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Legume nodule senescence: a coordinated death mechanism between bacteria and plant cells

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Abstract

In the legume-rhizobia symbiosis, atmospheric dinitrogen (N_2) fixation by rhizobia takes place in a specific root organ, called the nodule. During biological N_2 fixation, the plant provides photosynthetic carbohydrates to bacteria and, in exchange, bacteria feed the plant with reduced nitrogen (N) under ammonia (NH_3) form. Like most other organs, the root nodule has a limited lifespan, and eventually enters a senescence process characterized by a decline of N_2 fixation and the coordinated death of both bacteria and plant cells. This senescence process characterized by a metabolic switch from a carbon sink to a nutrient source either results from nodule aging (developmental nodule senescence) or can be triggered prematurely by adverse environmental conditions. Accumulating evidence suggests that redox and hormone signaling contribute to the triggering of senescence. Indeed, nodule senescence is a developmentally programmed process in which antioxidants, reactive oxygen and N species (ROS/RNS), hormones and proteinases play key roles. This chapter reviews recent knowledge on nodule senescence and proposes models by which ROS, RNS, and likely hormones intervene in the orchestration of this process.

1. Introduction

Plant-microbe interactions represent a major aspect of the formation and of the development of ecosystems. Plants often establish symbiotic relationships with specific fungi and bacteria to acquire the nutrients they need to support growth and development (Wang et al., 2017; Smith and Smith, 2011; van der Heijden et al., 2016; Venkateshwaran et al., 2013). Amongst the different nutrients, nitrogen (N) has a major importance as it represents a crucial constituent of proteins, nucleic acids and chlorophyll and is often in insufficient amounts in the soil under nitrate and/or ammonia forms. As a result, plants belonging to the legume family have developed a symbiotic association with soil bacteria collectively called rhizobia to increase their N nutrition via the biological fixation of atmospheric dinitrogen (N_2) (BNF). In BNF, the plant provides photosynthetic carbohydrates to the bacteria and, in exchange, bacteria feed the plant with reduced N under ammonia (NH_3) form. Legumes are a family of dicotyledonous plants of the *Fabales* order. It is the third most important family of flowering plants after the *Orchidaceae* and *Asteraceae* with more than 19,500 species (Christenhunsz & Byng, 2016). Economically, *Fabaceae* is the second largest family after *Poaceae* and is a valuable source of plant proteins for human food and animal feeding.

The success of the symbiotic interaction relies on the molecular dialog between the plant and the bacteria (Oldroyd, 2013). The plant root exudates which contain flavonoids and isoflavonoids induce the production by rhizobia of lipochitooligosaccharides, called Nodulation Factors (NFs; Fergusson et al., 2010). NFs are usually essential for the bacterial infection process and the organogenesis of the root nodule, the organ which hosts the N_2-fixing bacteria. During the infection process, rhizobia attach to root hairs in the susceptible zone of the host roots. The recognition of the NFs by the plant induces the root hair curling and the formation of an infection pocket (Fig. 1). Then, bacteria enter into the root hairs through a membrane invagination and the formation of tubular structures, the infection threads (Fig. 1), which allow the transit of the bacteria from the root surface to the cells of the neo-formed nodule (Timmers et al., 1999). Later, the bacteria are endocytosed into the plant cell in a new organelle-like structure, called symbiosome. Bacteria inside the symbiosome, called bacteroids, become able to reduce N_2 to NH_3 thanks to the activity of a specific enzymatic complex, the nitrogenase (Peters et al., 1995).

Two predominant nodule types exist in legumes (Fig. 1). The nodule type depends on the host plant and is determined by the nature of the nodule meristem which could be either transient or persistent (Oldroyd, 2013). Determinate nodules, with a transient meristem, are

spherical since cell divisions occur only during a short period of time. The final growth of the nodule is achieved by cell enlargement. In contrast, indeterminate nodules, with a persistent meristem, are cylindrical as they grow during the whole nodule life by increasing the number and the size of cells. Determinate nodules are generally formed in tropical legumes such as *Phaseolus*, *Vigna* and *Glycine*, whereas indeterminate nodules are found in temperate legumes such as *Medicago*, *Trifolium* or *Pisum*.

Beside the nodule types, the bacteroids possess also three different morphotypes named U, S and E (Kondorosi et al., 2013; Oono et al, 2010). The U-morphotype (for Undifferentiated) corresponds to bacteroids with minor morphological modifications compared to the free-living bacteria and the ability to reverse from the symbiotic lifestyle to the free-living state. The S- and E- morphotypes correspond to large Spherical and large Elongated bacteroids, respectively. These bacteroids cannot return to free-living life and are termed "terminally differentiated". This terminal differentiation is associated with the increase of permeability of the bacteroid membrane and of DNA content compared to free-living bacteria (Mergaert et al., 2006). The bacteroid differentiation is driven by the plant host via the production of a class of peptides called nodule-specific cysteine-rich peptides (NCRs; Van de Velde et al., 2010). To date, NCR peptides have been found to control the terminal differentiation of endosymbionts in inverted repeat-lacking clade (IRLC) legumes and in *Aeschynomene* spp. (Mergaert et al., 2003; Alunni et al., 2007; Czernic et al., 2015). At the functional level, NCRs induce membrane permeability (Lamouche et al., 2019) and have intracellular bacteroid targets such as the cell division protein FtsZ and the chaperone protein GroEL, suggesting that NCRs interact with the bacterial metabolism to induce the terminal differentiation (Farkas et al., 2014).

The cellular differentiation of both symbiotic partners is required to allow the symbiotic N_2 fixation. The production of the nitrogenase enzymatic complex in the bacteroid is a prerequisite for the reduction of N_2 to NH_3. This complex needs high amounts of energy, using sixteen ATP for each N_2 reduced to NH_3, and as a result demands a high respiration rate in the nodule. However, the nitrogenase activity is extremely sensitive to dioxygen (O_2) implying that free O_2 should be present at a very low level in bacteroids (Peters et al., 1995). Multiple strategies allow to deal with this paradox. To decrease the O_2 level in the infected cells, the nodule inner cortex cell layer acts as an oxygen barrier (Tjepkema and Yocum, 1974). In parallel, leghemoglobin (Lb), an efficient O_2-binding protein, present at millimolar concentration in the cytoplasm of infected cells (Appleby, 1992), delivers O_2 to the mitochondria and the bacteroids for respiration and decreases free O_2 to the nanomolar range

in the N_2-fixing zone (Soupène et al., 1995; Ott et al., 2005). Finally, the bacteroid is able to function at a very low oxygen concentration thanks to a specific high affinity *cbb3* cytochrome oxidase (Preisig et al., 1996). The reduction of N_2 to NH_3 is accompanied by the interuption of NH_3 assimilation into bacteroid amino acids. NH_3 is secreted from the bacteroid into the plant cytosol, for assimilation into the amides glutamine and asparagine, or into ureides (Day et al., 2001). In return, the plant provides carbon and energy sources to bacteroids in the form of dicarboxylic acids, particularly malate and succinate, which are produced from sucrose via sucrose synthase and glycolytic enzymes. Thus, the nodule presents multiple peculiarities, involving a plant-microbe crosstalk associated with a metabolism switch which needs a high energy level under microoxic conditions. These working conditions involve a specific cellular redox control in both symbiotic partners. The contribution of reactive oxygen and N species (ROS and RNS) and the importance of the antioxidant defence in the formation and the functioning of root nodules have been largely demonstrated (Alloing et al., 2018; Damiani et al., 2016; Frendo et al., 2013; Puppo et al., 2013).

