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INFLUENCE OF MUTATION IN PEA (*PISUM SATIVUM* L.) *cdt* (*cadmium tolerance*) GENE ON HISTOLOGICAL AND ULTRASTRUCTURAL NODULE ORGANIZATION

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❀ **Background.** A comparative analysis of the structural organization of the symbiotic nodules of the pea initial line SGE and the mutant line SGECd^t, characterized by increased tolerance to cadmium and increased its accumulation, was carried out. **Materials and methods.** Nodules of initial line SGE and mutant SGECd^t were analyzed using light and transmission electron microscopy. **Results.** The non-treated nodules of SGE and SGECd^t were characterized by a similar histological and ultrastructural organization. In the nodules of SGE exposed to 100 μM $CdCl_2$ in infected cells, the following abnormalities were observed: expansion of the peribacteroid space, destruction of the symbiosome membrane, fusion of symbiosomes and, as a result, the formation of symbiosomes containing several bacteroids. In the nodules of SGECd^t, infected cells did not undergo pronounced changes. In the nodules of SGE exposed to 1 mM $CdCl_2$, at the base of the nodule, senescent infected cells with completely destroyed cytoplasm and degrading bacteroids appeared. Also there were present cells in which the contents of symbiosomes were lysing, and only the "ghosts" of the bacteroids remained in them. In SGECd^t, in some infected cells, abnormalities were manifested in an increase in the peribacteroid space, partial destruction of symbiosome membranes, fusion of symbiosomes, and release of bacteroids into the vacuole. **Conclusions.** The tolerance of pea nodules to cadmium can be significantly increased due to a single recessive *cdt* mutation.

❀ **Keywords:** plant-microbe interactions; symbiotic nodule; tolerance to cadmium; symbiosome; bacteroid; infection thread.

ВЛИЯНИЕ МУТАЦИИ В ГЕНЕ ГОРОХА (*PISUM SATIVUM* L.) *cdt* (*cadmium tolerance*) НА ГИСТОЛОГИЧЕСКУЮ И УЛЬТРАСТРУКТУРНУЮ ОРГАНИЗАЦИЮ КЛУБЕНЬКОВ

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❀ Был проведен сравнительный анализ структурной организации симбиотических клубеньков исходной линии гороха SGE и мутантной линии SGECd^t, характеризующейся повышенной устойчивостью к кадмию и увеличенной его аккумуляцией. Было показано, что не обработанные $CdCl_2$ клубеньки SGE и SGECd^t имели сходную гистологическую и ультраструктурную организацию. При действии 100 мкМ $CdCl_2$ в клубеньках SGE в инфицированных клетках наблюдались следующие аномалии: расширение перибактероидного пространства, разрушение симбиосомной мембраны, слияние симбиосом и, как следствие, образование симбиосом, содержащих несколько бактероидов. В клубеньках SGECd^t инфицированные клетки в зонах инфекции и азотфиксации не были подвержены сильным изменениям. При действии 1 мМ $CdCl_2$ в основании клубеньков SGE появлялись стареющие инфицированные клетки с полностью разрушенной цитоплазмой и деградирующими бактероидами. Присутствовали также клетки, в которых содержимое симбиосом лизировалось, и в них оставались лишь «тени» бактероидов. У SGECd^t в некоторых инфицированных клетках аномалии проявлялись в увеличении перибактероидного пространства, частичном разрушении симбиосомных мембран, слиянии симбиосом и высвобождении бактероидов в вакуоль. Таким образом, устойчивость клубеньков гороха к кадмию может быть повышена благодаря единичной рецессивной мутации *cdt*.

❀ **Ключевые слова:** растительно-микробные взаимодействия; симбиотический клубенек; устойчивость к кадмию; симбиосома; бактероид; инфекционная нить.

INTRODUCTION

The release of cadmium (Cd) into the environment is conditioned by natural processes as well as by constantly increasing human activities [1]. Cd is a toxic element that causes various abnormalities in plant development [2, 3] and it is absorbed from the

soil by the roots of plants. Plants exhibit different levels of tolerance to Cd; most of them demonstrate common tolerance, while others show hypertolerance that can be accompanied by hyperaccumulation of Cd [4, 5]. Both types of tolerance have different underlying molecular, genetic, biochemical, and cellular mechanisms [6].

Cd affects not only the development and the functioning of plants but also the plant interaction with symbiotic microorganisms [7–12]. It was showed that the development of pea plants and especially rhizobial growth are inhibited at higher concentrations of Cd in comparison with the growth of nodules [13]. Therefore, in order to develop plant-microbe systems tolerant to Cd, the tolerance of nodule formation to Cd needs to be increased. Since rhizobia show high levels of Cd tolerance [13], the best way to increase the level of tolerance of the symbiotic system is modification in plant genome.

