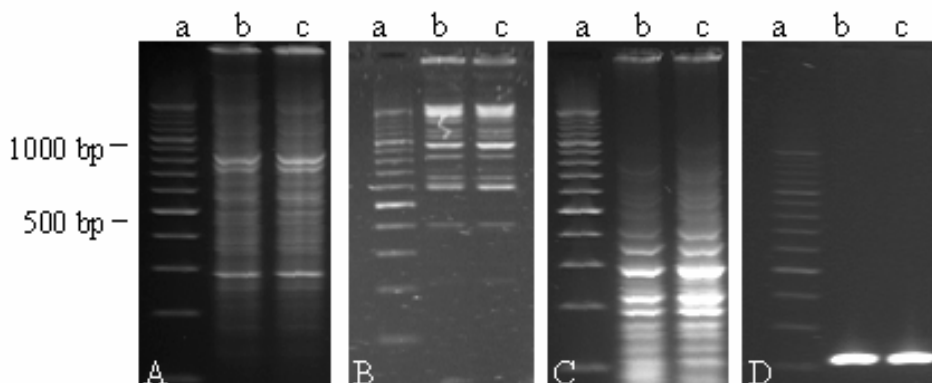


Fig. 1. PCR products for the sequences chosen for PRINS and C-PRINS reaction; (a) is the DNA ladder). A – The 27-bp *FokI* fragment: annealing temp. 40°C (b) and 45°C (c). B – The entire *FokI* element (59 bp): annealing temp. 40°C (b) and 45°C (c). C – The fragment from the cDNA of *L. luteus*: annealing temp. 57°C (b) and 59°C (c). D – The marker sequence for the anthracnose resistance gene from *L. angustifolius*: annealing temp. 54°C (b) and 57°C (c).

The *FokI* fragment, used as a single primer, was analysed on the chromosomes of *L. angustifolius* by PRINS and C-PRINS. The results of C-PRINS were not satisfactory because of the strong background and dispersed signals. The signals of the PRINS reaction were of variable intensity, and localised mainly in the centromeric regions (Fig. 2A). Some were visible as single dots, others as double dots (on both chromatids). The PRINS signals of the *FokI* fragment, observed on seven to eight chromosome pairs, may serve as cytogenetic markers for chromosomes of the narrow-leafed lupin. Furthermore, our results show the presence of a sequence in the genome of *L. angustifolius* similar to the *FokI* satellite of *V. faba*.

The *L. luteus* cDNA and the marker sequence for anthracnose resistance were first analysed by the PRINS reaction, but the intensity of signals obtained was very low. We therefore switched to the C-PRINS procedure, following which some signals were observed after only ten cycles. Twenty cycles provided strong, reproducible signals.

Signal quality was improved by performing two additional steps: preliminary denaturation (91°C, 5 min) before the C-PRINS cycles, and extension (72°C, 10 min) as the final step. It should be noted that with twenty-five and thirty cycles, non-specific labeling of chromosomes occurred. The cDNA sequence of yellow lupin was found in two loci on two different medium submetacentric chromosomes, one in the centromeric region and another on the short arm (Fig. 2B). Thus, that particular sequence provides a cytogenetic marker for two chromosome pairs of the narrow-leafed lupin.

