ГЕНЕТИЧЕСКИЕ ОСНОВЫ
ЭВОЛЮЦИИ ЭКОСИСТЕМ

ORGANIZATION OF THE ENDOPLASMIC RETICULUM IN CELLS OF EFFECTIVE AND INEFFECTIVE PEA NODULES (*PISUM SATIVUM* L.)

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❦ **Background.** The endoplasmic reticulum (ER) is the largest membrane-bound organelle, which plays an important role in the functioning of a plant cell and participates in its differentiation. **Materials and methods.** Using the methods of transmission electron microscopy, the morphological features and dynamics of structural changes in the ER in symbiotic nodules of pea (*Pisum sativum* L.) wild-type and mutants blocked at different stages of nodule development were studied. **Results.** The ER developed from a network of individual tubules in meristematic cells, to a developed network of cisterns around the nucleus and plasmalemma, and a network of rough and smooth tubules accompanying infection structures in colonized and infected cells and symbiosomes in infected cells. **Conclusions.** A correlation was found between the level of development of the ER network and the degree of bacteroid differentiation.

❦ **Keywords:** legume-rhizobial symbiosis; symbiotic nodule; ineffective mutants; cell organelles; *Pisum sativum* L.

ОРГАНИЗАЦИЯ ЭНДОПЛАЗМАТИЧЕСКОГО РЕТИКУЛУМА В КЛЕТКАХ ЭФФЕКТИВНЫХ И НЕЭФФЕКТИВНЫХ КЛУБЕНЬКОВ ГОРОХА (*PISUM SATIVUM* L.)

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❦ Эндоплазматический ретикулум (ЭПР) является самой большой, окруженной мембраной органеллой, которая выполняет важную роль в функционировании растительной клетки и участвует в ее дифференцировке. С помощью методов просвечивающей электронной микроскопии были исследованы морфологические особенности и динамика структурных изменений ЭПР в симбиотических клубеньках гороха (*Pisum sativum* L.) дикого типа и мутантов, блокированных на различных стадиях развития клубенька. ЭПР развивался от сети отдельных канальцев в меристематических клетках, к развитой сети цистерн вокруг ядра и плазмалеммы и сети гранулярных и гладких канальцев, сопровождающих инфекционные структуры в колонизированных и инфицированных клетках и симбиосомы в инфицированных клетках. Была выявлена корреляция между уровнем развития сети ЭПР и степенью дифференцировки бактероидов.

❦ **Ключевые слова:** бобово-ризобиальный симбиоз; симбиотический клубенек; неэффективные мутанты; клеточные органеллы; *Pisum sativum* L.

INTRODUCTION

All cells, including plant cells, contain basic cellular organelles, such as nuclei, plastids, mitochondria, the endoplasmic reticulum (ER), the Golgi, and other compartments [1, 2]. The ER is an interconnected network of cisternae and tubules located throughout the cytoplasm. This is the largest organelle surrounded by a membrane in eukaryotic cells. It plays an important

role in the synthesis, modification, and movement of both soluble and membrane proteins and also plays a crucial role in the biosynthesis and distribution of phospholipids and steroids and the detoxification of various toxins [3−5]. The different functions performed by the ER determine the diversity of its morphology in various types of cells. In developing plant cells, such as cells of the root cap and root hairs, the ER is mainly represented by cisternae, while ER tubules are most often found in mature plant cells. In addition, the ER undergoes transitions from cisternae to tubules and vice versa during cell development and also undergoes transitions in response to biotic and abiotic stresses, which gives it an extremely dynamic structure [5].

The ER takes part in the development of the symbiotic nodule of legume plants at both early and late stages [6, 7]. Thus, it has been shown that during the interaction of *Mesorhizobium loti* with the root hairs of *Lotus japonicus*, the formation of a rhizobial microcolony and the induction of an infection thread are associated with the condensed ER in which the tubules form a dense network [6]. The ER binds the tip of the infection thread to the nucleus in the root hair cell, co-participating with microtubules [8] in the formation of cytoplasmic bridges [6]. When the infection thread reaches the base of the root hair cell, the condensed ER changes its configuration to an open one represented by small cisternae [6]. For nodules of *Pisum sativum*, it has been shown that the ER network actively develops during the differentiation of infected cells; however, in the nitrogen fixation zone, the number of rough ER (RER) profiles decreases [7]. When studying the organization of the ER during the formation of ineffective nodules with mutant rhizobial strains, contradictory results were obtained. Thus, two strains of *Rhizobium meliloti*, namely, R21 *vio-r* and R21 *tum-r*, formed ineffective nodules on the roots of *Medicago sativa* in which the intensive development of the RER network was observed [9]. The mutants of *R. meliloti* in the *nifA*, *nifD*, *nifH*, *nifK*, and *fixA* genes also formed nodules in whose infected cells numerous RER profiles were present [10, 11]. In the nodules of the *R. meliloti* mutant in gene encoding succinate dehydrogenase, the expansion of the RER profiles occurred [12]. At the same time, strain 1019 of *R. leguminosarum* formed ineffective nodules on the pea variety Little Marvel in which the RER and polyribosome profiles were rare [13].

