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Development of molecular cytogenetics and physical mapping of ribosomal RNA genes in *Lupinus*

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Abstract

Identification of individual chromosomes in *Lupinus* is not possible due to gradient in size and similar morphology. To overcome this problem, molecular cytogenetics was developed for *Lupinus*. As an initial step in karyotype analysis, fluorescent *in situ* hybridization (FISH) was performed to determine genomic distribution of rRNA genes in *L. hispanicus*, *L. luteus* and *L.* × *hispanicoluteus*. It was found that all three diploid species posses two chromosome pairs carrying 18S-5.8S-25S rDNA and one chromosome pair carrying 5S rDNA. The use of probes for rDNA permitted unambiguous identification of three different pairs of chromosomes and revealed conservation of the number of rDNA loci among the three species. The study represents the first step in physical mapping of *Lupinus* genome through FISH by providing distinct chromosome landmarks.

Additional key words: fluorescent *in situ* hybridization, *Lupinus hispanicus*, *L. luteus*, *L.* × *hispanicoluteus*, rRNA genes.

Introduction

Genus *Lupinus* belongs to a family *Leguminosae* and comprises both wild species and economically important crops (Gladstones 1998). Lupin cultivars are valuable source of seed protein for human and animal nutrition. They are also used as green mass for forage as well as organic fertilizers. Because of large intraspecific and intrageneric variation, there is a great potential for improving cultivated forms of *Lupinus* by introducing wild gene pool *via* interspecific hybridization. However, until now there are only few examples of successful production of distant hybrids within the genus. A stable synthetic species *L.* × *hispanicoluteus*, which has been obtained after hybridization between wild *L. hispanicus* subsp. *hispanicus* and cultivated *L. luteus* (Święcicki *et al.* 1999), may be an example. *L. luteus* is characterized by a high protein content in seeds and low soil requirements but also a relatively low and unstable seed yield. Probably some characters of *L. hispanicus* (such as resistance to waterlogging, cold and diseases) could be used for *L. luteus* improvement.

Compared to other plant genera, cytological studies in *Lupinus* are lagging behind. Lupins are particularly difficult to karyotype by conventional methods due to difficulties in preparing chromosome spreads. Most of the chromosomes in *L. hispanicus*, *L. luteus* and *L.* × *hispanicoluteus* complements are median or submedian. Due to gradient in chromosome size and similar morphology, it is not possible to identify chromosome pairs and to distinguish individual species based on chromosome length or arm ratio (Naganowska and Ładoń 2000). However, the three species differ in their genome size (Obermayer *et al.* 1999). There is a 1.13-fold difference in nuclear DNA content between *L. luteus* and *L. hispanicus*. Interestingly, genome size of *L.* × *hispanicoluteus*, which is believed to be their hybrid, is not intermediate, it is close to *L. hispanicus*.

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Since karyotype differences among most of *Lupinus* species are small, lupins could benefit from the application of molecular cytogenetics. Fluorescent *in situ* hybridization (FISH) allows localization of DNA sequences on chromosomes and interphase nuclei (Heslop-Harrison 1991). It provides chromosome landmarks for identification of individual chromosomes and chromosome arms (Dong *et al.* 2000, Cheng *et al.* 2001), allows identification of alien chromosome segments (Schmidt *et al.* 1997, Pickering *et al.* 2000), investigation of long-range chromosomes organization (Cuadrado and Schwarzacher 1998, Nouzová *et al.* 1999) and enables construction of physical maps and their integration with genetic maps (Fuchs *et al.* 1998, Kulikova *et al.* 2001). This information is very useful in breeding programmes as well as to understand changes of chromosome structure during evolution and their role in speciation.

The application of FISH in chromosome studies relies on the availability of suitable DNA markers. Both the 18S-5.8S-25S rRNA and 5S rRNA genes are organised in tandemly repeated units ranging from 250 to 22 000 copies per genome in plants (Rogers and Bendich 1987). The clusters of rRNA genes are usually localized at few discrete chromosomal sites and can be easily visualised by FISH (Ohmido and Fukui 1995, Hanson *et al.* 1996, Doleželová *et al.* 1998). This makes rRNA genes suitable targets for initial studies on physical genome mapping. In this study, we have used FISH to examine the distribution of rDNA sites in *Lupinus*. The aim was to provide the first chromosome landmarks and to compare the genomic distribution of rDNA genes in three related species of *Lupinus*.

