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CELL BIOLOGY

Changes in the Extracellular Matrix Surface Network during Cyclic Reproduction of Proembryonic Cell Complexes in the *Fagopyrum tataricum* (L.) Gaertn Callus

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Intercellular interactions in multicellular organisms are an important factor of morphogenesis control. These interactions include exchange of signaling molecules; generation of hormonal, metabolic, ion, and electric gradients; and mechanical interaction of dividing cells with each other. The presence of proembryonic cell complexes (PECCs) or proembryonic cell masses (PEMs) is a typical feature of most plant cultures capable of embryoidogenesis [1]. According to the results of histological studies, PECCs are embryos whose growth was stopped at the preglobular stage by the addition of auxin to the culture medium [2]. Morphologically, PECCs are observed as lustrous white structures (nodules) on the callus surface. In earlier studies [3, 4], we obtained calluses of the Tatar buckwheat *Fagopyrum tataricum* (L.) Gaertn., whose nodular morphology was typical of embryogenic cultures [3, 4]. For a long period of cultivation (several years), these cultures retained their specific morphological features, diploid number of chromosomes, and capacity for embryoidogenesis [5]. We also found that PECCs were reproduced in the morphogenic callus of the Tatar buckwheat *F. tataricum* (L.) Gaertn. as a result the of restoration of the development cycle, including the formation and growth of PECCs, further loosening of these structures, and formation of new PECCs from individual cells of loosened PECCs [4]. According to the published data, nodular cultures were sustained as a result of proembryo cloning *in vitro* [1]. However, the mechanisms of this cloning are obscure. Therefore, the electron microscopic study of the surface of the morphogenic callus of the Tatar buckwheat *F. tataricum* (L.) Gaertn. during cyclic reproduction of PECCs was of considerable interest. It was also interesting to compare the specific features of the structural organization of morphogenic and nonmorphogenic calluses.

Morphogenic and nonmorphogenic calluses of the Tatar buckwheat *F. tataricum* (L.) Gaertn. were used. The morphogenic nodular callus was prepared from an immature embryo and consisted of PECCs and soft callus domains. The morphology and methods of preparation of these cultures were described elsewhere [3, 4]. The nonmorphogenic callus consisted only of parenchymatous cells; it was selected from the morphogenic callus. In contrast to the morphogenic callus, the nonmorphogenic callus was characterized by a loose structure, high growth rate, and completely inhibited capacity for morphogenesis. Callus cultures were cultivated at a constant temperature of 26 ± 2°C in the RX medium. The nonmorphogenic and morphogenic calluses were replated every two and four weeks, respectively.

Two methods of fixation of the pieces of callus tissue for scanning electron microscopy were used. According to the first method suggested in [4], pieces of callus were fixed in a 2.5% glutaric aldehyde, with a subsequent additional fixation in 1% OsO_4. According to the second method, pieces of callus were fixed in a 1% glutaric aldehyde and a 3.7% paraformaldehyde. The resulting samples were washed in PBS, dehydrated using a gradient of alcohols (up to absolute ethanol), and dried near the critical point with subsequent vacuum evaporation of gold. The resulting samples were viewed using a Jeol scanning electron microscope (Japan).

The results of our electron microscopic study showed that the size, shape, and surface structure of the *F. tataricum* embryogenic callus PECC were changed during the cycle of PECCs reproduction. Young PECCs (up to 250 μm in diameter) were of spherical shape (Figs. 1a and 1b) and produced by small rounded cells of about uniform size distribution (10 μm) (Fig. 1c). Individual fibrillar structures were also observed on the surface of PECCs (Fig. 1c). Mature PECCs had an oval shape (up to 1 mm in length). Larger cells were seen to be desquamated from the PECCs surface (Fig. 1d). Pro-