This work aims to study the formation of the ER during the pea symbiotic nodule development in effective and ineffective symbioses.

MATERIALS AND METHODS
Plant material and strain of bacteria
The pea (*P. sativum* L.) genotypes used in this study are shown in Table. For inoculation, a strain of *R. leguminosarum* bv. *viciae* 3841 was used [14].

Growing conditions and collection of material for analysis
The methods of sterilization and seed inoculation were described previously [23]. Plants were planted in plastic pots containing 200 g of vermiculite and 100 ml nitrogen-free nutrient solution [24]. Plants were grown in growth chambers (Sanyo Electric Co., Ltd., Moriguchi, Japan) under controlled conditions: day/night, 16/8 h; temperature, 21 °C; relative humidity, 75%; and photosynthetic photon flux density, ~280 μmol photons $\cdot$ m$^{-2} \cdot$ s^{-1}.

Material sample preparation
After collection, the nodules were transferred directly to a 2.5% aqueous solution of glutaraldehyde in 0.01 M phosphate buffer (pH 7.2). After fixing for 16 h at 4 °C, the nodules were dehydrated in a series of alcohols of increasing concentration (20 min each at 30%, 50%, 70%, 90%, and 100% ethanol at room temperature). They were then placed in a mixture of 100% ethanol and acetone in a ratio of 1:1 for 10 min, after which they were kept twice for 20 min in pure acetone. Then, the material was gradually infiltrated in three changes of a mixture of epoxy resin EMbed-812 (Honeywell Fluka, Thermo

Table

Wild-type and mutant lines of pea (Pisum sativum L.) used in the study

Genotype	Phenotype	Reference
SGE	Wild-type	[15, 16]
SGEFix⁻-1 (*sym40*)*	Hypertrophied infection droplets and infection threads, abnormal bacteroids	[15, 17, 18]
SGEFix⁻-2 (*sym33-3*)**	Abnormal infection thread growth inside nodule, no bacterial release***	[15, 17, 18]
SGEFix⁻-3 (*sym26*)	Early senescence	[19]
Sprint-2	Wild-type	[20]
Sprint-2Fix⁻ (*sym31*)	Undifferentiated bacteroids	[20]

Note. *The *Sym40* gene is orthologous to the *M. truncatula EFD* gene [21]. **The *Sym33* gene is orthologous to the *M. truncatula IPD3* gene [22]. ***The mutant line SGEFix⁻-2 (*sym33-3*) has leaky phenotype, and in some cells or some nodules, bacterial release occurs [17, 18].

Fisher Scientific, Longborough, UK) and acetone (in a ratio of 1:1, 2:1, and 3:1, respectively) for 1 h and then in a freshly prepared mixture of pure resin overnight at room temperature. The polymerization was carried out in an IN55 incubator (Memmert GmbH, Schwabach, Germany) at 60 °C for 48 h.

Transmission electron microscopy

For transmission electron microscopy, ultrathin sections (90−100 nm) cut on an ultramicrotome Leica EM UC7 (Leica Microsystems, Vienna, Austria) were collected on copper−palladium grids coated with 4% of pyroxylin and carbon. Sections were contrasted with a 2% aqueous solution of uranyl acetate for 1 h and lead citrate according to Reynolds for 1 min. Nodule tissues were examined and photographed using a JEM-1400 transmission electron microscope (JEOL Corporation, Tokyo, Japan) with an Olympus-SIS Veleta digital camera (Olympus Corporation, Tokyo, Japan) at an accelerating voltage of 80 kV.