Materials and methods

Plants and preparation of chromosome spreads: Seeds of *Lupinus hispanicus* subsp. *hispanicus* Boiss. et Reut. (2n=52), *L. luteus* L. (2n=52) and *L.* × *hispanicoluteus* Swiec. et Swiec. (2n=52) were germinated at 18 - 20 °C in Petri dishes on moistened filter paper. Root tips were excised, treated with ice-cold water for 24 h and fixed in a mixture of ethanol and glacial acetic acid (3:1, v/v) for 24 h. The meristems were washed in enzyme buffer (0.01 M citric acid-sodium citrate, pH 4.8) for 15 min and digested in 2 % (m/v) cellulase (*Calbiochem Corp.*, La Jolla, USA) and 20 % (v/v) pectinase from *Aspergillus niger* (*Sigma*, St. Louis, USA) in enzyme buffer, for 30 min at 37 °C. Digested root tips were squashed under glass coverslips in 45 % acetic acid. Immediately, after that the coverslips were removed on dry ice, preparations were air dried and stored at -20 °C.

DNA probes: The plasmid VER 17 (Yakura and Tanifuji 1983) containing 5.8S, parts of the 18S and most of 25S genes of *Vicia faba* was cloned into XL1 Blue *E. coli*. The probe was labelled with digoxigenin using a Random Primed DNA Labelling Kit (*Roche Diagnostics*, Mannheim, Germany) according to the manufacturer's instructions. The 5S rDNA probe was labelled with biotin using PCR with a pair of specific primers (RICRGAC1, RICRGAC2), which amplify 303 bp in rice (Ohmido and Fukui 1995).

***In situ* hybridization:** The slides were baked at 60 °C for 1 h, treated with 100 μg cm^{-3} RNase in 2×SSC (0.3 M NaCl, 0.03 M sodium citrate) for 1 h at 37 °C and washed 3 times in 2×SSC at room temperature (RT). After treatment with 500 μg cm^{-3} pepsin (*Sigma*) in 0.01 M HCl (10 min, 37 °C) and several washes in H_2O and 2×SSC, the preparations were fixed in freshly prepared 4 % (m/v) paraformaldehyde in water for 10 min at RT. The slides were washed twice in 2×SSC at RT, once at 60 °C, and then incubated in 70 % formamide (in 2×SSC) for 3 min at 76 °C to denature chromosomal DNA. Finally, the slides were dehydrated in ethanol series (70, 90 and 100 %) and air-dried at RT.

The hybridization mixture consisted of deionized 100 % formamide, 50 % dextran sulphate, 20×SSC, 10 % sodium dodecyl sulphate, sheared blocking salmon sperm DNA (*ca.* 100 × probe concentration) and labelled probe (50 - 100 ng per slide). The mixture was denatured at 95 °C for 10 min and then chilled on ice. 0.015 cm^3 of hybridization mixture was added to each slide and covered with a plastic coverslip. Hybridization was carried out in a humid chamber at 37 °C for 48 h. Post hybridization washes were performed at 42 °C: 2×SSC, stringent wash in 20 % formamide (v/v in 0.1×SSC), 0.1×SSC, 2×SSC; the last wash was performed at RT. In order to visualize the sites of probe hybridization, chromosome preparations were equilibrated in detection buffer (DB: 0.2 % Tween 20 v/v in 4×SSC) for 5 min at 37 °C, and incubated in 2 % (m/v) BSA (made in DB) for 5 min at RT. Then the slides were incubated with 20 μg cm^{-3} anti-digoxigenin-FITC (*Roche*) and 10 μg cm^{-3} avidin-Rhodamine (*Vector Laboratories*, Burlingame, USA) in 2 % BSA, in a humid chamber for 1 h at 37 °C, in the dark. Afterwards the slides were washed three times in DB (5 min each at 37 °C) to remove excess antibody. Chromosomes were counterstained with 1.5 μg cm^{-3} DAPI (made in DB) for 10 min at RT in the dark. Finally, the slides were briefly washed in DB and mounted in Vectashield antifade solution (*Vector Laboratories*).