Pea mutant line SGECd^t, characterized by increased tolerance to Cd and elevated Cd accumulation in the plant tissues, was obtained after ethyl methanesulfonate mutagenesis. The mutant line SGECd^t carries a recessive *cdt* mutation [14] localized in pea linkage group VI [15, 16]. Grafting experiments showed that this root genotype causes increased tolerance to Cd and increased Cd accumulation in the tissues of the mutant line [17].

The mutant line SGECd^t is highly tolerant to the effect of Cd in the process of nodule formation compared with the initial line SGE [13, 18]. However, the effect of Cd on the structural organization of mature nodules in the mutant line has not yet been studied.

The *aim of this study* was to analyze the effect of Cd on the structural organization of the symbiotic nodules in the initial line SGE and in the mutant line SGECd^t.

MATERIALS AND RESEARCH METHODS

Plant material

The pea (*Pisum sativum* L.) mutant line SGECd^t (*cdt*), which is characterized by increased tolerance to Cd and elevated Cd accumulation [14], and the initial line SGE [19] from the collection of the All-Russia Research Institute for Agricultural Microbiology were used.

Bacterial strain

The plants were inoculated with commercial strain of *Rhizobium leguminosarum* bv. *viciae* RCAM 1026 (=CIAM 1026) [20] from the collection of the All-Russia Research Institute for Agricultural Microbiology.

Growth conditions and collection of material for analysis

The seeds were sterilized in concentrated sulfuric acid for 30 min and were subsequently washed in sterile water 10 times. The seeds were placed on rafts floating in a hydroponic solution with the root system immersed in the solution and the cotyledons and shoots on the surface of the raft. The hydroponic solution had the following composition: KH_2PO_4, 110 mM; $Ca(NO_3)_2$, 50 mM; $MgSO_4$, 400 mM; KCl, 300 mM; $CaCl_2$, 70 mM; H_3BO_3, 1 mM; $MnSO_4$, 1 mM; $ZnSO_4$, 1 mM; Na_2MoO_4, 0.03 mM; and $FeC_4H_4O_6$, 2.5 mM. The hydroponic solution was exposed to permanent bubbling and it was changed every 3 days. The plants were grown in growth chamber MLR-352H (Sanyo Electric Co., Ltd., Japan) in the day/night 16/8 h mode at 21 °C, relative humidity of 75%, and illumination of 280 μmol m^{-2} s^{-1}. In 23 days after inoculation, $CdCl_2$ was added to the hydroponic solution up to a concentration of 100 μM and 1 mM. In 24 h after adding $CdCl_2$, the nodules from five plants were harvested for fixation.

Microscopic analysis

For electron microscopy analysis, the nodules were exposed to slight vacuuming with fixation in 2.5% solution of glutaraldehyde in 0.3 M phosphate buffer (pH 7.2) at 4 °C overnight. After fixation, the samples were washed four times in 0.3 M phosphate buffer with additional fixation in 1 % solution of osmium tetroxide in 0.3 M phosphate buffer for 2 h. After washing thrice for 15 min in distilled water, the samples were dehydrated in ethanol of increasing concentrations: 50%, 70% (leaving overnight at 4 °C), and 96% for 15 min in each solution. Then, the samples were dehydrated twice in absolute ethanol for 10 min, in a mixture of absolute ethanol and acetone in the ratio 1 : 1 for 10 min, and twice in acetone for 10 min.

Epon 812 resin with DMP-30 catalyst was used as the embedding mixture (Honeywell Fluka™, Fisher Scientific, Loughborough, UK). The tissues were infiltrated with increasing concentrations of embedding medium in the ratio 1 : 1 and 1 : 3 mixed with absolute acetone for 1 h and then in the pure resin overnight at room temperature. The nodules were placed in previously dried polyethylene capsules filled with fresh embedding mixture. Polymerization was carried out at 60 °C for 2 days in the incubator IN55 (Memmert GmbH, Germany).

Semithin sections (0.5–1 μm) were stained with toluidine blue and studied under the microscope Leica DM LB2 (Leica Microsystems, Austria) using 5×, 10× and 20× lenses. Ultrathin sections (90–100 nm)

were cut on ultratome Leica Ultracut UCT (Leica Microsystems, Austria) and collected on a copper grids covered with formvar. The ultrathin sections were contrasted with 1% uranyl acetate solution for 20 min and Reynold's lead citrate for 1 min. The ultrathin sections were observed and photographed using the transmission electron microscope LEO 910 (LEO Electron Microscopy Group, Germany) with accelerating voltage of 60 kV.

