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**SYMBIOTIC NODULE SENESCENCE IN LEGUMES:
MOLECULAR-GENETIC AND CELLULAR ASPECTS**

(review)

T.A. SEROVA, V.E. TSYGANOV*All-Russian Research Institute of Agricultural Microbiology, Russian Academy of Agricultural Sciences, 3, sh. Podbel'skogo, St. Petersburg, 196608 Russia, e-mail tsyganov@arriam.spb.ru**Supported by Ministry of Education and Science of the Russian Federation, by Grant of the President of the Russian Federation, Russian Fund for Basic Research**Received July 31, 2013***Abstract**

Senescence is the natural stage in development of symbiotic nodule. As a result of senescence, reutilization of different nutrients from nodule to the other plant organs occurs. Generally senescence in legumes is triggered after flowering finishing, although the first traits of senescence can be observed very early during nodule development. A delay of the triggering of senescence program will allow to prolong the active nitrogen-fixing period and therefore to increase the amount of symbiotrophic nitrogen in plants and, finally, to elevate legume productivity. That is why no wonder that in the recent years the senescence of nitrogen-fixing nodules is actively studied. In this review the main developmental stages of nitrogen-fixing symbiotic nodule of legumes, particularities of symbiotic nodule development of determinate and indeterminate types are considered. In legumes with indeterminate nodules, the symbiosomes are not long-leaving as the infected tissues are permanently renewing due to apical meristem. There are two subsequent stages identified in an indeterminate nodule senescent. First a bacteroid degradation and the death of some infected cells occur, and then both symbiosomes and all infected cells are destroyed. In determinate nodules, the senescence initiated in the central part of a nodule, then extends to the peripheral zone. In this review morphological characters of nodule senescence at ultrastructural level are analyzed. The role of cysteine and threonine proteases is discussed. Reutilization of nitrogen and other products of protein degradation are probably the most important during senescence. There are the evidences that in the root nodules of legumes the cysteine proteases are involved into nodule functions, adaptation of the host plant cells to physiological stresses, and the nodule senescence control. By a large-scale analysis of nodule transcriptome of *Medicago truncatula* Gaertn. several groups of genes expressed at successive stages of the senescence of indeterminate nodule are revealed. In this review the role of phytohormones, such as ethylene, abscisic acid, jasmonic acid, gibberellins and nitrogen monooxide in senescence of symbiotic nodule is considered. Nevertheless, until recent days our knowledge about hormonal control of nodule senescence is still incomplete. The oxidative stress, accompanying the process of nodule senescence is discussed. On the nodule aging, the concentrations of peroxides, protein carbonyls, modified DNA nucleotides and catalytic Fe increase. Iron activates lipids peroxidation in a peribacteroid space, resulting in degradation of the peribacteroid membrane in senescent nodules. The concentrations of oxidized glutathione and homogluthathione rise significantly during the nodule development, and the reduced forms decrease under senescence, indicating an oxidative stress in the senescing nodules. In this review the role of genes, encoding proteins involved in transport of wide-range of molecules, and genes, whose products are involved in regulatory and signal functions in cell; differences between stress-induced senescence and natural senescence are considered. Using model legumes, *Lotus japonicus* (Regel) K. Larsen and *M. truncatula*, several genes were cloned the mutations of which caused early senescence. It is emphasized that these genes encode different proteins involved into functioning of a symbiotic nodule. Until now, two transcription factors in *M. truncatula* are described, which are involved into nodule senescence. An induced senescence is more rapid, comparing to natural senescence, and manifests the signs of an oxidative stress and programmed cell death.

Keywords: legume-*Rhizobium* symbiosis, nodule development, genetic control, oxidative stress, proteases, ethylene, abscisic acid, jasmonic acid, gibberellins, nitrogen monoxide, rhizobia.

Senescence is the natural stage in development of symbiotic nodules. As a result of senescence, reutilization of different nutrients from nodule to the

other plant organs occurs. Generally senescence in legumes is triggered after the flowering has finished, although the first signs of senescence can be observed very early during nodule development.

