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HISTOLOGICAL AND ULTRASTRUCTURAL NODULE ORGANIZATION OF THE PEA (*PISUM SATIVUM*) MUTANT SGEFix⁻-5 IN THE *Sym33* GENE ENCODING THE TRANSCRIPTION FACTOR PsCYCLOPS/PsIPD3

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❀ **Background.** The transcription factor CYCLOPS/IPD3 is a key activator of the organogenesis of symbiotic nodules. Its participation in the development of infection threads and symbiosomes is also shown. In pea, three mutant alleles were identified for this gene (*sym33-1 – sym33-3*). The phenotypic manifestations of the *sym33-3* allele of the SGEFix⁻-2 mutant, characterized by a "leaky" phenotype (the formation of two types of nodules: white and pinkish) were the most studied. The *sym33-2* allele in the mutant SGEFix⁻-5 was described as a strong allele, however, its phenotypic manifestations have not been studied in detail. **Materials and methods.** In this study, the histological and ultrastructural nodule organization of the SGEFix⁻-5 mutant was analyzed using confocal laser scanning microscopy and transmission electron microscopy. **Results.** In the nodules "locked" infection threads were observed, from which no bacteria release into the cytoplasm of the plant cell occurs. In this case, in some infection threads, bacteria were degraded, which may indicate the activation of strong defense reactions in the nodules of the SGEFix⁻-5 mutant. **Conclusions.** The *sym33-2* allele in the mutant SGEFix⁻-5 is a strong allele, which triggers the severe defense reactions, when rhizobia are already perceived as pathogens in infection threads.

❀ **Keywords:** plant-microbe interactions; development of symbiotic nodule; transcription factor; infection thread.

ГИСТОЛОГИЧЕСКАЯ И УЛЬТРАСТРУКТУРНАЯ ОРГАНИЗАЦИЯ КЛУБЕНЬКОВ МУТАНТА ГОРОХА (*PISUM SATIVUM*) SGEFix⁻-5 ПО ГЕНУ *Sym33*, КОДИРУЮЩЕМУ ТРАНСКРИПЦИОННЫЙ ФАКТОР PsCYCLOPS/PsIPD3

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❀ Транскрипционный фактор CYCLOPS/IPD3 является ключевым активатором органогенеза симбиотических клубеньков, он также принимает участие в развитии инфекционных нитей и симбиосом. У гороха было выявлено три мутантные аллели по этому гену (*sym33-1 — sym33-3*). Наиболее изучены фенотипические проявления аллели *sym33-3* у мутанта SGEFix⁻-2, характеризующегося «leaky»-фенотипом — формированием двух типов клубеньков: белых и розоватых. Аллель *sym33-2* у мутанта SGEFix⁻-5 была описана как строгая аллель, тем не менее ее фенотипические проявления не были детально изучены. В данном исследовании проанализирована гистологическая и ультраструктурная организация клубеньков мутанта SGEFix⁻-5. В клубеньках наблюдались «запертые» инфекционные нити, из которых не происходил выход бактерий в цитоплазму растительной клетки. При этом в некоторых нитях отмечалась деградация бактерий, что может свидетельствовать об активации сильных защитных реакций в клубеньках мутанта SGEFix⁻-5.

❀ **Ключевые слова:** растительно-микробные взаимодействия; развитие симбиотического клубенька; транскрипционные факторы; инфекционная нить.

INTRODUCTION

The implementation of genetic program for symbiotic nodule development includes subprograms for root infection with rhizobia and nodule organogenesis [1]. In recent years many components of root rhizobial infection [2] and nodule organogenesis [3]

have been identified. Among the identified components, transcription factors, particularly CYCLOPS/IPD3 [4–7], play an important role.