RESULTS

Histological and ultrastructural organization of the pea symbiotic nodules in the initial line SGE and mutant line SGECd^t

The nodules in the SGE and SGECd^t lines grown in hydroponic cultures without $CdCl_2$ had similar histological organization, which was typical for nodules of the indeterminate type, in which the meristem, infection, and nitrogen fixation zones were distinguished (Fig. 1*a*, *b*). Nevertheless, separate cells were underwent to degradation, which was caused probably by the growth conditions in hydroponic cultures (Fig. 1*a, b*). The nodules of SGE and SGECd^t lines had similar ultrastructural organization, which was not different from that earlier described for pea nodules. We observed infection threads filled with bacteria (Fig. 1 *g*) and the symbiosomes containing individual pleomorphic bacteroids (Fig. 1*h*).

Histological and ultrastructural organization of pea symbiotic nodules of the initial line SGE and mutant line SGECd^t treated with 100 μM $CdCl_2$

Treatment of the root systems with different $CdCl_2$ concentrations affected the nodules of various ages. The mature nodules were characterized by bigger size com-

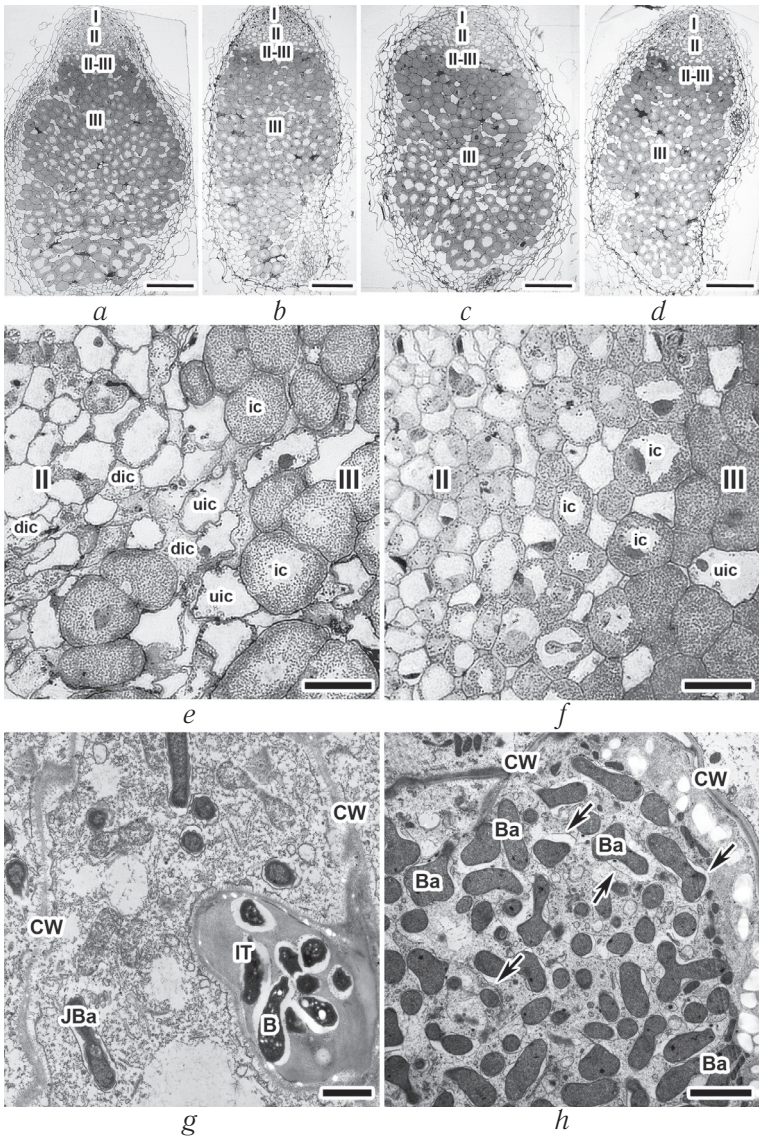

Fig. 1. Histological organization of pea nodules of the initial line SGE and mutant line SGECd^t, untreated and treated with 100 μM $CdCl_2$, and ultrastructural organization of untreated SGECd^t nodules: *a* – histological organization of untreated SGE nodules; *b* – histological organization of untreated SGECd^t nodules; *c*, *e* – histological organization of SGE nodules treated with 100 μM $CdCl_2$; *d*, *f* – histological organization of SGECd^t nodules treated with 100 μM $CdCl_2$; *g* – infected cell of SGECd^t from the infection zone with infection thread and juvenile bacteroids; *h* – infected cell of SGECd^t from the nitrogen fixation zone with pleomorphic bacteroids. I – meristem, II – infection zone, II-III – interzone between infection and nitrogen fixation zones, III – nitrogen fixation zone, ic – infected cell, uic – uninfected cell, dic – degrading infected cell, IT – infection thread, CW – cell wall, B – bacterium, Ba – bacteroid, JBa – juvenile bacteroid; arrows indicate symbiosome membrane. Scale bar: *a-d* – 100 μm, *e*, *f* – 20 μm, *g*, *h* – 1 μm

pared to the young ones. Moreover, the mature nodules were more tolerant to the toxic effect of Cd.

