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Nuclear genome size and karyotype analysis in *Papaver* for BAC library construction

T. K. Kyrylenko, O. I. Martynenko, O. G. Alkhimova

Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine
150 Zabolotnoho str., 03143 Kyiv, Ukraine

E. mail: alkhimiv@hotmail.com

The objective of the research carried out is study of the pathway of alkaloid production in Papaver species and cell lines, and integration data on physical mapping of newly developed marker DNA sequences with existing difference in expression of genes for key enzymes of alkaloid biosynthesis. This research requires the knowledge on genome structure and organization, and development of genomic resources for detailed characterization of opium poppy genome. The work is focused on the investigation of some features of genome organization of Papaver somniferum and related species, Papaver bracteatum and Papaver rhoeas. These characteristics are necessary for the construction of a BAC library which would be used as appropriate genomic resource for characterization of karyotype changes in poppy stocks with altered alkaloid biosynthesis pathway. Some of these stocks (cell lines) were generated and differed in the types of alkaloids they accumulated.

Key words: Papaver, alkaloids, genome size, bacterial artificial chromosome library, FISH.

Introduction. The data from several different alkaloid-producing plants suggest that their biosynthesis and accumulation involve a highly regulated process that includes cell-, tissue-, development and environment-specific controls [1, 2]. The evolution of alkaloid pathways together with their cellular compartmentation appears to be closely associated with the primary reactions from which they have evolved. Opium poppy, *Papaver somniferum*, is cultivated for its alkaloid-rich latex. Tyrosine decarboxylase (TyDC) is the first enzyme in poppy alkaloid biosynthesis and is encoded by a small gene family. Members of this family are differentially expressed in organs of the plant and cultivated cells [3]. With the availability of an increasing number of genes involved in alkaloid biosynthesis, increasing efforts would be made to identify the regulators [4] associated with the development of specialized cell types which relate to alkaloid biosynthesis and accumulation. Gene localization and isolation require a detailed knowledge of genome structure. While genetic maps provide information on relative order of molecular markers and genes along the chromosomes, physical mapping provides data on the physical position of DNA sequence within a genome. *P. somniferum*, *P. bracteatum* and *P. rhoeas* belong to one taxonomic section within genus *Papaver* and produce morphinan and benthophenanthridine type alkaloids which belong to different biosynthetic pathways.

Large-insert DNA libraries are one of the key resources that facilitate gene isolation by positional cloning and the analysis of genome organization, structure and evolution. The easy handling and propagation of the clones make BACs (bacterial artificial chromosomes) an invaluable tool in genomic research, used for a variety of applications, including physical mapping and genome sequencing [5]. The production of full-length cDNA molecules from genomic DNA libraries, representing genes of interest, is of paramount importance in basic plant biology re-

search as well as plant biotechnology [6]. Estimation of genome size, karyotype parameters, testing restriction endonucleases patterns are the necessary characteristics for BAC library construction.

This study was undertaken to determine nuclear genome size and genomic distribution of ribosomal DNA loci and two families of repetitive DNA sequences in *Papaver* species representing one taxonomic section within genus *Papaver*, with the aim to expand the number of species where these characteristics are known, and aid in the construction of a BAC library wich would be used as appropriate genomic resource for characterization of karyotype changes in poppy stocks with altered alkaloid biosynthesis pathway.

Material and Methods. *Plant material.* For DNA extraction and chromosome slide preparations we used seedlings and roots of poppy species, *P. somniferum*, *P. bracteatum* and *P. rhoeas* (accessions from Kyiv Central Botanic Garden collection). DNA was extracted from etiolated seedlings following Ausubel et al. [7].

