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The role of spherosome-like vesicles in formation of cytomictic channels between tobacco microsporocytes

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Abstract

The formation of cytomictic channels (CCs) during the tobacco microsporogenesis has been analyzed by microscopy and cytochemical methods. Starting from the pachytene stage, CCs were formed between microsporocytes with involvement of specific organelles, the so-called spherosome-like vesicles. The presence of the enzyme callase, able to degrade callose and form CCs in the cell wall of microsporocytes, has been demonstrated for the first time in the spherosome-like vesicles. An active form of callase was detectable in the spherosome-like vesicles and cell wall but not in the endoplasmic reticulum and Golgi apparatus. The release of callase from spherosome-like vesicles into the cell wall was described. Two ways in formation of the CCs in the tobacco microsporogenesis, the primary formation in the cell wall composed of pectins and cellulose (leptotene-zygotene) and secondary formation in the cell wall of callose (after the pachytene stage), were compared.

Additional key words: callase, cytomixis, microsporogenesis, *Nicotiana tabacum*, plasmodesmata.

Introduction

It is known that intercellular channels, which directly connect the cytoplasms of adjacent cells, play a key role in the regulation of plant growth and development. Two types of such channels are usually distinguished in the plant tissues, namely plasmodesmata (Lucas and Lee 2004) and cytomictic channels, CCs (Heslop-Harrison 1966, Wang *et al.* 2002, 2006). Plasmodesmata are present in almost all types of plant cells, whereas CCs are, as a rule, formed between microsporocytes during male meiosis (Bellucci *et al*. 2003, Negron-Ortiz 2007, Kumar and Singhal 2011). Unlike plasmodesmata, CCs has no internal structure as a desmotubule and may exceed over 20-fold the size of plasmodesmata (Wang *et al*. 1998, 2004, Mursalimov *et al*. 2010). Large CC sizes allow large organelles, including the nucleus, to migrate along them (Wang *et al*. 2004, Song and Li 2009, Mursalimov and Deineko 2011). The migration of a nucleus or its fragments from one cell to another *via* CCs is referred to as cytomixis (Gates 1911). Occurrence of cytomixis between plant cells unambiguously suggests that CCs have been formed in them since the nucleus is unable to migrate between cells *via* plasmodesmata (Maule 2008, Mursalimov *et al*. 2010).

As it has been demonstrated in different plant species, the CCs between microsporocytes are formed at the very beginning of a meiotic division (Feijo and Paris 1989, Polowick and Sawhney 1992, Wang *et al*. 2002). In early prophase I (leptotene-zygotene), the primary CCs are formed between microsporocytes *via* two pathways, namely utilizing plasmodesmata (Wang *et al*. 1998, Mursalimov *et al*. 2010) and *de novo* in the cell wall regions where plasmodesmata are absent (Wang *et al*. 1998, 2002, Yu *et al*. 2004). It has been shown that the enzymes cellulase and pectinase, destroying the cell wall and middle lamella, are involved in the primary CC formation. These enzymes are produced in the endoplasmatic reticulum and are then transported to the cell wall in the vesicles detached from it (Wang *et al*. 1998, Yu *et al*. 2004). However, starting from the pachytene stage, the primary cell wall becomes inaccessible for these enzymes because of a callose deposition on its inner surface. The intensively deposited callose layer also stops the CCs formed earlier (Wang *et al*. 2002, Mursalimov and Deineko 2012).

Despite that the primary CCs are completely closed by callose from the pachytene stage, researchers have

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Abbreviation: CCs - cytomictic channels.
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repeatedly observed intercellular migration of nuclei during the entire microsporogenesis until the stage of tetrads (Ressayre *et al*. 2003, Sheidai and Bagheri-Shabestarei 2007, Song and Li 2009, Rani *et al*. 2010) suggesting both the connection of microsporocytes *via* CCs after pachytene and, correspondingly, existence of the mechanisms of secondary CC formation with a local destruction of the callose wall. However, there is currently no data how the secondary CCs are formed and which organelles may be involved in this process.

We assume that spherosome-like vesicles, earlier detected in the tobacco microsporogenesis (Mursalimov *et al*. 2010, Mursalimov and Deineko 2012), may take part in the secondary CC formation *via* a local destruction of the callose wall. This hypothesis is confirmed by detection of the callose lysis zones in the vicinity of these organelles (Mursalimov *et al*. 2010). However, to unambiguously infer that the spherosome-like vesicles are involved in the secondary CC formation, it is necessary to prove that these organelles contain the enzyme callase, capable of destroying the callose wall. Once the presence of callase in spherosome-like vesicles is confirmed, it is of a considerable interest to clarify the callase delivery pathway from spherosome-like vesicles to the sites of secondary CC formation.