RESULTS

The ER organization in wild-type pea symbiotic nodules

A similar organization of the ER was observed in nodules of the wild-type lines SGE and Sprint-2. Many free ribosomes and rare RER profiles were in the cytoplasm of meristematic cells (Fig. 1, a). In colonized meriste-
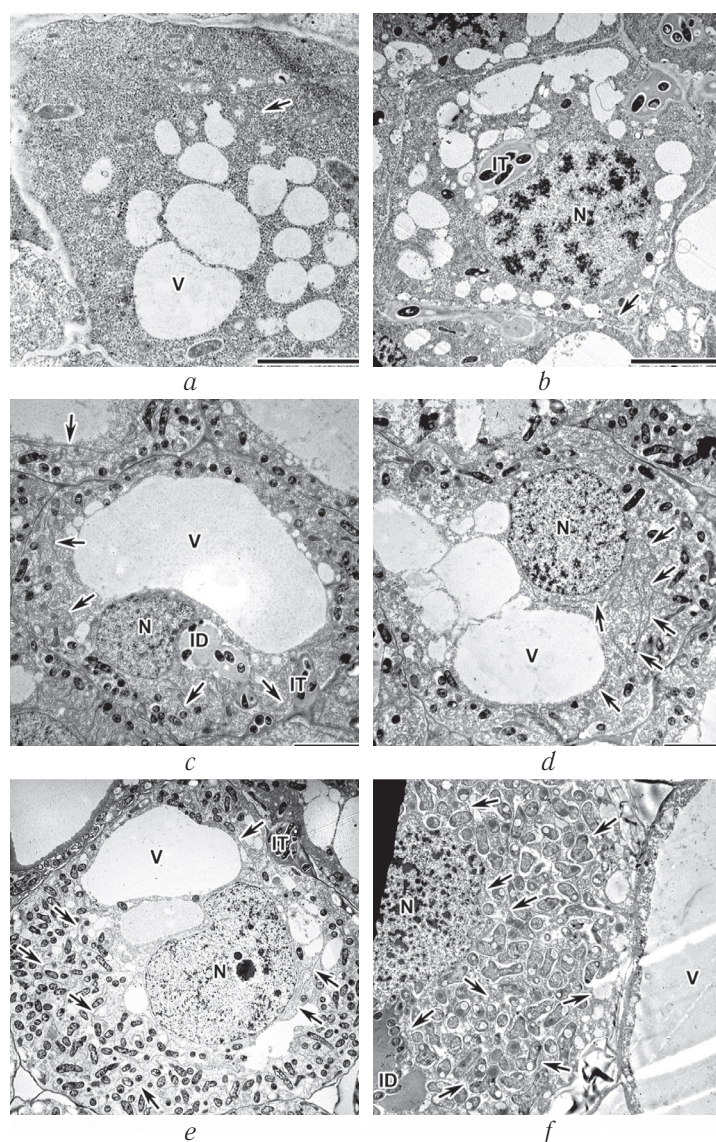

Fig. 1. The endoplasmic reticulum in the wild-type symbiotic nodules of *P. sativum*: *a* − cell of the meristem; *b* − cell of the meristem with infection threads; *c* − cell from the early infection zone; *d* − cell from the late infection zone; *e* − infected cell from the early nitrogen fixation zone; and *f* − mature infected cell from the nitrogen fixation zone. *a*, *b*, *e*, *f* − Sprint2; *c*, *d* − SGE. N − nucleus, V − vacuole, IT − infection thread, ID − infection droplet; arrows indicate profiles of the RER. Bar: 5 μm

matic cells with infection threads, the number of RER profiles was also infrequent (Fig. 1, *b*). In the infection zone, numerous free ribosomes were still present in young infected cells with rare juvenile bacteroids located on the cell periphery, with RER profiles becoming more extended (Fig. 1, *c*). The differentiation of infected cells in the infection zone was accompanied by an increase in the number of polyribosomes and the extension of RER profiles (Fig. 1, *d*). In the center of the cell around the nucleus, RER profiles were represented by long strands of tubules of the RER (Fig. 1, *d*). In mature infected cells in the infection zone, the RER was observed be-

tween numerous bacteroids and near the nucleus, where it formed parallel strands of tubules (Fig. 1, *e*). In infected cells in the nitrogen fixation zone, elements of the RER network were also present among numerous bacteroids (Fig. 1, *f*).