Determination of genome size. According to Dolezel et al. [8], approximately 50 mg of midrib was cut from a poppy, *P. somniferum*, young leaf and transferred to a glass Petri dish. About 10 mg of a young leaf of maize (*Zea mays* cv. CE-777) with 2C = = 5.43 pg DNA was added and served as an internal reference standard. The tissues were chopped simultaneously in 1 ml of Otto I buffer (0.1 M citric acid, 0.5 % v/v Tween 20). Crude suspension of isolated nuclei was filtered through a 50 μm nylon mesh. Nuclei were then pelleted (300 *g*, 5 min), resuspended in 200 μl Otto I and incubated for 1 h at room temperature. Finally, 600 μl Otto II buffer (0.4 M Na_2HPO_4), supplemented with 50 μg/ml RNAse and 50 μg/ml propidium iodide (PI), was added. Samples were analysed using Partec PAS flow cytometer («Partec GmbH», «Munster», Germany) equipped with 488-nm argon laser. The gain of the instrument was adjusted so that peak representing maize G1 nuclei appeared approximately on channel 100 on histogram of relative fluorescence intensity when using 512-channel scale. About 5,000 nuclei were analysed at rate 10—25 nuclei/s. Three plants were measured per accession. Analysis of each plant was repeated three times on different days. Nuclear DNA content was calculated from individual measurements.

Fluorescence in situ hybridization (FISH). FISH probe for ribosomal DNA was obtained by labelling a pTa71 DNA clone containing 18S, 5.8S and 26S rRNA genes [9] with biotin-16-dUTP («Roche», Germany) by Nick Translation. Along with rDNA probe two new clones were used. These were made by sonicating total genomic DNA from *P. somniferum* (Danish flag accession) to an average length of 200 to 600 bp. The fragments were denatured at 100 °C in 0.1 M sodium phosphate buffer, pH 7.5, for 10 min and incubated at 60 °C to C_0t of 0.02. The samples were treated with S1 nuclease. The resulting highly repeated double-stranded DNA sequences were cloned into *pUC18* at the *PstI* site. From several thousand clones, 200 were chosen randomly for dot hybridization with ^{32}P-labelled total DNA from the representatives of four *Papaver* taxonomic sections (*Meconella*, *Pilosa*, *Macrantha*/*Papaver*, *Argemonidium*). Two clones, pPs21 and pPs41 gave very strong signals with *P. somniferum* DNA and no visible signals with three other sections members. These clones were selected for further studies on *in situ* and southern hybridizations. Digoxigenin-labelled probes for these repeats were prepared using PCR with M13 direct and reverse primers and pPs21 and pPs41 clones as a templates.

Metaphase spreads were prepared according to Alkhimova et al. [10]. The slides were treated with 100 mg/ml RNAse in a 2 × SSC solution at 37 °C for 1 h in a humid chamber, washed 3 × 5 min in 2 × SSC at room temperature. After two washes in 2 × SSC the slides were treated in 4 % paraformaldehyde for 10 min at room temperature, washed in 2 × SSC, dehydrated in ethanol series, and air dried. Prior to hybridization, the probes were mixed in a solution containing 50 % formamide, 10 % dextran sulphate, 0.12 % SDS in 2 × SSC and 50 ng/μl salmon sperm DNA. 1 μl of probe in 30 μl hybridization mixture per slide was used. The hybridization mixture was denatured at 70 °C for 10 min and incubated on ice for 10—15 min before being added to the preparations. The chromosomes together with the probes were denatured at 70 °C for 5 min and the hybridization was performed overnight at 37 °C in a humid chamber. The slides were then washed in 2 × SSC at 42 °C and rinsed in a stringent washing solution of 20 % formamide in 0.1 × SSC at 42 °C for 10 min, followed by several washes in 2 × SSC and 4 × SSC (0.2 % Tween). The sites of digoxigenin- and biotin-labelled probe hybridization were detected using anti-digoxigenin fluorescein («Roche») and streptavidin conjugated to Cy3 («Sigma», USA), respectively. Finally, the preparations were counterstained with DAPI (0.2