The aim of this paper was to analyse the mechanism involved in the secondary CC formation in tobacco microsporocytes from the moment of callose wall formation (pachytene) to the end of the first mitotic division using light and electron microscopies in combination with cytochemical methods with a special focus on the spherosome-like vesicles and their involvement in CC formation. The localization of callase activity and the evidences for involvement of spherosome-like vesicles in local callose destruction was described as well as the process of secondary CC formation with participation of spherosome-like vesicles.

Materials and methods

Three tobacco (*Nicotiana tabacum* L.) lines were used in this work, namely control diploid ($2n = 48$) line SR1 with a rate of cytomixis about 2 - 5 % and two mutant polyploid lines ($2n = 96$), Res79 and 16.70, with cytomixis rates about 20 - 60 %. The mutant lines Res79 and 16.70 were obtained from line SR1; characteristic of them is a constantly high cytomixis rate, which suggested these lines as model objects for studying the specific features of this process (Zagorskaya *et al*. 2001, Sidorchuk *et al*. 2004, 2007). Except for different cytomixis rates, we have not detected any principle differences in the mechanisms of CC formation and function in the mutant and control plants; correspondingly, the microphotographs of microsporogenesis of different lines are assembled together. All the plants were grown in a hydroponic culture in a greenhouse with a 16-h photoperiod, irradiance of 600 μmol m^{-2} s^{-1}, day/night temperature of 22/18 ºC, and relative humidity of 70 %.

For light microscopy examination, the flower buds of analyzed tobacco lines containing anthers with microsporocytes at different meiotic stages were fixed in a solution of ethanol:acetic acid (3:1) at 4 ºC for 24 h. The fixed samples were stored in 70 % (v/v) ethanol. Cytological analysis of microsporogenesis was conducted in squash preparations stained with acetocarmine using an *Aksioskop 2 plus* (*Carl Zeiss*, Jena, Germany) microscope. The images were recorded with an *AxioCam HRc* (*Carl Zeiss*) CCD camera.

Ultrastructural analysis was conducted using the anthers of Res79 and SR1 plants at different stages of microsporogenesis, previously determined by light microscopy. Anthers were cut into pieces with a size of 2 - 3 mm and fixed with 2.5 % glutaraldehyde (*Serva*, Germany) in phosphate buffer (pH 7.2 - 7.4) at room temperature for 4 h . Then the samples were washed three times in the phosphate buffer for 10 min and postfixed with 1 % (m/v) osmium tetroxide (*Azurite*, Russia) at a room temperature for 2 h and again washed twice with the phosphate buffer for 15 min. Then the samples were successively dehydrated with ethanol at increasing concentrations. The samples were transferred to acetone for 1 h and embedded in the epoxy resin *Araldite* (*Fluka*, Switzerland).

Ultrathin sections with a thickness of 80 nm were cut using an *Ultracut UCT* (*Leica*, Switzerland) ultramicrotome and successively stained with lead citrate and uranyl acetate (*Serva*, Germany). The stained sections were examined using a *JEM 100S* (*Jeol*, Japan) and a *Libra120* (*Carl Zeiss*) transmission electron microscopes at an acceleration voltage of 80 kV. The microscopy was conducted at the Joint Access Center for Microscopy of Biological Objects with the Siberian Branch of the Russian Academy of Sciences.

The cytochemical localization of callase (3-β-D-glucan-glucanohydrolase, EC 3.2.1.39) was determined by a modified technique proposed by Bal (1974). The anthers of 16.70 and SR1 plants were sampled according to their size to determine their developmental stage by light microscopy. Then the anthers were cut into pieces of 1 - 2 mm and fixed with mixture of 1 % glutaraldehyde and 4 % (m/v) paraformaldehyde (*Sigma*, St. Louis, USA) in 0.1 M phosphate buffer (pH 7.2), at 0 ºC for 1 h. The fixed tissue was 10 times washed with 0.1 M phosphate buffer (pH 7.2) and left in this buffer at 0 ºC overnight. Then the material was incubated in 0.05 M citrate buffer (pH 5) containing 1 % laminarin (the callase substrate extracted from *Laminaria digitata; Sigma*) at a tempe-rature of 25 ºC for 25 min and placed into Benedict's solution (1.73 % cupric sulfate, 17.3 % sodium citrate and 10 % sodium carbonate; *Fluka*) at 80 ºC for 15 min. In the control specimens, the treatment

with either laminarin or Benedict's solution was omitted. The tissue was three times washed with distilled water, postfixed with 1 % OsO_4 at a room temperature for 2 h, washed with distilled water, dehydrated, and embedded in *Araldite*. Ultrathin sections were obtained, contrasted, and examined.