Development of a symbiotic nodule. The development starts with a signal interaction between legumes and bacteria known as rhizobia. Various aspects of the development of symbiotic nodules were widely discussed in numerous reviews (1-5). In brief, the legumes produce flavonoids, which trigger the synthesis of lipochitooligosaccharides, or Nod-factors, in rhizobia. The Nod-factors are perceived by specific receptors of plants, and activate the signal transduction mechanisms. As a result, the infection of host plant by rhizobia occurs and the formation of symbiotic nodule primordium is induced (1, 5). The infection process starts with the deformation and curling root hairs, which form an infection pocket containing rhizobia (2, 5). Hence they move deeper into the root hairs and root cells by special channel, an infection thread, formed by the plant (3-5). Simultaneously, in the root cortex and pericycle the cell divisions are induced, leading to formation of a nodule primordium. The infection thread reaches primordium, where the special outgrowths appear. They are surrounded by a plasma membrane but have no wall, which normally forms the border of an infection thread (3, 4). Due to a process resembling endocytosis the rhizobia are released from these outgrowths, or the infection droplets, into the plant cell cytoplasm (4), wherein the bacteria are surrounded by a symbiosome (peribacteroid) membrane of plant origin with the inclusion of bacterial proteins (1, 4). Then the differentiation of bacteria into a specialized form, the bacteroid, occurs. The bacteroid with symbiosome membrane is the main nitrogen-fixing unit, a symbiosome (1, 4, 5). In legumes from temperate latitudes (i.e. *Pisum sativum* L., *Medicago sativa* L.), the meristematic activity is maintained over the life of nodules (they earned the name «indeterminate»). Due to permanent meristematic activity in the nodule, there are different zones: I — meristem; II, III and IV — zones of infection, nitrogen fixation and senescence, respectively (Fig. 1, A, C). The indeterminate nodules are typically elongated in shape. In the nodules of the southern legumes (*Glycine max* L., *Phaseolus vulgaris* L.) the meristem remains active for a short period, leading to no zone formation and spherical shape of nodules, earned the name «determinate» (1, 4, 5).

Infected plant cells from the nitrogen-fixing zone remain functionally active for a limited time, and then senescence begins as a programmed cell death.

In the legumes with indeterminate nodules the symbiosomes are short-lived, because the central infected tissues are constantly renewed due to apical meristem. Thus, the first signs of senescence of the symbiosomes in pea and alfalfa were observed 14 days after the inoculation (6), while the intensive senescence started much later, after the end of flowering. In the legume plants that have determinate nodules the senescence also coincides with the end of flowering and early ripening, as it was shown in *G. max* (7) and *Vigna mungo* (L.) Hepper (8). Often the senescence is accelerated if the symbiosis is not effective due to mutations in plants (9, 10) or in bacteria (11). Senescence can be also induced by different abiotic stresses (12).

Morphological signs of senescence. Visually the senescence in nodules manifests in change of color in the nitrogen-fixing zone from pink, associated with the leghemoglobin functioning, to green due to the destruction of the heme group of the protein and the formation of biliverdin (13).

During senescence the cells of both symbiotic partners are destroyed (Fig. 1, B-D, 2, A, B). But in case of indeterminate nodules the zone of nitrogen fixation is constantly renewed due to cell transition from the meristem. Nev-

ertheless, the senescence zone increases with age, and the ultrastructural changes can be found in symbiosomes and cell organelles. Thus, the electron density of cytoplasm decreases, and there are vesicles and membrane fragments due to destruction of the plant cells and symbiosomes (14, 15).

Fig. 1. Symbiotic nitrogen-fixing nodules in *Pisum sativum* L., the parental line SGE (A, B, 4 and 6 weeks, respectively) and its derivate, the symbiotic mutant SGEFix-8 (*sym25*) with early senescent nodules (C, D, 4 and 6 weeks, respectively): I — meristem, II, III, IV — zones of infection, nitrogen fixation, and senescence, respectively (Scale bars: 200 μ m).

In determinate nodule the senescence starts in its center and extends to a peripheral zone (16).

