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INFLUENCE OF MUTATION IN THE GENE *Sym26* OF THE GARDEN PEA (*Pisum sativum* L.) ON THE ORGANIZATION OF TUBULIN CYTOSKELETON IN NODULES

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Abstract

Symbiotic nodule is a unique organ forming on legume roots. Indeterminate nodules (with prolonged meristem activity) (F. Guinel, 2009) are characterized by differentiation of both the nodule cells and the bacteria that infect nodule and are converted into a form specialized for nitrogen fixation — bacteroids. Bacteroids surrounded by a membrane of plant origin, form organelle-like symbiosomes (A. Tsyganova et al., 2018; T. Coba de la Peca et al., 2018). Cell differentiation leads to appearance of uninfected (free of bacteria) and infected cells filled with many thousands of symbiosomes formed in the central part of nodule (A. Tsyganova et al., 2018). A prolonged activity of the meristem results in histological zonation of the indeterminate nodule. A meristem, an infection zone, a nitrogen fixation zone are distinguished, and a senescence zone appears in the basal part of a mature nodule (F. Guinel, 2009). Obviously, the tubulin cytoskeleton plays an important role in the development of a nodule, but until now researchers had a focus on the early stages of nodule development (A. Timmers, 2008). Only recently it was revealed that the tubulin cytoskeleton plays a key role in the differentiation of nodule cells (A. Kitaeva et al., 2016). It was shown that in nodules of garden pea (*Pisum sativum* L.) and barrel medic (*Medicago truncatula* Gaertn.) the release of bacteria into the cytoplasm of a plant cell prevents the formation of a regular pattern of cortical microtubules, oriented parallel to each other and perpendicular to the longitudinal axis of the cell, typical for uninfected cells. This leads to an irregular pattern of cortical microtubules, the appearance of which contributes to the transition of infected cells to isodiametric growth (A. Kitaeva et al., 2016). Endoplasmic microtubules build a mold for the growth of infection threads, and support the location of infection droplets and symbiosomes in infected cells (A. Kitaeva et al., 2016). However, changes in the organization of the tubulin cytoskeleton during senescence of nodule cells have not been studied. In this study, using immunocytochemical analysis and confocal laser scanning microscopy, the organization of the tubulin cytoskeleton in the nodules of the pea mutant SGEFix$^-$-3 (*sym26*) (V. Tsyganov et al., 2000) was studied. This mutant is characterized by the formation of ineffective nodules with premature degradation of symbiotic structures (T. Serova et al., 2018). It was shown that in the mutant line, the formed patterns of cortical and endoplasmic microtubules did not differ from those of the initial line SGE. Cortical microtubules formed an irregular pattern in meristematic and infected cells and regular pattern in uninfected and colonized cells. Endoplasmic microtubules surrounded the nucleus in interphase cells, formed spindles and preprophase bands during mitosis, and also surrounded infection threads. At the same time, in the senescence zone in degrading cells, complete depolymerization of the tubulin cytoskeleton occurred in both infected and uninfected cells. In the initial line, senescence was induced only in four-week-old nodules, and microtubule depolymerization was also observed in senescent cells. Thus, the complete depolymerization of microtubules in various types of nodule cells can be a cytological marker of its senescence.

Keywords: legume-rhizobial symbiosis, microtubules, symbiosome, bacteroid, infection thread, nodule senescence, immunolocalization, *Pisum sativum*

