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SHORT COMMUNICATION

A rise of ploidy level influences the rate of cytomixis in tobacco male meiosis

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Abstract The effect of plant ploidy level on the rate of cytomixis in microsporogenesis has been analyzed with the help of a unique model, the collection of tobacco plants of different ploidies ($2n = 2x = 24$, $4x = 48$, $6x = 72$, and $8x = 96$). As has been shown, the rate of cytomixis proportionally increases in 6x and 8x cytotypes, being rather similar in 2x and 4x plants. The rate of cytomixis is highly variable, differing even in the genetically identical plants grown under the same conditions. The cytological pattern of cytomixis in the microsporogenesis of control 4x plants has been compared with the corresponding patterns of 2x, 6x, and 8x plants. Involvement of cytomixis in production of unreduced gametes and stabilization of the newly formed hybrid and polyploidy genomes is discussed.

Keywords Cytomixis · *Nicotiana tabacum* · Microsporogenesis · Polyploids · Haploids

Introduction

Cytomixis is migration of nuclei between plant cells through intercellular channels of a special type (cytomictic channels), differing from the plasmodesmata in their structure and size. This unique phenomenon was discovered over a century ago (Arnoldy 1900; Gates 1911), and to date, cytomixis has been observed in microsporogenesis of hundreds of plant species (for review, see Lone and Lone 2013; Mursalimov et al. 2013b).

Despite that the mechanisms underlying formation of the cytomictic channels and the migration of nuclei through these channels have been so far studied in some detail (Mursalimov et al. 2013a; Mursalimov and Deineko 2012; Sidorchuk et al. 2007; Wang et al. 2002, 2004; Yu et al. 2004), the cause of cytomixis is still unknown. It is assumed that cytomixis can contribute to the changes in karyotype of produced pollen (Falistocco et al. 1995; Ghaffari 2006; Lavia et al. 2011; Negron-Ortiz 2007; Pécrix et al. 2011; Kumar et al. 2014). On the one hand, cytomixis-based acquisition of additional chromosomes by a cell may result in generation of unreduced pollen (Falistocco et al. 1995; Lavia et al. 2011; Mursalimov and Deineko 2015). On the other hand, it is postulated that cytomixis is the way to get rid of "excess" chromatin for genome stabilization and restoration of pollen fertility in meiosis of genetically unbalanced plants, such as aneuploids and polyploids (Baptista-Giacomelli et al. 2000; Kalinka et al. 2010; Zhou 2003). A high rate of cytomixis in the microsporogenesis of genetically unbalanced plants, such as polyploids and hybrids, has been repeatedly described (Guan et al. 2012; Kumar et al. 2011; Negron-Ortiz 2007; Peng et al. 2003); however, the data are contradictory. An essential shortcoming of the works on cytomixis in microsporogenesis of different plant forms is that, as a rule, they involve genetically heterogeneous plants grown under nonuniform conditions, while it is known that the external conditions, such as temperature, may significantly influence the rate of cytomixis (Barton et al. 2014; Lattoo et al. 2006; Pécrix et al. 2011).

Thus, analysis of the effect of ploidy level on the rate of cytomixis in microsporogenesis of a polyploid series of model

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plants bred involving a genetically uniform line and grown under the same conditions, thereby excluding the difference resulting from the impact of external factors, is the task of an utmost importance. Here, we report the results of analysis of the rate of cytomixis and the cytological pattern in microsporogenesis of 2, 4, 6, and 8x tobacco plants bread using the SR1 line and grown under uniform greenhouse conditions.

Materials and methods

Plant material

The tobacco plants of different ploidy levels ($2n = 2x = 24$, $4x = 48$, $6x = 72$ and $8x = 96$) were obtained using ($2n = 4x = 48$) line *Nicotiana tabacum* L. cv. Petit Havana SR1. 8x ($2n = 96$) plants were produced by treating 4x seeds with 0.5 % (*w*/*v*) colchicine for 48 h; 6x plants ($2n = 72$), by crossing 4x (♂) and 8x (♀) tobacco plants; and 2x plants ($2n = 24$), by in vitro androgenesis (Anagnostakis 1974; Nitsch and Nitsch 1969).

The ploidy level of all plants was determined by counting the number of chloroplasts in the stomatal guard cells and examining metaphase plates in the root meristems stained with 6 % acetocarmine (Fig. 1a, d, g, and j).

All plants were grown in a hydroponic greenhouse with a photoperiod of 16/8 h (day/night) at a temperature of 22/18 °C (day/night).

