



Genetic variability of tobacco plants regenerated from cell lines resistant to heavy metal ions

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Abstract. One of the main elements of cytological observations carried out at all stages of creating new forms of plants is the determination of ploidy, both of the crossing components and of the resulting hybrids, varieties, and variants. The traditional method of determining ploidy by counting chromosomes on pressurized or permanent preparations under a microscope is laborious, and other methods of determining ploidy by the size of oysters, pollen or the number of chloroplasts in epidermal stomatal cells are not very accurate, especially if the ploidy of the samples does not differ significantly.

The most reliable method for determining plant ploidy is the cytophotometric method of ploidy determination based on changes in the amount of DNA in the cell. The essence of flow cytometry (FCM) is that the fluorochrome light pulse recorded by a flow cytometer is proportional to the amount of DNA of each individual cell nucleus, in turn, the amount of DNA of the interphase nucleus reflects the ploidy of the latter.

Data on the analysis of fertility in experimental plants are presented. The genomes of tobacco (*Nicotiana tabacum* L.) regenerants obtained from cell lines resistant to barium, cadmium, and vanadium ions were studied by cytometric analysis. It was found that the regenerant obtained from the cadmium (Cd^{2+}) resistant line is a triploid. The regenerants obtained from cell lines resistant to barium (Ba^{2+}) and vanadium ions are mixoploids. Control tobacco plants have a diploid genome. Regenerants retain the phenotypic characteristics of resistant cell lines. In our opinion, these characteristics are probably related to the phenomenon of polyploidy. However, other types of mutations in the studied tobacco plant variants are not excluded.

Key words: *Nicotiana tabacum* (L.), cell selection, heavy metal ions, cytometric analysis, polyploidy.

Генетична мінливість рослин тютюну, регенерованих з клітинних ліній, стійких до іонів тяжких металів

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Резюме. Одним з основних елементів цитологічних спостережень, що проводяться на всіх етапах створення нових форм рослин, є визначення плідності, як компонентів схрещення, так і отриманих гібридів, сортів, варіантів. Традиційний метод визначення плідності шляхом підрахунку хромосом на давлених або постійних препаратах під мікроскопом є трудомістким, а інші методи визначення плідності за розміром устриць, пилку або числа хлоропластів в епідермальних устичних клітинах не відзначаються високою точністю, особливо, якщо плідність зразків відрізняється не суттєво.

Найбільш надійним методом визначення плідності рослин є цитофотометричний метод визначення плідності, оснований на зміні кількості ДНК в клітині. Суть проточної цитометрії (ПЦМ) полягає в тому, що світловий імпульс флуорохому який фіксується проточним цитометром, пропорційний кількості ДНК кожного індивідуального ядра клітини, в свою чергу кількість ДНК інтерфазного ядра відображає плідність останнього.

Наведені дані по аналізу плідності у дослідних рослин. За допомогою методу цитометричного аналізу досліджували геноми рослин-регенерантів тютюну (*Nicotiana tabacum* L.), одержаних з клітинних ліній, стійких до іонів барію, кадмію, ванадію. Встановлено, що регенерант, отриманий із кадмій (Cd) – стійкої лінії – триплоїд. Отримані регенеранти з клітинних ліній, стійких до іонів барію (Ba^{2+}) та ванадію – міксоплоїди. Контрольні рослини тютюну мають диплоїдний геном. Регенеранти зберігають фенотипічні особливості стійких клітинних ліній. Ці характеристики, на наш погляд, можливо, пов'язані з явищем поліплоїдії. Однак, не виключено інших типів мутацій у досліджуваних варіантах рослин тютюну.

Ключові слова: *Nicotiana tabacum* (L.), клітинна селекція, іони важких металів, цитометричний аналіз, поліплоїдія.

INTRODUCTION

The phenomenon of genome variability in cell cultures in vitro has long been a subject of study and the goal of many experimental and applied research efforts. A number of reasons have already been identified that contribute to this process. Among them are both the initial functional heterogeneity of explant cells (endogenous factors) and the conditions of the isolated culture (exogenous factors). Genetic variability is the basis of cell selection. This method has been used to obtain cell mutants with various properties: auxotrophs, supersynthetics of certain substances, cell lines that differ in the level of resistance to pathogens, xenobiotics, and stress [19, 20]. Such variants are valuable both in themselves and as a starting material for further biotechnological manipulations. However, altered cellular genotypes are particularly relevant for plant regeneration.