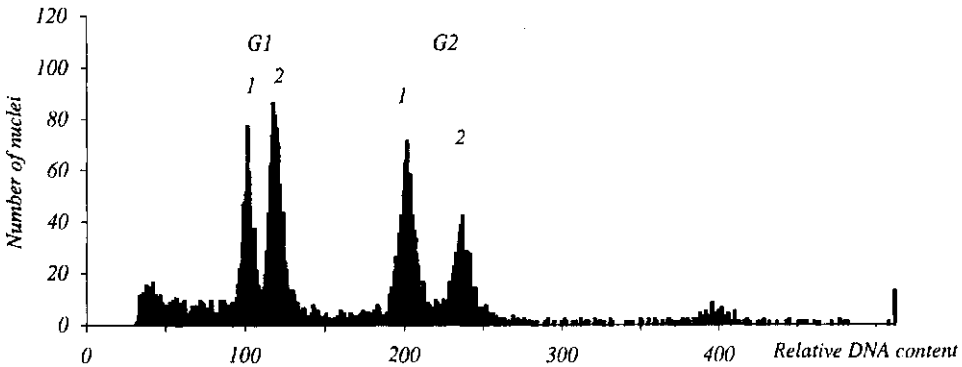

Fig. 1. Estimation of nuclear DNA content using flow cytometry. Simultaneous analysis of nuclei isolated from *Zea mays* (*1*) ($2n = 22$) used as internal standard and from *Papaver somniferum* (*2*)

μg/ml) and mounted in Vectashield antifade solution («Vector Laboratories», USA).

The preparations were evaluated using Olympus BX60 microscope equipped with optical filter sets appropriate for DAPI, fluorescein and Cy3 fluorescence. The images of DAPI, fluorescein and Cy3 fluorescence were acquired separately with a b/w CCD camera, which was interfaced to a PC running the ISIS software («Metasystems», «Altlussheim», Germany). The images were superimposed after contrast and background optimization.

Results and Discussion. *Estimation of genome size of Papaver somniferum.* Flow cytometry is a rapid technique that allows accurate estimation of nuclear DNA content [11]. To determine nuclear DNA content in absolute units, fluorescence intensity of nuclei is compared with that of nuclei isolated from a species with known nuclear genome size. Fluorescence of PI-stained nuclei were analysed using a Partec PAS II flow cytometer. Nuclei isolated from *Z. mays* cv. CE-777 with known nuclear genome size (2C = = 5.43 pg DNA) were used as internal standard to estimate nuclear DNA content of *P. somniferum* in absolute units. Small amounts of leaf tissues (standard and sample) were simultaneously chopped in buffer, supplemented with PI and RNAse. Suspension of nuclei was filtered through 50 μm nylon and stored on ice prior to analysis. 2C DNA content of *P. somniferum* was calculated according to formula:

$$2C \text{ nuclear DNA content} = \frac{5.43 \times G_1 \text{ peak mean of } Papaver}{G_1 \text{ peak mean of } Zea} = 6.46 \text{ pg.}$$

Genome size, which represent one copy of nuclear DNA equal to 1C, was further determined as:

$$\text{Genome size} = \frac{2C \text{ nuclear DNA content (pg)}}{2} \cdot 0.978 \cdot 10^9 \text{ bp [11].}$$

The results of the study showed (Fig. 1) that the size of *P. somniferum* nuclear genome equal 6.46 pg is smaller than previously estimated [12].

Karyotype of P. somniferum and related species, P. bracteatum and P. rhoeas. A karyotype, which is characterized by the number and morphology of chromosomes, is an important characteristic of a species. Methods for chromosome preparation and *in situ* hybridization essentially followed Heslop-Harrison et al. [13]. Root tips were fixed, partially digested with enzymes and cells were spread on slides. At least ten well-spread metaphase plates with similar degree of chromatin condensation were used to make chromosomal measurements. For constructing the karyotype, the chromosomes were arranged in order of decreasing size and increasing asymmetry (Fig. 2).

Localization of rDNA loci and repetitive DNA clones. Fluorescence *in situ* hybridization on *P. somniferum* chromosomes showed that pPs21 and pPs41 DNA sequences were distributed mostly uniformly along all chromosomes with gaps near centromeres and nucleolar organizer regions (data not shown). FISH with *P. bracteatum*, the representative of the same taxonomic group *Macrantha/Papaver*, is shown in Fig. 3. DNA sequences have been shown to be dispersed over the *P. bracteatum* genome. It had substantially more copies of pPs41 dispersed along most chromosomes (Fig. 3, *b*) than pPs21 which gave less number of dots.

