=== **PLANT GENETICS** ===

Chromosome Variability in Grape (*Vitis amurensis* Rupr.) Cells Transformed with Plant Oncogene *rolB*

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Abstract—The numbers of chromosomes and nucleoli in cultured cells of *Vitis amurensis* transformed with the *rolB* oncogene from *A. rhizogenes* have been studied. In general, the integration of the *rolB* gene in grape DNA mostly caused the elevation of the level of the chromosome variability, as well as higher numbers of nucleoli in the cultured cells. The possible influence of the observed chromosomal modifications on the productivity parameters of the grape cell cultures is discussed.

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INTRODUCTION

The karyotype characteristics (chromosome number and size, shape, and individual features) reflect the structural organization of the cellular genetic material and, therefore, represent the most important species trays. It is generally assumed that karyotype modifications lead to strong morpho-anatomical variations. Hence, these processes are considered to be associated with speciation, particularly in plants [1, 2].

Polyploidy (multiple increase in chromosome numbers) is one of the most widespread karyotype modifications. Another frequent modification is aneuploidy, which is a change in the number of individual chromosomes. It is known that around 45% of plant species on the planet Earth is represented by polyploids [3]. The most valuable cultivars, such as wheat, oats, potato, plum, strawberry, tobacco, alfalfa, sugar cane, and many others, are polyploids. Unlike plants, diploids prevail among animal species. A change in the diploid status generally leads to severe genetic diseases in offsprings.

The chromosome number among various species is variable. In some Radiolaria, this number is as high as 1000–1600 chromosomes. A champion in the plant world is a fern *Ophioglossum reticulatum* (2n approximately equals to1200) [3]. In monocot plants the highest chromosome number is found in the palm *Voanioala gerardii* (2n is around 596) [3]. In eudicots, the lowest and the highest chromosome numbers (2n = 4 and 2n ~ 640) are described in the *Sedum* genus of the Crassulaceae family [4]. The lowest chromosome number among Asteraceae (2n = 4) was observed in *Haplopappus gracilis* [5].

Many factors can cause multiple increases in the chromosome number, including high or low temperature, ionizing irradiation, chemicals, and changes in the physiological status of the cell [6, 7]. An alkaloid colchicine that prevents the formation of the mitotic spindle and normal chromosome disjunction is the most effective amongst chemicals.

Gigantism is not unusual among polyploid plants. This suggests oversized cells and organs (leaves, flowers, and fruits), the elevated concentration level of some chemicals, and changes in both flowering and fruiting times [8, 9]. Since economically useful properties of polyploids has always drawn attention from plant breeders, this resulted in artificially bred polyploids, which represented an important source of the variability and could be used as the initial selection material [10–12]. The usual disadvantage of autopolyploids, however, is low fertility.

As was shown previously, the transformation of gingseng (*Panax ginseng*) cells with the *rolC* gene from agrobacteria *Agrobacterium rhizogenes* can also cause the elevation of both the polyploidy and aneuploidy levels. In this case, cell cultures accumulated their biomass and secondary metabolites, ginsenosides, much faster [13, 14]. The agrobacterial *rol* locus includes four genes called A, B, C, and D with unknown functions [15]. The *rolB* gene is of particular interest for biotechnology studies, as its expression in plant cells can considerably augment the level of biologically active compounds, as well as the level of expression of defensive genes [16–18].

In this work, the *rolB* gene was integrated in the DNA of grape (*V. amurensis* V2) callus cells, which resulted in two types of cell cultures, i.e., high-level transgene expression (VB1) and low-level transgene expression (VB2). In general, the transformation led to a considerable increase in resveratrol (3,5,4'-trihydroxy-trans-stilbene) production [19], a valuable anti-

cancer drug that belongs to the small group of secondary metabolites, i.e., stilbenes [20].