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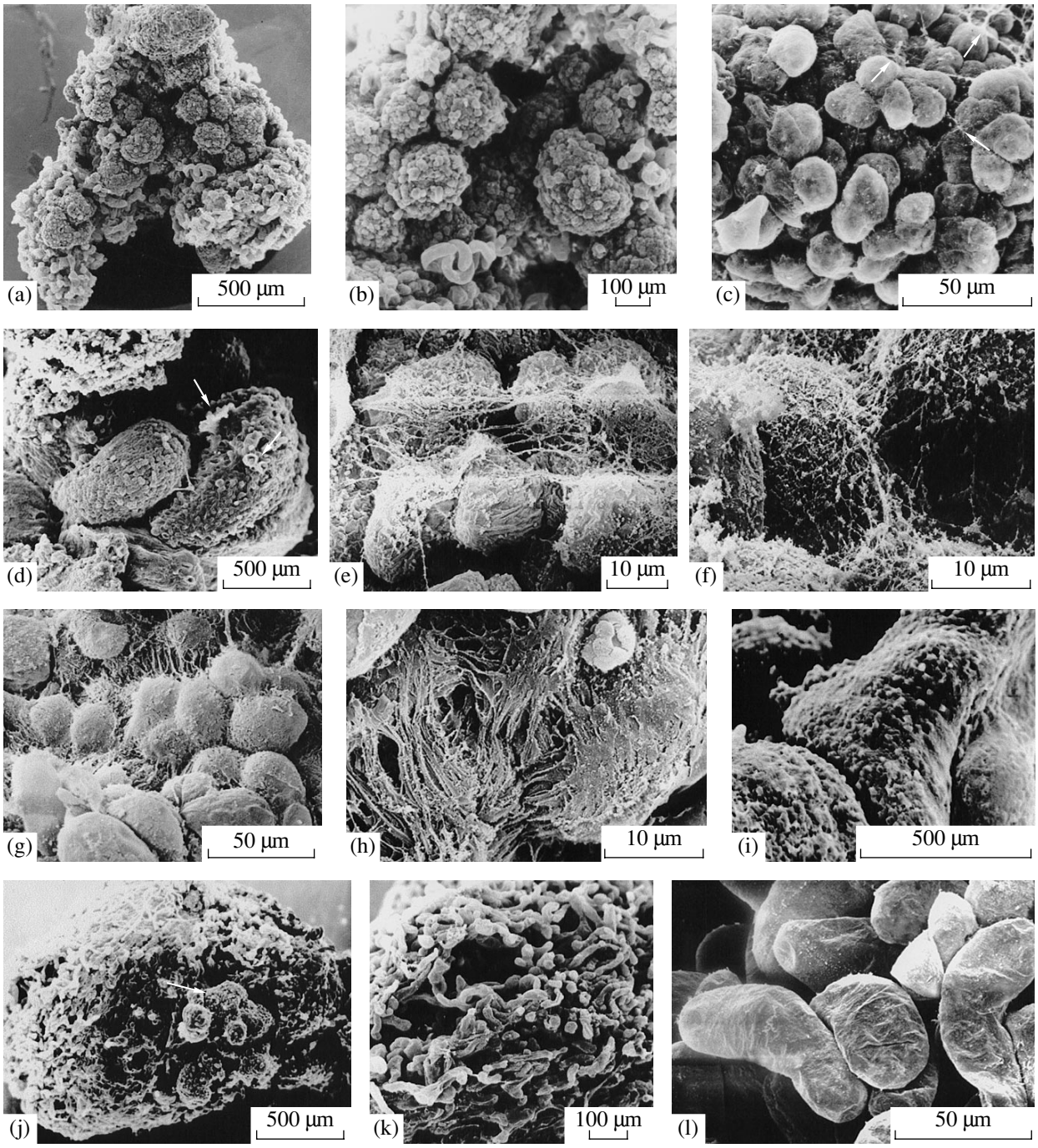

Fig. 1. Specific features of the changes in the structural organization of the morphogenic callus of *F. tataricum* during the formation of proembryonic cell complexes (PECCs). Scanning electron micrographs: (a) a general view of a morphogenic callus; (b) young PECCs; (c) cells constituting young PECCs, individual fibrils of the surface network are indicated by arrows; (d) a mature PECC (left) and a loosening PECC (right), large cells on the surface of the loosening PECC are indicated by arrows; (e)–(f) the structure of the surface network of a mature PECC; (g)–(h) the structure of the surface network of a loosening PECC; (i) the layer of secretion on the PECC surface before the formation of soft callus and the appearance of a new PECC; (j) disintegration (loosening) of the parent PECC and formation of a new PECC (indicated with an arrow); (k)–(l) cells of a soft callus devoid of surface network.

embryonic cell complexes were coated with a fibrillar network, elements of which were exposed at the surface of PECC cells and located between the cells (Figs. 1e and 1f). Separate strands connect closely located PECCs with one another (Fig. 1d). Note that the aging of individual PECCs was accompanied by changes in the number and thickness of the fibrils. During the transition to the stage of loosening and formation of new PECCs, the surface of mature PECCs (with lengths of 1.5 mm and more) was formed by larger cells; and