In mature SGE and SGECd^t nodules, all the histological zones were observed (Fig. 1 *c*, *d*), but in the nodules of the initial line some infected cells with signs of degradation were observed (Fig. 1 *e*). However, these abnormalities were not observed in the nodules of the mutant line (Fig. 1 *f*).

In the mature SGE nodules, the infected cells in the nitrogen fixation zone were filled with numerous bacteroids (Fig. 2 *a*). The membranes of the bacteroids were characterized by increased rugosity (Fig. 2 *b*). The peribacteroid space in the symbiosomes was also increased. Frequent destruction of symbiosome membranes and symbiosome fusion that led to the appearance of symbiosomes containing several bacteroids

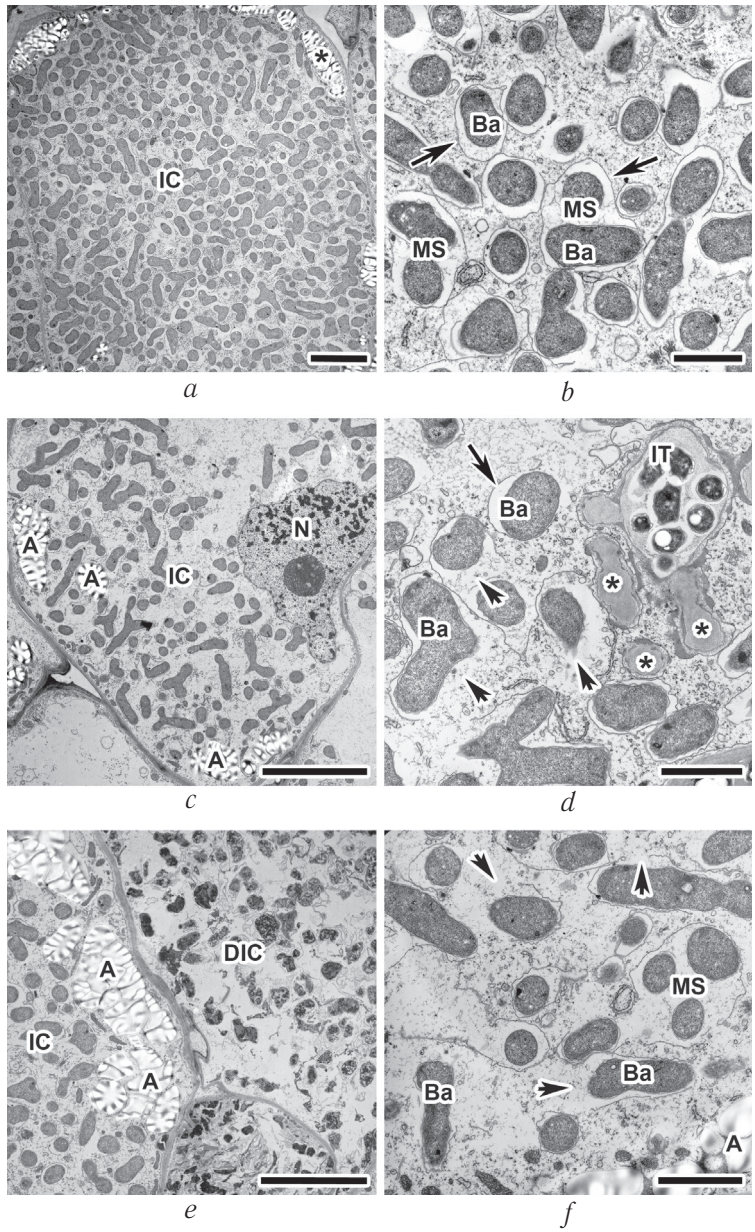

Fig. 2. Ultrastructural organization of pea nodules of the initial line SGE, treated with 100 μM $CdCl_2$: *a*, *b* – infected cells from the nitrogen fixation zone with pleiomorphic bacteroids; *c*, *d* – infected cells from the nitrogen fixation zone with the primary signs of senescence (degradation of the cytoplasm and developmental abnormalities of the infection thread); *e*, *f* – degrading infected cells from the senescence zone. IC – infected cell, DIC – degrading infected cell, N – nucleus, A – amyloplast, IT – infection thread, Ba – bacteroid, MS – "multiple" symbiosome, formed as a result of symbiosome fusion and containing several bacteroids; arrows indicate a symbiosome membrane, arrowheads indicate destruction of symbiosome membranes, asterisks indicate outgrowths of infection thread containing a matrix without bacteria. Scale bar: *a*, *c*, *e* – 5 μm; *b*, *d*, *f* – 1 μm