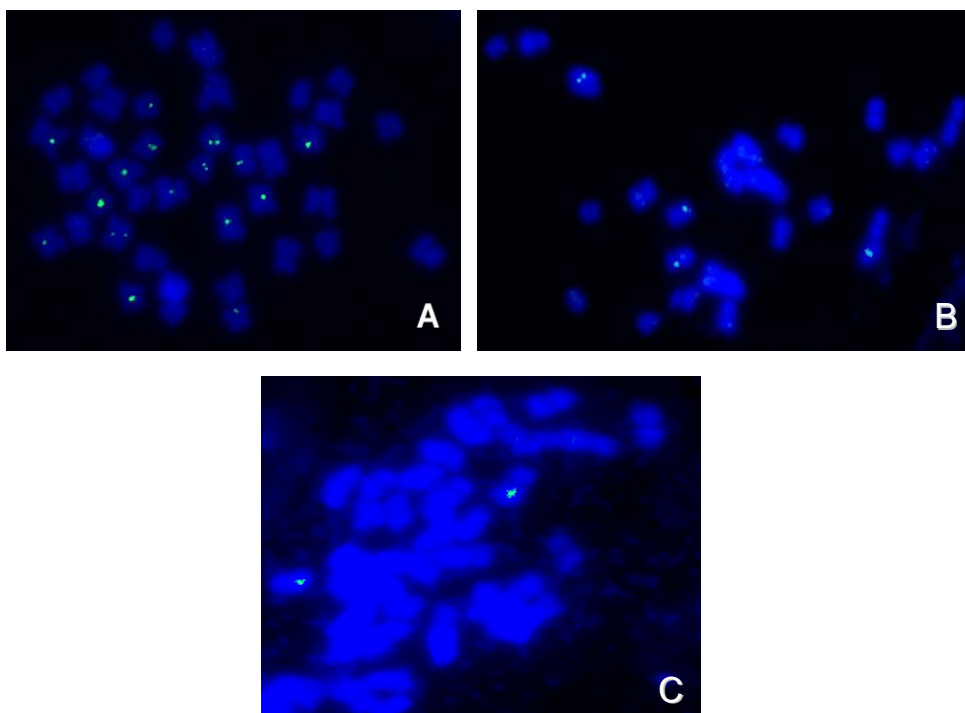


Fig. 2. *Lupinus angustifolius* metaphase chromosomes with signals. A – *FokI* fragment (PRINS). B – sequence from the cDNA library of *L. luteus* (C-PRINS). C – marker sequence for the anthracnose resistance gene of *L. angustifolius* (C-PRINS). [green – FITC, blue – DAPI]

The second DNA fragment analysed was connected with the anthracnose disease resistance gene in that species [19]. The C-PRINS signals were observed in one locus on a medium submetacentric chromosome, on the long arm near the centromere (Fig. 2C).

The procedure of cell cycle synchronization provided a high number (up to 50%) of metaphase plates. The use of oryzalin solution improved the distribution of chromosomes. According to some authors [12-14], prior to PRINS or C-PRINS, slides should be treated with RNase, post fixed, then pepsin treated and denatured (as is done prior to FISH); however, others skip these steps [16] and obtain slides with distinct signals and without any background. We compared these variant methods and found that pre-treatment was unnecessary.

It is noteworthy that some attempts had been made to localise a BAC clone connected with the anthracnose resistance gene on *L. angustifolius* chromosomes. This clone was selected and isolated from a genomic BAC library of *L. angustifolius* cv. Sonet [21], labeled and used as a molecular probe for FISH. As here with the PRINS reaction, signals in one locus were observed, in the same region of a medium, submetacentric chromosome [Naganowska and Kaczmarek, unpublished results].

The double C-PRINS procedure was also performed simultaneously using primers for both sequences (the sequence from the cDNA *L. luteus* library and the marker for the anthracnose resistance gene from *L. angustifolius*). The preliminary results show that each of these sequences was mapped on different chromosomes, providing markers for three chromosome pairs altogether [Kaczmarek and Naganowska, unpublished results].

Our earlier results from FISH studies allowed us to localise the first cytogenetic landmarks in the *L. angustifolius* genome. Physical mapping of BAC clones by FISH is in progress at the moment. Current results indicate that indirect PRINS and C-PRINS techniques are suitable tools for the localisation of short DNA sequences as new markers for chromosome identification. All these cytogenetic chromosome markers together will enable us to construct the karyotype of *L. angustifolius*. PRINS and C-PRINS will also be useful for the localisation of marker sequences from the genetic maps of the narrow-leafed lupin [22, 23] on its chromosomes. That will allow the verification of linkage groups and the integration of the physical and genetic maps of *L. angustifolius*.

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