In young nodules of *Medicago truncatula* Gaertn., the model plant, it was shown (15), that in the central part of nitrogen-fixing zone there are several senescent infected cells of type I, in which the symbiosome degradation occurs with an increase of vesicular activity and amount of rough endoplasmic reticulum. Plant cells thus remain intact and have no visible signs of aging. In the same zone there are cells of type II with more severe senescence signs, in which integrity of a cell wall is disrupted. In older nodules, additionally to the cells of types I and II there are the cells of type III with destroyed symbiosomes and the signs of plant cell death (e.g. loosening of plasma membrane from the cell wall).

Рис. 2. Zone of senescence in nitrogen-fixing nodules in *Pisum sativum* L. of the parental line SGE (A) and its derivate, a symbiotic mutant SGEFix-8 (*sym25*) with early senescent nodules (B): IC — infected cell; early degradation of bacteroids and host plant cells (type II, small arrows) and their full degradation (type III, big arrows) are shown (Scale bars: 100 μm).

Based on ultrastructural analysis, two stages can be indicated in the senescence of indeterminate nodules, namely an initial degradation of bacteroids and death of several plant cells, and full degradation of both symbiosomes and infected cells (12, 15).

In the nodules of ineffective pea mutants with early aging in the senescent cells there were the lysosome-like compartments with degrading bacteroids (9).

A senescence zone in mature indeterminate nodules of *M. truncatula* is cone-shaped and oriented towards direction of the nodule growth. The infected cells are destroyed before uninfected cells, which are involved into the transport of substances to the vascular tissues. The cone shape probably optimizes a remobilization of catabolites, because the cells with a peripheral localization near the vascular bundles can remain viable for longer time. Thus, a central position of the point where the senescence is initiated resulted from the largest distance from peripheral vascular system and environment that probably is due to the role of concentration gradients of oxygen or molecules delivered from the vascular bundles (12).

In alfalfa nodules after 7 weeks, the zone V located proximally to the senescence zone was described besides of zone IV (14). In zone V the next stage of releasing rhizobia from the remaining infection threads was observed that leads to reinvasion of old plant cells. This zone is an ecological niche where intracellular rhizobia act as exclusively saprophytic partners (14).

Proteases. Senescence functionally means a reutilization of substances accumulated in tissues and then transported into another parts of the plant to be used for new organ formation. Protein degradation that allows reutilizing nitrogen is probably the most important during senescence. Therefore, it is not surprising that the majority of genes with positive regulation that are involved in senescence processes are the protease genes (17).

There are several proteases involved into senescence in plants. Thus, it was shown that the cysteine and threonine proteases mainly participate in the senescence of nodules (12, 15, 18).

There are evidences that in the root nodules of legumes the cysteine proteases are involved in the functioning of the nodule, in the adaptation of the host cells to physiological stress and in the control of nodule senescence. Proteases and their inhibitors were identified in the infected cells of the nodules (7, 19). Cysteine proteases with the acidic optimal pH were described in the nodules of *P. vulgaris* (19), and their activity increased to the beginning of senescence. Similar investigations were performed on *G. max* (7), *V. mungo* (8) and *M. sativa* (10).

In *P. sativum* an expression of the genes of cysteine protease 1 (*PsCyp1*) and cysteine protease 15a (*PsCyp15a*) was examined (18), using *Rhizobium leguminosarum* bv. *viciae* 3841, the effective strain, and *R. leguminosarum* bv. *viciae* B661, defective in the lipopolysaccharide synthesis and forming early senescent nodules under inoculation. Expression of both genes, *PsCyp1* and *PsCyp15a*, increased during nodule development (18).

By using wide-range transcriptome analysis of alfalfa *M. truncatula* nodules, few groups of genes specifically activated at successive stages of indeterminate nodule senescence were found. An increased expression was identified for genes from protease gene family during the later stages of senescence under a significant degradation of both symbiotic partners (15).

Among the identified molecular markers of the later stages of senescence, there was the vacuolar processing enzyme (VPE) gene from family C13 of cysteine protease group, responsible for maturation of vacuolar proteins, and genes encoding threonine proteases involved in F-box-specific ubiquitin/26S proteasome pathway (15).