Whereas formation and functioning of the root nodule has been extensively analysed the rupture of the interaction leading to the nodule senescence has not been much characterized and only few reviews have been dedicated to nodule senescence (Puppo et al., 2005; Dupont et al., 2012; Serova and Tsyganov, 2014; Bruand and Meilhoc, 2019). Some investigators have used the initial decline in nitrogenase activity and BNF efficiency as an indication that nodule senescence has begun (Klucas, 1974). Others authors have defined nodule senescence as the period of rapid nodule decay which occurs at a plant physiological maturity stage, i.e. at leaf yellowing (Streeter, 1981), after flowering has finished (Serova and Tsyganov, 2014), or at pod filling (Lawn and Brun, 1974; Bethlenfalvay and Phillips, 1977). The nodule senescence represents a strong agronomical interest through the possible improvement of N_2 fixation efficiency under adverse environmental conditions to maintain or even increase the yield of legume crops. In this review, we summarize the different modifications of nodule functioning observed during the set up and the progress of nodule senescence, and we focus on the role of antioxidants, ROS/RNS, hormones and proteases in regulating this process.

2. The regulation of nodule senescence

2.1 Physiological and structural modifications

Although the lifespan of nodules of most legumes is 10-12 weeks, their ability to fix N starts to decline 3-5 weeks post-inoculation (Puppo et al., 2005). Nodule senescence is a highly organized and genetically controlled process that begins with metabolic changes. As nodules and leaves are tightly connected in a reciprocal sink-to-source relationship for carbon and N, any defect in photosynthetic activity will indeed impair the metabolic functions of the nodule associated with N assimilation and consequently provoke nodule senescence (Fig. 2). Developmental senescence (also known as natural senescence) occurs because of organ aging, but exposure to various environmental changes also induces nodule senescence (Fig. 2), e.g. when plants are submitted to drought (Gogorcena et al., 1997; Aranjuelo et al., 2013; Gil-Quintana et al., 2015), cadmium stress (Balestrasse et al., 2004; Redondo et al., 2009), photosynthesis inhibitors (Müller et al., 2001), prolonged darkness (Matamoros et al., 1999; Hernandez-Jimenez et al., 2002; Perez-Guerra et al., 2010; Pierre et al., 2014), defoliation (Vance et al. al., 1979), or treatments with argon (Curioni et al., 1999), nitrate (Matamoros et al., 1999; Pierre et al., 2014) or abscisic acid (ABA; Gonzalez et al., 2001). Alternately, senescence can result from a deficient recognition between the two partners (Banba et al., 2001). Depending on the type of triggering factor, the senescence process is more or less rapid and implement variable responses (Van de Velde et al., 2006), but a number of common typical features are found in most nodule senescence processes.

During developmental senescence, the first sign of nodule senescence is the drop in measurable N_2 fixation activity that occurs in parallel with a decrease in Lb content (Dalton et al., 1986; Lahiri et al., 1993). However, during induced senescence, such as after dark treatment, drought stress, or ABA treatment, the rapid decline in N_2 fixation occurs well before a decrease in Lb transcripts or amounts is observed (Escuredo et al., 1996; Gogorcena et al., 1997; Matamoros et al., 1999; Gonzalez et al., 2001). The most visible sign of senescence is a change of the nodule color, from pink to green, caused by bilirubin and biliverdin accumulation after disruption of the Lb structure (Lehtovaara and Pettilä, 1978; Swaraj and Bishnoi, 1996). In senescent soybean nodules, the green color comes partially from Lb nitration, which highlights the critical role of ROS and RNS in the senescence process (Meakin et al., 2007; Navascués et al, 2012; Sainz et al., 2015).

In nodules of the determinate type, senescence develops radially (Fig. 1), starting from the center of the nodule and then progressively extending to the peripheral tissues in a few weeks (Puppo et al., 2005). In nodules of indeterminate type, the N_2 fixation zone is constantly renewed and the senescence zone (zone IV) grows as the nodule grows (Fig. 1); it

increases from the root proximal zone towards the apical zone of the nodule (Puppo et al., 2005; Perez-Guerra et al., 2010; Montiel et al., 2016). Because this zonation is not strictly defined, a senescence inter-zone is sometimes described between zone III and zone IV. Histologically, early nodule senescent cells exhibit a less dense cytoplasm containing numerous vacuoles, altered peroxisomes, mitochondria, starch granules, and late senescent cells ultimately accumulate damaged "ghost" membranes and cell walls (Puppo *et al*., 2005; Pérez-Guerra *et al*., 2010). In common bean nodule developmental senescence, mitochondria were found to be early targets of oxidative modifications and a potential source of ROS/RNS (Matamoros et al., 2013). A dynamic evolution of both plant and bacteroid cell structures is observed: bacteroids are first degraded whereas plant cells remain alive during the early senescence stage, allowing nutrient remobilization, even though at later stages plant cells are also degraded (Vasse et al., 1990; Van de Velde *et al*., 2006). In senescent cells, the degradation of symbiosomes is correlated with the loss of the acidity of the peribacteroid space (Pierre *et al*., 2013), this acidification being an indicator of the bacteroid functioning in the nodule N fixing zone (Pierre *et al*., 2013).

The senescence process was analyzed at gene expression level in both leaves and nodules, particularly in *Medicago truncatula* (Van de Velde et al., 2006, De Michele et al., 2009). It appeared that nodule senescence has a significant degree of overlap with leaf senescence, arguing for the recruitment of similar pathways. A strong redox regulation, through antioxidants decrease and ROS/RNS accumulation, as well as hormonal regulations such as abscisic acid, ethylene or jasmonic acid, was evidenced to correlate with catabolic and remobilization processes during nodule senescence (Puppo et al., 2005; Van de Velde et al., 2006).

2.2 Redox state, ROS and RNS

Changes in the redox balance, which modify the antioxidant defence and the level of ROS and RNS, is directly associated with the transition between N_2 fixation and senescence in nodules (Fig. 2; Puppo et al., 2005).

Ascorbate (ASC) and glutathione (GSH) are the main reported soluble antioxidants within nodules and their regulation was supposed to control the organ lifespan (Loscos *et al*., 2008; Becana *et al.*, 2010). In soybean plants, drought-induced nodule senescence is characterized by a decrease in ASC reduction state and an increase in GSH content (Marquez-Garcia *et al.*, 2015). In bean nodules, the progression of developmental senescence correlates

with a decrease in both ASC and GSH concentrations and reduction states (Loscos *et al.*, 2008). Moreover, the decrease in ASC and GSH concentrations in mitochondria correlates to an increase of oxidative modifications of lipids and proteins, supporting the hypothesis that the mitochondria is an early target of senescence in nodules (Matamoros *et al.*, 2013). In contrast, an increased level of GSH in the N_2-fixing zone is correlated with a higher N_2 fixation efficiency (El Msehli *et al.*, 2011). GSH content is notably involved in the control of glutathione *S*-transferases (GSTs) activities, which are enzymes involved in the cell protection from an oxidative damage, by catalyzing the conjugation of GSH with oxidative degradation products. The level of the GST9 protein, the most prevalent glutathione-S-transferase in soybean nodules, is accordingly increased during senescence (Dalton *et al*., 2009). A decrease in N_2 fixation associated with an increase of the oxidatively damaged protein content is observed in GST9-RNAi silenced soybean nodules, illustrating the role of GSTs in nodule senescence through the regulation of antioxidant defences (Dalton *et al*., 2009). In parallel, the importance of GSH during symbiosis of *Sinorhizobium meliloti* and *Rhizobium tropici* has been also demonstrated (Harrison et al., 2005; Muglia et al., 2008). The *S. meliloti* and *R. tropici* strains deficient in *gshB*, the gene encoding the enzyme catalysing the second step of GSH synthesis, have a reduced N_2 fixation capacity associated with early nodule senescence. Similarly, mutation in the *S. meliloti* glutathione reductase gene, *gor*, impaired N_2 fixation efficiency (Tang et al., 2018).