A characteristic feature of legumes is their interaction with nodule bacteria, rhizobia, which results in formation of symbiotic root nodules. A symbiotic nodule is a unique organ, in which a plant creates ecological niches for rhizobia, which gain the ability to fix atmospheric nitrogen [1]. In various species of *Fabaceae* symbiotic nodules differ in structure. There are two types of nodules, determinate and indeterminate [2]. Determinate nodules have a meristem that is active during a short period, resulting in a spherical shape of mature nodules. Meristem of indeterminate nodules remains active for a long time leading to appearance of histological zonation in the central part of nodule having elongated shape. As a result there are nodule meristem, the infection zone, where rhizobia are released into the cell cytoplasm, and the nitrogen fixation zone, in which they acquire nitrogen fixation ability by differentiating into the specialized forms, called bacteroids [2, 3]. After four weeks post inoculation, the senescence zone appears proximal to the nitrogen fixation zone [4]. Along with bacteroid differentiation, a pronounced differentiation of nodule cells is observed [5]. A number of cells remain uninfected while infected cells grow in size significantly and become filled with numerous symbiosomes. Symbiosome contains a bacteroid separated from plant cell cytoplasm by a symbiosome membrane of plant origin [5, 6]. Apparently, plant cell differentiation during symbiotic nodule development is accompanied by significant cytoskeleton reorganization [7].

The reorganization of actin microfilaments was described as one of early responses triggered by Nod factors [8-10]. Actin microfilaments are also required for the formation of infection thread [8-13]. Recently, it was shown that actin microfilaments are involved in release of rhizobia into the cytoplasm of the plant cells from infection droplets and facilitate symbiosome accommodation [14].

Numerous microtubules were identified in infection sites in curled root hairs as early as in the mid-1980s [15, 16]. Later on, the engagement of microtubules in root hair curling, initiation and growth of the infection thread was discovered [17-22]. In mature nodules the organization of microtubules was studied in alfalfa (*Medicago sativa* L.) [23], soybean (*Glycine max* L.) [24], garden pea (*Pisum sativum* L.) [25] and white lupin (*Lupinus albus* L.) [26]; however, the three-dimensional organization of tubulin cytoskeleton was not described in detail, specifically around infection threads and infection droplets [7]. Only recently we showed changes in the pattern of cortical microtubules during cell differentiation in nodules of pea and *Medicago truncatula* Gaertn. [27]. For instance, in uninfected cells the cortical microtubules are parallel to each other and perpendicular to longitudinal axis of the cell [27]; a similar pattern is characteristic of pea root cells in the transition zone [28, 29]. In infected cells of pea and *M. truncatula* nodules the cortical microtubules form irregular pattern promoting an isodiametric growth of these cells [27]. In nodules of both analyzed species the endoplasmic microtubules formed a dense network around infection structures, i.e. infection threads and droplets, creating a matrix for their development [27]. Significant differences were observed in the pattern of endoplasmic microtubules forming the network between symbiosomes. For instance, in pea the microtubules were randomly located between symbiosomes, which coincided with the lack of order in the location of symbiosomes themselves. At the same time, in infected cells of *M. truncatula* nodules microtubules were parallel to the symbiosomes, which, in turn, were perpendicular to the cell wall [27].

In this report we show for the first time that both natural and induced nodule senescence is accompanied by depolymerization of microtubules.

The aim of the study was to evaluate the impact of natural senescence and senescence induced by mutation in the pea *Sym26* symbiotic gene leading to premature degradation of symbiotic structures on organization of tubulin cyto-

skeleton in nodules.

Techniques. In our work we used the initial line SGE of garden pea (*Pisum sativum* L.) [30] and its mutant SGEFix$^-$-3 (*sym26*) [31] forming ineffective nodules with premature degradation of symbiotic structures (the senescence zone is formed as early as 2 weeks after inoculation), i.e., with early senescence phenotype [32]. The plants were inoculated with *Rhizobium leguminosarum* bv. *viciae* 3841 strain [33].

The seeds were surface sterilized for 15 min with concentrated sulphuric acid and rinsed 10 times with sterile water. The plants were grown in plastic pots, filled with 100 g of sterile vermiculite, at 21 °C, 75% relative humidity, illumination of 280 μM photons $m^{-2}\,c^{-1}$ and day/night mode of 16/8 h (a growth chamber MLR-352H, Sanyo Electric Co., Ltd, Japan). Nitrogen-free nutrient solution was used for watering [34]. The nodules for analysis were collected from 10 plants 2 weeks after inoculation, for SGE line also 4 weeks after inoculation. Three independent experiments were performed.