MATERIALS AND METHODS

V. amurensis callus cell culture. The V2 callus culture was initiated in the Biotechnology Laboratory of the Institute of Biology & Soil Science, Far East Branch, Russian Academy of Sciences from a young *V. amurensis* stem in 2004 [16]. Moreover, a transgenic *V. amurensis* cell cultures carrying the *rolB* gene from *A. rhizogenes* were used. These cultures were of two types with both weak (VB1) and strong (VB2) *rolB* expression. The VB2 cell culture also demonstrated a high level of resveratrol biosynthesis (around 0.5–1.5% of the dry weight) [21, 22].

In this work, V2, VB1, and VB2 cells were grown in standard 15-mL test tubes on solid $W_{B/A}$ medium [18, 23] containing 2 mg/L BAP and 0.5 mg/L NAA at 24°C in the dark [18]. Cells were transferred to fresh medium with a 35-day period. Media components used were produced by ICN Biochemicals, United States.

Karyological analysis. Karyological analysis was carried out according to standard procedures applicable to this study [25]. Small pieces of calli were treated by a 0.2% colchicine solution for 2 h. As a fixative, acetic alcohol (1 : 3) was used. Just before staining, the material was pretreated with 4% ferric ammonium sulfate. Next, the material was placed in aceto-hematoxylin for 12–24 h at room temperature. The stained material was placed on a microscope slide in a drop of a saturated chloral hydrate solution, covered by a cover-glass, and used for preparation of a squashed sample. Ready samples were preliminary analyzed using a Leica DMLS (Leica Microsystems, Germany) microscope. Then, samples were photographed in the oil-immersion system using an Axioskop-40 microscope with a built-in Axiokam HRc (Zeiss, Germany) camera. In order to define the nucleolus activity, samples were stained in a solution of 50% silver nitrate under 42–45°C for 6–7 h according to E.N. Muratova [24]. The following parameters that are characteristic of the nucleolus activity have been determined: the number of cells with different numbers of nucleoli, the average number of nucleoli per cell, area, and the size of nucleoli (summarized), as well as the the nucleus-to-nucleolus ratio (nucleus area/total area of nucleoli).

Statistical analysis. The statistical treatment of the results was carried out using the Statistica v. 10.0 program. All data are presented as average values with their standard errors. The significance of the data was found using the Student's paired test. The minimal significance level was 0.05 in all experiments.

RESULTS

Chromosome Numbers in V2, VB1, and VB2 Cell Cultures

The number of chromosomes in the *V. amurensis* cells is found to be $2n = 38$ [26]. An analysis of the chromosome variability showed that the chromosome mosaicism was usual in the control group of the V2 cells, which is characteristic of cell cultures of many plants [27, 28]. The chromosome numbers in the dividing cells of the callus control line were 8–52 (Fig. 1a), although some cells contained up to 120 chromosomes, which makes up less than 6% of all cells analyzed. Most cells contained 38 chromosomes (9%).

The cell transformation by the *rolB* gene significantly affects the chromosome number. With a low level of expression of the *rolB* gene in the VB1 cell culture, the chromosome numbers in dividing cells was 8–124 (Fig. 1b). An increase in the maximal chromosome number also persists in the VB2 cell culture with the high level expression of the *rolB* gene. In this case, the chromosome numbers were 8–160 (Fig. 1c). Furthermore, it should be noted that necrosis was observed in many cells that originated from the VB1 and VB2 cell cultures; in addition, the number of metaphase nuclei was much lower compared to the V2 cells. All of this made working with the VB1 and VB2 cell cultures more difficult.

Nucleolus Numbers in V2, VB1, and VB2 Cell Cultures

The analysis of the nucleolus numbers in interphase nuclei of the V2 callus control group revealed this parameter to be 1–7 (Fig. 2c). Cells that contain one, two, three, and four nucleoli consisted 71, 21, 5, and 2%, respectively. Altogether, cells that contain five, six, and seven nucleoli made up 1% (Fig. 2c).