Parallel strands of RER tubules were located along the nucleus (Fig. 2, *a*) and on the cell periphery along the plasma membrane (Fig. 2, *b*). The ER was closely associated with infection structures. For example, RER tubules were in contact with the wall of the infection thread (Fig. 2, *c*), and smooth ER (SER) vesicles were located near the infection droplets and contained material
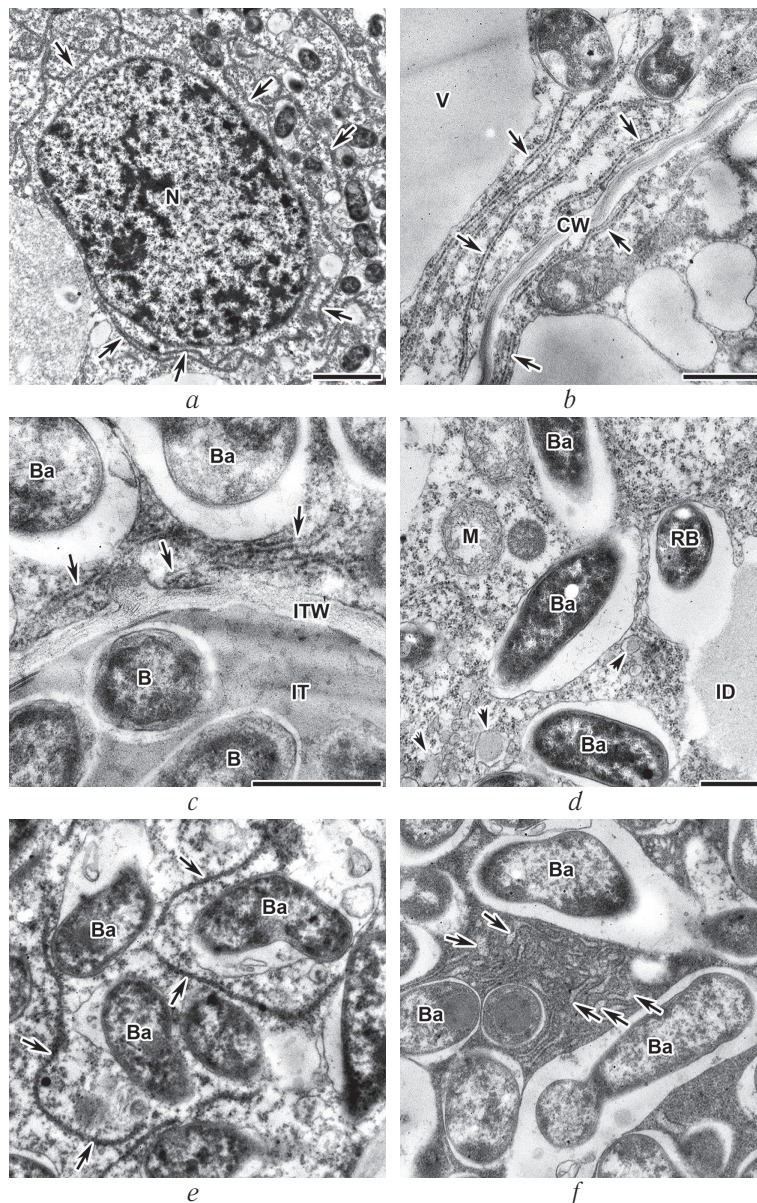