Fluorescence microscopy: The slides were examined with *Olympus* (Hamburg Germany) *AX 70 Research System Microscope* and the images of DAPI, FITC and rhodamine fluorescence were acquired sparately with black and white CCD camera. The camera was interfaced to a PC running the *AnalySIS 3.0* software (*Soft Imaging System*, Muenster, Germany). Image processing consisted exclusively of signal intensity, contrast, and background adjustments that affected the whole image.

Results and discussion

Fluorescent *in situ* hybridization revealed four intercalary sites of 18S-5.8S-25S rDNA in all three *Lupinus* species (Fig. 1*A*,*B*,*C*). The two pairs of signals differed in intensity. Two major signals appeared to be localized on a pair of the longest chromosomes. As a rule, 18S-5.8S-25S rDNA is a marker for nucleolar organizer region (NOR), which is normally visible on mitotic chromosomes as secondary constriction. Although secondary constrictions were not noticeable regularly on mitotic chromosomes of *L. hispanicus*, *L. luteus* and *L.* × *hispanicoluteus*, they were consistently observed on the largest pair of chromosomes. Thus a probe for 18S-5.8S-25S rDNA may be used as a marker for the largest NOR-bearing chromosome pair in all three *Lupinus* species. The second pair of 18S-5.8S-25S rDNA hybridization sites that exhibited lower intensity identified a pair of chromosomes, which did not reveal any marked morphological feature. Thus FISH with the rDNA probe provides the first cytogenetics landmark that can be used to identify this pair of chromosomes.

In contrast to the 18S-5.8S-25S rDNA probe, only two sites (one locus) were revealed after FISH with the probe for 5S rDNA in *L. hispanicus*, *L. luteus* and *L.* × *hispanicoluteus*. The two sites were present on chromosomes, which did not carry the 18S-5.8S-25S rDNA (Fig. 1*A*,*B*,*C*). Based on this observation it may be concluded that 5S rDNA provides a cytogenetics marker for unambiguous identification of a third pair of chromosomes in all three *Lupinus* species.

The application of FISH to other genera of *Leguminosae*, which are difficult to karyotype, gave very interesting results. For example, Raina and Mukai (1999) analysed 21 diploid and tetraploid species of *Arachis* (with little variation of karyotype), using FISH with probes for 5S and 45S rDNA. They found a great interspecific diversity concerning the number of rDNA loci and the intensity of hybridization signals. The differences in the number of 5S and 25S rDNA loci were also revealed by two-colour FISH among seven diploid *Lathyrus* species, which have similar chromosome morphology but different nuclear DNA content (Ali *et al.* 2000).

Although genus *Lupinus* is believed to be of polyploid origin, its ploidy level is not clear (Wolko and Weeden 1989, Atkins *et al.* 1998 and references therein). The observation of only one 5S rDNA locus in the present