In the transformed grape cells, the maximal nucleolus number increased and was between 1 and 11. The percentage of cells with one nucleolus was diminished to 54–62%, whereas the percentage of cells with 2–11 nucleoli was increased compared to the V2 cell culture. Thus, in the VB1 and VB2 cultures, the percentage of cells with 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 nucleoli was 22–27, 8–10, 4–5, 1.3–2, 0.7–1, 0.4–0.5, 0.3, 0.1–0.8, 0.1–0.3, and 0.1%, respectively (Fig. 2c).

It can be suggested that, in the control group of cells, four pairs of chromosomes are functional. In the transformed cells, based on the maximal nucleolus number, one can suggest as many as six of these pairs. In this case, a two- or threefold decrease in the nucleus-to-nucleolus ratio was observed because of the growth in the total nucleolus area compared to the control V2 culture.
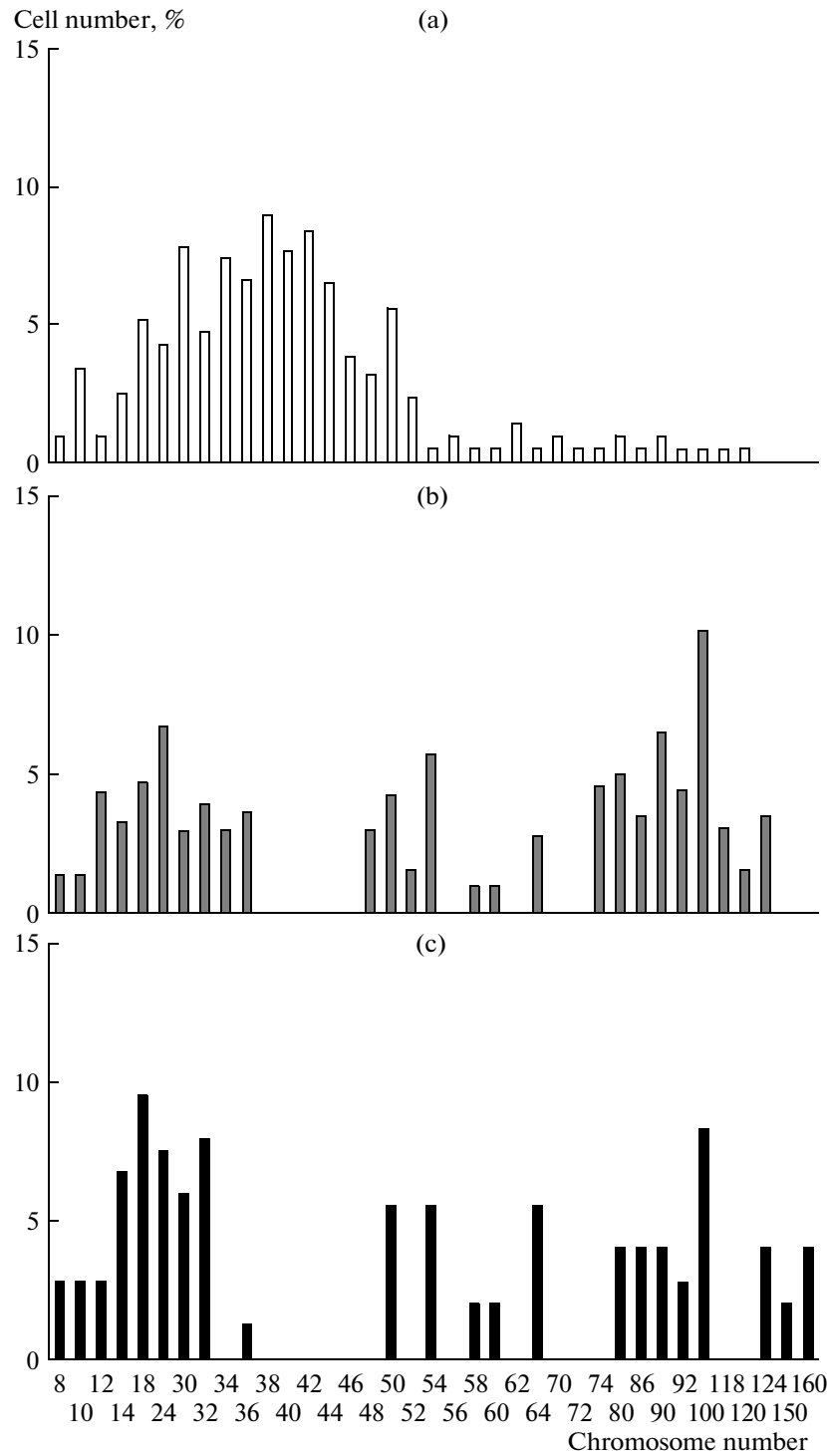
Fig. 1. Diagram of average values of chromosome numbers in grape cells originated from the cell cultures of (a) V2, (b) VB1, and (c) VB2. The last two cultures are *rolB* transgenic. Each average was calculated using more than 100 cells.