In *Lotus japonicus,* the *CYCLOPS* gene encodes a protein with a short C-terminal coiled-coil domain and a functional nuclear localization signal [5]. CYCLOPS acts as a phosphorylation substrate for the calcium- and calmodulin-dependent protein kinase (CCaMK). Investigation of a series of allelic *cyclops* mutants revealed that these mutants form nodule primordia but no further nodule development occurs. The *cyclops-3* mutant exhibits the colonization of curled root hairs, without developing infection threads. Detailed analysis of *cyclops-3* mutant revealed five serine phosphorylation sites, two of which (S50 and S154) are important for symbiosis [7]. Substitution of serine at these positions by phosphomimetic aspartic acid in transgenic roots expressing a modified *CYCLOPS* variant led to the formation of spontaneous nodules not only in wild-type plants but also in the *cyclops-3* mutant and mutants in the gene encoding LRR-containing receptor kinase SYMRK, *symrk-3* and *symrk-13*. This suggests that CYCLOPS phosphorylation is sufficient for the initiation of nodule organogenesis, indicating that the *CYCLOPS* gene is a master regulator, whereas CYCLOPS protein is a CCaMK-regulated DNA-binding transcriptional activator. CYCLOPS phosphorylation at S50 and S154 positions leads to conformational changes, resulting in the release of the DNA-binding domain of CYCLOPS from autoinhibition by its regulatory N-terminal domain. Subsequently, CYCLOPS binds to the CYC-box of the *NIN* gene promoter, inducing its expression, which leads to nodule organogenesis [7].

Medicago truncatula IPD3 and pea (*Pisum sativum*) *Sym33* genes are orthologous to the *L. japonicus CYCLOPS* gene. Analysis of *ipd3* and *sym33* mutants showed that *IPD3* and *Sym33* regulate the development of infection threads and symbiosomes [6], indicating an important role of CYCLOPS/IPD3 transcription factor in nodule development.

Using the pea laboratory line SGE and variety Finale, three mutants of the *Sym33* gene have been obtained [8–10]. However, the phenotype of only the mutant line SGEFix$^-$-2 (*sym33-3*) has been described in detail [9].

The *aim of this study* was to investigate the histological and ultrastructural organization of the mutant line SGEFix$^-$-5 (*sym33-2*) nodules and to examine the phenotypic effects of mutation.

MATERIAL AND RESEARCH METHODS

Plant material

In this study, we used the mutant line SGEFix$^-$-5 (*sym33-2*), which forms white ineffective nodules [6, 11] from the collection of All-Russia Research Institute for Agricultural Microbiology.

Bacterial strains

To study the ultrastructural and histological organization of nodules, plants were inoculated with *Rhizobium leguminosarum* bv. *viciae* commercial strain RCAM1026 (=CIAM 1026) [12] and strain 3841 [13], respectively.

Growth conditions and sample collection

Seeds were sterilized with concentrated sulfuric acid for 15 min and washed with sterile water 10 times. Plants were grown in plastic pots containing 100 g of sterile vermiculite and watered with nitrogen-free nutrient solution [14]. Plants were grown in the growth chamber MLR-352H (Sanyo Electric Co., Ltd., Moriguchi, Japan) under the following conditions: 21 °C temperature, 75% relative humidity, 16 h light/8 h dark photoperiod, and 280 microphotons m^{-2} s^{-1} light intensity. The nodules were collected from 10 plants in 2 weeks after inoculation.

Sample fixation and confocal microscopy

The nodules were fixed, and 40–50 μm serial sections were prepared using a microtome with a vibrating blade HM650V (Microm, Waldorf, Germany), as previously described [15]. To visualize cell nuclei and bacteria, the sections were washed with TBS buffer (50 mM TrisHCl, 150 mM NaCl, pH 7.5) thrice for 10 min each and then stained with propidium iodide (0.5 μg/ml) for 7 min. Then, the sections were washed with TBS buffer twice for 10 min each and placed under glass coverslips using mounting medium Prolong Gold antifade reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Images were captured using LSM780 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany).

Electron microscope analysis

For electron microscopy analysis, nodules were subjected to a vacuum (–0.9 bar) and fixed in 2.5% glutaraldehyde in 0.3 M Millonig's phosphate buffer (pH 7.4) at 4 °C overnight. The fixed samples were washed with the new Millonig's buffer four times and then fixed in 1% osmium tetroxide in 0.3 M Millonig's phosphate buffer for 2 h. The fixed samples were washed with distilled water thrice for 15 min each and

then dehydrated in a series of increasing ethanol concentrations: 50% ethanol, 70% ethanol (overnight at 4 °C), 96% ethanol for 15 min, and 100% ethanol twice for 10 min each. Lastly, the samples were treated with a 1:1 mixture of absolute ethanol and acetone for 10 min, followed twice by acetone for 10 min each.