During the legume-rhizobium symbiosis, both ROS and RNS, and especially nitric oxide (NO), appear to play crucial roles in the establishment and functioning of the interaction (Puppo et al., 2013; Hichri et al., 2015). ROS including superoxide radicals ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are produced in high amounts in nodules due to elevated rates of respiration in bacteroids and plant mitochondria (Matamoros et al., 2013). Plant NADPH oxidases, known as Respiratory Burst Oxidase Homologues (RBOHs), are plasma membrane enzymes catalyzing the reduction of O_2 to generate $O_2^{\bullet-}$ using the reducing power of NADPH. Seven *Rboh* genes have been identified in legume genomes (Marino et al., 2011) and these oxidases are involved in legume-rhizobium symbioses (Arthikala et al., 2014; Marino et al., 2011; Montiel et al., 2016). Other possible sources for H_2O_2 are cell wall peroxidases, germin-like oxalate oxidases, and diamine oxidases (Smirnoff and Arnaud, 2019). On the other hand, NO is a reactive diffusible gas known to have important functions as inter- or intra-cellular signaling molecules in plant growth, development and defence against abiotic or biotic stresses (Brouquisse, 2019, and references therein). For a decade, NO has been also

identified at all stages of legume-rhizobium symbiotic interactions (Hichri et al., 2015, 2016; Berger et al., 2019). The way NO is produced in legumes is not completely clear. The best characterized sources of NO are the nitrate reductase (NR) and the mitochondrial transfer chain (Horchani et al., 2011). Interestingly, it has been shown in soybean and *M. truncatula* nodules that the micro-symbiont accounts for 30% up to 90% of NO production thanks to a nitrite reductase (Nir) belonging to the denitrification pathway (Calvo-Begueria et al., 2018; Horchani et al., 2011; Sánchez et al., 2010). Even though ROS and NO are different types of molecules issuing from distinct synthesis pathways, some parallels can be established when looking at their occurrence during symbiosis. Strikingly, NO and ROS have comparable production patterns during symbiosis such as an early production burst in the root in response to rhizobia inoculation, later in the infection thread, then during nodule organogenesis and finally in mature nodules (Hichri et al., 2016; Montiel et al., 2016).

Interestingly, NO and ROS play a role in nodule senescence. At a late stage of the nodulation process (e.g. 6 weeks post-inoculation), H_2O_2 is detected in ultrathin sections of nodules (Puppo et al., 2013). At the ultrastructural level, the senescent nodules of *Glycine max* and *Pisum sativum* plants display alterations in the symbiosome structure accompanied by the presence of H_2O_2, supporting the idea that ROS have roles also during senescence (Montiel et al., 2016). In addition, it has been suggested that NO is present in higher amounts during the late symbiotic stages than at earlier stages (Bruand and Meilhoc, 2019). Indeed, NO detected by means of a bacterial biosensor is more abundant in the root-proximal part of *M. truncatula* nodules, where senescence is known to initiate (Cam et al., 2012). Similarly, NO detected by means of a fluorescent probe accumulates in old *Lotus japonicus* nodules and Lbs with nitrated heme groups are found in old soybean nodules, indicating the presence of NO in ageing nodules (Chungopast et al., 2017; Navascués et al., 2012; Sainz et al., 2015). Remarkably, exogenous addition of a NO donor on mature nodules of *M. truncatula* lead to an accelerated senescence of the nodules (Cam et al., 2012). Also, several *S. meliloti* mutant strains, altered on *hmp*, *nnrS1*, *nnrS2* and *norB* genes (Table 1) and showing either increased or decreased NO degradation capacity compared to the wild-type, were used to modulate NO levels within nodules and, strikingly, strains deficient in NO degradation induced an early nodule senescence, whereas strains with higher degradation activity led to a delayed nodule senescence (Blanquet et al., 2015; Cam et al., 2012; Meilhoc et al., 2013). NO is also involved in stress-induced *M. truncatula* nodule senescence (Cam et al., 2012). Comparable results were recently obtained in determinate *L. japonicus* nodules (Fukudome et al., 2019).

Indeed, overexpression of a phytoglobin gene *LjGlb1-1* (Table 1) decreased NO levels in nodules and delayed nodule senescence.

By analogy, Redondo et al. (2009, 2012) reported that overexpression in *S. meliloti* of a cyanobacterial flavodoxin involved in the response to oxidative stress in bacteria and cyanobacteria improves the oxidative balance, delays developmental nodule senescence in *Medicago sativa*, and enhances tolerance to cadmium. Overexpression of this flavodoxin also permitted to avoid the decline in nitrogenase activity associated with a salinity stress whether the flavodoxin was expressed in *S. meliloti* or in *M. truncatula* (Coba de la Peña et al., 2010). In *S. meliloti*, various ROS scavenging enzymes (katA and katC catalases particularly) are expressed in the N_2-fixing zone (Roux et al., 2014). The *S. meliloti katA katC* double mutant displays a reduced N_2 fixation activity and bacteroids underwent faster senescence (Puppo et al., 2013). *R. etli* and *B. japonicum* possess only one catalase gene and corresponding mutants were not affected in nodule functioning, but ROS scavenging enzymes other than catalases might reduce H_2O_2 (Puppo et al., 2013). This might also suggest a different role for ROS and antioxidant defence components in the senescence of determinate and indeterminate nodules.

A drastic reduction in N_2 fixation was observed in *Phaseolus vulgaris* nodules silenced in NADPH oxidase (*Rboh*) genes probably linked to an early senescence phenotype (Arthikala et al., 2014). By contrast, overexpression of *RbohB* delays senescence of the nodule. Hence, these elements suggest that ROS produced by RBOHs may have either a promoting, or an inhibiting role during nodule senescence in *P. vulgaris*. Two *Rboh* genes (*MtRbohA and MtRbohB)* were found specifically expressed in the N_2-fixing zone of *M. truncatula* nodules (Marino et al., 2011; Montiel et al., 2016; Roux et al., 2014). A decrease in *MtRbohA* expression *via* a RNA interference (RNAi) approach led to a reduction in the N_2 fixation capacity, showing again the link between MtRBOHA and nodule function (Marino et al., 2011). However, none of these genes displays any detectable promoter activity in the senescence zone of indeterminate nodules, suggesting that they do not directly act in nodule senescence. In addition, RBOHs might have a more prominent role in determinate nodules than in indeterminate nodules, even though the possibility that they participate in nodule senescence should not be eliminated before each gene has been studied in detail in different plants.