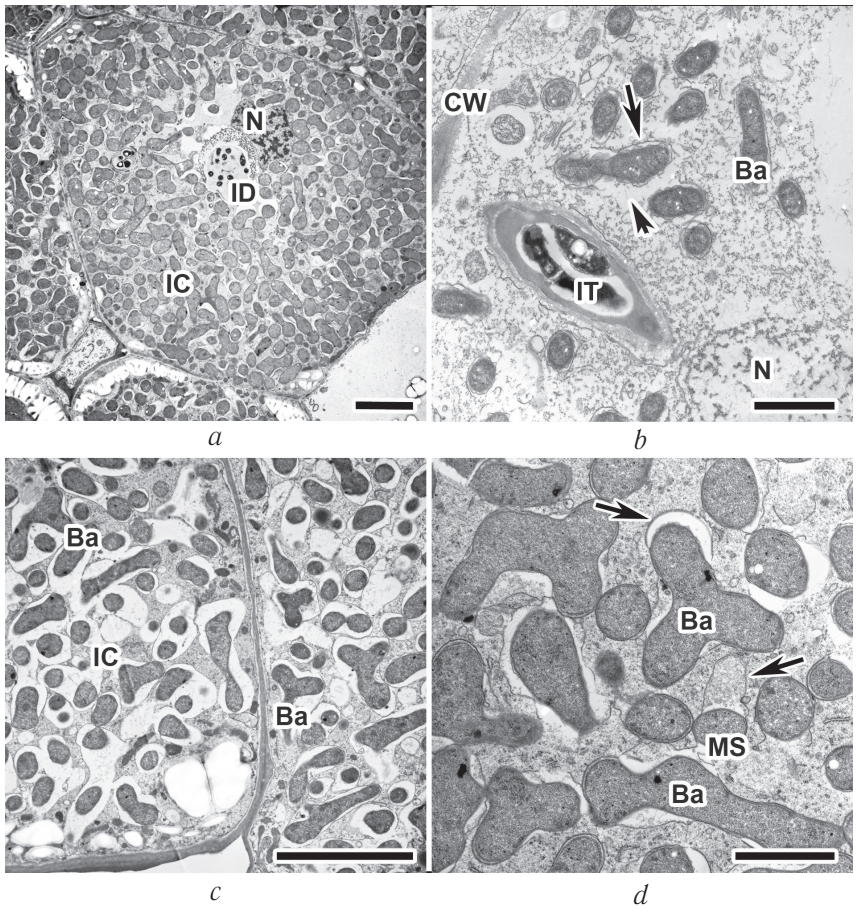

Fig. 3. Ultrastructural organization of pea nodules of the mutant line SGECd^t, treated with 100 μM $CdCl_2$: *a* – infected cell from the nitrogen fixation zone with infection thread and infection droplet; *b* – infected cell from the infection zone with infection thread and juvenile bacteroids; *c* – infected cells from the nitrogen fixation zone, filled with symbiosomes with expanded peribacteroid spaces; *d* – pleomorphic bacteroids from the nitrogen fixation zone. IC – infected cell, N – the nucleus, CW – cell wall, IT – infection thread, ID – infection droplet, Ba – bacteroid, MS – "multiple" symbiosome, formed as a result of symbiosome fusion and containing several bacteroids; arrows indicate symbiosome membrane, arrowheads indicate the destruction of symbiosome membranes. Scale bar: *a* – 5 μm; *b*, *d* – 1 μm; *c* – 2 μm

were observed (Fig. 2 *b*). The ultrastructural organization of the infection threads was similar to the normal; however, some of them had numerous outgrowths surrounded by a cell wall and filled with matrix without bacteria (Fig. 2 *d*). In the infected cells with signs of degradation, symbiosome membranes were destroyed (Fig. 2 *d*), which resulted in the release of bacteroids into the clear cytoplasm (Fig. 2 *c*, *f*) followed by their complete degradation (Fig. 2 *e*).