Epon 812 resin with DMP-30 catalyst was used as a embedding mixture (Honeywell Fluka™, Fisher Scientific, Longborrow, UK). Tissues were infiltrated in a mixture of absolute acetone and embedding mixture (1:1 and 1:3) for 1 h and then in clean resin overnight at room temperature. The nodules were placed to the previously dried polyethylene capsules filled with fresh embedding mixture. Polymerization was carried out at 37 °C for 24 h, 45 °C for 6 h, and at 60 °C for 2.5 days in the incubator Memmert IN55 (Memmert GmbH, Schwabach, Germany).

Ultrathin (90–100 nm) sections of nodules were cut on the Leica EM UC7 ultratome (Leica Microsystems, Vienna, Austria) using a diamond knife (Diatome, Biel, Switzerland) and collected on nickel grids covered with pyroxylin and carbon. The ultrathin sections were contrasting with 2% water solution of uranyl acetate for 20 min and with Reynolds' lead citrate for 1 min. Images of ultrathin sections were captured using JEM-1400 transmission electron microscope (JEOL Corporation, Tokyo, Japan) with the digital camera OlympusSIS Veleta (Olympus Corporation, Tokyo, Japan) at an accelerating voltage of 80 kV.

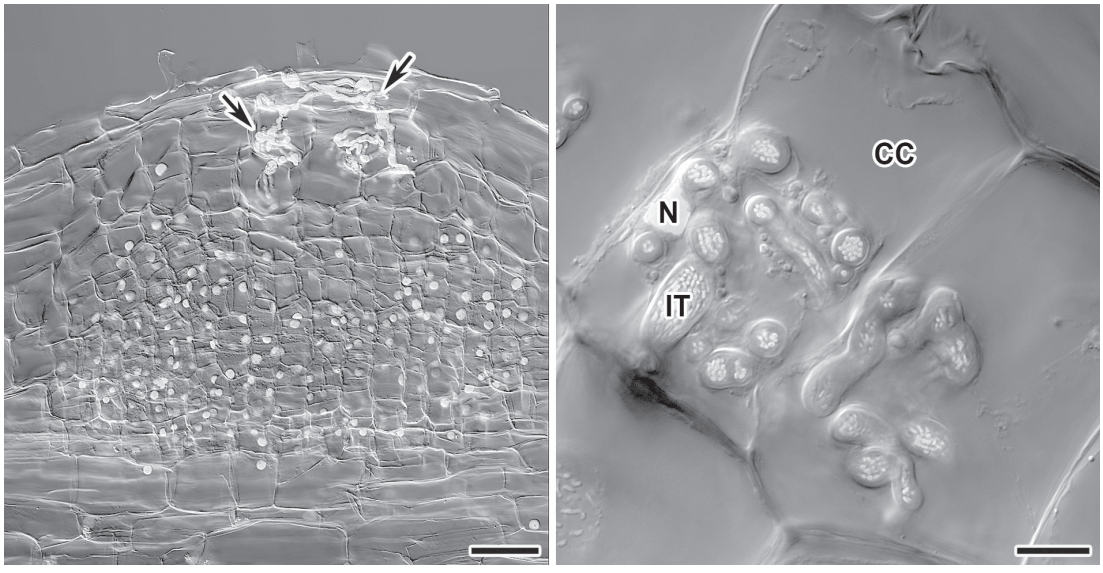
RESULTS

Histological organization of nodules

Most nodules of the mutant line SGEFix⁻-5 (*sym33-2*) were characterized by developmental arrest at the early stages. Ramified infection thread, which was blocked in cells of the root outer cortex, did not penetrate deep into the emerging nodule (Fig. 1 *a*). Strong branching of the infection thread was observed within an individual colonized cell of the nodule (Fig. 1 *b*).