At the same time, this part of the problem can significantly limit cell selection as a method. First, the morphogenetic potential is constantly decreasing with increasing age of the culture. Secondly, the nature of genetic changes as such negatively affects the totipotency of cells, in particular polyploidization reduces the frequency of regeneration. Thirdly, regenerants often do not stand out for the original characteristics of cell lines, which may primarily indicate an epigenetic cause of the phenomenon [21]. On the other hand, if a trait that is selected at the cellular level is expressed in the whole plant, it is natural to expect that the source of the phenotypic response is genetic changes.

Tobacco cell lines resistant to heavy metal ions Ba^{2+} , Cd^{2+} , and VO^{3-} were previously obtained by cell selection [20, 22]. In addition to resistance to the selected ions, the clones were resistant to modeled osmotic stresses and tungstate. Plants were regenerated from these lines, which were also characterized by an increased level of stress resistance. The genotoxic effect of heavy metal ions is an established fact [20]. Therefore, it is advisable to study the genomes of the obtained regenerants. For this purpose, the fluorescence cytometry method was used, which allows to measure the DNA content and thus determine the fertility, revealing the proportion of aneuploids and mixoploids [8].

The first automatic methods for determining fertility were used in medicine to analyze cell fertility in human cancer [5, 16, 1718]. Then this method was used to determine the DNA content in the nuclei of cells of various plant species [6, 12, 14, 15]. In 1999, the method was used in Ukraine to create tetraploid multi-seeded pollinators for sugar beet [1, 10].

METHODS AND MATERIALS

The content of nuclear DNA in plant cells was determined using a Partec ploidy analyzer, using a specific DNA reaction, namely, AT-pairs of nucleotides and fluorochrome (propidium iodide or DAPI - 4,6-diamidino-2-phenylidene).

The essence of the method is to measure the fluorescence intensity of the labeled cell, which is directly proportional to the amount of DNA in each individual cell nucleus. The fluorescence intensity and, at the same time, the amount of DNA are divided into 4096 quantitative classes. The result (histogram) shows the number of measured classes (x-axis; a channel) and the number of cells belonging to each class (y; count).

The flow cytometer allows to measure the DNA content and, accordingly, the fertility of 150–300 thousand cells in 2-3 minutes, depending on the flow rate of the liquid.

For the study, tobacco regenerants were cultivated in vitro on hormone-free Murashige-Skoog medium [2]. By the time of the experiment, the plants had a developed root system and several (4 or more) leaves. For the analysis, a suspension of nuclei was prepared, the upper and middle parts of the leaves (1–2 cm²) were selected and ground with extraction buffer. After grinding in a Petri dish, a fluorochrome solution was added to the tissue. The mixture was incubated for 5 minutes at room temperature and filtered to separate the coarse fraction. The measurement was performed in a volume of 1 cm³ on a Partec cytometer with a multichannel analyzer. Buffer and staining solutions are patented by the original manufacturer. The control plants were diploid plants of Samsun and Dubec varieties. The results were processed using a computer program developed by the manufacturer.

The DNA peak on the histograms of the Partec AP was determined by the device and corresponded to 102,29 units on channel 100; the coefficient of variation, which determines the variability of the fluorescence intensity of cell populations, CV = 10,6 %.

RESULTS AND DISCUSSION

The study of ploidy in plant cells provides valuable information about changes that can cause phenotypic reactions. This is especially true if the changes affect the resistance of the organism. In the cytometry of formed leaves (as in our case), cells that have completed division and are actively functioning are evaluated. The cytometric analysis of tobacco regenerants obtained from cell lines resistant to lethal concentrations of Ba^{2+} ; Cd^{2+} ; VO^{3-} ions is presented in

histograms. Fig. 1 illustrates the DNA content in a wild-type plant of the Dubec variety. A similar picture

was observed for the study of Samsun plants, so due to the lack of differences, the histogram is not attached.