Hormones. Development of a legume-*Rhizobium* symbiosis is also subject to hormonal regulation by the host plant. Among the phytohormones, ethylene, abscisic and jasmonic acids, gibberellins and nitrogen oxide NO were shown to be involved in the senescence of symbiotic nitrogen-fixing nodules. Nevertheless, it should be noted that to date our knowledge about the hormonal regulation of this process remains quite incomplete.

Ethylene acts as an activator of senescence in the symbiotic nodules of legume plants that is confirmed by upregulation of transcription factors (ERF) and the genes of ethylene biosynthesis, such as S-adenosylmethionine (SAM) synthetase, and 1-aminocyclopropane-1-carboxylate oxidase (15).

Induction of lipoxygenase genes suggests the involvement of jasmonic acid in different stages of aging symbiotic nodule. The oxidation of polyunsaturated fatty acids, with the participation of lipoxygenase enzymes is the first step in the biosynthesis of oxylipins such as jasmonate (15, 20).

During the natural senescence of the nodule, there is a decrease of ascorbate-glutathione antioxidant pool, combined with a reduction in carbon-to-nitrogen ratio in the nodule tissues. These changes can be perceived and transmitted by signaling mechanisms associated with abscisic acid (ABA), mobilizing proteolytic activity in the senescent symbiotic nodule (15, 21). ABA may be involved in the induction of the enzyme, which enhances the acceptor capability,

and thus contribute to the early development of individual nodules (22). However, an increased rate of synthesis of ABA during aging can cause the death of nodule. The content of ABA in the nodules of pea (*P. sativum*) is quite high during the first 2 weeks, but then reaches a plateau, and again increases at the later stages of development (23).

Gibberellic acid can inhibit senescence in nodules as it was illustrated by activation of genes which encode gibberellin-2-oxidase converting active phytohormone to inactive state (15, 24).

Recently, using the model legume plant *M. truncatula*, the regulation of aging in symbiotic nodule by nitrogen monoxide was revealed (25). Bacterial flavogemoglobin has been shown to play an important role in NO control in the nodules. In *hmp* mutant defective in flavogemoglobin biosynthesis, early senescence was observed with an increased amount of NO indicated in nodule tissues, while the *hmp*⁺⁺ strain with flavogemoglobin overexpression was characterized by slower senescence and decreased amount of NO in nodules. Endogenous NO also caused a premature senescence of nodules (25).

Oxidative stress under senescence. Functioning of Legume-*Rhizobium* symbiosis contributes to the formation of reactive oxygen species (ROS). A slow autooxidation of oxyhemoglobin was shown to occur resulting in formation of superoxide anion O₂⁻ which disproportionates and produces hydrogen peroxide H₂O₂ (26). Besides, leghemoglobin reacts with H₂O₂, as a result the oxidized forms, particularly proteins with ferric iron and protein radicals, are produced (27). Hydrogen peroxide can also cause the protein destruction with releasing catalytic iron (i.e. iron in a molecular form) which is capable to activate lipid peroxidation and hydroxyl radical formation (28). ROS also are produced in reduction processes under conditions required for nitrogen fixation and due to activity of some proteins, including ferredoxine, uricase and hydrogenase (29).

Nevertheless, in nodules there are high concentrations of antioxidants (e.g. ascorbate, glutathione, superoxide dismutases, catalases and enzymes of ascorbate-glutathione pathway, peroxiredoxine) (30). Antioxydants provide for keeping ROS in low concentrations. Free radicals were detected in the nodules (31, 32), and their concentrations increased during nodule development. Different rhizobial antioxidative systems play important role in ROS control. Moreover, the extracts from senescent nodules can cause the lipid peroxidation in plant cell membranes and peribacteroid membranes (26). In the cytochemical analysis with CeCl₃ the generation of cerium perhydroxide precipitates was observed in the senescent cell walls and around the peribacteroid and bacteroid membranes, confirming H₂O₂ involvement in the senescence of a symbiotic nodule (33, 34).