Method of nodule fixation and tubulin immunolocalization was described earlier [27]. Monoclonal mouse antibody to tubulin (DM1A clone, Sigma-Aldrich, USA) was used for visualization of microtubules (1:1000 dilution, incubation during the night at 4 °C). Goat antibody conjugated with Alexa Fluor 488 (Life Technologies, USA) was used as secondary antibody to mouse γ-globulin (1:500 dilution, incubation during 90 min at 28 °C). To identify nuclei and bacteria sections were stained for 7 min with propidium iodide (0.5 μg/ml). After rinsing the sections were embedded into ProLong Gold® antifade reagent (Thermo Fisher Scientific, USA) under cover glasses.

The microtubule pattern in nodule cells was analyzed using laser confocal scanning microscope LSM780 and ZEN2012 software (Carl Zeiss, Germany).

Results. The histological organization of 2-week-old nodules of SGE line did not differ from that described earlier [35], the meristem, the infection zone and the nitrogen fixation zone were identified (Fig. 1, A). In 2-week-old ineffective nodules of SGEFix$^-$-3 mutant (*sym26*) we observed the meristem, the infection zone and a zone corresponding to the nitrogen fixation zone, along with the senescence zone (Fig. 2, B), as it was described earlier [32].

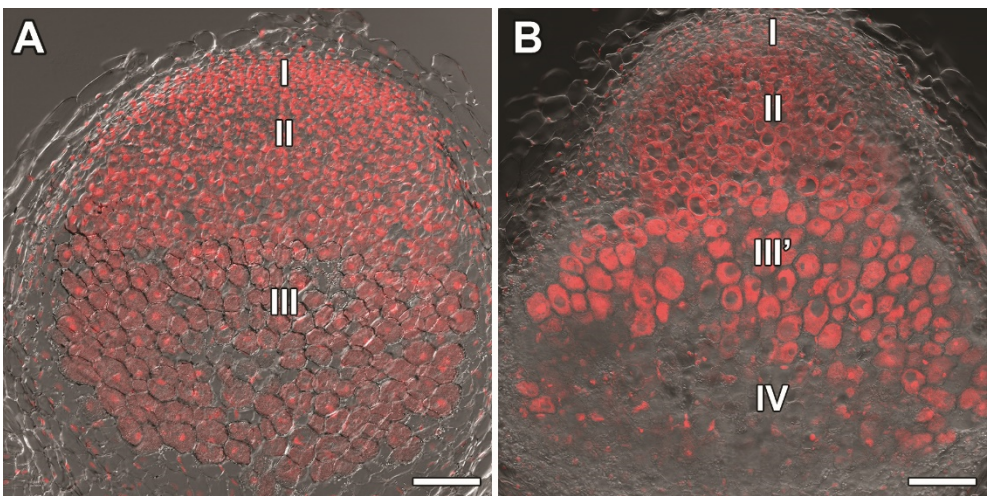

Fig. 1. Histological organization of 2-week-old nodules of pea (*Pisum sativum* L.) initial line SGE (A) **and mutant line SGEFix$^-$-3 (*sym26*)** (B)**:** I — meristem, II — infection zone, III — nitrogen fixation zone, III' — zone corresponding to the nitrogen fixation zone in the initial line, IV — senescence zone (longitudinal sections). Confocal laser scanning microscopy (microscope LSM780, ZEN2012 software, Carl Zeiss, Germany). Merge of single optical sections of differential interference contrast

and red channel. Scale bar 100 μm.