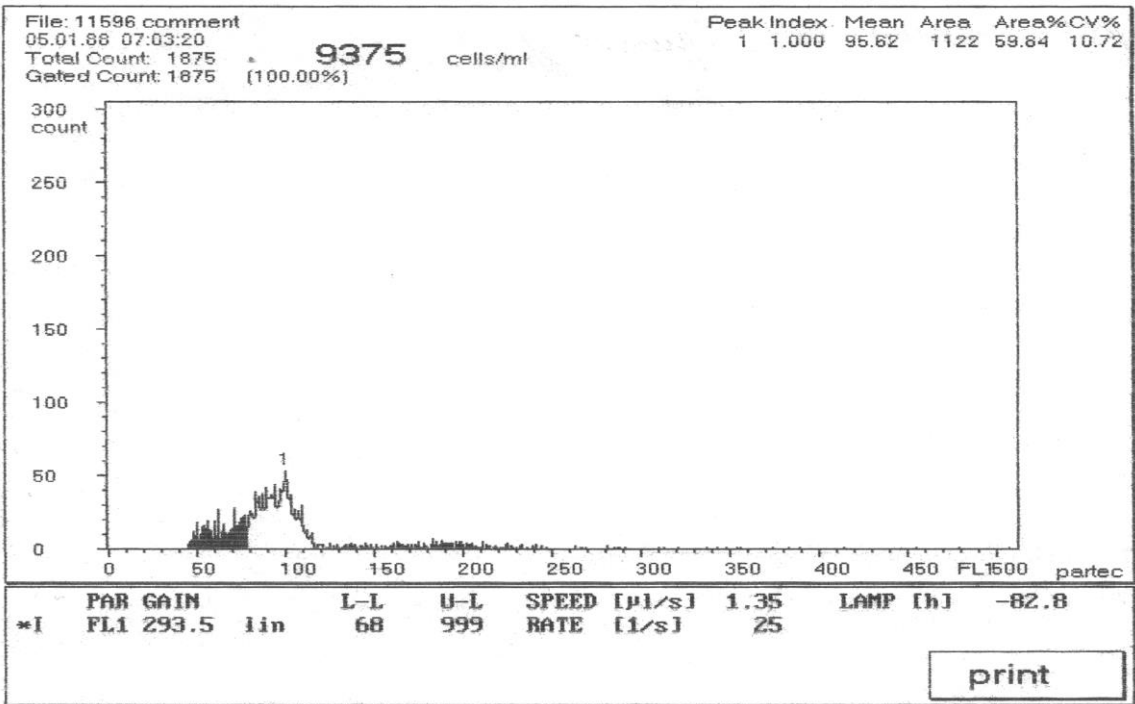


Fig.1. DNA content in cells of control tobacco plants; diploid plant

The histogram shows the diploid level of the standard plant, which corresponds to the peak on channel 100. A different picture is observed in Figs. 2–4. Fig. 2 shows the results of cytometric analysis of DNA content in the cells of a regenerated Cd-resistant cell

line. Fig. 3 characterizes the same parameter in a plant derived from a vanadate-resistant cell line, while Fig. 4 shows the genome of a regenerant from a Ba-resistant tobacco line.

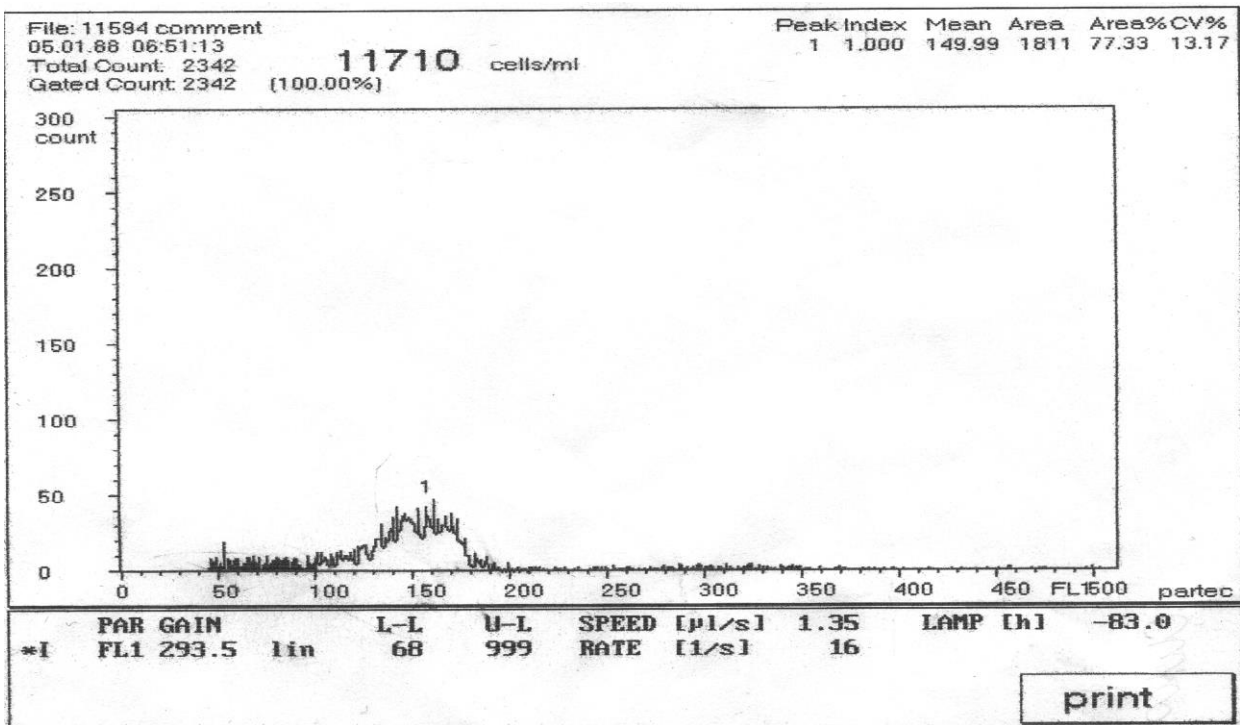


Fig. 2. DNA content in regenerated plants from a Cd-resistant cell line; triploid plant