Study of 2- and 10-weeks nodules of *G. max* showed the oxidative stress during symbiotic nodule senescence in legumes. During nodule aging, concentration of peroxides, protein carbonyls, modified DNA, and catalytic iron increased. The iron from peribacteroid space provides for activation of lipid peroxidation, that can contributed to peribacteroid membrane degradation in the senescent nodules. The amounts of glutathione and homoglutathione increased significantly in the course of development of the nodules, while the concentration of their reduced forms decreased during senescence, indicating the development of oxidative stress. For the first 24 hours of a nodule development, a significant DNA and protein destruction was found, thus there is the likelihood of oxidative stress during the formation of symbiosis (35).

Diffusion barrier responsible for an oxygen gradient in active nodules is probably lower under senescence. This may cause an increased oxygen flow to the tissues and an accelerated ROS generation (35).

Genetic control. Using experimental mutagenesis, in different legume plants a number of mutants unable to fix atmospheric nitrogen (Fix^- phenotype) were obtained. The histological and ultrastructural study indicated their Nop^- phenotype (no nodule persistence) as they were unable to maintain a stable structure and function of nodules. As a result, the early senescence is triggered in the nodules (Fig. 1, C, D; 2, B). In pea, the early senescence is characteristic for mutants in the genes *sym13*, *sym25*, *sym26*, *sym27* and *sym42* (36). Early senescence may be obviously considered as a specific reaction of the host plant to ineffective symbiosis.

In the experiments on model legume plants, *Lotus japonicus* (Regel) K. Larsen and *M. truncatula*, some genes have been cloned. Being defective, they led to early senescence. It must be emphasized that these genes encode quite different proteins involved in a symbiotic nodule functioning.

The first of them, *LjSST1*, encodes a nodule-specific transporter which is located on symbiosome membrane and transfers sulphates from a plant cell cytoplasm to bacteroids (37). In mutants defective in this gene the early senescent nodules and lytic vacuoles are observed (37).

The gene *LjIGN1* encodes an ankyrin-repeat membrane protein containing transmembrane regions, necessary for differentiation and keeping up bacteroids, although its exact role remains unknown (38). In mutant in gene *LjIGN1*, the aggregation of bacteroids was observed in infected cells with disintegrated intracellular compartments, and the senescence starts much earlier than in the other Fix^- *L. japonicus* mutants (38).

The gene *LjSEN1* encodes the integral membrane protein, homologous to nodulin-21 of *G. max*, Fe/Mn transporter CCC1 of *Saccharomyces cerevisiae* and Fe transporter VIT1 of *Arabidopsis thaliana* L. *LjSEN1* expresses only in infected cells of the nodule (39), and the mutants demonstrate early senescence (40).

In *M. truncatula* the gene *MtDNF2* is cloned, for which, due to alternative splicing, the five mRNA transcripts can be transcribed. A predominant transcript encodes PI-PLC-like protein, similar to phosphoinositide phospholipase C. Presumably it can bind phosphatidylinositol or phosphorylated phosphatidylinositol, preventing their decay into inositol phosphate and diacylglycerol, which are the secondary messengers or the precursors of secondary messengers that trigger defense reactions. The mutant plants have nodules with the signs of early senescence (41).

In *M. truncatula* the mutant *esn1* with early senescence is recently reported. For this mutant, the differentiation of bacteroids, *nifH* (a nitrogenase subunit) gene expression, and *LHG* of leghemoglobin are characteristic (42).

It should be noted that to date there are no induced mutants with later senescence. Nevertheless, the transgenic alfalfa *M. truncatula* plants were obtained defective in expression of cysteine protease 15a (gene *Cyp15a*), which are characterized by a delayed senescence of the symbiotic nodules (43).