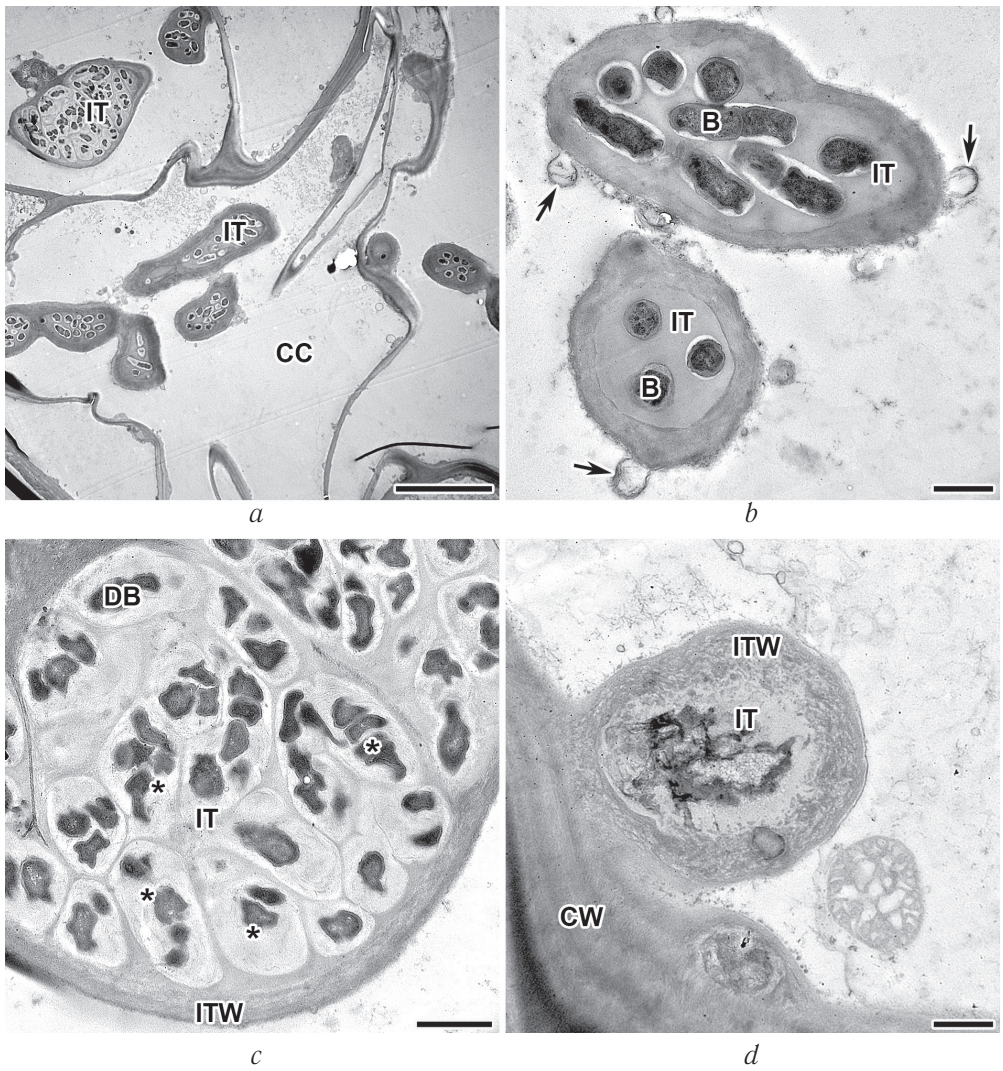
Ultrastructural organization of nodules

Colonized cells of nodules of the mutant line SGEFix⁻-5 (*sym33-2*) were filled with a ramified network of infection threads, and bacteria from these infection threads did not release into the cytoplasm of the plant cell (Fig. 2 *a*). Further analysis showed that infection threads could be subdivided into two types. The first type was represented by threads surrounded by a thickened wall, wherein intact bacteria were immersed into a homogeneous matrix (Fig. 2 *b*). Moreover, vesicles associated with the thickened cell wall could also be observed (Fig. 2 *b*). The second type included infection threads, in which clusters comprising 2–6 bacteria and light matrix were encompassed within typical dark matrix (Fig. 2 *c*). Herewith, bacteria forming the clusters showed signs of degradation (Fig. 2 *c*). Consequently, bacteria inside the infection thread were completely destroyed (Fig. 2 *d*).