In the mature SGECd^t nodules, the infected cells in the infection and nitrogen fixation zones did not undergo major changes (Fig. 3 *a*). The structure of the infection threads and droplets did not differ from those of untreated plants (Fig. 3 *a*, *b*). However, in some infected cells from the infection and nitrogen fixation zones, we found symbiosomes with enlarged peribacteroid space (Fig. 3 *b*, *c*). Besides, in the infected cells from the nitrogen fixation zone, we observed signs of symbiosome membrane destruction and symbiosome fusion (Fig. 3 *d*). In the base of nodule in some cells, we detected noticeable signs of degradation such as destruction of the cytoplasm and organelles of the plant cells and release of bacteroids from symbiosomes (data not shown).

Histological and ultrastructural organization of pea symbiotic nodules in the initial line SGE and mutant line SGECd^t treated with 1 mM $CdCl_2$

In mature SGE nodules, the infected cells in the nitrogen fixation zone showed a rough and folded surface (data not shown). The structure of the mature SGECd^t nodules was similar to that of the nodules treated with 100 μM $CdCl_2$ (data not shown).

The ultrastructural organization of the mature SGE nodules showed signs of premature degradation of the

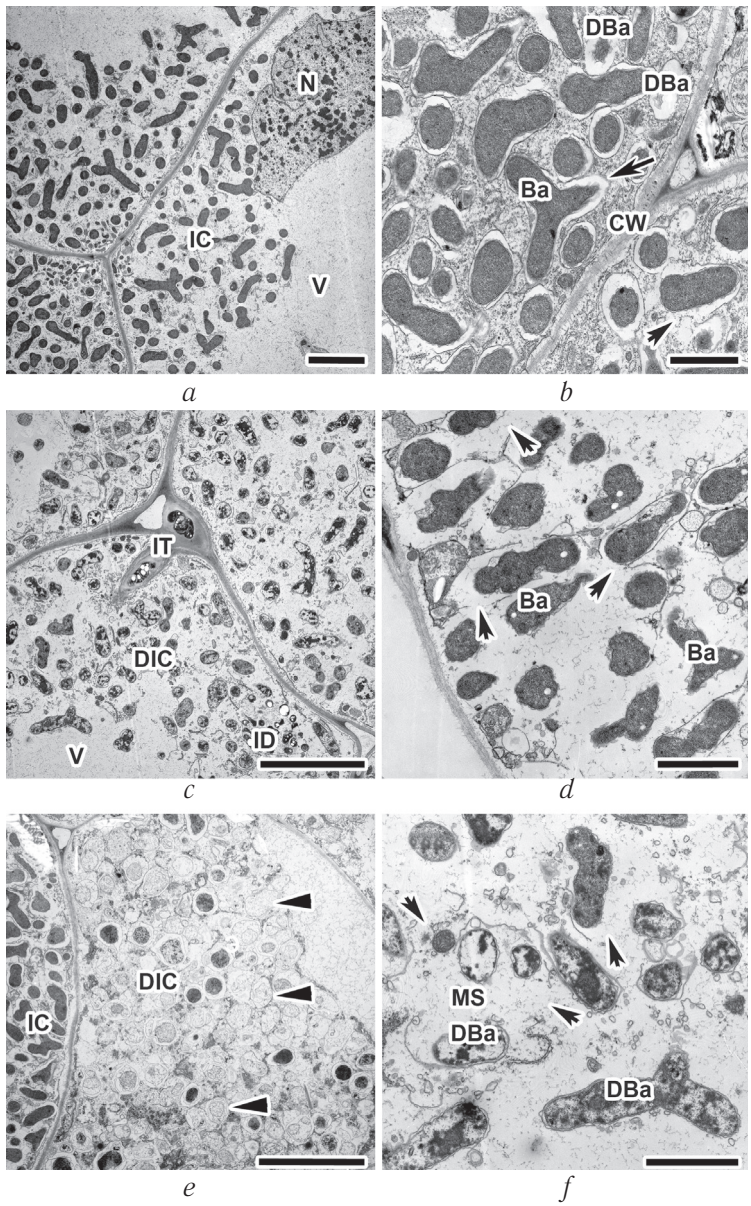

Fig. 4. Ultrastructural organization of pea nodules of the initial line SGE, treated with 1 mM $CdCl_2$: *a* – infected cells from the nitrogen fixation zone with premature signs of degradation; *b* – pleomorphic bacteroids from the nitrogen fixation zone; *c* – degrading infected cell from the senescence zone with infection thread and infection droplet; *d* – bacteroids of irregular shape and with rugose surface in degrading infected cell from the senescence zone; *e* – degrading infected cell from the senescence zone, filled with the "ghosts" of bacteroids; *f* – degrading bacteroids with a partly cleared matrix and destroyed symbiosome membrane. IC – infected cell, DIC – degrading infected cell, N – nucleus, V – vacuole, CW – cell wall, IT – infection thread, ID – infection droplet, Ba – bacteroid, DBa – degrading bacteroid, MS – "multiple" symbiosome, formed as a result of symbiosome fusion and containing several bacteroids; arrows indicate symbiosome membrane, arrowheads indicate the destruction of symbiosome membranes, and triangles indicate the "ghosts" of bacteroids. Scale bar: *a*, *c*, *e* – 5 μm; *b*, *d*, *f* – 1 μm