DISCUSSION

Despite intensive study, the mechanism of initiation of the neoplastic transformation of plant cells by the *rol* oncogenes has not yet been elucidated. As was suggested earlier, *rol* genes affect the physiological processes in plant cells by changing their hormonal balance. Estruch et al. showed that the *rolB* gene encodes for an enzyme that breaks glycosides of indole acetic acid [29]. However, it was found later that the concentration levels of both unbound and bound indole acetic acid were constant in plants that express *rolB* [30]. At present, most researchers rather believe
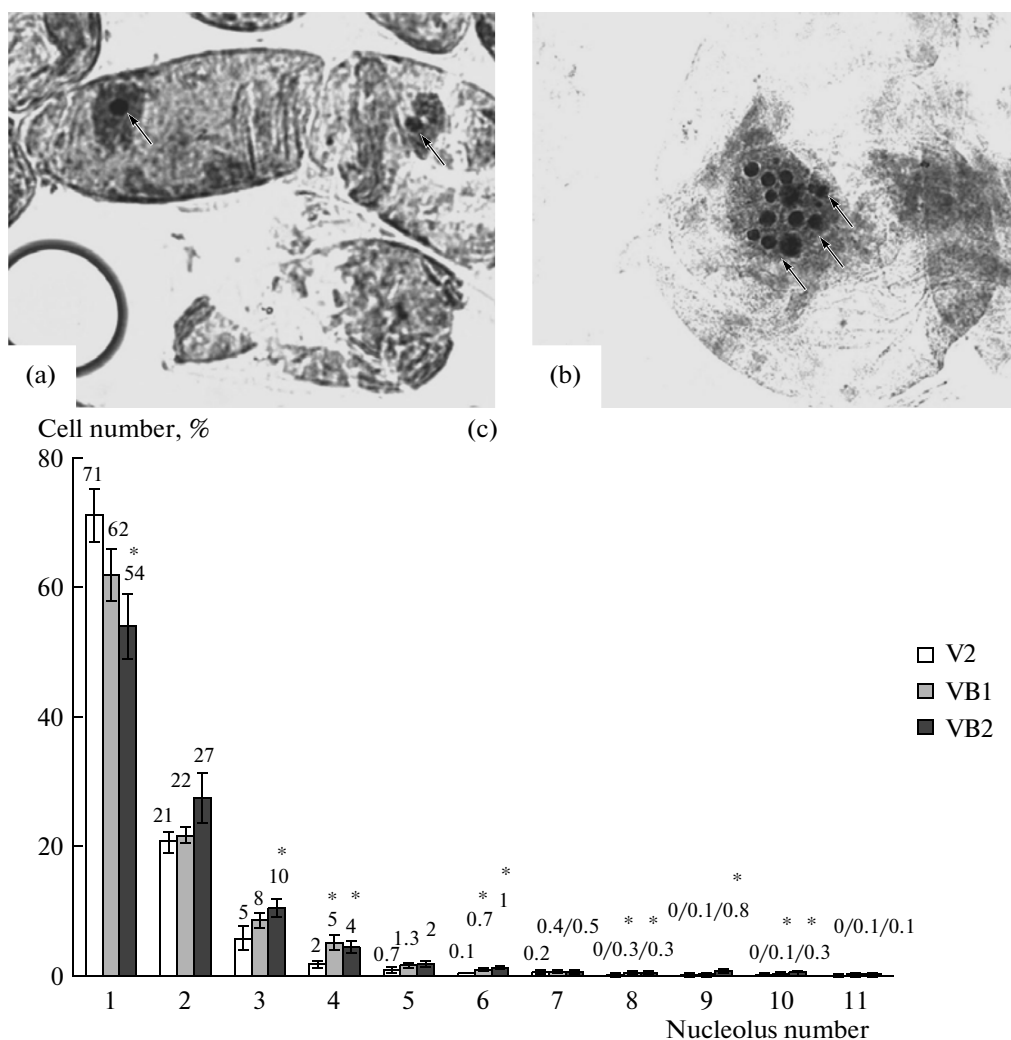
Fig. 2. (a) Singular nucleoli (marked by arrows) in the nuclei of the V2 cells. (b) Numerous nucleoli (marked by arrows) that are located in the same nucleus of the VB2 cell. (c) Average values of the nucleolus numbers. A total of 1000 cells were analyzed. Numbers designate the percentage of cells referred to all analyzed cells. Asterisks mark statistically significant difference in the nucleolus numbers originated from the *rolB* transgenic cell cultures of VB1 and VB2 compared to the V2 cell culture ($p < 0.05$).