Analysis of microsporogenesis

The flower buds carrying microsporocytes at the necessary meiotic stages were fixed with freshly prepared acetic alcohol (3:1) and stored in 70 % ethanol at 4 °C. Squash preparations stained with acetocarmine were used for cytological analysis of microsporogenesis and counting of the rate of cytomixis by routine light microscopy (Aksioskop 2 plus microscope, Carl Zeiss, Germany). The images were recorded using an AxioCam HRc camera with the AxioVision (Carl Zeiss, Germany) software.

Statistical data processing

For statistical analysis, five plants of each ploidy level were used; three buds each containing five anthers were processed. Totally, 305,149 cells were examined. The percentages of the

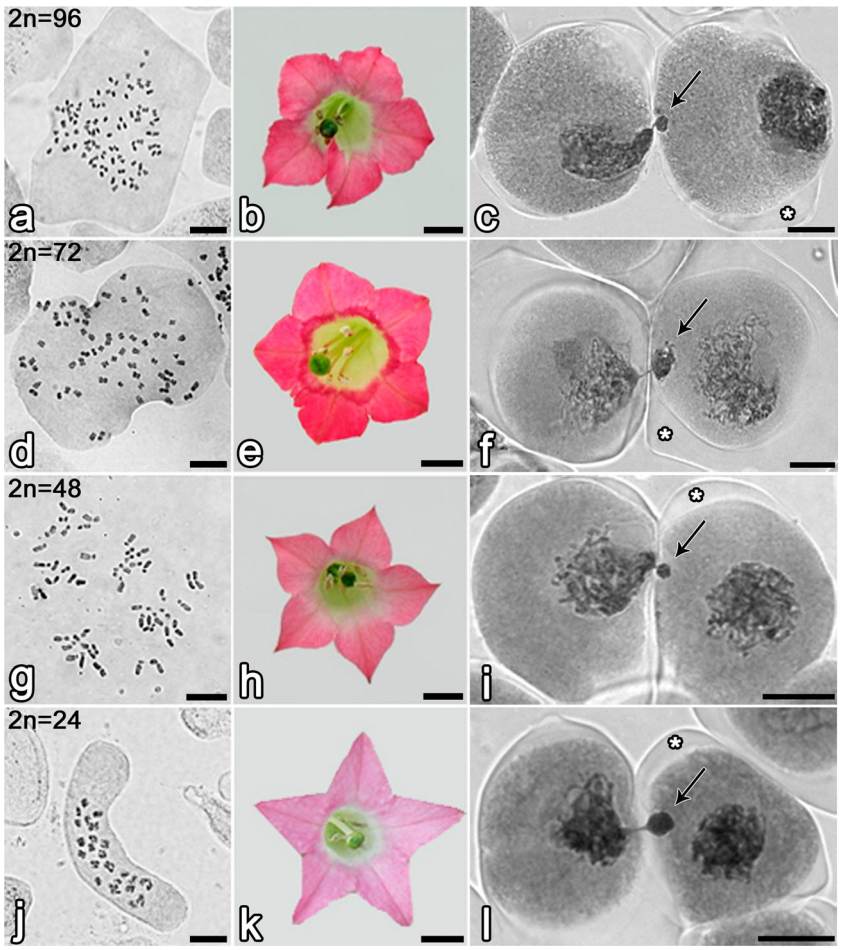

Fig. 1 Karyotype, flower morphology, and cytomixis in **a**–**c** 8x, **d**–**f** 6x, **g**–**i** 4x, and **j**–**l** 2x tobacco plants. *Arrows* denote migrating chromatin and *asterisks* denote callose wall. *Scale bars* are 5 mm in (**b**, **e**, **h**, and **k**) and 10 μm in the remaining panels

microsporocytes with cytomixis for each bud were compared between the different ploidy levels with the non-parametric Kruskal-Wallis test and subsequent pairwise multiple comparison with the Student-Newman-Keuls Method. The calculations were performed with the statistical program SigmaStat (Systat Software, Inc.).

Results and discussion

The tobacco has a complex genome and is assumed to be an ancient allotetraploid (Lim et al. 2000; Shibata et al. 2013). However, despite its origin, the tobacco now is an established species with a normal meiosis and high pollen fertility; that is why here we regarded the tobacco *Nicotiana tabacum* L. cultivar Petit Havana SR1 ($2n = 4x = 48$), used for production of the polyploid series, as a control.