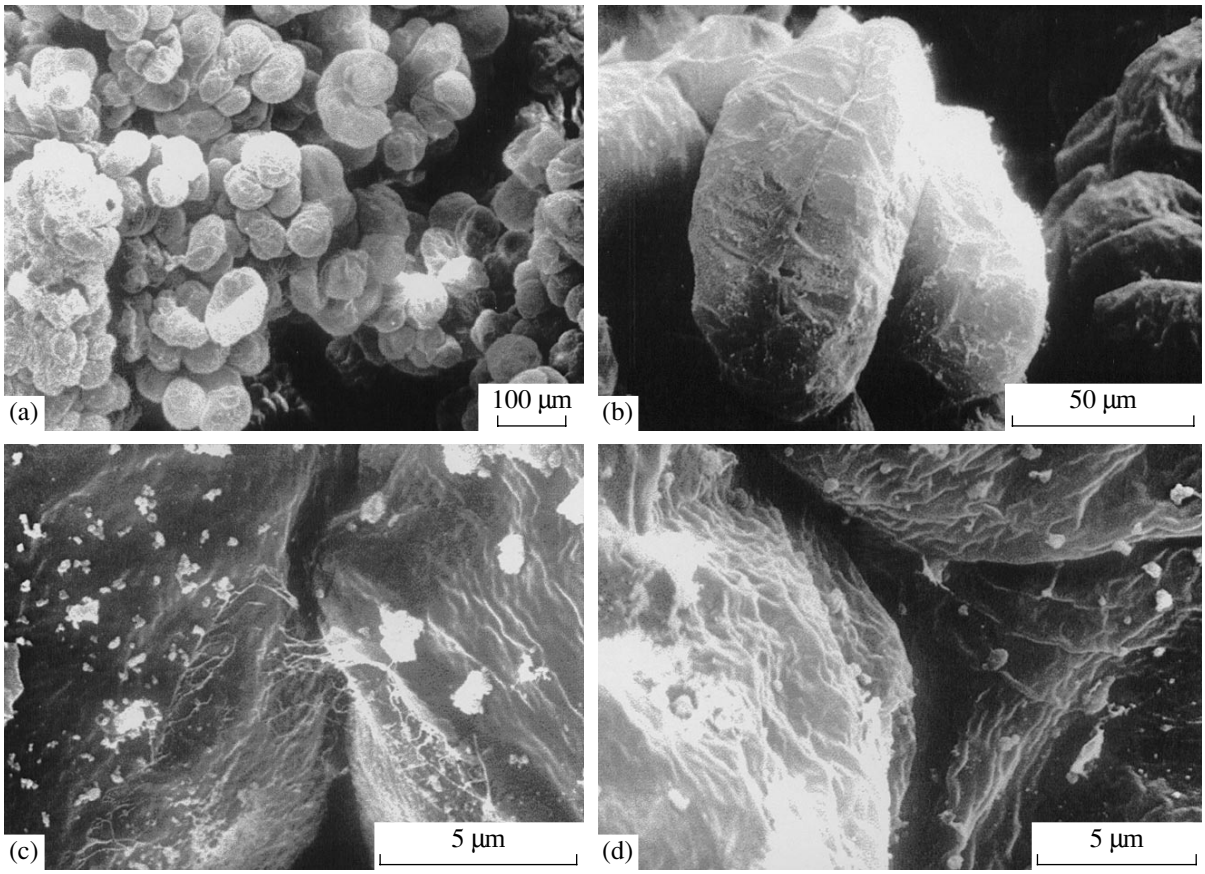

Fig. 2. Specific features of the structural organization of a nonmorphogenic callus of *F. tataricum*. Scanning electron micrographs: (a) a general view of a nonmorphogenic callus; (b) individual cells of the nonmorphogenic callus; (c, d) the cell surface structure in the nonmorphogenic callus.

newly formed PECCs were coated with bundles of secret (Figs. 1g and 1h).

Changes in the size of the surface cells are clearly seen in Fig. 1d, where two closely located PECCs are at different stages of development: mature (left) and loosening (right). Before loosening, the whole surface of the PECCs was coated with a layer of secretion (Fig. 1i). The stage of formation of soft callus cells was preceded by enhanced secretion of polymer on to the PECC surface. Probably, this secretion was due to disintegration of the dense PECC structure. A complete disintegration of PECCs was as the loss of intercellular contacts and multiple elongation of PECC cells (Figs. 1j and 1k). Not only the cells of the surface layer, but also the cells of the subsurface layer underwent elongation (Fig. 1k). It is seen from Fig. 1j that new PECCs were induced from individual cells of loosening PECCs, the main mass of PECCs being disintegrated, giving rise to a soft callus. Elongated baloonlike cells of the soft callus did not divide, and their contacts were weak (Figs. 1k and 1l). In addition, the surface of the soft callus cells was almost completely devoid of the fibrillar surface network (Fig. 1l). Note that no fibrillar network was observed on the cell surface of the nonmorphogenic callus. Individual fibrils between cells were observed only occasionally (Fig. 2).