Hypotheses on mechanisms by which NO and ROS could trigger nodule senescence are multiple. These molecules can both act at the transcriptional and post-translational levels. Indeed, NO is known to activate the expression of bacterial as well as plant genes (Boscari et al., 2013a; Ferrarini et al., 2006; Meilhoc et al., 2010). However, it is unknown yet which

genes or transcription factors (TFs) could be involved at the onset of nodule senescence. One of the main downstream effects of NO is the post-translational modification of proteins such as S-nitrosation of cysteine residues or nitration of tyrosine residues, which might cause conformational changes in the protein with concomitant changes in their activities, turnover or localization. About 80 proteins were found to be S-nitrosated in *M. truncatula* nodules*;* among them 20 and 8 plant and bacterial proteins, respectively, were also found to be sulfenylated by ROS (Puppo et al., 2013). These protein targets remain to be characterized to establish a potential link with the nodule senescence. However, the activity of many of them, involved either in the glycolysis, the tricarboxylic acid cycle, the energy regeneration or the N_2 assimilation, were found to be inhibited by NO, suggesting that an increased NO production could directly down-regulate N, carbon and energy metabolism in senescent nodules (Boscari et al., 2013b). Interestingly, the enzymatic activity of the plant glutamine synthetases (GS) involved in N assimilation decreases when GS is subjected to NO-mediated tyrosine nitration (Melo et al., 2011). Nevertheless, methionine sulfoxidation of GS is also important for its regulation in response to ROS, showing again that both NO and ROS can be involved in the regulation of protein activity (Matamoros et al., 2013). It has also been shown that the nitrogenase activity is inhibited by NO (Trinchant and Rigaud, 1982).

NO and ROS are generated in cells at the same time and in response to the same cues, so that it is important to consider their possible interaction and associated consequences. One of the best known interaction between NO and ROS is the production of peroxynitrite ($ONOO^-$) from NO and superoxide anion ($O_2^{\bullet-}$). This has two potentially important outcomes: first, the reaction removes both $O_2^{\bullet-}$ and NO from the cell environment, thereby reducing their bioavailability; and second, there is a new compound produced ($ONOO^-$) which itself can act as a signaling/regulatory molecule, potentially giving different responses than that resulting from ROS or NO signaling alone. As an example, peroxynitrite is more potent than NO alone to trigger tyrosine nitration (Astier and Lindermayr, 2012).

NO/$ONOO^-$-dependent modifications may also alter signaling pathways mediated by other reactive signals. Indeed, Tyr nitration of superoxide dismutase can inhibit its activity and hence alter ROS signaling (Holzmeister et al., 2015). NO has also been proposed as a negative regulator of RBOH activity in nodulation (Damiani et al., 2016). Indeed, it has been reported that the AtRBOHD enzyme is S-nitrosated which thus reduces its ROS generating activity (Yun et al., 2011). Therefore, NO has a role in controlling ROS levels and hence their associated downstream signaling. In addition, the *B. japonicum* key regulator of the N_2-fixing

enzymes $FixK_2$ is reversibly susceptible to oxidation, which might indirectly have an influence on the expression of bacterial genes involved in the NO metabolism regulated by $FixK_2$ (Mesa et al., 2009). Similarly, LsrB, a TF involved in the nodulation and redox regulation, is also regulated by its redox state and might be involved in the senescence process (Tang et al., 2017).

2.3 Hormones

Plant hormones are other obvious candidate regulatory molecules to control the transition between N_2 fixation and nodule senescence, some of these signals being previously associated with leaf senescence, such as cytokinins that delay leaf senescence (Zwack & Rashotte, 2013). In white lupin, expression of the cytokinin receptor *LaHK1*, closely related to *AHK4/CRE1* in *Arabidopsis thaliana*, is increased during nodule senescence (Coba de la Peña *et al*., 2008). In *L. japonicus*, expression of the *LjIPT3* (*IsoPentenyl-adenine Transferase 3*, Table 1) gene encoding a rate-limiting cytokinin biosynthetic enzyme, increases during nodule aging and is associated with vascular bundles of mature nodules. *LjIPT3* down-regulation by an RNAi approach triggers a decrease in N_2 fixation, consequently associated with premature nodule senescence (Chen *et al*., 2014). In addition, a maintained N_2 fixation associated with delayed nodule senescence was observed in drought-stressed nodules of alfalfa inoculated with an *S. meliloti* strain engineered to overexpress an *IPT* cytokinin biosynthesis gene and therefore producing increased levels of cytokinins (Xu *et al*., 2012). Cytokinin may therefore act as a positive regulator of N_2 fixation (Boivin *et al.*, 2016; Reid *et al.*, 2016), and possibly as a negative regulator of root nodule senescence as reported during leaf senescence (Zwack *et al*., 2013).

Besides cytokinins, several gene expression analyses revealed putative functions for other plant hormones (Van de Velde *et al*., 2006; De Michele *et al*., 2009). Among those, genes encoding enzymes of the ethylene biosynthesis pathway, such as an 1-aminocyclopropane-1-carboxylate (ACC) oxidase, are induced in senescent nodules (Van de Velde *et al*., 2006). Interestingly, the lack of an ACC deaminase activity in the *Sinorhizobium* strain sp. BL3 accelerates nodule senescence in mung bean, suggesting a positive role for ethylene in nodule senescence similarly to what is observed during leaf senescence (Tittabutr *et al*., 2015). Lipoxygenase genes associated with Jasmonic Acid (JA) biosynthesis and GA2-oxidase genes associated with Gibberellic Acid (GA) degradation are also up-regulated during nodule senescence, suggesting that JA may positively and GA negatively regulate nodule

senescence (Van de Velde *et al*., 2006; Benedito *et al*., 2008). Abscisic acid (ABA) could be also involved in nodule senescence since some ABA responsive genes such as *ABR17* (*ABA Responsive 17*) are induced during nitrate-induced senescence (Liese *et al*., 2017). In addition, ABA, which is associated with various environmental stress responses, improves nodule N_2 fixation capacity in alfalfa salt-stressed nodules where antioxidant defenses mediated by the polyamine spermidine and spermine are induced (Palma *et al*., 2014), and might therefore regulate senescence progression. Such putative negative effect of ABA on nodule senescence is however not observed in leaves where ABA is a positive regulator of senescence (Lee *et al*., 2011).

Considered together, if each of these studies suggests that hormones can intervene in the regulation of senescence, in most cases, functional analyses are lacking to clearly identify how these hormones regulate nodule senescence and more specifically how they may interact with ROS and RNS signaling pathways that are the most extensively linked to nodule senescence. These limited data may nevertheless suggest that common hormonal regulations between leaf and nodule senescence may exist, which could be surprising considering their differences in carbon *versus* N metabolic status.