Results and discussion

Analysis of the microsporogenesis in mutant lines Res79 and 16.70 demonstrated that the CCs between microsporocytes were present not only in prophase I but also at later meiotic stages including the prophase of the second division. The cytomixis was observed at these stages in Res 79 and 16.70 (Fig. 1*A-C*), but in the control line SR1, cytomixis was undetectable after completion of prophase I. However, the presence of CCs between micro sporocytes at least until the stage of metaphase I was observed despite the absence of migrating nuclei (Fig. 1*D,E*). CCs were confined by the plasma membrane and filled with cytoplasm. Despite that the cells at this stage of microsporogenesis were separated, they retained their connection *via* the CCs linking the cells through the intercellular space. The plasmodesmata between tobacco microsporocytes at this meiotic stage were undetectable. The distinction between the CCs and plasmodesmata in the cell wall of tobacco microsporocytes has been comprehensively described earlier (Mursalimov *et al*. 2010).

Thus, it has been demonstrated that the CCs between microsporocytes still exist after completion of prophase I in both control and mutant tobacco plants. On the other hand, it was shown earlier that plasmodesmata and CCs formed in the tobacco microsporogenesis in early prophase I were stopped with callose at the stage of late pachytene (Mursalimov and Deineko 2012) suggesting the existence of a secondary mechanism providing the CC formation.