Preparation of BAC library construction. BAC library is a source of DNA clones, which will be used as landmarks for chromosome identification, and also for the search of clones consisted of single copy sequences. An important step toward the structural analysis of a functional DNA domains is the construction of a large-insert libraries. Their inserts represent large DNA fragments that can be easily localized on mitotic chromosomes using FISH and allow selec-

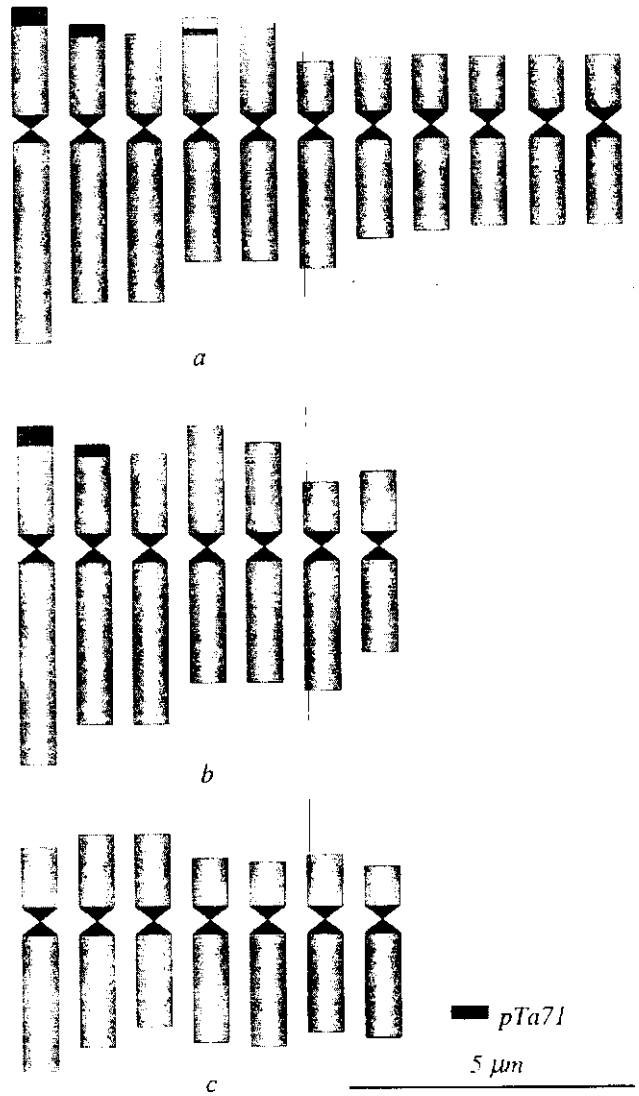

Fig. 2. Karyoidiogram of *Papaver somniferum* (*a*), *P. bracteatum* (*b*) and *P. rhoeas* (*c*) showing the length of individual chromosomes and centromere position. Distribution of rDNA loci revealed by *in situ* hybridization with pTa71 probe

tion of BAC clones with deficiency in repetitive DNA sequences. This approach requires determination of restriction endonucleases suitable for library construction, vector selection, appropriate ratio of high molecular weight DNA and chosen vector, optimal ligation and transformation conditions.

Testing conditions for different restriction enzymes. *EcoRI*, *BamHI*, or *HindIII*, the restriction enzymes most frequently used in BAC cloning [5]. High molecular weight DNA of *P. somniferum*, obtained by purification of protoplasts from the seedlings in enzyme solution followed by embedding on low-melting-point agarose, was digested with the five restriction enzymes, which can be ranged according to