2.4 Proteases associated with bacteroid and plant cell degradation

A hallmark of cell aging is the progressive decline in proteostasis and the accumulation of misfolded, oxidized and damaged proteins (López-Otín *et al.*, 2013). Therefore, proteolytic activities are under strict regulation to ensure protease activation and degradation at appropriate time and space. In plants, proteases have been associated with global protein turnover, protein processing, degradation of regulatory proteins, cell growth and differentiation, stress response and developmental senescence, but to date functional evidence of their mode of action remains still limited (Buono *et al*., 2019). There are hundreds of protease-encoding genes; for example, more than 800 have been identified in Arabidopsis (van der Hoorn, 2008). Such a large number of proteases can be grouped into families according to their catalytic residues, aspartic-, cysteine-, serine-, threonine-, glutamic-, asparagine-, or metalloproteases (Oda, 2012). A description of the protease classification can be retrieved in the MEROPS database (https://www.ebi.ac.uk/merops/; Rawlings et al., 2010).

To date, many reports have elucidated the roles of cysteine proteases (CPs) in nodule senescence and in response to detrimental environments (Cilliers et al. 2018; Perez-Guerra et al. 2010; Pierre et al. 2014; Pladys et al., 1991; Pladys and Vance, 1993; Van de Velde et al.

2006). The synthesis of CP precursors is orchestrated by a complex transcriptional regulation network. The post-translational activation of many CPs relies on either self-processing or on the interaction with other enzymes. Their activity is also modulated by inhibitors and co-factors. CPs encoding genes are among the most strongly and specifically induced nodule senescence genes, either developmentally or environmentally triggered (Van de Velde *et al*., 2006; Seabra *et al*., 2012; Chungopast *et al*., 2014; Cabeza *et al*., 2014). Their activity is directly correlated to the protein degradation occurring during senescence, and was documented in various legume plants including soybean (Alesandrini *et al*., 2003), *Astragalus sinicus* (Naito *et al*., 2000), pea (Kardailsky & Brewin, 1996), and *M. truncatula* (Pierre *et al*., 2014).

In soybean, 18 cysteine proteases were identified as actively transcribed in nodules during the developmental senescence (van Wyk *et al*., 2014). This expression was increased at specific time points of the senescence process with a majority of *CP* genes highly expressed in 14 week-old nodules. In *Trifolium repens*, the identified *CP* encoding genes were specifically expressed in the senescent zone of the nodule as revealed by a spatio-temporal analysis (Asp *et al*., 2004). In *A. sinicus* indeterminate nodules, the papain cysteine protease *AsNodF32* expressed in senescent tissues of the nodule was identified from a differential screening of a nodule cDNA library (Naito *et al*, 2000). An RNAi silencing of *AsNodF32* extended the period of the active N_2-fixation, delaying nodule senescence (Li *et al*., 2008). In *M. truncatula* nodules, the Cysteine Protease 6 (*MtCP6*), (MEROPS family C1A; https://www.ebi.ac.uk/merops/), and a Vacuolar Processing Enzyme (*MtVPE*; MEROPS family C13), belonging respectively to papain and legumain families, were strongly up-regulated in response to both developmental and environmentally-induced nodule senescence (Van de Velde *et al*., 2006; Pierre *et al*., 2014; Table 1). A detailed spatio-temporal analysis of the *MtCP6* and *MtVPE* expression patterns during nodule developmental senescence revealed an expression associated with the transition region between the N_2-fixing and senescence zones (Fig. 3 A and B, Pierre *et al*., 2014). In nitrate-induced senescent nodules, *MtCP6* and *MtVPE* expression was detected in the whole nodule except in the basal senescence zone (Fig. 3 C and D). Subcellular localization of both *Mt*CP6 and *Mt*VPE in senescent nodules was associated with vacuoles of cells non-infected by rhizobia and to symbiosomes of infected cells, suggesting a role of these CPs in symbiosomes and plant cell degradation (Pierre *et al*., 2014) Silencing of *MtCP6* or *MtVPE* genes delayed nodule senescence and increased N_2 fixation, while their ectopic expression in the N_2-fixing zone promoted nodule senescence and hindered N_2 fixation (Pierre *et al*., 2014). In pea, both

PsCyp1 and *PsCyp15a* (CP) genes transcript levels are increased during nodule aging. However, laser capture microdissection of pea nodule zones III and IV revealed a different pattern of expression: the *PsCyp1* gene is activated at an earlier stage of nodule senescence as transcripts are found exclusively in zone III, whereas the *PsCyp15a* gene is expressed in both zone III and senescent zone IV of pea nodule (Serova et al., 2017).

CPs were reported to interact with CP inhibitors called cystatins, which act as proteolysis regulators (Benchabane *et al*., 2010; Martínez *et al*., 2012; van Wyk *et al*., 2014; Kunert *et al*., 2015; Quain *et al*., 2015). In soybean developmental senescent nodules, three cystatin genes are co-expressed with CPs (van Wyk *et al*., 2014). Remarkably, despite no direct evidence for a role of cystatins during nodule senescence was yet reported, an ectopic overexpression of a rice oryzacystatin in soybean leads to a strong decrease in cathepsin L-like CP activities that are linked to nodule senescence (Quain et al., 2015). The coordinated expression of transcripts encoding cystatins and CPs interacting partners suggests their involvement as a fine-tuning control of proteolysis events associated with nodule senescence. In *M. truncatula* nodules, serpins (serine protease inhibitors) have also been found to be activated in drought-induced senescence and might play a role as modulators of papain-like CPs (Dhanushkodi *et al*., 2018).

Increasing data show that the symbiotic establishment relies on the suppression of plant immunity (Liang et al. 2013, Yu et al. 2018). Transcriptomic studies have revealed transient elicitation of legume plant defence related genes upon *rhizobium* inoculation (Lohar et al. 2006; Libault et al. 2010; Puciariello et al., 2009), followed by their suppressions in the subsequent symbiotic stages (Aslam et al. 2008). Besides being known as an ending stage of organ development, nodule senescence is also proposed to involve a reversion of the plant innate immunity (Puppo et al. 2005). CPs have also been shown to be involved in salicylic acid induced plant defence to cope with microbial pathogens (Shindo et al. 2012; van der Linde et al. 2012; Müeller et al. 2013). Thus, we could hypothesize that activation of CPs during nodule senescence might be linked to increasing plant defence responses linked to the declining nodule N_2 fixation capacity.

Genes encoding components of the SCF (skp1-cullin-F-Box protein) complex, also known as the E3 ubiquitin ligase complex, and genes encoding 26S proteasome AAA-ATPase subunits and the ubiquitin carrier protein 4 are up-regulated during *M. truncatula* developmental nodule senescence (De Michele et al., 2009; Perez-Guerra et al., 2010; Van de Velde et al., 2006). This suggests that the 26S proteasome pathway may be acting during nodule senescence.

3. Transcriptomic characterization of nodule senescence

To get insights into transcriptional regulations associated with symbiotic nodule senescence, several transcriptomic approaches were performed initially on *M. truncatula* nodules. First, a cDNA-AFLP (Amplified Fragment Length Polymorphism) technique was applied on developmental senescent nodules (Van de Velde *et al*., 2006). A second approach used microarrays on nodules treated for 8h with phosphinotricin, an inhibitor of GS activity and therefore affecting N_2 fixation (Seabra *et al*., 2012). More recently, an RNAseq was performed on nodules exposed to nitrate for 4h or 28h (Cabeza *et al*., 2014). Similar studies have been also performed in other legumes species than *M. truncatula*, such as in *L. japonicus* using microarrays and in soybean using RNAseq, at different time-points during nodule aging (Chungopast *et al*., 2014; Yuan *et al*., 2017).