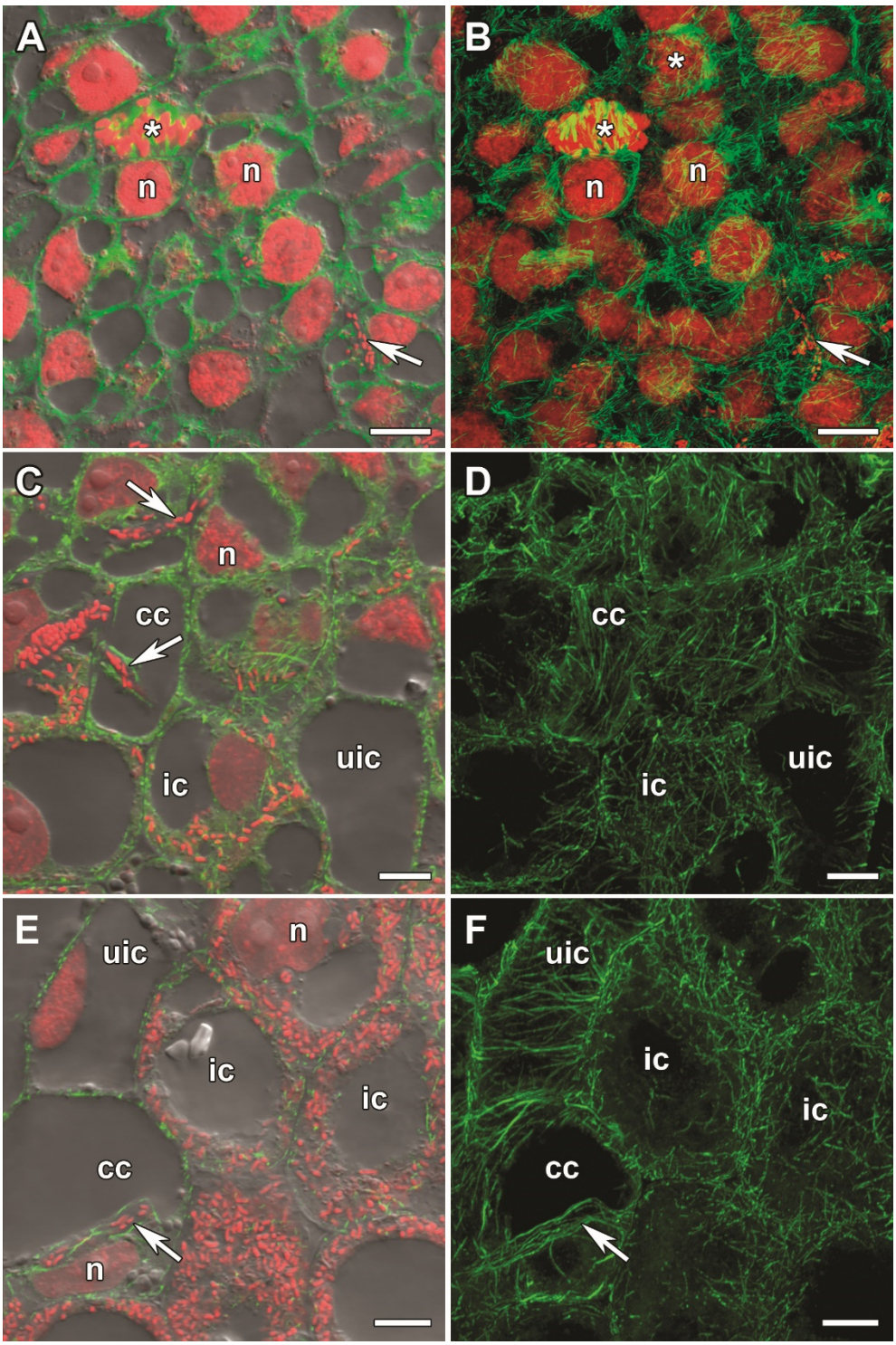

Fig. 2. Organization of tubulin cytoskeleton in meristematic cells (A, B)**, and in cells of the early** (C, D) **and the late** (E, F) **infection zone of 2-week-old nodules of pea (*Pisum sativum* L.) mutant SGEFix$^-$-3 (*sym26*):** n — nucleus, ic — infected cell, uic — uninfected cell, cc — colonized cell, arrows indicate infection threads, asterisk marks mitotic structures (longitudinal sections). Confocal laser scanning microscopy (microscope LSM780, ZEN2012 software, Carl Zeiss, Germany). Scale bar 10 μm.