that morphogenic effects of the RolB protein are only associated with the change in sensitivity of the transformed cells to auxins but not with a change in the concentration of these phytohormones [15]. It was shown that the RolB protein has the activity of Tyr phosphatase and is localized in the cell membrane of the transformed plants [31]. An important discovery was made in recent years, i.e., the product of the *rolB* gene is capable of interacting with the 14-3-3 protein, which leads to the modulation of its function [32]. Interestingly, Moriuchi et al. [32], unlike Filippini et al. [31], reported the nuclear localization of RolB, which makes its function as a transcriptional coactivator/mediator more probable.

As was shown earlier, the *V. amurensis* transformation with the *rolB* gene considerably increases the concentration level of a secondary metabolite, resveratrol [16]. This increase was due to the selective elevation of expression levels of stilbene synthases (*STS*). Furthermore, changes in the functioning of the calcium signal system were observed compared to the control nontransgenic cells [17, 18]. Special interest in the calcium signal system can be explained by its key role in the *rol* gene function [33].

In this work, we demonstrate that the transformation of *V. amurensis* with the *rolB* gene leads to an increase in the chromosome and nucleolus numbers in these plant cells. Higher nucleolus numbers suggest the activation of the rRNA genes by *rolB*, which can in turn reflect the activation of other genes that are localized elsewhere on grape chromosomes. A similar increase in the secondary metabolite production (monoterpene unsaturated fats) was obtained when ploidy was elevated in plants of the *Cymbopogon* genus using colchicine [34]. Thus, we demonstrated that the transformation of the plant cells with the *rolB* gene

from *A. rhizogenes* leads to polyploidy of the plant genome. It is possible that, as a result of a *rolB* transfer, polyploidy is one of the basic reasons for all effects found earlier, including the increase in the concentration level of secondary metabolites and biomass accumulation by the transformed cells [16—19]. The suggestion is supported by the complete lack of data on the direct influence of *rolB* on the biosynthesis of plant secondary metabolites, although the final confirmation would be finding the RolB function.

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