a *b*

Fig. 1. Histological organization of nodules of the mutant line SGEFix⁻-5 (*sym33-2*): *a* – sagittal nodule section; *b* – colonized cells with infection threads. Merge of single optical sections of differential-interference contrast and red channels (nuclei and bacteria), presented in grayscale. CC –colonized cell, N –nucleus, IT –infection thread, arrows indicate infection threads. Scale bar: *a* – 50 μm, *b* –10 μm



a *b* *c* *d*

Fig. 2. Ultrastructural organization of nodules of the mutant line SGEFix⁻-5 (*sym33-2*): *a* – colonized cells; *b* – infection threads with thickened walls and intact bacteria in the matrix; *c* – infection thread with thickened walls and degraded bacteria assembled in clusters; *d* – infection thread with completely degraded bacteria in the lumen. CC – colonized cell, IT – infection thread, ITW – infection thread wall, CW – cell wall, B – bacterium, DB – degrading bacterium, arrows indicate vesicles, asterisks indicate clusters of bacteria inside the infection thread. Scale bar: *a* – 10 μm, *b-d* – 1 μm

DISCUSSION

For the pea *Sym33* gene, three independently obtained mutants have been described: SGEFix⁻-2 [9] and SGEFix⁻-5 [11] using the laboratory line SGE and RisFixU, induced on the variety Finale [10]. All *sym33* mutants formed ineffective nodules. SGEFix⁻-5 and RisFixU mutants form white nodules, whereas the SGEFix⁻-2 mutant form white and pinkish nodules. The SGEFix⁻-2 mutant was phenotypically characterized in great detail. Previously, it has been shown that "locked" infection threads, from which the bacteria do not release into the cytoplasm of the plant cell, are formed in white nodules [9]. Subsequently, it has been shown that in some white nodules in certain cells bacterial release occurs [16], accompanied by the formation of infection droplets [17]. Bacteria are released in pinkish nodules but remain undifferentiated [9]. Mutants RisFixU and SGEFix⁻-5 were investigated to a lesser extent. Ultrastructural organization of the nodules of these mutants was not studied; however, it was previously shown that in nodules of these mutants "locked" infection threads are formed [6, 16].

The pea *Sym33* gene encodes CYCLOPS/IPD3 transcription factor [6], which is crucial for symbiosis [6, 7]. Therefore, characterization of *sym33* mutants is of great interest because these mutants enhance our understanding of the functions of this gene.

Previously, it was shown that the SGEFix$^-$-5 mutant forms predominantly large white nodules, with a developed network of "locked" infection threads [6]. In this study, SGEFix$^-$-5 mutant plants mainly formed small white nodules, the development of which was blocked at the stage preceding the penetration of infection threads deep into the nodule. This may indicate that these differences are associated with the strain used. Analysis of the ultrastructural organization of the nodules revealed thickened walls surrounding infection threads and the vesicles supplying material to them. The bacteria from infection threads do not release into the cytoplasm of the plant cell. "Locked" infection threads, similar to those described in this study, have been previously described for the mutant SGEFix$^-$-2 [9]. However, in the previous study, the mutant SGEFix$^-$-2 showed the formation of infection droplets on some infection threads and bacterial release [16, 17]. The SGEFix$^-$-5 mutant did not show such phenotypic features. This indicates that unlike *sym33-3*, *sym33-2* is a strong allele. Additionally, the SGEFix$^-$-5 mutant is characterized by some infection threads, in which bacteria do not separate after dividing, thus forming clusters. This process is followed by complete bacterial degradation. The phenotype can be explained by activation of the strong plant defense. Indeed, in the nodules of the mutant SGEFix$^-$-2 the suberization of cell walls and infection thread walls as well as the activation of a number of defense-related genes was detected [18]. Additionally, the deposition of pectins, particularly rhamnogalacturonan I, both in the infection thread walls and in the matrix surrounding the bacteria was revealed [19]. The manifestation of defense reactions in the mutant SGEFix$^-$-5 is likely to be even stronger affecting the bacteria inside infection threads.

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