A-F: immunolocalization of tubulin (microtubules) — green channel, DNA staining with propidium iodide (nuclei and bacteria) — red channel.

A, C, E: merge of single optical sections of differential interference contrast, green and red channels.

B: maximum intensity projections of green and red channels based on z-stacks from 50 optical sections.

D, F: maximum intensity projections of green channel based on z-stacks from 30 (D) and 70 (F) optical sections.

Previously, we have described the tubulin cytoskeleton organization in 2-week-old nodules of SGE line; therefore, in this study the tubulin cytoskeleton organization was analyzed only in SGEFix$^-$-3 mutant (*sym26*). To compare microtubules organization in senescent wild type cells, 4-week-old nodules of SGE line were also analyzed, in which such cells could have been identified.

The meristem cells in SGEFix$^-$-3 mutant nodules (*sym26*) were small in size and had centrally located nucleus. The cortical microtubules lying at different angles to each other and often criss-crossing formed irregular pattern (see Fig. 2, A, B). The endoplasmic microtubules in interphase cells enveloped the nucleus connecting it with the cell periphery (see Fig. 2, A, B). In mitotic cells, endoplasmic microtubules formed a mitotic spindle and a preprophase band (see Fig. 2, A, B). The observed patterns of cortical and endoplasmic microtubules of meristematic cells in SGEFix$^-$-3 (*sym26*) nodules were similar to those in SGE [27].

Three cell types observed in the early infection zone were i) uninfected cells, ii) colonized cells where infection structures (infection threads and infection droplets) were present but no bacterial release into the cytoplasm occurred, and iii) infected cells (see Fig. 2, C, D). The uninfected cells were lack of endoplasmic microtubules, whereas cortical microtubules formed a regular pattern, i.e. they were parallel to each other and perpendicular to the longitudinal axis of the cell (see Fig. 2, C, D). In colonized cells the pattern of cortical microtubules was identical to that of uninfected cells (see Fig. 2, C, D). Furthermore, endoplasmic microtubules located along the infection thread were observed (see Fig. 2, C, D). In infected cells cortical microtubules formed an irregular pattern (see Fig. 2, C, D). Previously, for pea wild type line SGE and *M. truncatula* wild type line A17 it was demonstrated that microtubules serve as a matrix for infection thread growth and surround infection droplets thereby preparing them for bacterial release into the nodule cell cytoplasm [27].

In the late infection zone, the uninfected and especially infected cells increased in size; moreover, they retained the microtubular patterns described above (see Fig. 2, E, F). Furthermore, a well-defined network of randomly positioned endoplasmic microtubules was observed between symbiosomes (see Fig. 2, E, F).

In the zone corresponding to the wild type nitrogen fixation zone the infected cells further increase in size; moreover, they continued to support irregular patterns of cortical and endoplasmic microtubules passing among symbiosomes (Fig. 3, A, B). The involvement of tubulin cytoskeleton in the positioning of symbiosomes in nitrogen-fixing cells of pea and *M. truncatula* was previously identified; it was shown that the positioning of endoplasmic microtubules between symbiosomes varied for the analyzed species [27]. The observed pattern of endoplasmic microtubules around symbiosomes in the infected nodule cells of SGEFix$^-$-3 (*sym26*) mutant did not differ from that of SGE nodules.

The similarity of patterns in nodules of initial line and its mutant is indicative of the fact that up to a point the development of infected cells in both genotypes is identical. Indeed, it was reported earlier that morphologically differentiated bacteroids that undergo premature degradation are typical for SGEFix$^-$-3

(*sym26*) mutant line [32].