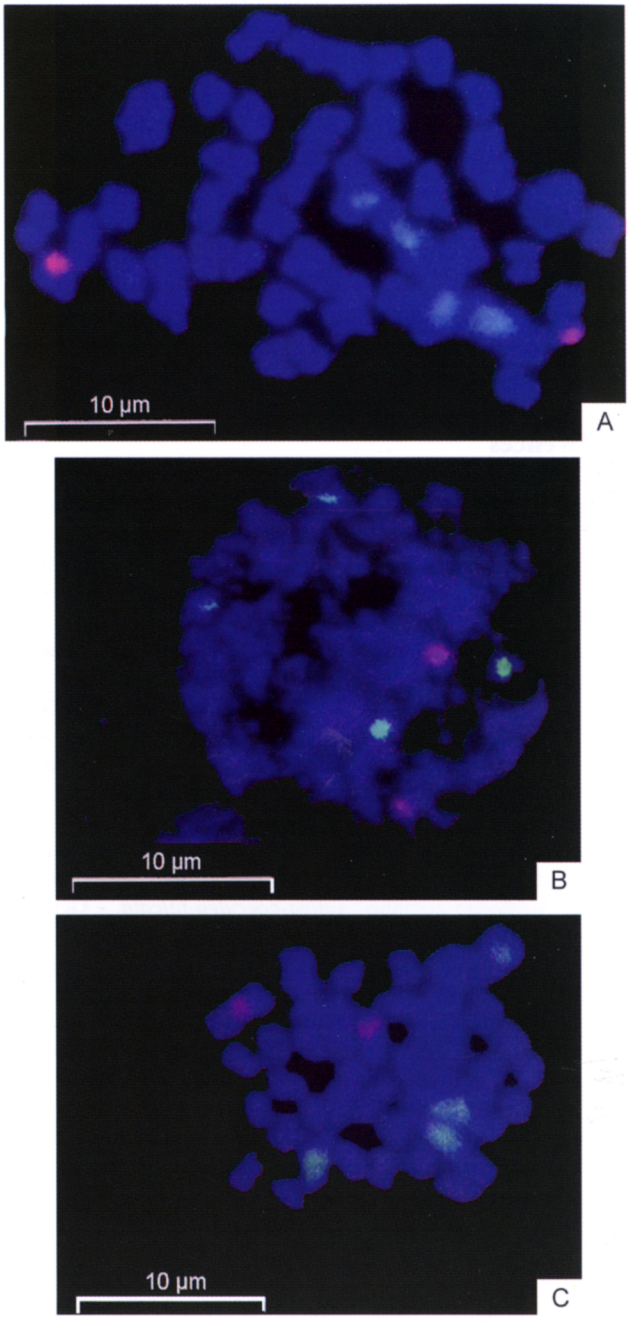

Fig. 1. Mitotic metaphase (*A*, *C*) and prometaphase (*B*) plates of diploid (2n=52) *Lupinus* species after *in situ* hybridization with rDNA probes. The chromosomes were counterstained with DAPI (blue colour), the probe for 18S-5.8S-25S rDNA was detected by FITC (yellow-green signals) and the probe for 5S rDNA was detected by rhodamine (red signals). *A* - *L. hispanicus*, *B* - *L. luteus*, *C* - *L.* × *hispanicoluteus*.

study contradicts the hypothesis of polyploid origin. However, it is also possible that the number of 5S rDNA sites was reduced after a polyploidization event. The results obtained in other plant species indicate that rDNAs may not be reliable markers to proof the origin of putative polyploids. On the one hand, Calderini *et al.* (1996) found that the number of 18S rDNA sites in tetraploid *Medicago sativa* was double the number found in diploid *M. coerulea* and *M. falcata*, suspected progenitors of *M. sativa*. On the other hand, tetraploid *Brassica* species have fewer 18S-5.8S-25S rDNA loci than the sum of their diploid ancestors (Maluszyńska and Heslop-Harrison 1993). Similarly, Hanson *et al.* (1996) reported variation in the number and size of 18S-28S rDNA loci and the lack of additivity between the tetraploid *Gossypium hirsutum* and its putative diploid ancestors. Adachi *et al.* (1997) found that in *Brachyscome linearilobа* the number of 5S rDNA sites increased linearly with the ploidy level. However, the number of 45S rDNA sites remained constant regardless of ploidy level.

This study represents the first step in physical mapping of *Lupinus* genome through FISH by providing the first physical chromosome landmarks. The use of probes for 18S-5.8S-25S rDNA and 5S rDNA permitted unambiguous identification of three pairs of chromosomes and revealed conservation of the number of rDNA loci among *L. hispanicus*, *L. luteus* and *L.* × *hispanicoluteus*. Future work should concentrate on comparative mapping of other repetitive DNA sequences and large DNA fragments. Such work could help not only to characterize new materials obtained in breeding programmes but also to reveal interspecific relationships and improve the knowledge of the evolution of *Lupinus* species.

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