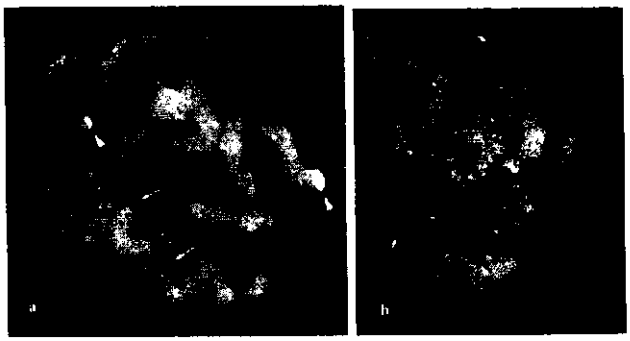

Fig. 3. *In situ* hybridization showing the localization of repetitive sequences, pPs21 (*a*) and pPs41 (*b*), derived from opium poppy, on chromosomes of *Papaver bracteatum*. Arrows mark NORs, arrowheads indicate major pPs21 sites at the terminal region of two chromosomes

increase of recognition sites, in the next order: *KpnI*, *XbaI*, *BamHI*, *EcoRI*, *HindIII*. As *HindIII* restriction fragments contained TyDC sequence (hybridization data not shown), and *HindIII* digestion gave more fragments, this enzyme is chosen for the construction of *P. somniferum* BAC library.

Logistics. The pIndigo BAC-5 (*HindIII*-Cloning Ready) vector has been chosen for the *P. somniferum* BAC library construction. The vector is provided in a «ready-to-use» state. It has been linearized at the unique restriction enzyme recognition site (*HindIII*), dephosphorylated and rigorously tested for purity and recombinant cloning efficiency (Epicentre). Competent *Escherichia coli* DH10B cells («Gibco BRL», USA) and Gibco BRL Cell-Porator System («Life Technologies», USA) have been used to get effective and reliable result. The vector to DNA ratio has been calculated to be in proportion approximately 1:10.

Conclusions drawn. The cultured cell lines which accumulated alkaloids of only one type, morphinan (thebaine) or benthophenanthridine (sanguinarine) have been generated from *P. bracteatum* (Fig. 4). As the members of the TyDC gene family are differentially expressed in cells and organs accumulating predominant alkaloid type [1], one can assume that different genes of this family are «responsible» for the alkaloid type produced. Southern hybridization with TyDC probe have revealed the different organization of restriction fragments in unorganized callus culture [3, 14]. The opium poppy TyDC gene family is composed of about 15 members that are divided into two subfamilies with regard to the sequence identity [3]. These two groups distinguish in organ-specific expression, namely, the members of the TyDC1-like subgroup are expressed abundantly in roots, while the members of the TyDC2-like subgroup — in roots and

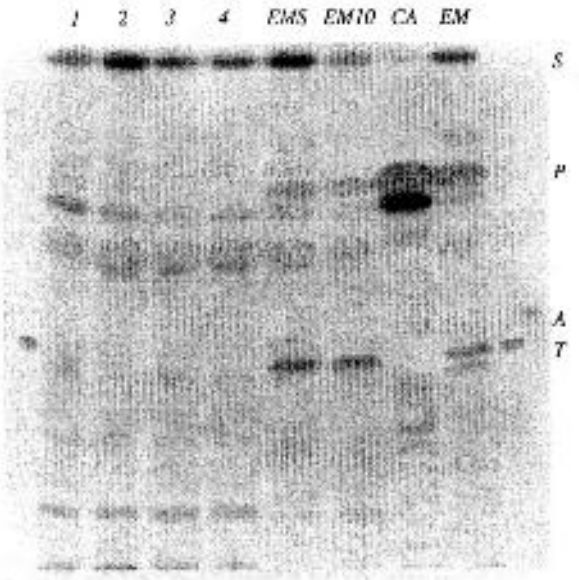

Fig. 4. Thin-layer chromatographic analysis of alkaloids in *Papaver bracteatum* cultured cell clones: *1—4* — primary cell cultures (up to 5 months in culture); *EM*, *EM5*, *EM10* — morphogenous lines; *CA* — dedifferentiated callus. Authentic standards of sanguinarine (*S*), protopine (*P*), allocryptopine (*A*) and thebaine (*T*) were also run

stems. The members of the *P. somniferum* TyDC gene family show also differences in the developmental, tissue-specific, and inducible expression patterns. This gene family can be used as a molecular marker to study the regulation and localization of alkaloid biosynthesis in the poppy species.