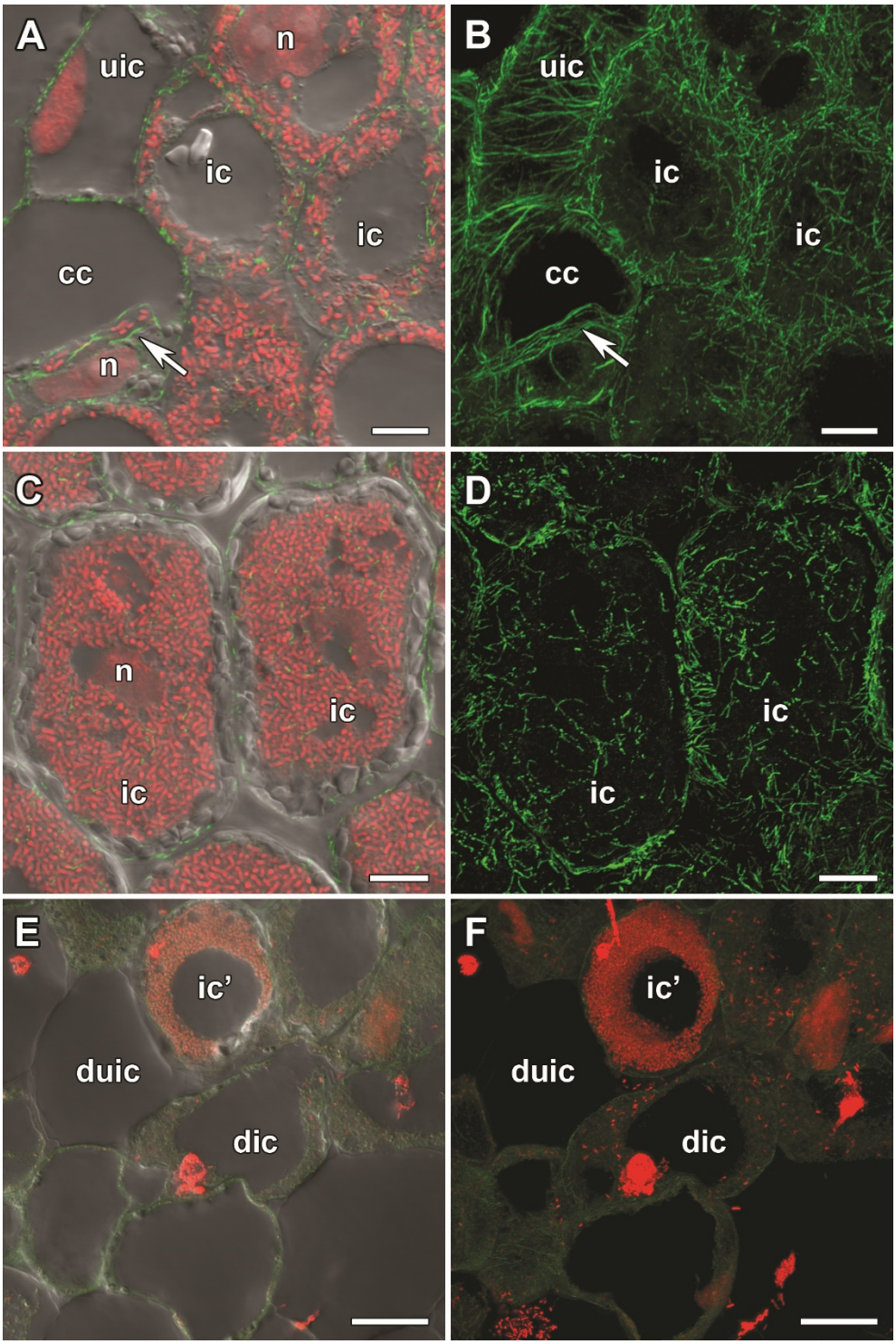

Fig. 3. Organization of tubulin cytoskeleton in cells of a zone corresponding to the nitrogen fixation zone in the initial line (A, B)**, in cells of the senescence zone** (C, D) **of 2-week-old nodules in SGEFix$^-$-3 (*sym26*) mutant, and in senescent cells** (E, F) **of 4-week-old nodules of pea (*Pisum sativum* L.) initial line SGE:** n — nucleus, ic — infected cell, ic' — infected cell with early signs of degradation, uic — uninfected cell, cc — colonized cell, dic — degrading infected cell, duic — degrading uninfected cell, arrows indicate infection threads, asterisk marks mitotic structures (longitudinal sections). Confocal laser scanning microscopy (microscope LSM780, ZEN2012 software, Carl

Zeiss, Germany). Scale bars 10 μm (A, B) and 20 μm (C-F).

A-F: immunolocalization of tubulin (microtubules) — green channel, DNA staining with propidium iodide (nuclei and bacteria) — red channel.

A, C, E: merge of single optical sections of differential interference contrast, green and red channels.

B. D: maximum intensity projections of green channel based on z-stacks from 50 optical sections.

F: maximum intensity projections of green and red channels based on z-stacks from 50 (D) и 45 (F) optical sections.

The senescence zone was observed in the distal part of the nodule (see Fig. 1, B). In this zone degradation of symbiotic structures in cells was observed, which was accompanied by complete depolymerization of both cortical and endoplasmic microtubules (see Fig. 3, C, D). As reported earlier, the pattern of endoplasmic microtubules located between symbiosomes in Sprint-2Fix$^-$ (*sym31*) pea mutant with undifferentiated bacteroids was similar to that of wild type [36], whereas in *M. truncatula dnf1-1* mutant [37], which also formed undifferentiated bacteroids, fast depolymerization of microtubules occurred [27]. Apparently, depolymerization of microtubules in SGEFix$^-$-3 (*sym26*) mutant in cells of the senescence zone is related to degradation of symbiotic structures [32] and