The regenerant from the Cd-resistant cell line is a triploid. The peak on channel 150 reliably indicates this (Fig. 2). The other two plants are mixoploids.

However, in the regenerant from the vanadate-resistant cell line, the genome is dominated by cells of modal class 3x (Fig. 3).

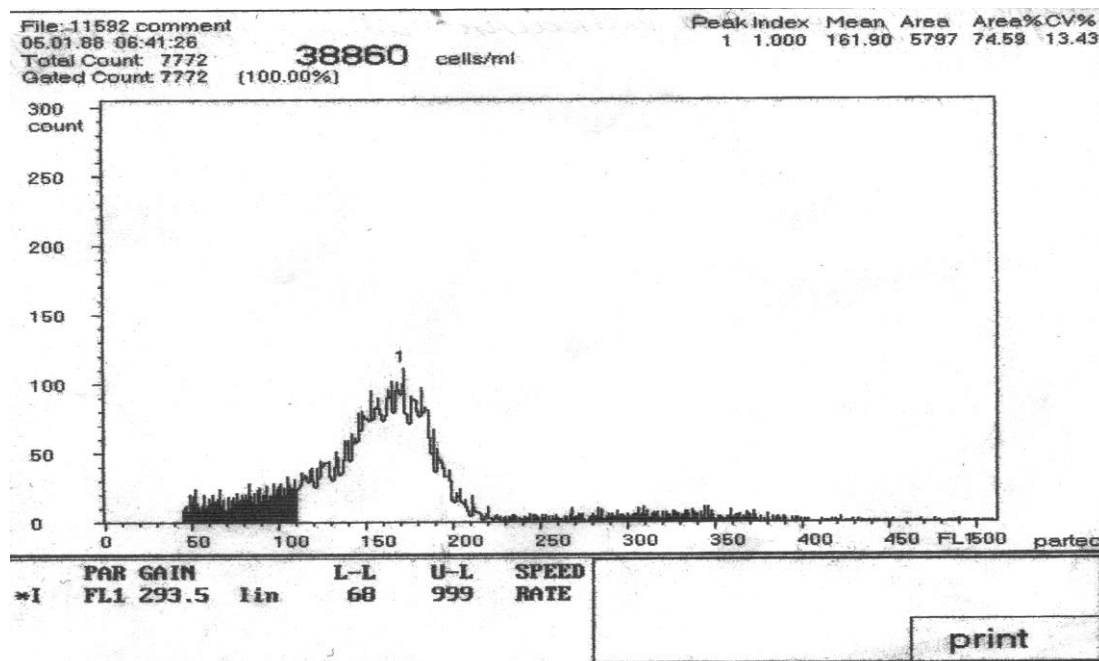


Fig. 3. DNA content in cells of a regenerant from a vanadate-resistant cell line; plant triploid

At the same time, the mixoploid genome of a regenerant from a Ba-resistant cell line is more likely to correspond to the 4x-n class (Fig. 4).

Notes: Figures 1–4 are original histograms analyzed, calculated and statistically processed automatically, where:

1. The x-axis indicates the classes of measured cells; the y-axis indicates the number of cells belonging to each class;

2. The upper part of the file contains: the total number of cells counted during the analysis (Total Count); the number of peaks that determine ploidy (Peak) and their values (Index - the number of cells of the same ploidy; mean - their total number; area - the total area of the peak; area % - its value in percent; CV % - the coefficient of variation).