Fluorescence *in situ* hybridization has been performed to investigate the physical distribution of repetitive clones along the chromosomes of *P. somniferum* and *P. bracteatum* species. Two new clones have been used, pPs21 and pPs41. *In situ* hybridization on *P. somniferum* chromosomes has shown that pPs21 and pPs41 DNA sequences are distributed mostly uniformly along all chromosomes with gaps near centromeres and nucleolar organizer regions (data not shown). These repetitive sequences have been shown to be dispersed over the *P. bracteatum* genome distinguishing by the copy numbers of their repeats (Fig. 3). For the first time, the localization of the ribosomal DNA on *P. bracteatum* chromosomes is shown.

BAC library is a source of new DNA clones, which will be used as landmarks for the chromosome identification, and also for searching the clones consisted of single copy sequences. An important step toward the structural analysis of functional DNA domains is the construction of large-insert libraries. The construction of the representative BAC library requires the determination of the genome size to obtain the appropriate amount of clones. The results of the study show that the size of the *P. somniferum* nuclear genome equal to 6.46 pg is smaller than previously estimated. The genome size of *P. somniferum* ($2n = 22$) which represents one copy of nuclear DNA equal to 1C, has been determined as 3100 Mb. Based on this haploid genome size and average insert size of fragments around 150 kb that can be cloned efficiently in the BAC vector [5], the library representing five genome equivalents would consist of 100000 clones. Using cDNA probes will allow the verification of the coverage. The results obtained will be used for the construction of the large insert size genomic library as a new molecular resource.

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Т. К. Кириленко, О. І. Мартиненко, О. Г. Алхімова

Визначення розміру ядерного геному і каріотипічний аналіз видів *Papaver* для створення BAC бібліотеки

Резюме

Роботу присвячено вивченню шляхів біосинтезу алкалоїдів у видів Papaver та інтегруванню даних фізичного картування нових маркерних послідовностей ДНК з відмінностями в експресії генів ключових ферментів біосинтезу алкалоїдів. Таке дослідження потребує знання структури геному та його організації, а також передбачає наявність геномних ресурсів задля детального аналізу геному опійного маку. У даному повідомленні визначено розмір геному опійного маку та зроблено каріотипічний аналіз Papaver somniferum і його споріднених видів, P. bracteatum і P. rhoeas. Отримані характеристики є необхідними для створення BAC бібліотеки, яка слугує базисом для цитогенетичного картування та пошуку змін каріотипу в лініях маку, що відрізняються за шляхами біосинтезу алкалоїдів. Вже одержано декілька клітинних ліній, які накопичують різні типи алкалоїдів.

Ключові слова: Papaver, алкалоїди, розмір геному, BAC бібліотека, флуоресцентна гібридизація in situ.

Т. К. Кириленко, Е. И. Мартыненко, Е. Г. Алхимова

Определение размера ядерного генома и кариотипический анализ видов *Papaver* для создания BAC библиотеки

Резюме

Работа посвящена изучению пути биосинтеза алкалоидов у видов Papaver и интегрированию данных по физическому кар-

тированию новых маркерных последовательностей ДНК с различиями в экспрессии генов ключевых ферментов биосинтеза алкалоидов. Такое исследование нуждается в знании структуры генома и его организации, а также предполагает наличие геномных ресурсов для детального анализа генома опийного мака. В данном сообщении определен размер генома опийного мака и сделан кариотипический анализ Papaver somniferum и его родственных видов, P. bracteatum и P. rhoeas. Полученные характеристики необходимы для создания BAC библиотеки, предоставляющей базис для цитогенетического картирования и поиска изменений кариотипа у линий мака, различающихся путями биосинтеза алкалоидов. Уже выделены несколько клеточных линий, накапливающих разные типы алкалоидов.

Ключевые слова: Papaver, алкалоиды, размер генома, BAC библиотека, флуоресцентная гибридизация in situ.

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