Transcriptome analysis is one more fruitful approach to detect genes involved in nodule senescence. In 2006, the transcriptome was studied in *M. truncatula* plants of different age to find genes associated with the nodule senescence (15). Using cluster analysis, three groups of genes were revealed according to subsequent stages of senescence. To the first cluster the regulatory genes were assigned which are active when the senescence is initiated. These are the genes of APETALA/Ethylene Response Factor (AP2/ERF), also involved in defense against diseases and stresses; the gene homologous to that of abscisic acid-insensitive transcription factor with DNA-binding domains for AP2/ERF and B3; the genes of protein kinases and MAP-kinases encoding products involved in

signal transduction associated with stresses and reaction to external conditions; the DEAD-box genes of RNA-helicase, participating in mRNA export at stresses and plant development (15). The genes from second and third clusters are involved in senescence at stage I, when the bacteroids are destroyed, and at stage II, when plant cell degradation occurs, respectively. In both these clusters there are genes with regulatory and signal functions. Besides, the degradation of proteins, nucleic acids, membrane lipids and carbohydrates is subject to transcriptional regulation. Intensification of catabolic gene expression results in destruction of symbiosomes at early stage of senescence and plant cells at a later stage. Induction of genes encoding proteins that are involved in transfer of different molecules (e.g. ATP-binding proteins and specific transporters of phosphates, amino acids, ions of metals) suggests that the degradation of macromolecules and mobilization are closely related, and under senescence of nodules the reutilization of metabolites occurs. Due to these catabolic events associated with transport of metabolites, the nodule is reversed from a carbon recipient to the donor of nutrients (15).

To date in *M. truncatula* two transcription factors are found involved in the senescence of symbiotic nodule. Thus, an increased expression of *MtATB2* gene encoding transcription factor of bZIP-type is described. The transcripts were found in apical zone and in vascular bundles of nodules. *MtATB2* transcription is repressed by saccharose, and MtATB2 protein is involved in the regulation of metabolism of amino acids (44). Another transcription factor, MtNAC969, was involved in repression of the genes activated in roots at saline stress, and also it was shown that RNA interference of transcripts led to manifestation of early senescence signs in symbiotic nodules (45).

The responses associated with nodule senescence are also subject to regulation during translation, since some of the studied genes (e.g., the gene encoding 40S ribosomal protein S8) encode ribosomal proteins, elongation factors and other proteins involved in regulation of translation (15).

Stress-induced and natural senescence. Premature nodule senescence can be induced by stresses. An induced senescence is faster and has the signs of oxidative stress and programmed cell death (15).

In a comparative ultrastructural study of natural and stress-induced nodule senescence in *M. truncatula*, some characteristic features were revealed, in particular, the bacteroids were more dense, peribacteroid space increased, and symbiosomes often fused. Nevertheless, the peribacteroid membrane remains intact even if internal part of bacteroid disappears, thus being evidently different from natural senescence when the symbiosomes are completely destroyed. Vesicular transport is less, too. After vacuole destruction, a complete degradation of cytoplasm occurs, mitochondria disappear, with the remnants of peribacteroid membranes and bacteroids being observed. Saprophytic bacteria from infection threads colonize the old cells at an early stage of degradation (12).

The darkness-induced senescence is faster and accompanied by destruction of the symbiosome contents in absence of remobilization cellular signals, and also by rapid colonization by saprophytic bacteria (12).

Comparing natural and darkness-induced senescence, not only characteristic morphological features but specific gene expression was found. At a transcriptional level, 50 % of genes activated under natural senescence are not involved in stress-induced senescence. They are genes responsible for regulation and transport, degradation of membranes and proteins, and also for stress tolerance (e.g., the genes encoding syntaxin protein and two phosphatidylinositol-4-phosphate 5-kinases, necessary for specific vesicle trafficking) (46). In certain other genes, just temporary positive regulation under darkness-induced nodule senescence was reported. Genes that are not likely to be involved in such in-

duced senescence encode many functions related to the degradation of proteins by means of the proteasomes, and some cysteine proteases, probably due to a simplified degradation at stress-induced senescence if compared to natural senescence. Recently a sharp acidification of peribacteroid space was shown in plants after nodule senescence induced by darkness (47).

Thus, currently the molecular, genetic and cell mechanisms underlying symbiotic nodule senescence are actively studied. Their understanding allows to start the programs for creating varieties of legumes with long nitrogen fixation to improve providing the soils with biological nitrogen and to increase yields with decreased application of mineral fertilizers.

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