symbiotic compartments (Fig. 4 *a, c, e)*. In the nitrogen fixation zone in the most cells, symbiosome membranes were partially or completely destroyed and degrading bacteroids were observed (Fig. 4 *b*). The senescence zone contained cells with completely destroyed cytoplasm and degrading bacteroids (Fig. 4 *c*, *d*). The bacteroids acquired irregular shapes and folded surfaces (Fig. 4 *d*), and their matrix was partially or completely cleared (Fig. 4 *c*, *f*). We identified the cells wherein the content of the symbiosomes was lysed. They contained membranes of the bacteroids only (Fig. 4 *e*).

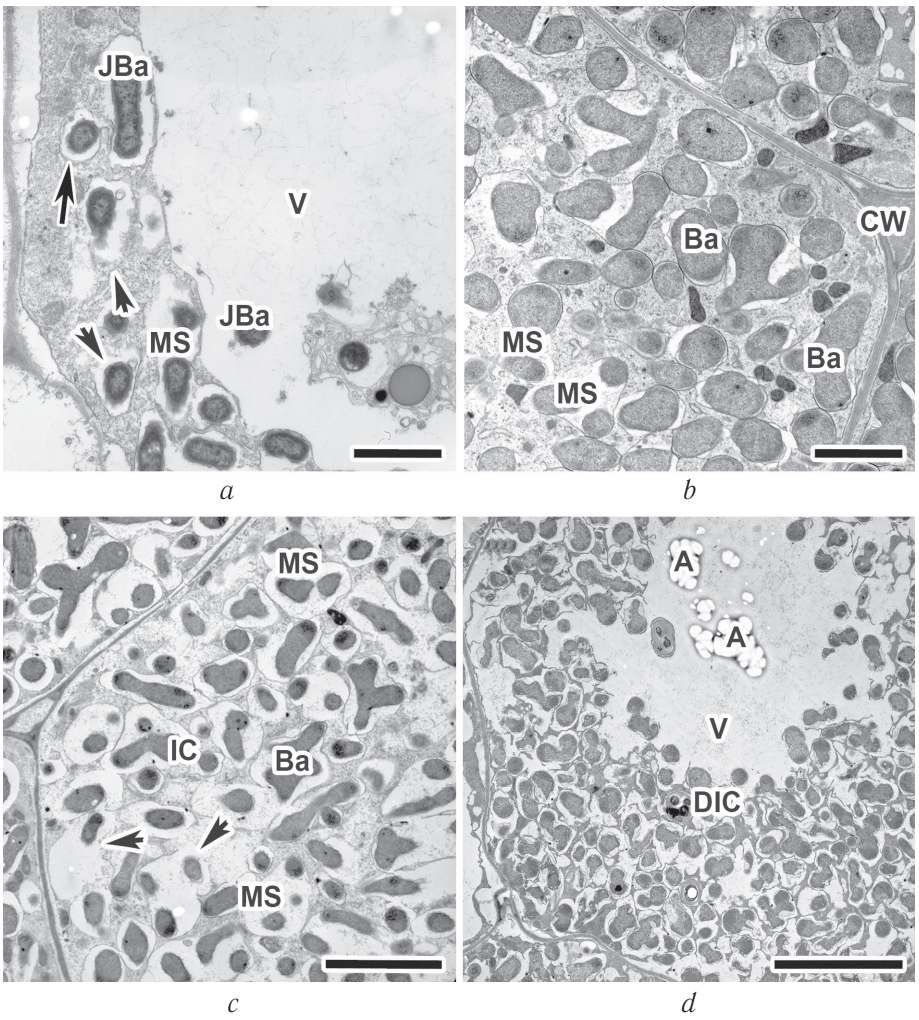

Fig. 5. Ultrastructural organization of pea nodules of the mutant line SGECd^t, treated with 1 mM $CdCl_2$: *a* – infected cell from the infection zone with juvenile bacteroids; *b* – pleomorphic bacteroids in the infected cell from the nitrogen fixation zone; *c* – infected cell from the nitrogen fixation zone with primary signs of symbiosome degradation: expansion of the peribacteroid space, destruction of the symbiosome membrane and fusion of symbiosomes; *d* – degrading infected cell from the senescence zone. IC – infected cell, V – vacuole, CW – cell wall, A – amyloplast, Ba – bacteroid, JBa – juvenile bacteroid, MS – "multiple" symbiosome, formed as a result of symbiosome fusion and containing several bacteroids, arrows indicate symbiosome membranes, arrowheads indicate the destruction of symbiosome membranes. Scale bar: *a*, *b* – 1 μm; *c* – 2 μm; *d* – 5 μm