Fig. 2 . Distribution of the endoplasmic reticulum in infected cells of the wild-type nodules of *P. sativum*: *a* − around the nucleus; *b* − along the plasma membrane; *c* − along infection threads; *d* − SER network with the matrix material of infection droplets; *e* − around individual symbiosomes or groups of symbiosomes; and *f* − an extension of ER profiles in senescent cells. N − nucleus, M − mitochondrion, V − vacuole, CW − cell wall, IT − infection thread, ITW − infection thread wall, ID − infection droplet, B − bacterium, RB − releasing bacterium, Ba − bacteroid; arrows indicate RER profiles, arrowheads − SER profiles. Bar: *a* − 2 μm, b−f − 500 nm
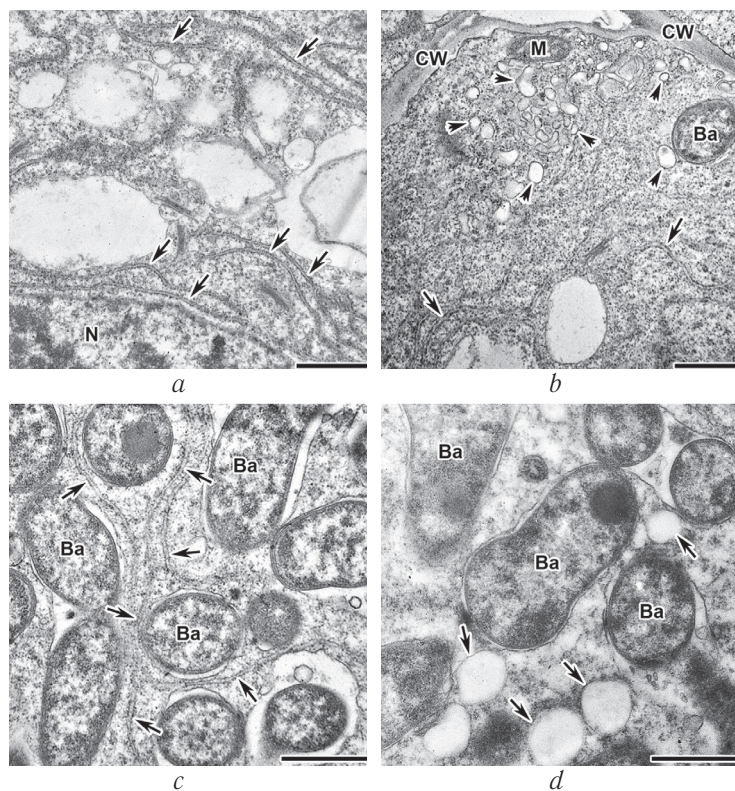
Fig. 3. The endoplasmic reticulum in the nodules of *P. sativum* mutant line SGEFix⁻-3 (*sym26*): *a* − parallel cisternae around the nucleus; *b* − SER network; *c* − around individual symbiosomes or groups of symbiosomes; and *d* − an extension of RER profiles and partial ribosome loss in some cells. N − nucleus, M − mitochondrion, CW − cell wall, Ba − bacteroid; arrows indicate RER profiles, arrowheads − SER profiles. Bar: 500 nm

with an electron density similar to the matrix of infection droplets (Fig. 2, *d*). Separate RER profiles were observed around individual symbiosomes or groups of symbiosomes, which formed a network of tubules (Fig. 2, *e*). During the senescence of the infected cell in wild-type nodules, a noticeable expansion of RER tubules was observed (Fig. 2, *f*).

The ER organization in symbiotic nodules of pea mutant lines

In the mutant line SGEFix⁻-3 (*sym26*), the character of the distribution of ER profiles mainly coincided with that in the symbiotic nodules of wild-type pea (Fig. 3). In infected cells in the late infection zone and the zone corresponding to the early nitrogen fixation zone in wild-type nodules, parallel extended RER tubules were located along the nucleus (Fig. 3, *a*), and the SER was well developed (Fig. 3, *b*). RER profiles were observed in close association with both individual symbiosomes and groups of symbiosomes (Fig. 3, *c*). Often, there were infected cells with signs of degradation of symbiotic structures and expanded RER cisternae that had lost ribosomes on membranes (Fig. 3, *d*).

In the mutant line SGEFix⁻-1 (*sym40*), in colonized cells containing hypertrophic infection droplets and in-

fection threads, several RER tubules were observed in a thin layer of cytoplasm (Fig. 4, *a*). In infected cells from the late infection zone and the zone corresponding to the nitrogen fixation zone in wild-type nodules, RER tubules accompanied symbiosomes containing several undifferentiated bacteroids (Fig. 4, *b*). There were infected cells in which RER cisternae were expanded and fragmented, and partial loss of ribosomes on their membranes was also observed (Fig. 4, *c*).