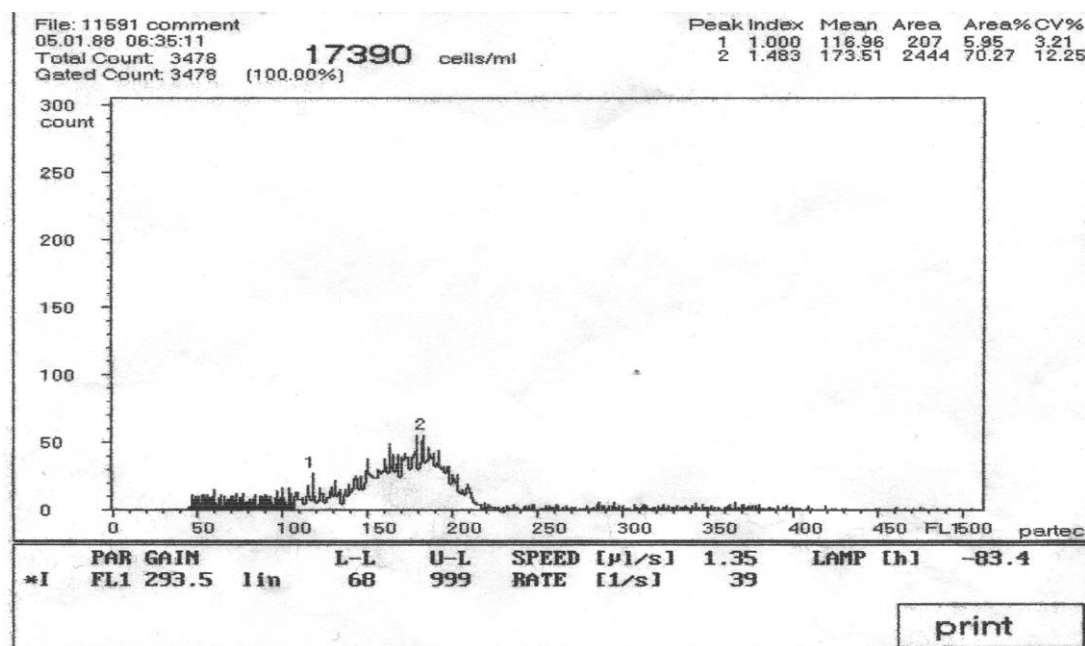


Fig. 4. DNA content in cells of a regenerant from a Va-resistant cell line; a plant mixoploid with an unbalanced number of chromosomes

CONCLUSION

Thus, despite the existing individual differences, regenerants from cell lines resistant to heavy metal ions differed in genome polyploidization.

This event was the result of selection under severe stress conditions. Under normal conditions, a cell culture is a mixoploid population regardless of the fertility level of the original explant from which it was obtained [4, 7, 8, 9, 11, 13]. This status is the most optimal for maintaining the system's homeostasis. Obviously, in the presence of lethal concentrations of selective ions (cell selection was carried out only in this way), unstable cells were eliminated, but this was the vast majority, since the frequency of formation of viable clones was 10^{-6} . It is quite logical to assume that the cells with the altered genome were able to proliferate. Moreover, there were no specific differences between tobacco regenerants obtained from the two varieties: Dubek and Samsun were not noted, and there were no differences between the types of stressor ions. This is probably due to the intensity of the stress pressure. For example, it was previously reported that 1.0-1.5% NaCl and 1.5-2% Na_2SO_4 concentrations increased the number of polyploid cells in beet culture by up to 30% compared to the control [15, 20]. However, in this case, it was only a question of changing the ratio of cell population components, first of all. Secondly, the publication did not indicate the production of regenerants from polyploid cells, which removed the question of regenerant stability. Other authors noted a similar reaction in a stable culture of *C. capillaris* [11]. There are also numerous data on the stabilization of

cell cultures under the influence of a number of mutagens, before the formation of haploid calli [7]. In our studies, it was previously found that a long-term cultivated soybean cell line resistant to lethal concentrations of vanadium and tungsten oxyanions was represented mainly by tetraploid cells [3, 11]. However, even in this case, the goal of the study was regenerants. In our cell breeding experiments, we have an experimental system of resistant cell line - resistant regenerant. Tobacco cell lines resistant to heavy metal ions have been continuously tested under various stresses. Clones selected on selective media with barium and cadmium ions grew in the presence of lethal concentrations of seawater salts, sodium sulfate, and mannitol. The regenerants of these lines were continuously cultured in vitro under normal conditions. However, after transferring them to stressful conditions, the plants showed an increased level of resistance to osmotic stress [20, 22]. A similar pattern was observed for the study of a vanadate-resistant cell line and the corresponding regenerant. Both at the cellular level and in the plants, increased nitrate reductase activity was observed [23]. These facts are in favor of the genetic nature of the changes that occurred. The cytometric analysis showed that the regenerated plants are triploids or mixoploids, in which, however, the DNA content does not exceed 4x. Probably, this is the most optimal cell fecundity, at which the simultaneous realization of two events is possible: regeneration/expression of a trait. At the same time, quantitative changes in DNA content do not exclude the presence of other types of mutations that may cause new phenotypic reactions associated with resistance.

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