Among the differentially expressed genes identified, the downregulation of specific N_2 fixation associated genes, such as Lb, late nodulins and NCR peptide encoding genes attests the inhibition of the N_2 fixation at the molecular level (Van de Velde *et al*., 2006; Seabra *et al*., 2012; Cabeza *et al*., 2014). Genes related to energy production, such as mitochondrial ATP synthase subunit encoding genes, or amino-acid biogenesis encoding genes, were accordingly repressed (Seabra *et al*., 2012; Cabeza *et al*., 2014). An upregulation of genes encoding proteins involved in macromolecule degradation, such as the CPs previously mentioned, and nutrient remobilization (phosphate, amino-acid, ion transporters and ABC [ATP-Binding Cassette] transporters) was observed, whereas ammonium, potassium and most of the sulfate transporters were down-regulated (Van de Velde *et al*., 2006; Cabeza *et al*., 2014). Genes associated with redox stress responses such as catalases, oxidoreductases, hydrolases, peroxidases, esterases, glutathione transferases were also upregulated, showing the relevance of redox regulations during nodule senescence (Van de Velde *et al*., 2006; Seabra *et al*., 2012), as previously described.

In order to distinguish at the molecular level environmentally induced-senescence from developmental senescence, selected genes up-regulated during a *M. truncatula* developmental nodule senescence were tested by qRT-PCR in nodules exposed to darkness for 3 to 5 days (Van de Velde *et al*., 2006; Pérez-Guerra *et al*., 2010). Only 50% of the genes selected as up-regulated during developmental senescence were up-regulated during a dark-induced nodule senescence. Genes not regulated in the dark-induced nodule senescence were notably related to the 26S proteasome-mediated protein degradation (Pérez-Guerra *et al*.,

2010), suggesting either a less organized, or more probably an accelerated cellular degradation in dark-induced conditions. To refine the spatio-temporal expression profiles during nodule senescence, *M. truncatula* nodules were sectioned into apical *versus* basal zones and pea nodules were microdissected (Van de Velde et al., 2006; Serova et al., 2017). In this way, the *M. truncatula* or pea *ATB2* genes, encoding a bZIP (basic leucine ZIPper) transcription factor (TF), were identified as reaching a peak in fully senescent nodules (Van de Velde *et al.*, 2006; D'haeseleer *et al.*, 2010; Serova *et al*., 2017).

To evaluate if genes were specifically regulated during the senescence of nodule organs, a transcriptomic analysis was performed using a cDNA-AFLP approach in *M. truncatula* senescent leaves (de Michele et al., 2009). The overlap between nodule and leaf senescence associated genes was relatively low (23%). When excluding N-metabolism related genes, a higher degree of overlap was nevertheless observed in the early phase, including specific MAP kinases, ubiquitin cycle components, phosphatases, glutathione transferases, and transcription factors; as well as in the late phase, i.e. several CPs and components of the 26S proteasome (Van de Velde *et al.*, 2006; de Michele *et al*., 2009).

Based on transcriptomic analyses previously described, transcription factors (TF) encoding genes differentially regulated during nodule senescence were identified, mainly from MYB (MYeloBlastosis), bHLH (basic Helix-Loop-Helix), and NAC (NAM ATAF CUC) families (Van de Velde *et al*., 2006; Seabra *et al.*, 2012; Cabeza *et al.*, 2014; Chungopast *et al.*; 2014; Yuan *et al.*, 2017). Among those, only few TFs were selected for further functional analyses, with the aim of identifying regulatory pathways controlling the transition between N_2 fixation and nodule senescence, either during aging or in response to environmental stress conditions. The NAC TF family was previously largely associated with leaf senescence based on transcriptome and genetic studies performed in Arabidopsis (Breeze *et al*., 2011), and is also enriched in nodule senescence transcriptomes. Based on RNAseq or microarray experiments, approximately one third of *NAC* genes expressed in nodules are induced by nitrate (16 / 59 in Cabeza *et al.* 2014; and 21 / 57 in Benedito *et al.*, 2008) whereas over 30 out of 90 *NAC* genes are upregulated during *Arabidopsis* leaf senescence (Breeze *et al*., 2011). Expression of the *M. truncatula* TF *MtNAC969*, closely related to the well-known *Arabidopsis* leaf senescence associated gene *AtNAP* (*NAC-LIKE ACTIVATED BY AP3/PI*), is induced by nitrate and associated with the central region of symbiotic nodules including the N_2-fixation zone (de Zélicourt et al., 2012). In addition, both *AtNAP* and *MtNAC969* genes are induced during *Arabidopsis* and *M. truncatula* leaf senescence, respectively (de Michele et al., 2009; Balazadeh *et al*., 2010). An RNAi silencing of the *MtNAC969* TF promotes nodule

563 senescence (Table 1), based on the increased expression of *CP* genes used as senescence
564 markers, concomitantly to an accumulation of amyloplasts suggestive of an altered nodule
565 metabolism (de Zélicourt *et al*., 2012). Expression of the *M. truncatula MtATB2* bZIP TF is
566 increased during nodule aging senescence, as well as by sucrose but not by a nitrate treatment
567 (Van de Velde *et al*., 2006; D'haeseleer et al., 2010). Despite this transcriptional regulation
568 pattern, ectopic expression of *MtATB2* reduced the number of nodules formed but no
569 senescence phenotype could be observed (D'haeseleer *et al.*, 2010). This highlights that an
570 increased expression in senescent nodules is not a sufficient criteria to identify genes
571 functionally linked to nodule senescence. As *MtATB2* is expressed in the peripheral region of
572 nodules containing the vascular bundles and not in the N_2 fixation or senescence central
573 zones, and because of its regulation by sucrose and not by nitrate, this suggests that this TF
574 might sense carbon sources availability and regulate remobilization processes linked to
575 senescence (D'haeseleer *et al*., 2010). Finally, no TF was yet directly linked to the
576 upregulation of genes encoding CPs or other cell degradation related enzymes, or to the
577 downregulation of Lb or N-assimilation metabolic enzymes encoding genes, or to components
578 of redox pathways. Similarly, a symbiotic bacterial contribution to the regulation of nodule
579 senescence associated genes has not been explored even if some toxin/antitoxin bacterial
580 systems, such as the vapBC-5 (Table 1), have been showed to alter nodule senescence
581 progression (Oláh et al., 2001; Lipuma et al., 2014).
582
583 **4. Concluding remarks - future prospects**
584
585 Senescence affecting the legume-rhizobia symbiotic nodule organ during aging or in
586 response to environmental conditions remains poorly documented. The best functionally
587 characterized senescence mechanisms are related to the redox dependent inhibition of the N_2
588 fixation and assimilation. Physiological and molecular approaches show that nodule
589 senescence is followed by a decrease in the level and/or reduction state of the main cellular
590 antioxidants ASC and GSH, and by an increase in the production of ROS and RNS, such as
591 H_2O_2 and NO. This results in an increase of oxidized/denatured proteins and subsequently in
592 an inhibition of N, carbon and energy metabolisms. In addition from being harmful to cellular
593 processes, both ROS and NO production are also important components of the signaling
594 network used by legumes and their symbionts to communicate at the various stages of the
595 symbiotic interaction. The evolution of efficient ROS/NO degrading systems has most likely
596 enabled plant cells and bacteroids inside nodules to overcome ROS and NO toxicity. Diverse

bacterial and plant systems contribute to the control of NO and ROS levels in nodules. Only when these systems are overcome by NO and ROS, these molecules can act as signals to trigger senescence (Puppo et al., 2013). Alternatively, senescence could be a consequence of NO-induced modifications of proteins essential for N_2 fixation. Further studies are needed to link the modifications of these proteins by NO or ROS to nodule senescence.