Spherosome-like vesicles, which according to our

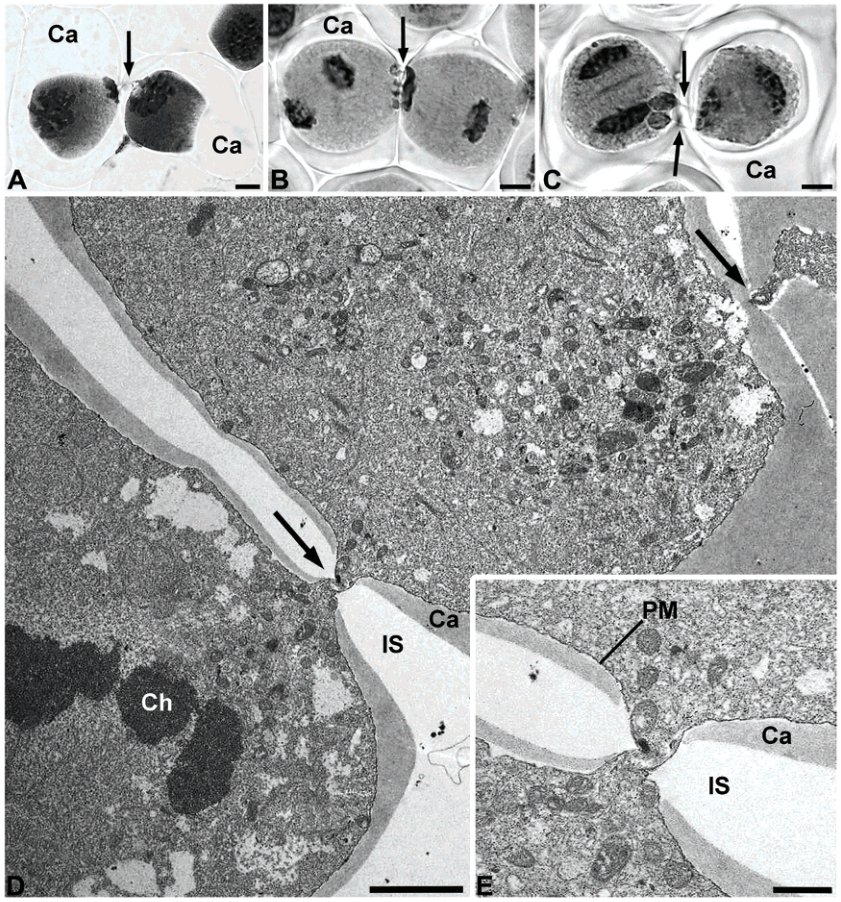

Fig. 1. Cytomixis and the cytomictic channels between microsporocytes after callose cell formation. *A* - The cytomixis between microsporocytes of line Res79 at the stage of metaphase I. *B* - Cytomixis between microsporocytes of line 16.70 in telophase I. *C* - Cytomixis between microsporocytes of line 16.70 in prophase II. *D* - Cytomictic channels between microsporocytes of line SR1 in metaphase I. *E* - A cytomictic channel, enlarged. *Black arrows* denote cytomictic channels, Ca - callose, Ch - chromatin, IS - intercellular space, PM - plasma membrane. *Scale bars* represent 2.5 μm in *A* - *C*, 1 μm in *D*, and 500 nm in *E*.

hypothesis may play a key role in the secondary CC formation, have been detected in all the studied lines (Figs. 2 and 3). In pachytene, the spherosome-like vesicles were round or oval and were in contact with the callose wall (Fig. 2*A*,*C*) or, rarer, lying free in the cytoplasm or near the nucleus (Fig. 2*B*,*D*). A high osmiophilicity suggested a possible lipid content of these organelles which were visually very similar to spherosomes (Yatsu and Jacks 1972). When these organelles were formed, the callose wall was already well developed and the characteristic sites of callose lysis were observed in the contact zones with spherosome-like vesicles (Fig. 2*D*) which confirmed our earlier results (Mursalimov *et al*. 2010).

In order to prove the involvement of spherosome-like vesicles in the CC formation as well as to find out how the secondary CCs were formed, it was necessary to localize the sites of callase (the enzyme hydrolyzing callose) activity, since callose at this stage was the only cell wall component of the tobacco microsporocytes (Mursalimov and Deineko 2012). To solve this problem, we selected a modified technique for cytochemical localization of the enzymes destroying polysaccharides (Bal 1974). After the corresponding staining procedures, the reaction products of callase activity in the microsporocytes of tobacco SR1 and 16.70 lines were detectable as small electron-dense crystals (Fig. 3) complying with the results obtained by this method in analogous works (Wang *et al*. 1998, Yu *et al*. 2004). The reaction products were observed only in the samples that passed all the stages of preliminary processing and were undetectable in the control samples prepared omitting one of the processing stages. The callase reaction products were detected in spherosome-like vesicles (Fig. 3*A,F*) and within the cell wall (Fig. 3*G*) and were undetectable in the Golgi apparatus and endoplasmic reticulum. Thus, these data suggested that the content of spherosome-like vesicles displayed an enzyme activity and that these organelles were able to destroy the callose wall forming new CCs and participating in remodeling the microsporocyte cell wall. Both the presence of the enzyme callase in spherosome-like vesicles and their role in the CC formation between microsporocytes have been demonstrated for the first time.

Based on these results, we suggest the following pattern of callase release from spherosome-like vesicles during the CC formation. Initially, the spherosome-like vesicle contacts a vacuole filled with callose and enters this vacuole (Fig. 3*A*). The proof for the presence of callose in these vacuoles is that they further fuse to the callose wall as well as that the callase reaction products are present in the contact zone of a spherosome-like vesicle and the mentioned vacuole (Fig. *3A*). Presumably, the release of callase is necessary for these organelles to penetrate into the callose wall. Then the callose vacuole, containing inside a spherosome-like vesicle, is transported to the cell wall (Fig. 3*B,C*). When contacting the plasma membrane, the content of callose vacuole is released beyond the cell boundary *via* exocytosis; correspondingly, the callose transported by it as well as the spherosome-like vesicle find themselves incorporated into the cell wall (Fig. 3*D,E*). Note that the reaction products of callase activity are undetectable during the transport of spherosome-like vesicles and their