The literature contains only a few works describing the results of electron microscopic studies of plant cell cultures [6–10]. It was shown in these works that the PECCs of the embryogenic cultures or the proembryo formed in explant tissues contained a typical structural marker, which was termed the extracellular matrix surface network (ECMSN) [11]. The ECMSN is a fibrillar structure and is, at least partially, of protein origin [8]. Although the functions of this structure remain obscure, it was shown that disappearance of the fibrillar network preceded the formation of the protoderm and transition to the globular stage of embryo development [7, 8, 10]. Our findings suggest that ECMSN is the marker inherent only in the embryogenic cells, because no such structure was observed on the surface of either nonmorphogenic cells or cells of soft callus, which also had no morphogenic potential. We also found that changes in the morphology of ECMSN during cyclic reinitiation of PECCs in the morphogenic callus of *F. tataricum* were determined by the age (or the stage of development) of individual PECCs. Young PECCs were covered with a fibrillar network composed of indi-

vidual fibrils. The structure of the ECMSN changed with the age (and size) of the PECC: the number of fibrils and strands producing the ECMSN increased as the PECC age increased, and the whole surface of the PECC before disintegration was coated with a layer of secretion. It may be suggested that various substances fulfilling different functions were secreted on to the surface at different stages of PECC development. The molecules required to maintain the existence of PECCs as a structural unit and performing a protective function are synthesized first. These molecules are represented by arabinogalactane proteins (AGPs) and proteoglycans, the presence of which on the surface of PECCs was demonstrated in maize [11] and chicory [12]. The protective function of AGPs can be determined by considerable water-holding capacity of these molecules, which is of cardinal importance for providing the proembryo growth in the absence of the protoderm. As a certain critical mass of PECCs is attained in the absence of appropriate conditions for transition to the globular stage of development, disintegration (loosening) of PECCs in the callus of *F. tataricum* begins. Separation of PECC cells is determined by large-scale changes in the composition and structure of cell walls, because we observed an increase in the cell size and modification of cell shape.

Scanning electron microscopy data also demonstrated the degradation of the middle lamellae and weakening of intercellular contacts during the disintegration of PECCs and formation of soft callus. Probably, the processes of separation of PECC cells are due to the activation and secretion of various enzymes capable of catalyzing the hydrolysis of the middle lamella and modifying cell wall structure. According to the scanning electron microscopy data, disintegration of PECCs was preceded by the modification of the ECMSN structure from fibrillar to gluelike. It was found that initial stages of cell elongation in embryogenic cultures were accompanied by an increase in the rate of exocytosis [13]. A similar pattern of enhanced exocytosis (formation of a layer of secretion on the surface of PECCs) was observed in our experiments at the initial stages of PECC loosening in *F. tataricum*. The absence of ECMSN on the surface of the soft callus cells is probably due to the fact that surface polymers have already been cut into fragments and partially metabolized. In addition, the absence of ECMSN can also be explained by the fact that the long cells of soft callus are incapable of active secretion anymore. Probably, the processes of disintegration of tissue structures play an important role in separation of individual embryogenic cells.

The processes of secretion of certain molecules capable of triggering embryoidogenesis are also of considerable interest. It has been proven by now that soluble secreted molecules, such as oligosaccharides AGPs, and lipochitooligosaccharides, are involved in the regulation of embryoidogenesis [14]. Probably, the metabolism of ECMSN components and median plate pectins in the nodular callus of *F. tataricum* are accompanied by the formation of a pool of regulatory molecules, which are able to reinitiate a new cycle of PECC formation by inducing the division of embryogenic cells. In addition, ECMSN molecules are able to fulfil the function of a "nurse" that takes care of the growth of new PECCs. The results of our earlier studies also provide evidence for an important role of glycosylated forms of proteins in the maintenance of the embryogenic state of cultivated cells [15]. It was found in several lines of callus cell cultures with different morphogenic potentials that calluses with a larger morphogenic potential synthesized a broader spectrum of glycoproteins.

Because the appearance and disappearance of ECMSN are associated with cyclic reinitiation of PECCs, identification of substances involved in the formation of this structure could provide a deeper insight into the processes of regulation of embryoidogenesis.

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