The obtained tobacco plants with different ploidy levels display considerable phenotypic differences, first and foremost, related to the flower morphology (Fig. 1). The corona of 6x and 8x plants (Fig. 1b, e) is waved with widened and shortened petals warping outside as compared with that of 4x (Fig. 1h). On the contrary, the corona of 2x (Fig. 1k) has a distinct star-like structure with triangle petals rather than drop-shaped ones, which are characteristic of 4x (Fig. 1h). Besides the altered corona shape, the flowers of 2x and 8x plants develop the pistil that is considerably longer than the anther filaments and corona (Fig. 1e, k).

Microscopic examination has demonstrated that, in general, the nucleus migrates between cells in plants of different ploidy levels in a similar manner (Fig. 1c, f, i, and l). Microsporocytes of all experimental plants are enclosed into a normal callose wall (Fig. 1c, f, i, and l). In cytomixis, the nucleus leaves the cell central zone, approaches one of the walls, and passes to the neighboring cell through the cytomictic channel (Fig. 1c, f, i, and l).

Analysis of the rate of cytomixis in the microsporogenesis of tobacco plants with different ploidies demonstrates statistically significant differences (except 2x versus 4x) in this rate between examined ploidy levels. The average rate of cytomixis in the microsporogenesis of 4x plants is about 0.6 and 1.6 % in 2x versus 18.6 and 38.4 %, observed in the microsporogenesis of 6x and 8x plants, respectively.

A high rate of cytomixis in the microsporogenesis of genetically unbalanced plants (hybrids, aneuploids, and polyploids) has been repeatedly reported for various species. In particular, compare the rate of cytomixis of 1.3 and 1.7 %, respectively, observed in the microsporogenesis of *Aegilops tauschii* and *Triticum turgidum* with the rate of 20.2 %, reported for their hybrid (Peng et al. 2003). In *Ranunculus hirtellus*, cytomixis is observed at a high rate (on the average, 20 %) in tetraploids and is almost undetectable in diploids (Kumar et al. 2011). Hexaploid and octaploid plants of the genus *Consolea* display a high rate of cytomixis in their microsporogenesis (Negron-Ortiz 2007). This pattern fits well the rates of cytomixis observed in the microsporogenesis of 6x and 8x tobacco plants, displaying a drastic increase in this rate.

Since most of the authors relate an increase in the rate of cytomixis to an unbalanced state of the plant genome (Ghaffari 2006; Guan et al. 2012; Kumar et al. 2011; Peng et al. 2003), we expected to observe the increase in this rate in 2x comparable to the increase in 6 and 8x cytotypes; however, the results for tobacco 2x plants meiosis appeared to be quite unexpected. An increase in the rate of cytomixis in the microsporogenesis of 2x tobacco plants is rather insignificant as compared with 6x and 8x variants.

Thus, our results suggest that an increase in the rate of cytomixis in the tobacco microsporogenesis is primarily associated with the increase in the ploidy level and amount of DNA in the cell rather than with the state of genome balanced or an increase in the pollen sterility rate, since the rate of cytomixis in 2x plants, completely unable to form bivalents and provide normal chromosome segregation in meiosis, is significantly lower as compared with 8x plants, able to produce fertile pollen, although in a small amount.

Similar results have been described for the *Triticum* species with different ploidy levels (Sidorchuk et al. 2015). The authors observed that the rate of cytomixis in the microsporogenesis increased with the number of chromosomes in the karyotype of these plants. In particular, the rate of cytomixis amounted to approximately 3 % for *Triticum monococcum* ($2n = 2x = 14$) versus about 10 % for *Triticum flaksbergeri* ($2n = 8x = 56$) (Sidorchuk et al. 2015).

At the first glance, the conclusion clearly emerges that an increase in the rate of cytomixis in polyploids (6x and 8x cytotypes) results from the attempts of the cell to get rid of "excess" chromosomes in order to stabilize the newly constructed genome. However, we believe that this is an unjustified interpretation. It should be taken into account that a drastic increase in the rate of cytomixis with mass chaotic migration of nuclei is observed in an abnormal meiosis of polyploids and is associated with other numerous division abnormalities, first and foremost, involving spindle formation and cytokinesis. Correspondingly, we are not inclined to regard the mass cytomixis in polyploids as a real mechanism for altering the pollen karyotype. We believe that this is a mere side effect associated with the general discoordination of meiosis caused by the presence of surplus chromosomes in the cell.