In the mutant line SGEFix⁻-2 (*sym33-3*), cells with and without bacterial release into the cytoplasm were observed. In infected cells from the zone corresponding to the nitrogen fixation zone in the wild-type pea nodules, RER tubules were located along the plasma membrane (Fig. 4, *d*) and surrounded by symbiosomes containing several undifferentiated bacteroids or a group of symbiosomes (Fig. 4, *e*). The SER was also developed in infected cells of the mutant line SGEFix⁻-2 (*sym33-3*) (Fig. 4, *f*). In nodules in which no bacterial release was detected, the cytoplasm was in a thin layer along the cell walls, nuclei, and infection threads, and a few RER profiles were observed in this layer (Fig. 5, *a*). Separate RER tubules accompanied infection threads (Fig. 5, *a*) and infection droplets that did not contain bacteria (Fig. 5, *b*).
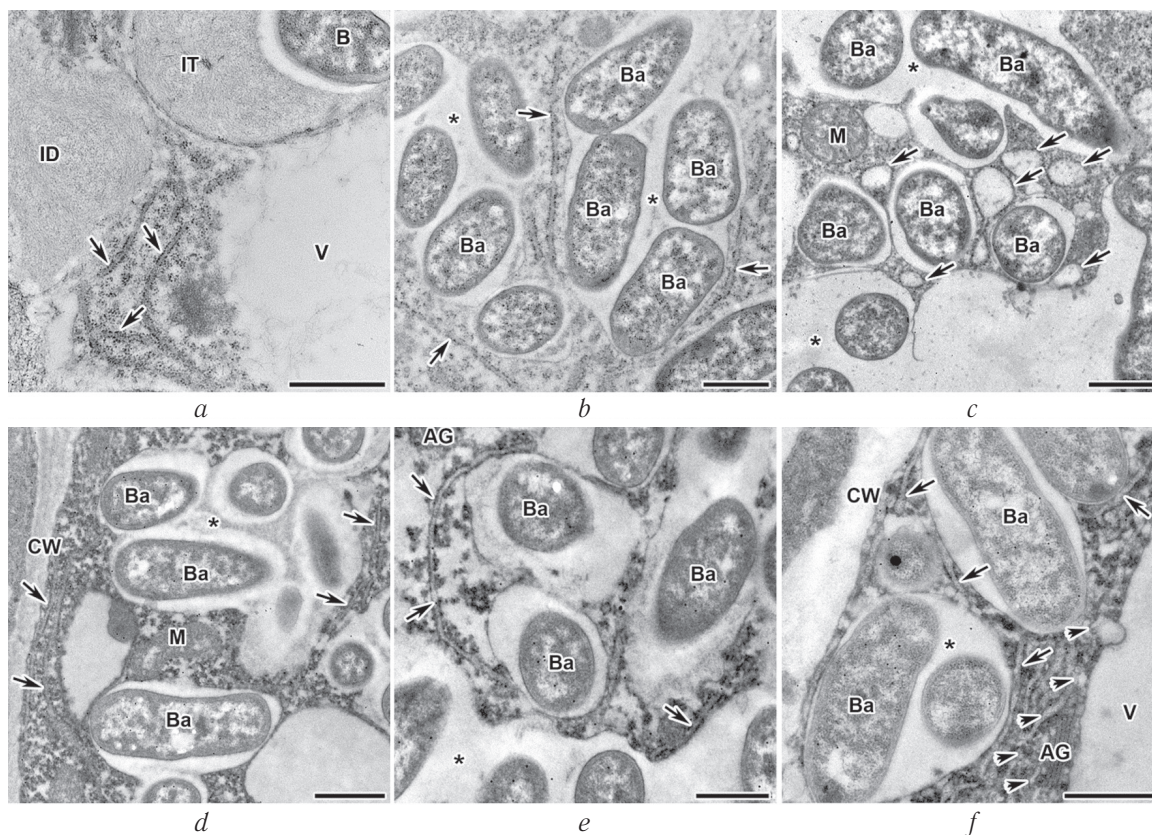
Fig. 4. The endoplasmic reticulum in the nodules of *P. sativum* mutant lines SGEFix⁻-1 (*sym40*) (*a−c*) and SGEFix⁻-2 (*sym33-3*) (*d−f*): *a* − around the vacuole and infection threads and droplets; *b* − around symbiosomes; *c* − extension and fragmentation of RER profiles with partial loss of ribosomes; *d* − along the plasma membrane; *e* − around symbiosomes; and *f* − SER network. M − mitochondrion, AG − Golgi apparatus, V − vacuole, CW − cell wall, IT − infection thread, ID − infection droplet, B − bacterium, Ba − bacteroid, * − symbiosome containing several bacteroids surrounded by a common symbiosome membrane; arrows indicate RER profiles, arrowheads − SER profiles. Bar: 500 nm

In the mutant line Sprint-2Fix⁻(*sym31*), which was characterized by undifferentiated bacteroids, there was a certain developmental gradient: from infected cells with symbiosomes with single bacteroid in the late infection zone to infected cells with symbiosomes containing several bacteroids in the zone corresponding to the nitrogen fixation zone in wild-type nodules (Fig. 5, *c, d*). In both types of cells, ER profiles were rare and represented by single tubules along the plasmalemma (Fig. 5, *c*) and among symbiosomes (Fig. 5, *d*).