An increased proteolysis and the induction of plant vacuolar proteases such as CPs and VPEs during senescence are also well documented, and used as a hallmark to follow the senescence progression. Identifying how these nodule specific proteases are regulated and what are their targets remains a challenge to understand better nodule senescence. Both 26S and 20S proteasome proteolytic pathways are additionally induced during nodule senescence, and their contribution should be established. Finally, the nature of bacterial proteases as well as their contribution to the degradation of bacteroid proteins remains to be better documented.

Large-scale transcriptomic analyses from the last decade allowed identifying new candidate genes that may act in the senescence processes. These approaches were however focused on the plant transcriptome, and it would be interesting to analyze in parallel the transcriptional response of symbiotic bacteria to determine if they could contribute to the regulation of nodule senescence progression. In the model legume *M. truncatula*, partially overlapping plant regulatory pathways were identified between environmental, developmental and leaf senescence. This suggests that a core of common genes is conserved between the senescence of different organs, but also that a subset of genes may specifically regulate senescence in nodules, which may be notably related to the symbiotic and metabolic features of this organ hosting numerous N_2 fixing bacteria. Such putative plant-microbe gene network interactions remain however to be investigated. Only few regulatory genes directly regulating nodule senescence have been yet functionally characterized, such as TFs and hormone-related genes. A future prospect would be to integrate how the diversity of bacteria hosted inside the nodules, beyond rhizobia, could control senescence progression. Indeed, promising recent data (Rodrigues *et al*., 2013) reported that other soil bacteria than rhizobia might indeed modify the nodule lifespan, as cowpea nodules co-inoculated with *Bradyrhizobium* sp. and *Paenibacillus graminis* or *Paenibacillus durus* plant growth-promoting bacteria present an increased N availability and delayed nodule senescence. Finally, an applied perspective of studying nodule senescence would be, as achieved with “staygreen” leaves, to engineer “staypink” nodules that would have maintained, and even improved, capacities to fix N_2 under environmental stress conditions.

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Figures & Legends

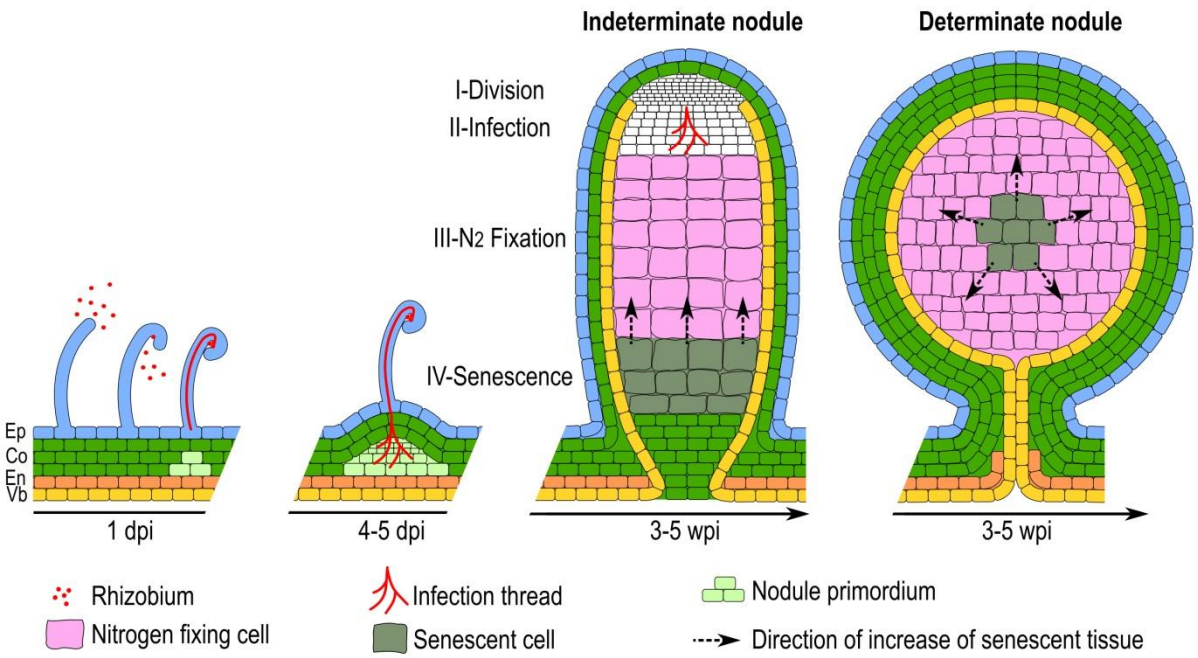
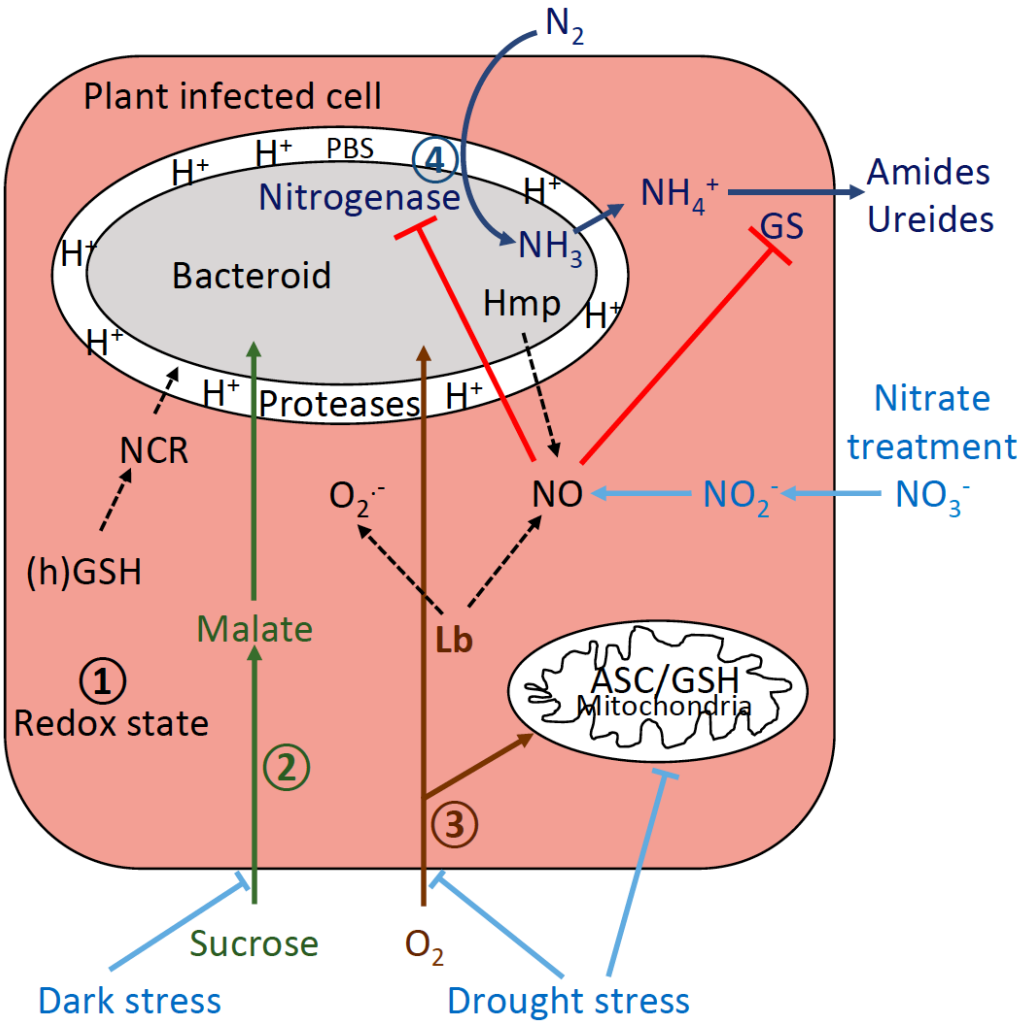