activation of nutrient reutilization. It is possible that the fast depolymerization of microtubules in *M. truncatula dnf1-1* mutant is due not to lack of bacteroid differentiation in this mutant, but rather to activation of degradation of symbiotic structures accompanied by a depolymerization of tubulin and actin cytoskeleton [38].

The senescent infected and uninfected cells with signs of degradation of symbiotic structures (see Fig. 3, E, F) were identified in SGE line in the basal part of 4-week-old nodules, and in such cells we observed depolymerization of microtubules (see Fig. 3, E, F). In SGE line, the senescence only starts in 4-week-old nodules and peaks in 6-week-nodules [32], whereas 4-week-old nodules of SGEFix$^-$-3 (*sym26*) mutant display almost complete degradation of symbiotic structures and expansion of the senescence zone that often fills the entire nodule [32].

To summarize, we have studied the impact of natural senescence on microtubule pattern in nodules of the initial line SGE. We have also investigated the influence of a mutation in the pea *Sym26* symbiotic gene on tubulin cytoskeleton. The mutation *sym26* leads to formation of ineffective nodules and induced premature degradation of symbiotic structures. It was shown that in SGEFix$^-$-3 (*sym26*) mutant the patterns of microtubules in cells of meristem, the infection zone and zone, which corresponds to the nitrogen fixation zone of wild type do not differ from the initial line SGE. This is indicative of normal development of nodule cells in the mutant line until induction of premature degradation of symbiotic structures followed by complete destruction of tubulin cytoskeleton both in infected and uninfected cells. Identical depolymerization of microtubules occurs during natural senescence of nodule cells in SGE line. Therefore, complete depolymerization of microtubules in different nodule cells is observed both in case of natural and induced senescence, and can be a cytological marker of senescence.

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