DISCUSSION

We used transmission electron microscopy to study the morphological features and dynamics of structural changes of the ER in the pea symbiotic nodules of the wild-type and mutant lines blocked at different stages of the infection process.

The distribution of the RER in various cell types and different histological zones of the wild-type pea symbiotic nodule was previously described [7]. Thus, many free ribosomes and rare RER profiles were observed in meristematic cells. In newly infected cells that contained a

small number of bacteroids, the number of RER profiles began to increase, with numerous polyribosomes being present on them. The differentiation of the infected cells and their filling with bacteroids in the infection zone was accompanied by a considerable increase in RER profiles located between the bacteroids. In infected cells in the nitrogen fixation zone, the number of RER profiles decreased again. In uninfected cells, the small number of RER profiles was also detected [7]. In this study, we observed a similar dynamic in the development of the ER, from the network of individual tubules in meristematic cells, actively dividing cells to the developed network of cisternae around the nucleus and plasmalemma and the network of rough and smooth tubules accompanying developing and mature symbiosomes and infection structures (see Fig. 1). However, in this study, we did not observe a considerable decline in the intensity of RER network in the nitrogen fixation zone, as described earlier, which may be due to differences in the pea genotypes and rhizobial strains used and the difficulty in identifying ER profiles in infected cells filled with numerous bacteroids (see Fig. 1, *f*).
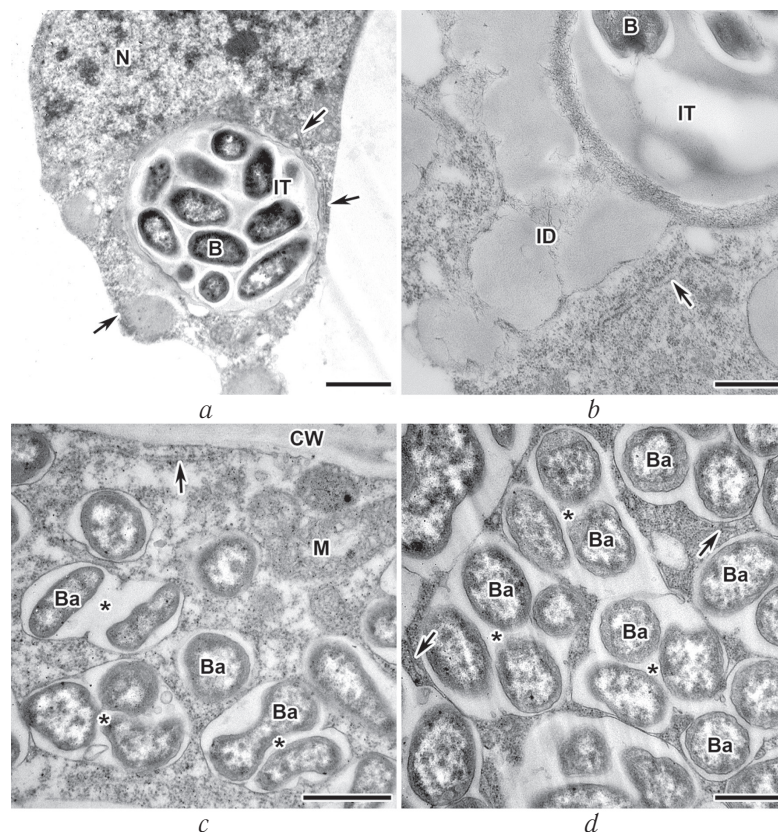
Fig. 5. The endoplasmic reticulum in the nodules of *P. sativum* mutant lines SGEFix⁻-2 (*sym33-3*) (*a*, *b*) and Sprint-2Fix⁻ (*sym31*) (*c*, *d*): *a* − in a narrow layer of the cytoplasm around the nucleus and infection threads; *b* − along bacterial free infection droplets; and *c* − along the plasma membrane; *d* − around symbiosomes. N − nucleus, M − mitochondrion, CW − cell wall, IT − infection thread, ID − infection droplet, B − bacterium, Ba − bacteroid, * − symbiosome containing several bacteroids surrounded by a common symbiosome membrane; arrows indicate RER profiles. Bar: *a*, *c* − 1 μm, *b*, *d* − 500 nm

It was previously explained that the enhanced development of the ER observed after bacterial release into the plant cell cytoplasm in pea and soybean nodules is accompanied by the formation of ER vesicles that merge with symbiosome membranes; it was subsequently suggested that this process could promote bacteroid differentiation [25]. It was later shown that the ER plays an important role in the synthesis of NCR peptides that provide terminated differentiation of bacteroids [26].