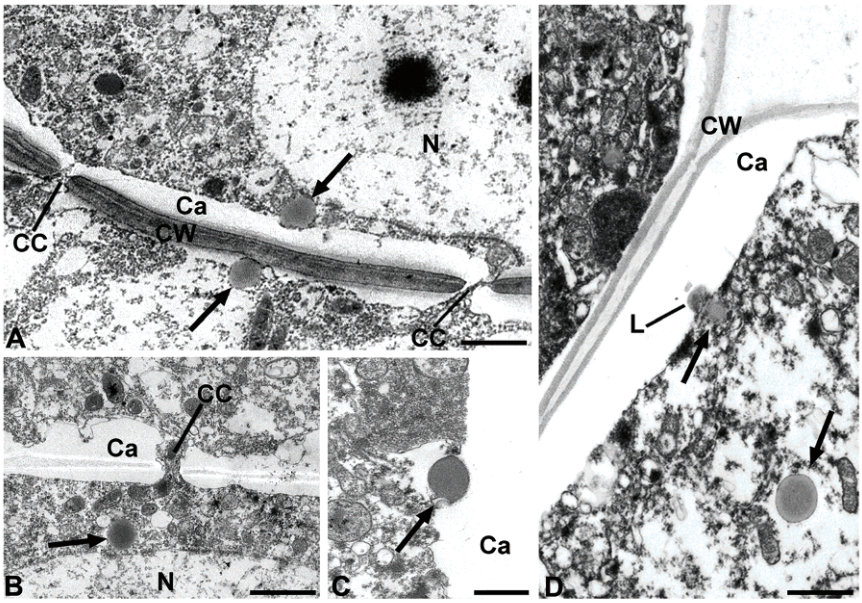

Fig. 2. Spherosome-like vesicles in tobacco line Res79 microsporocytes (pachytene). *A* - Spherosome-like vesicles contacting the cell wall. *B* - Spherosome-like vesicles near the nucleus. *C* - A spherosome-like vesicle partially submerged in callose. *D* - Spherosome-like vesicles near a site of the callose wall lysis (L). *Arrows* denote spherosome-like vesicles, Ca - callose, CW - primary cell wall, CC - cytomictic channel, N - nucleus. *Scale bars* represent 1 μm in *A*, *B* and *D*, and 500 nm in *C*.

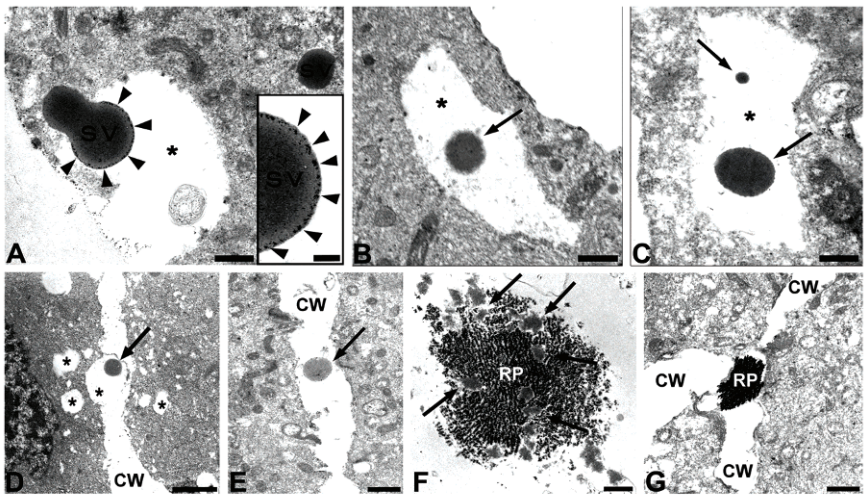

Fig. 3. Spherosome-like vesicles in tobacco microsporogenesis at the stages of pachytene - metaphase I after cytochemical localization of callase activity in SR1 (*D*, *F*) and 16.70 (*A,B,C,E,G*). *A* - A spherosome-like vesicle enters a vacuole with callose (magnified in inset, *arrowheads* denote reaction products of callase activity). *B, C* - Transport of spherosome-like vesicles within the vacuole with callose. *D* - Fusion of the vacuoles with callose to the cell wall. *E* - Spherosome-like vesicle within the cell wall. *F* - An explosion-like callase release upon destruction of spherosome-like vesicle (*arrows* denote fragments of the organelle). *G* - Reaction products of callase activity within the cell wall, the area where the reaction products are observed fits the size of a cytomictic channel. *Asterisks* denote vacuoles with callose, *arrows* denote spherosome-like vesicles, CW - cell wall, RP - reaction products. *Scale bars* represent 250 nm in *A* and 100 nm in the inset, 250 nm in *B,C,F,* 1 μm in *D* and 500 nm in *E*,*G*.

incorporation into the cell wall, suggesting the absence of enzyme activity of these organelles at the considered stage. Presumably, after spherosome-like vesicles are already inside the cell wall, they are destroyed and release their content into the ambient space in an explosion-like manner (Fig. 3*F*). The released callase locally lyse the cell wall (Fig. 3*G*) forming wide intercellular channels, which, according to our opinion, make possible the CC existence after formation of the callose wall.