The ultrastructural organization of the mature SGECd^t nodules showed that in the infected cells in the infection zone, the bacteroid membranes were characterized by elevated rugosity (Fig. 5 *a*). The peribacteroid space in the symbiosomes was increased; the symbiosome membranes in some cells of the infection zone were partially destroyed that resulted in the release of juvenile bacteroids into the vacuole (Fig. 5 *a*). In the nitrogen fixation zone, we observed symbiosome fusion in the infected cells (Fig. 5 *b*), wherein peribacteroid spaces were increased with symbiosome membrane destruction (Fig. 5 *c*). In some senescent cells, we observed visible destruction of symbiosomes and tonoplast of infected cells (Fig. 5 *d*).

DISCUSSION

The effect of Cd on the development and functioning of symbiotic nodules at the structural level has not been studied in detail. A study showed that treatment with 18 μM Cd^{2+} during 49 days entailed changes in the histological and ultrastructural organization of white lupin (*Lupinus albus* L.) nodules [8]. The intercellular spaces in the nodule cortex were filled with glycoproteins and some infected cells contained degraded bacteroids [8]. Soybean (*Glycine max* (L.) Merr.) nodules exposed to increasing Cd concentrations showed a decrease in the number of infected cells in the nitrogen fixation zone and in the number of bacteroids in the symbiosomes [7]. Another study elucidated the effect of Cd ex-

posure on the structure of nodules in alfalfa (*Medicago sativa* L.) [9]. Cd treatment in that study was shown to affect the shape of the infected and uninfected cells and cause disappearance of the organelles; however, the infection threads were preserved in the infected cells. Several small vacuoles instead of the central one were formed in the infected cells of nitrogen fixation zone. In the infection zone, the presence of cells with normal infection threads and bacteroids as well as severely damaged ones with very dark cytoplasm and destroyed bacteroids was shown by the ultrastructural analysis of the nodules. The nitrogen fixation zone contained cells with numerous vacuoles and plasmolysis manifestations. However, organelles other than symbiosomes and mitochondria were not observed. The bacteroids underwent degradation while the symbiosomes developed irregular forms. In some nodules, the Cd effect was less pronounced, and the degradation symptoms in the infected cells of nitrogen fixation zone were only peribacteroid space extension and intensified symbiosome fusion. Therefore, they contained more than one bacteroid [9]. In that study the tolerance to Cd exposure of nodules formed by *Sinorhizobium meliloti* strain with flavodoxine overexpression was investigated. The nodules were more tolerant to Cd, and the symptoms of damage in these nodules were less pronounced. Some cells of the infection zone showed expansion of the peribacteroid space and vacuolar capture of bacteria, whereas in the nitrogen fixation zone, degradation extended to the symbiosomes containing several bacteroids [9]. Thus, in the alfalfa and pea nodules, Cd induces symbiosome fusion and the appearance of symbiosomes containing several bacteroids that can be considered as a sign of the symbiotic nodule senescence [21]. Besides, in the cells of both species, it was found the presence of bacteria in vacuoles, which indicated disintegration of the tonoplast. This is one of the manifestations of the programmed cell death [22], which is often observed during the nodule senescence [23, 24].

The genetic models have not almost been used for studying nodule functioning under Cd. In pea the effect of Cd on the development of nodules was studied for two contrasting genotypes – VIR8456 and VIR3429 – which differed by the level of tolerance to Cd [25]. At Cd concentration of 4.4 ppm in the soil, VIR8456 nodules were less tolerant. In the infected cells, degradation of peribacteroid membranes and accumulation of electron-dense inclusions were observed. At Cd concentration of 22 ppm in the soil, abnormalities at the ultrastructural level were observed in the nodules of both genotypes, although they were pronounced more strongly in VIR8456 [26].

Thus, this work is a pioneer research in the genetic control of symbiotic nodule functioning under Cd stress. Our results showed that the tolerance of pea nodules to Cd can be increased significantly by the single recessive *cdt* mutation.

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