It should be noted that in the root hairs, the pattern of the ER network in infected cells repeats the microtubular cytoskeleton pattern [6]. A similar correlation is probably observed in infected cells in the nodule. It is known that symbiosomes in mature infected cells of pea symbiotic nodules are arranged in a disordered manner, and in these cells, bundles of microtubules do not support the organization of individual symbiosomes but rather groups of symbiosomes [27]. This study revealed the profiles of the RER between groups of symbiosomes (see Fig. 1, *e*).

The analysis of pea mutants forming inefficient nodules revealed the differences in the degree of ER development. For the mutant line SGEFix⁻-3 (*sym26*), characterized by the formation of morphologically differentiated bacteroids that undergo premature degradation [19], the degree of ER development in nodule infected cells was similar to that of the wild-type (see Fig. 3). In the nodules of the mutant line SGEFix⁻-1 (*sym40*), which forms hypertrophic infection droplets and abnormal bacteroids, and in the nodules of the mutant line SGEFix⁻-2 (*sym33−3*) in which bacterial release occurs, the ER was represented by extended tubules (see Fig. 4). In general, it was less developed, and the degree of its development corresponded to the degree of ER development in infected cells of wild-type nodules from the infection zone (see Fig. 1, *d*).

The nodules of the mutant line SGEFix⁻-2 (*sym33-3*), characterized by "locked" infection threads and lack of bacterial release, and the mutant line Sprint-2Fix⁻ (*sym31*), characterized by undifferentiated bacteroids, showed the least degree of ER development (see Fig. 5). It was represented by separate segmented tubules, which are observed in newly infected cells of wild-type nodules prior to their differentiation (see Fig. 1, *b, c*).

Based on the analysis of mutant genotypes, a correlation between the degree of differentiation of symbiosomes and the level of ER development was observed. Previously, in ineffective pea nodules formed by *R. leguminosarum* strain 1019, a sharp decrease in the level of the ER was detected, while bacteroids were characterized by a reduced degree of differentiation compared with nodules formed by an effective strain [13]. At the same time, for several strains of *R. meliloti* that form ineffective nodules on *M. sativa*, an increase in ER development was shown in comparison with effective strains [9–12]. For example, nodules formed by *R. meliloti* strains R21 *vio-r* and R21 *tum-r* were characterized by the intensive development of RER network, including stacks of parallel-oriented tubules associated with degrading bacteroids, among others [9]. It was previously suggested that in ineffective nodules of *M. sativa*, the ER is actively developed in response to nitrogen starvation, leading to an increased synthesis of lytic enzymes that destroy rhizobial cells for the usage of released nitrogen by plants [9]. Another explanation for the active development of the ER suggests that in ineffective nodules, the repression of the synthesis of nodulins, proteins specifically synthesized in nodules, is absent [10]. At the same time, the pea did not show an enhanced development of the ER during the formation of ineffective nodules by a mutant strain of rhizobia [13] and plant mutants. Moreover, in the nodules of the mutant SGEFix⁻-1 (*sym40*), the expansion of RER profiles was observed (see Fig. 4, *c*), and in the mutant SGEFix⁻-3 (*sym26*), characterized by early senescence of nodules [19], the expansion of the RER with loss ribosomes occurred in cells with signs of degradation of symbiotic structures (see Fig. 3, *d*). A similar expansion was observed in the *R. meliloti* mutant in the gene encoding succinate dehydrogenase [12].

The revealed differences in the organization of the ER in nodules of *P. sativum* and *M. sativa* may indicate the crucial importance of the plant species in the development of the ER. Indeed, a well-developed ER network has been described for various species of *Astragalus* and *Oxytropis* as an adaptation to growth under arctic conditions [28]. Recently, the intensive development of the ER was described for nodule cells of the relict legume *O. popoviana*, formed by the *M. japonicum* strain Opo-235 [29].

Thus, in the nodules of *P. sativum* during the differentiation of infected cells, a gradual formation of ER fine structure was observed, with the intensity of its development correlating with the degree of bacteroid differentiation.

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