A study of the mutant tobacco lines with a high rate of cytomixis using the SR1 line as a control have allowed us to discover previously unknown way of the CC formation as well as to detect specific organelles responsible for this process, the spherosome-like vesicles. The osmiophilic organelles, which are similar to the spherosome-like vesicles that we discovered, have been observed in the cytoplasm of microsporocytes of higher plants (Echlin and Godwin 1968); however, we have not found any data on their enzyme activity or involvement in the CC formation.

Our data demonstrating that the plant cell containing a special class of spherosome-like organelles enriched in lipids and displaying a hydrolytic activity towards callose are in agreement with analogous organelles previously detected in the stem apical meristem of *Betula pubescens* (Rinne *et al*. 2001). In the last case, these organelles are involved in destruction of the callose plugs on plasmodesmata when plants emerge from dormancy. Using immunogold labeling, the presence of the enzyme callase in the mentioned organelles has been confirmed (Rinne *et al*. 2001).

Summing up the results of our studies and the relevant literature data, it is possible to represent a general scheme for the CC formation in the microsporogenesis of higher plants as follows. Presumably, the primary CCs are formed in meiotic prophase I at the stages of leptotene - zygotene, when the cell wall is represented by middle lamella and a thin layer of cellulose; moreover, the primary CC formation may involve plasmodesmata or take place *de novo* with the help of the hydrolytic enzymes released by the Golgi apparatus and endoplasmic reticulum (Wang *et al*. 1998, Yu *et al*. 2004, Mursalimov *et al*. 2010). Then, starting from pachytene, the pectin-cellulose cell wall is replaced with the callose wall (Mursalimov and Deineko 2012) which covers the microsporocytes until the stage of tetrads. The secondary CC formation is associated with the cell wall remodeling since it is likely that the CCs are necessary not only at the early stages of microsporogenesis. The spherosome-like vesicles able to release callase are involved in the secondary CC formation. Approximately after metaphase I, microsporocytes start separating from one another to become isolated and CCs to gradually disappear. It is likely that the primary and secondary CC formations in different plant species may considerably differ from the pattern that we have described for tobacco.

Thus, the issue of how the CCs are formed between plant cells is being gradually clarified; however, there is almost no progress in the understanding what is the role of these channels. By the analogy to the channels between animal cells, some authors believe that the CCs in plant tissues fulfill the exchange of informational molecules for synchronization of microsporogenesis (Heslop-Harrison 1966). There is a hypothesis suggesting that CCs may be used for transporting nutrients to the

actively developing cells at the expense of weaker cells (Milyaeva 1965). These assumptions are rather logical since the CCs appear in a considerable amount at a strictly determined time moment in the development of microspores, namely in the early prophase I of meiosis when cell synchronization is of a paramount importance. Our observation of a secondary CC formation between microsporocytes after the primary CCs and plasmodesmata are stopped with callose depositions fits this hypothesis which implies the need in interconnection between microsporocytes to a certain moment in their development.

Consideration of CCs only as the pathways for exchanging of some molecules and metabolites between cells brings about the question on the nature of intercellular migration of nuclei. Under the proposed hypotheses, this phenomenon may be regarded as a random deviation from the norm. However, the papers describing the presence of cytomixis in all the individuals of a population (Banerjee and Sharma 1988) contradicts this statement as well as the existence of the plant genotypes with an inherited rate of cytomixis (Falistocco *et al*. 1995), formation of the pollen with different ploidy levels in the plants display a high rate of cytomixis in microsporogenesis (Falistocco *et al*. 1995, Ghaffari 2006, Lattoo *et al*. 2006, Negron-Ortiz 2007, Singhal and Kumar 2008, Song and Li 2009, Kumar *et al*. 2010, Kumar and Singhal 2011), retention of the structure by the migrating chromatin during cytomixis, and formation of "nuclear bridges" (the structures providing a direct contact between the nuclei of adjacent cells *via* CCs) as a result of cytomixis (Mursalimov and Deineko 2011). Thus, it is possible to assume that the role of CCs does not reduce to a mere traffic of nutrients and informational molecules and that the existence of large intercellular channels allowing migration of whole nuclei provide the plant with a new supplementary pathway for an increase in genetic diversity of gametes. Unfortunately, all the currently available evidences for an evolutionary significance of cytomixis are indirect, however, we believe that a considerable attention to this issue will now allow novel intriguing data on the mechanisms underlying cytomixis and its role in the plant life.

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