On the other hand, we admit that cytomixis can be involved in the normal meiosis, where its rate is rather low, taking part in formation of aneuploids and unreduced gametes, which was confirmed by experimental data for control ($2n = 4x = 48$) tobacco plants (Mursalimov et al. 2015; Mursalimov and Deineko 2011, 2015). In particular, we have repeatedly observed the formation of binucleate microsporocytes in the first

meiotic prophase of control tobacco plants as a result of cytomixis. In this process, both nuclei independently continued their meiotic division, remaining observable until the first metaphase, and the meiotic products of these plants contained unreduced pollen (Mursalimov and Deineko 2015).

A more detailed analysis of the rates of cytomixis in the microsporogenesis of individual tobacco plants demonstrates a high variation in this process even in genetically uniform plants (Table 1). For example, the rate of this trait may vary in the range of 0–2.42 % in individual 4x plants, and a very similar pattern is observed in 2x plants (0–5.01 %). Rather a low rate of cytomixis, 0.17 and 3.64 %, is detectable in individual 6x plants; however, the average rate is considerably higher, amounting to 18.6 %. In turn, 8x cytotype lack any individual plants with a low rate of cytomixis, and the average rate of this trait is significantly increased as compared with the remaining three variants (Table 1).

Thus, analysis of the rate of cytomixis has demonstrated a high variation of this trait for individual plants and statistically significant differences between the plants of different ploidy levels. Note here that a decrease in the ploidy level and reduction in the number of chromosomes in the nucleus of 2x plants have no significant effect on the rate of cytomixis; however, an increase in the number of chromosomes in the nucleus results in a manifold growth in the rate of cytomixis during microsporogenesis of 6x and 8x tobacco plants.

The very first papers on the rate of cytomixis demonstrated that cytomixis is an extremely variable trait (Soodan and Wafai 1987). As was shown for the almond microsporogenesis, only six of the 20 examined trees displayed cytomixis at the moments they were assayed and the amount of sporogenous tissue involved in the chromatin migration significantly differed in individual plants: 12.5 to 96.0 % of the anther microsporocytes were involved in the migration of nuclei (Soodan and Wafai 1987). The studies with *Chlorophytum comosum* demonstrate that the change in the rate of cytomixis in microsporogenesis may display a distinct seasonal dependence. In particular, this rate in the microsporocytes in April amounted to 9 % versus only 3 % in July. The authors assume that the difference between these values in this case results from the changes in environmental temperature and humidity (Lattoo et al. 2006). Analogous seasonal variations have been described for *Corchorus fascicularis* (Mandal and Datta 2012).

In our work with tobacco plants, we eliminated the differences determined by the impact of climate and genetic factors using the plants obtained from a uniform line and grown under uniform conditions of a greenhouse. However, the rates of cytomixis even in this situation displayed a considerable variation within each group of plants with different ploidies. Presumably, this is explainable by the effect of certain unaccounted factors, such as circadian rhythms or hormonal changes in the examined plants.

Table 1 The rate of cytomixis in individual tobacco plants with different ploidy levels

Ploidy level	Plant	Microsporocytes observed	Microsporocytes with cytomixis	
			Number	%
2n = 2x = 24	1	20,772	0	0
	2	19,528	28	0.14
	3	24,867	325	1.3
	4	17,140	470	2.74
	5	14,785	742	5.01
2n = 4x = 48	1	23,019	0	0
	2	18,434	4	0.02
	3	9680	12	0.12
	4	8017	61	0.76
	5	15,576	378	2.42
2n = 6x = 72	1	12,480	22	0.17
	2	16,883	615	3.64
	3	6186	1014	16.39
	4	13,769	4816	34.97
	5	11,551	4852	42.00
2n = 8x = 96	1	11,871	2416	20.35
	2	16,430	4243	25.82
	3	10,633	4161	39.13
	4	16,485	7303	44.30
	5	17,043	9682	56.80

A serious problem when studying the rate of cytomixis and associated features in microsporogenesis is that any molecular markers of cytomictic chromatin have not been so far discovered. Otherwise, it would be possible to utilize flow cytofluorometry for assessing the abundance of this phenomenon in plant cells.

So far, cytomixis has been observed in the microsporogenesis of over 400 plant species (Mursalimov et al. 2013b), and although the evolutionary significance of this phenomenon is still controversial, cytomixis as a permanent companion of meiosis in higher plants undoubtedly deserves attention in the perspective of the specific features in intercellular communication of higher plants.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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