Figure 1 : Schematic representation of the establishment and of the development of the legume-rhizobium symbiosis, and of the structure of mature indeterminate and determinate nodules.

En, endodermis; Co, cortex; dpi, day post-inoculation; Ep, epidermis; Vb, vascular bundle; wpi, week post-inoculation.



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1125 **Figure 2: Major processes triggering nodule senescence in response to environmental**
1126 **constraints.**

1127 (1), Modification of the redox balance; (2), alteration of the bacteroid carbon nutrition; (3),
1128 alteration of O_2 homeostasis; (4), alteration of N_2 fixation (nitrogenase). Red lines indicate
1129 down-regulation. Black dotted lines indicate scavenging of NO or O_2 by Lb or Hmp proteins.
1130 The pink color of the plant cell cytoplasm illustrates the Lb content. ASC: Ascorbate; Hmp:
1131 Flavohemoglobin; GS: Glutamine; (h)GSH: (homo)Glutathione; synthetase; Lb:
1132 Leghemoglobin; PBS: Peribacteroid space; Proteases: Cysteine proteases; NCR: Nodule
1133 cysteine-rich peptides.

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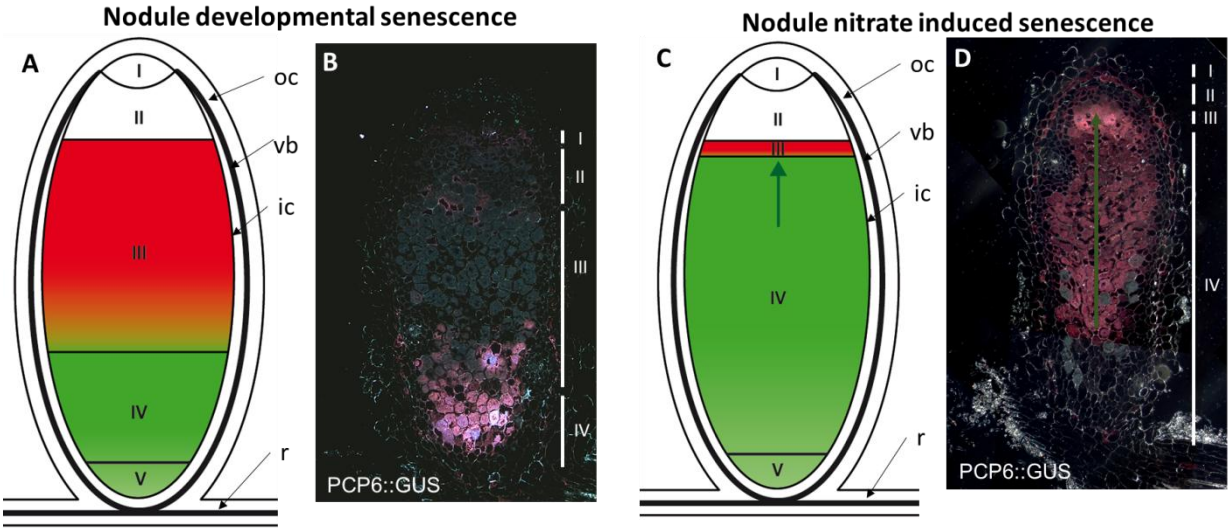


Figure 3: Developmental and induced senescence in the *M. truncatula* nodule.

A) Schematic representation of developmental/aging senescence in indeterminate nodules. I, meristematic zone ; II, rhizobial infection zone ; III, nitrogen fixing zone (in red); IV, senescence zone (in green); V, saprophytic zone (in green). oc, outer cortex; ic, inner cortex; vb, vascular bundle; r, root. **B**) Section of a nodule expressing the reporter gene *uidA* (GUS) under control of the *MtCP6* promoter 6 weeks post-inoculation. **C**) Schematic representation of nitrate induced-senescence in indeterminate nodules. The arrow shows the extension of the senescent tissues revealed by the p*MtCP6*:GUS reporter. **D**) Section of a nodule expressing the reporter gene *uidA* (GUS) under control of the *MtCP6* promoter 3 days after the nitrate treatment.

Gene (mutant)	Species	Gene characteristics	Reference
LjIPT3	*Lotus japonicus*	Reduced nitrogen fixation observed in RNAi plants. Coding for an Isopenthyl-Transferase, a rate limiting step in cytokinin biosynthesis	Chen *et al.*, 2014
MtCP6 and *MtVPE*	*Medicago truncatula*	RNAi plants are delayed in the apparition of senescence in nodule	Pierre *et al.*, 2014
MtNAC969	*Medicago truncatula*	Accumulation of senescence marker transcripts in RNAi plants	de Zélicourt *et al.*, 2012
Hmp (hmp^{++})	*Sinorhizobium meliloti*	Hmp overexpression delays nodule senescence. Hmp encodes a flavohemoglobin involved in NO detoxication	Cam *et al.*, 2012
nnrS1 *nnrS2*	*Sinorhizobium meliloti*	nnrS1/2 mutants induce early nodule senescence. nnrS1/2 encode for cooper-containing membrane proteins involved in NO detoxication	Blanquet *et al.*, 2015
norB	*Sinorhizobium meliloti*	norB mutants induce early nodule senescence. norB encodes for a NO reductase in the denitrification pathway	Meilhoc *et al.*, 2013
LjGlb1-1	*Lotus japonicus*	Glb1-1 overexpression delays nodule senescence. Glb1-1 encodes a Phytoglobin involved in NO detoxification	Fukudome *et al.*, 2019
vapBC-5	*Sinorhizobium meliloti*	Delayed senescent phenotype. Coding an active bacteria toxin	Lipuma *et al.*, 2014

Table 1: Genes involved in symbiotic nodule senescence.

IPT : IsoPentenyl Transferase, CP : Cystein Protease, VPE: Vacuolar Processing Enzyme, NAC : NAM ATAF CUC transcription factor, Hmp : flavohemoglobin, nnrS: nitrite and nitric oxide reductase S, norB: NO reductase B, Glb1 : Class 1 Phytoglobulin, vapBC : toxin-antitoxin module.